

UNIVERSIDADE FEDERAL DE SÃO CARLOS - UFSCAR
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

CARLOS ALEXANDRE BREYER

**Investigação dos determinantes moleculares envolvidos na
interação com os substratos de Tsa1 e Tsa2 de *Saccharomyces
cerevisiae***

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interação com os substratos de Tsa1 e Tsa2 de *Saccharomyces
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Tese de doutorado apresentada ao Programa
de Pós-Graduação em Biotecnologia.

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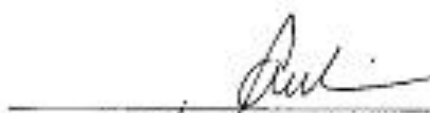
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Eu aprendi...

...que ignorar os fatos não os altera;

Eu aprendi...

...que quando você planeja se nivelar com alguém, apenas está permitindo que essa pessoa continue a magoar você;

Eu aprendi...

...que o AMOR, e não o TEMPO, é que cura todas as feridas;

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Eu aprendi...

...que a vida é dura, mas eu sou mais ainda;

Eu aprendi...

...que as oportunidades nunca são perdidas; alguém vai aproveitar as que você perdeu.

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Eu aprendi...

...que quanto menos tempo tenho, mais coisas consigo fazer.

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RESUMO

Peroxirredoxinas (Prx) são proteínas antioxidantes capazes de decompor uma grande variedade de hidroperóxidos, são muito abundantes na célula e em eucariotos estão distribuídas nos diversos compartimentos celulares. As Prx são capazes de reduzir seus substratos utilizando um resíduo de cisteína altamente reativa, denominada de cisteína peroxidásica (C_P-S^*), que se apresenta na forma de tiolato. No processo de decomposição de hidroperóxidos é oxidada a cisteína ácido sulfênico (C_P-SOH). A grande maioria destas proteínas se apresenta como dímeros obrigatórios e apesar de algumas Prx apresentarem somente uma cisteína, grande parcela possui uma segunda cisteína envolvida no ciclo catalítico que recebe o nome de cisteína de resolução (C_R), a qual forma um dissulfeto com C_P , que frequentemente é reduzido pela enzima tiorredoxina (Trx). Adicionalmente à redução das Prx, as Trx estão envolvidas em diversos outros processos biológicos como crescimento celular, inibição de apoptose, ativação de transcrição e síntese de DNA. Quando a célula é exposta a elevado estresse oxidativo, pode ocorrer a superoxidação de C_P formando espécies superoxidadas como a cisteína ácido sulfínico (C_P-SO_2H) e sulfônico (C_P-SO_3H) que não podem ser reduzidas por Trx. Entretanto, também resulta na alteração da estrutura quaternária das Prx, levando à formação de estruturas de alto peso molecular (HMW) que possuem propriedade de chaperona molecular e estão relacionadas a transdução de sinal desencadeada por hidroperóxidos. Em *Saccharomyces cerevisiae*, há duas isoformas citosólicas de Prx (Tsa1 e Tsa2), que possuem grande semelhança (86% de identidade e 96% de similaridade), mas apresentam diferenças na concentração celular e nível de expressão. Entretanto, muitas vezes são consideradas proteínas redundantes. Este trabalho teve como objetivos uma análise comparativa aprofundada buscando um maior entendimento das diferenças funcionais e estruturais de Tsa1 e Tsa2. Foram avaliados a relação de Tsa1 e Tsa2 com seus substratos oxidantes (hidroperóxidos) e substratos redutores (Trx1 e Trx2). Análises estruturais e cinéticas demonstraram uma importante relação entre a manutenção da estrutura decamérica a atividade das enzimas a qual ocorre por uma série interações polares similares mas não iguais entre aminoácidos do sítio ativo. Com destaque para a Thr44 de Tsa1 (Ser em Tsa2) que possui importância na estabilização da estrutura decamérica através de uma interação do tipo C-H- π com Tyr⁷⁷ e através do átomo de O γ que possui interações polares com Phe⁴⁵ e Leu⁴¹. Também foram realizadas análises de susceptibilidade a superoxidação de Tsa1 e Tsa2, e demonstrou-se que peróxidos orgânicos são capazes de promover mais eficientemente a superoxidação quando comparados a H₂O₂, sendo que Tsa1 apresenta uma maior sensibilidade a superoxidação. Essa diferença foi também relacionada à substituição Thr/Ser e análises de susceptibilidade a superoxidação dos mutantes Tsa1^{T44S} e Tsa2^{S44T} demonstraram que a substituição recíproca tornou Tsa1^{T44S} mais resistente a superoxidação e Tsa2^{S44T} mais sensível. A formação de estruturas de alto peso molecular (HMW) de Tsa1, Tsa2 e mutantes foram investigadas através de cromatografia de exclusão molecular (SEC) e microscopia eletrônica de transmissão (TEM). Os resultados demonstraram um aumento da formação de HMW após reações de superoxidação utilizando o sistema Trx em altas concentrações de CHP e foi verificada a presença de empilhamentos de decâmeros, além de esferas, descritas na literatura. Os resultados obtidos neste trabalho demonstram de forma clara que as proteínas Tsa1 e Tsa2 diferem de forma significativa tanto na suscetibilidade a superoxidação quanto na formação de complexos HMW distintos, indicando fortemente que estas proteínas possuem papéis biológicos não redundantes e que alterações sutis de aminoácidos no sítio ativo promovem grandes alterações funcionais e estruturais nas enzimas.

Palavras chave: Peroxirredoxinas. Reatividade. Superoxidação. Estruturas de alto peso molecular.

ABSTRACT

Peroxiredoxins (Prx) are antioxidant proteins capable of decomposing a variety of hydroperoxide are very abundant in the cell and in eukaryotes are distributed in many cell compartments. The Prx are able to reduce their substrates using a highly reactive cysteine residue, named peroxidatic cysteine (C_P-S^-), which is maintained in the thiolate form as a consequence of the active site environment. In hydroperoxide decomposition process the C_P is oxidized to cysteine sulfenic acid (C_P-SOH). These vast majorities of these proteins are obligate dimers and has a second cysteine involved in the catalytic cycle, named resolution cysteine (C_R), which forms a disulfide with C_P and is often reduced by the enzyme thioredoxin (Trx). In addition to the reduction of Prx, the Trx are involved in several other biological processes such as cell growth, inhibition of apoptosis, transcriptional activation and DNA synthesis. When cell are exposed to oxidative stress, the CP can suffer overoxidation, forming species such as cysteine sulfinic acid (C_P-SO_2H) and cysteine sulfonic acid (C_P-SO_2H), which are not reduced by Trx. However, the C_P overoxidation results in a change of the of Prx quaternary structure, resulting in the formation of high molecular weight structures (HMW) with molecular chaperone property and related to signal transduction triggered by hydroperoxides. *Saccharomyces cerevisiae*, have two isoforms of cytosolic Prx (Tsa1 and Tsa2), which shares high similarity (86% identity and 96% similarity), but differ in cellular abundance and expression levels. However, these enzymes are often considered redundant proteins. This study aimed the comparative analysis searching for functional and structural differences between Tsa1 and Tsa2. We evaluated Tsa1 and Tsa2 relationships with its oxidizing (hydroperoxide) and reducing substrates (Trx1 and Trx2). We also performed structural and kinetic analysis showed a significant relationship between maintaining the decameric structure the activity of enzymes which occurs by a series polar interactions but not equal to the active site amino acids. Highlighting the Thr44 of Tsa1 (Ser in Tsa2) which is involved in decamer structure stabilization by a CH- π interaction type with the Tyr77 and through the O γ atom (from Thr) polar interactions with Phe45 and Leu41. Overoxidation susceptibility assays were performed using Tsa1 and Tsa2, and our results revealed that organic hydroperoxides were able to promote the C_P overoxidation more efficiently when compared to H_2O_2 . In turn, Tsa2 was very more resistant to C_P overoxidation than Tsa1. This difference was also investigated concerning the catalytic triad Thr (Tsa1) to Ser (Tsa2). We generated mutants carrying reciprocal substitutions Tsa1^{T44S} and Tsa2^{S44T} and our results revealed that Tsa1^{T44S} become more resistant to C_P overoxidation and Tsa2^{S44T} become more sensitive. The formation of high molecular weight structures (HMW) of Tsa1, Tsa2 and mutants were also investigated by size exclusion chromatography (SEC) and transmission electron microscopy (TEM). The Tsa1, Tsa2 and mutants HMW formation were analyzed by size exclusion chromatograph (SEC) and transmission electron microscopy (TEM). The results showed an increase of HMW formation after Trx system reaction using high concentrations of CHP. Were observed the stacking of the ring-shaped structures besides spherical species. Our results demonstrate that Tsa1 and Tsa2 proteins differ significantly in overoxidation susceptibility and HMW complex formation, indicating that these proteins have not redundant biological roles and small changes in active site promote high functional and structural changes in these enzymes.

Keywords: Peroxiredoxins, Overoxidation, Reactivity, High Molecular Weight Complexes.

LISTA DE ABREVIATURAS

·OH	Radical hidroxila
AhpC	Alquil hidroperóxido redutase subunidade C
Cat	Catalases
CHP	Cumeno hidroperóxido
C _P	Cisteína peroxidásica
C _P -S ⁻	Tiolato
C _R	Cisteína de resolução
Cys-SO ₂ H	Ácido sulfínico
Cys-SO ₃ H	Ácido sulfônico
Cys-SOH	Ácido sulfênico
DLS	Espalhamento dinâmico de luz
DTPA	Ácido dietileno triamino pentacético-4
DTT -	1,4-ditiotreitol
EDTA	Ácido etilenodiamino tetra-acético
ERos	Espécies reativas de oxigênio
FAD	Flavina adenina dinucleotídeo
FF	Totalmente enovelado (<i>Folly folded</i>)
Gpx	Glutathionas peroxidases
H ₂ O ₂	Peróxido de hidrogênio
Hepes	Ácido 4-(2-hidroxiethyl)piperazina-1- etanosulfônico
HMW	Estruturas de alto peso molecular (<i>High molecular weight</i>)
HPLC-	Cromatografia líquida de alta eficiência (<i>High performance liquid chromatography</i>)
HRP	Peroxidase de raiz forte (<i>Horseradish peroxidase</i>)
IPTG	isopropil β-D-1 –tiogalactopiranosídeo
LMW	Estruturas de baixo peso molecular (<i>Low molecular weight</i>)
LU	Desenovelamento Local (<i>Locally unfolded</i>)
MES	Ácido 2-(N-morfolino) etanosulfônico
NADPH	β-nicotinamida adenina dinucleotídeo 2'-fosfato reduzido
NEM	N-etilmaleimida
Mxr1	Metionina sulfóxido redutase
NAD(P)H	Nicotinamida adenina dinucleotídeo fosfato
O ₂ ⁻	Ânion superóxido
PDB	<i>Protein Data Bank</i>
PEG	Polietileno glicol
PMSF	Fluoreto de fenilmetilsulfonila
Prx	Peroxirredoxina
PrxQ	Peroxirredoxina Q
SDS	Dodecil sulfato de sódio
SDS-PAGE	Eletroforese em gel de poliacrilamida sob condição denaturante
SAXS	Espalhamento de raio-X de baixo ângulo (<i>Small-angle X-ray scattering</i>)
SDS	Dodecil sulfato de sódio
SEC	Cromatografia de exclusão molecular (<i>Size exclusion chromatography</i>)
SOD	Superoxido Dismutase
<i>t</i> -BOOH	<i>tert</i> -butil hidroperóxido
TEM	Microscopia eletrônica de transmissão (<i>Transmission electron microscopy</i>)
TEMED	Tetrametiletlenodiamina
Tris	Tris(hidroximetil) aminometano
Trx	Tiorredoxina
Tsa	<i>thiol-specific antioxidant</i>
WT	Selvagem (<i>wild type</i>)
TrxR	Tiorredonina redutase

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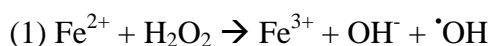
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1 INTRODUÇÃO

1.1 Espécies Reativas de Oxigênio

Espécies reativas de oxigênio (EROs) são formadas durante o metabolismo normal de organismos aeróbicos através da redução incompleta do oxigênio molecular, seu acúmulo em um organismo pode levar ao chamado estresse oxidativo (Halliwell; e Gutteridge, 2007). Estresse oxidativo pode ser definido como um desequilíbrio entre a produção de EROs e a capacidade de um organismo decompor essas espécies (Freinbichler *et al.*, 2011; Halliwell e Gutteridge, 1990). A ocorrência de estresse oxidativo tem sido relacionada há várias patologias, incluindo doenças neurodegenerativas, lesões cerebrais traumáticas ou isquêmicas, câncer, diabetes, lesões hepáticas e AIDS (Finkel, 2003; Halliwell e Gutteridge, 1990; Valko *et al.*, 2006). Também se acredita que o estresse oxidativo pode desempenhar um papel importante no processo de envelhecimento (Boisnard *et al.*, 2009; Dalle-donne *et al.*, 2007; Freinbichler *et al.*, 2011; Nelson *et al.*, 2011a).

Dentre as EROs, grande destaque dá-se ao peróxido de hidrogênio (H_2O_2), radical ânion superóxido ($\text{O}_2^{\cdot-}$) e o radical hidroxila ($\cdot\text{OH}$). A cadeia transportadora de elétrons consiste no principal processo metabólico responsável pela geração de EROs, sendo que cerca de 0.1 a 5% do oxigênio molecular escapa na forma de $\text{O}_2^{\cdot-}$ e H_2O_2 (Halliwell e Gutteridge, 2015). Algumas destas espécies podem transitar livremente nas células, como exemplo o $\text{O}_2^{\cdot-}$, que apesar da sua baixa permeabilidade pela membrana lipoproteica consegue atravessá-la por canais aniônicos e reagir com grupos prostéticos metálicos de proteínas (Halliwell e Gutteridge, 2015; Sies, 2014). O H_2O_2 , por sua vez, pode se difundir pelas membranas celulares, ou mesmo ser transportado através de canais homólogos às aquaporinas (Bienert, Schjoerring e Jahn, 2006; Bienstock, 2011; Malinouski *et al.*, 2011; Miller, Dickinson e Chang, 2010) e é um oxidante proteico, em especial, de resíduos de metionina e cisteína (Kang *et al.*, 2005). Apesar de não ultrapassar as membranas celulares, o radical $\cdot\text{OH}$ é extremamente reativo e pode danificar lipídeos, ácidos nucleicos, proteínas e outros compostos orgânicos (Halliwell e Gutteridge, 2015; Valko *et al.*, 2006). O radical $\cdot\text{OH}$ pode ser formado a partir de reações entre o $\text{O}_2^{\cdot-}$ e o H_2O_2 com metais de transição por meio da reação de Fenton (1) ou diretamente através da reação de Harber-Weiss (2) (Halliwell e Gutteridge, 2015; Kehrer, 2000).





Através do processo evolutivo, os organismos desenvolveram diferentes estratégias para impedir a formação e decompor as EROs e adicionalmente, passaram também a utilizar estas espécies na defesa e sinalização celular (Halliwell e Gutteridge, 2015; Mittler *et al.*, 2011). Desta forma, enzimas como as lipoxigenases, NAD(P)H oxidases, xantina desidrogenase, entre outras, são capazes de produzir EROs, as quais podem desempenhar importantes papéis na sinalização celular (Halliwell e Gutteridge, 2007). Segundo Mittler e colaboradores (2011), várias são as vantagens da utilização destas moléculas em sinalização celular. Dentre elas, destaca-se a capacidade da célula rapidamente produzir e degradar estas espécies de forma simultânea, permitindo assim mudanças rápidas em seus níveis de forma dinâmica. Outra vantagem seria um controle específico sobre a localização subcelular de sinais de EROs nas células (Mittler *et al.*, 2011). EROs controlam importantes eventos celulares em eucariotos, uma vez que agem como mensageiras para a transdução de sinal e controle da expressão gênica (Gamaley e Klyubin, 1999; Kamata e Hirata, 1999; Kang *et al.*, 2005; Pastori e Foyer, 2002) sendo o H_2O_2 um componente essencial desse processo (Toledano, Planson e Delaunay-Moisan, 2010). O H_2O_2 é capaz de oxidar resíduos de cisteína presentes em proteínas que agem como sensores, levando à formação do ácido sulfênico (Cys-SOH), sulfínico (Cys-SO₂H) ou sulfônico (Cys-SO₃H) e ocasionando mudanças estruturais de proteínas que respondem pela especificidade da sinalização celular (Chiu *et al.*, 2011; Kumsta e Jakob, 2009; Poole, Karplus e Claiborne, 2004). É importante ressaltar que concentrações intracelulares superiores a 1 μM de H_2O_2 são tóxicas às células e ineficazes para os propósitos reguladores (Antunes, Cadenas e Brunk, 2001).

As enzimas responsáveis pela degradação das EROs podem ser divididas em dois grandes grupos principais. O primeiro se caracteriza pela conversão de radicais superóxido ($\text{O}_2^{\bullet-}$) a peróxido de hidrogênio (H_2O_2), o qual é efetuado pela enzima superóxido dismutase (SOD) (Kato *et al.*, 1999). O segundo é capaz de decompor o H_2O_2 e suas principais representantes são as catalases (Cat), as glutatona peroxidases (Gpx) e as peroxirredoxinas (Prx) (Figura 1) (Halliwell e Gutteridge, 2015; Kang *et al.*, 2005).

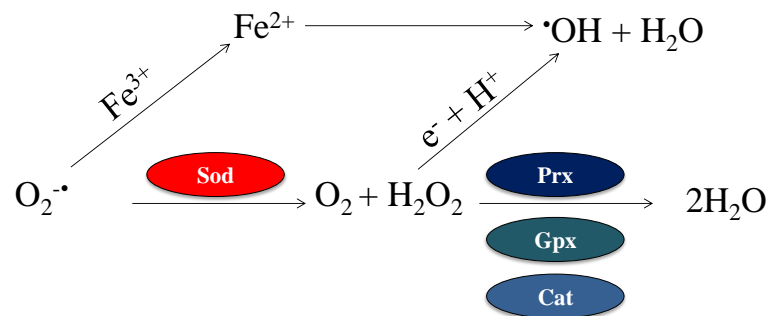


Figura 1. Formação e decomposição enzimática de espécies reativas de oxigênio. Processo de formação de algumas das espécies reativas de oxigênio mais abundantes nos organismos e as principais enzimas antioxidantes envolvidas na sua decomposição.

1.2 Peroxirredoxinas

As Peroxirredoxinas (Prx), também denominadas de tiorredoxinas peroxidases (Tpx), tem despertado o interesse de muitos pesquisadores voltados à compreensão dos mecanismos celulares antioxidantes, uma vez que estas são muito abundantes e largamente distribuídas nos mais diversos compartimentos celulares (Demasi, Pereira e Netto, 2001; Flohé e Harris, 2007). As Prx compreendem uma ampla família de peroxidases portadoras de grupamentos tiol, que são utilizados na redução dos substratos (Dietz, 2007; Hofmann, Hecht e Flohé, 2002; Lukosz *et al.*, 2010; Wood *et al.*, 2003). Todas elas possuem ao menos um resíduo de cisteína na porção N-terminal, denominada de cisteína peroxidásica (C_P), correspondente ao sítio de oxidação dos hidroperóxidos. Estas enzimas são encontradas em praticamente todos os organismos e largamente investigadas na levedura *Saccharomyces cerevisiae*, devido à alta similaridade entre as enzimas deste organismo e as de mamíferos.

As Prx podem ser subdivididas em três grupos distintos quanto às estruturas proteicas e pelo número de cisteínas presentes em seus sítios ativos (SA): as 1-Cys Prx são proteínas diméricas que apresentam apenas um resíduo de cisteína na região N-terminal; as 2-Cys típicas, também diméricas, com dois resíduos de cisteína dispostos nas regiões N e C-terminais. Estes durante o ciclo catalítico formam dissulfeto intermolecular e, finalmente, as 2-Cys atípicas, que se apresentam como monômeros e também possuem dois resíduos de cisteína que durante o ciclo catalítico formam ponte dissulfeto intramolecular (Choi *et al.*, 2005; Hall, Karplus e Poole, 2009; Hall *et al.*, 2009).

Recentemente uma nova classificação foi proposta para as Prx que leva em consideração o aspecto estrutural e aminoácidos conservados em torno do sítio ativo. Baseados nessa disposição as Prx são classificadas em seis subfamílias: AhpC-Prx1, BCP-PrxQ, Tpx, Prx5, Prx6 e AhpE. Todos os representantes das 2-Cys típicas, que são objeto deste estudo, foram incluídos na subfamília AhpC-Prx1 (Nelson *et al.*, 2011b).

Adicionalmente às formas diméricas, já foram observadas estruturas decaméricas de Prx 2-Cys típicas as quais são estabelecidas através da associação de cinco dímeros resultando na formação de decâmeros do tipo $\alpha_2[5]$. Alguns autores sugerem que esta organização poderia levar a decomposição cooperativa de moléculas de peróxido, semelhante ao caso muito bem estudado da molécula de hemoglobina (Cox e Nelson, 2008; Tairum *et al.*, 2015). Desta forma, a decomposição de peróxidos por uma subunidade do decâmero poderia aumentar a afinidade por peróxidos das demais subunidades (Cox *et al.*, 2009; Parsonage *et al.*, 2005). Aspectos adicionais referentes ao estado de oligomerização das Prx 2-Cys típicas serão discutidos posteriormente.

No processo de decomposição dos hidroperóxidos pelas Prx 2-Cys típicas, a cisteína peroxidásica (C_P) é oxidada e forma uma cisteína ácido sulfênico (C_P -SOH) que reage com o H da cisteína de resolução (C_R) do monômero adjacente, formando uma ligação dissulfeto intermolecular entre as cisteínas e eliminando H_2O (Chae, Ho Zoon, Chung e Rhee, 1994; Collin *et al.*, 2003; Hall, Karplus e Poole, 2009; Perkins *et al.*, 2015; Poole, Hall e Nelson, 2011; Wilson *et al.*, 1998; Wood *et al.*, 2003) (Figura 2).

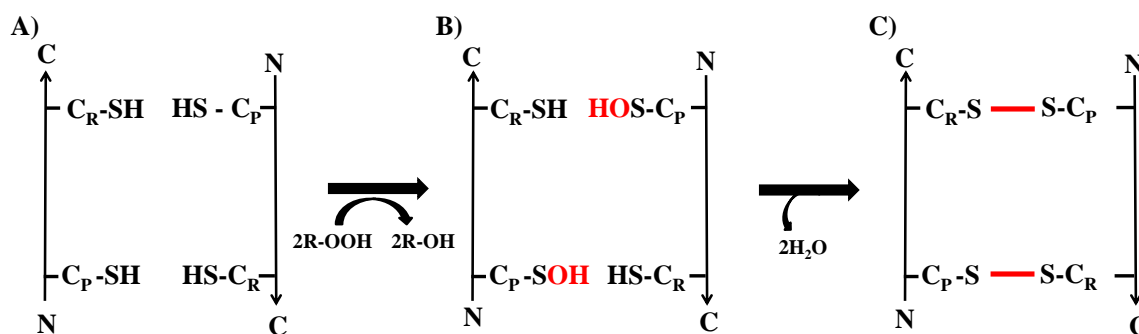


Figura 2. Mecanismo de decomposição de hidroperóxidos pelas Prx 2-Cys típicas. A) Inicialmente as cisteínas peroxidásicas dos diferentes monômeros reagem com moléculas de hidroperóxido. No caso de reação com um peróxido orgânico, ocorre à liberação de um álcool ou água se a reação ocorrer com H_2O_2 . B) Após a reação as C_P são oxidadas a cisteína ácido sulfênico (C_P -SOH). C) Ocorre uma reação de condensação liberando H_2O e formam-se ligações dissulfeto entre C_P e C_R dos diferentes monômeros.

As Prx diferentemente de outras proteínas antioxidantes não requerem cofatores para sua atividade catalítica. Durante a catálise a C_P é mantida na forma de tiolato (C_P-S^-) em um motivo conservado PXXXTXXC (em alguns casos a T pode ser substituído por S), ao reagir com um hidroperóxido o tiolato fica oxidado na forma de C_P -ácido sulfênico (C_P -SOH). Após a reação ocorre à formação de um dissulfeto intramolecular (2-Cys Prx típicas), intermolecular (2-Cys Prx atípicas) ou a forma C_P -SOH é diretamente reduzida (1-Cys Prx). Normalmente grupos sulfidrila de proteínas em sistemas biológicos tendem a ser manter protonados (tiol, R-SH), pois o pK_a da Cys é de 8.5 acima do pH neutro. Mas muitas vezes o pK_a dos resíduos de Cys pode ser influenciado pelo microambiente do sítio ativo e ser

estabilizado na forma de tiolato, sendo que o pK_a observado para diferentes Prx varia de 5.1 a 6.3. Sendo assim, estas proteínas mesmo em pH 7.0 apresentam-se ativas na forma C_P-S^- .

Recentemente através de análises estruturais e trabalhos computacionais foi proposto um mecanismo de catálise para as Prx baseado nas características do sítio ativo. Neste mecanismo a Prx reage com o peróxido ($HO_A-O_{B,R}$) através de uma substituição nucleofílica (S_N2), o tiolato ataca o oxigênio terminal (O_A) do hidroperóxido, levando quebra da ligação entre O_A-O_B , e a formação da C_PSOH (Figura 3A). Neste mecanismo também é proposto à participação dos resíduos de Thr e Arg os quais orientam a entrada do hidroperóxido no sítio ativo através de duas ligações de hidrogênio. Após o direcionamento do hidroperóxido no sítio ativo ocorre a estabilização do chamado estado de transição ($C_P - S\gamma^{\ddagger}O_A$), forma-se C_PSOH e ocorre a saída do grupo abandonador ($O_{B,R}$) (Hall *et al.*, 2010; Perkins *et al.*, 2015).

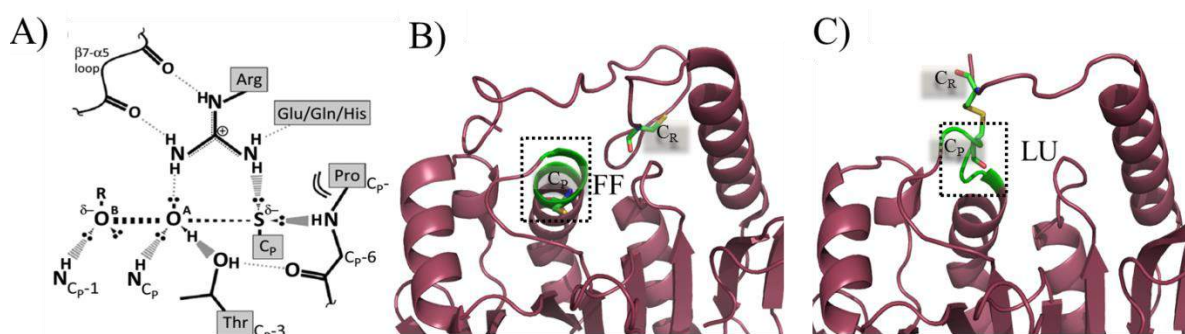


Figura 3. Mecanismo de reação proposto para as 2-Cys Prx e representação das conformações FF e LU. A) Representação em *cartoon* do mecanismo de reação, neste o hidroperóxido é direcionado ao tiolato através de ligações de H com a Thr e Arg, assim ocorre à reação do O_A com o tiolato levando a formação de SOH e liberação do grupo abandonador ($O_{B,R}$) (Compilado de Perkins *et al.* 2015). B) Representação do estado *Folly folded* (FF) da proteína AhpC de *S. typhimurium* na forma reduzida (código PDB: 4MA9). C) Representação do estado *Locally unfolded* (LU) da proteína AhpC de *S. typhimurium* na forma oxidada (código PDB: 1YEP). Nas figuras B e C a região que sofre um desenovelamento parcial é destacada pelo retângulo pontilhado. A C_P e C_R são representadas no modo *sticks* coloridas por CPK (C: verde; O: vermelho, N: azul e S: laranja). As figuras B e C foram geradas através do programa pyMOL.

Após a reação com um hidroperóxido nas 2-Cys Prx ocorre a formação de uma ligação dissulfeto entre a C_P e C_R . Para que ocorra essa ligação é necessário o desenovelamento parcial de uma α -hélice (Figura 3B e 3C). Neste contexto, quando a C_P encontra-se reduzida a α -hélice encontra-se enovelada em uma conformação denominada de *fully folded* (FF), após a reação com um hidroperóxido ocorre o desenovelamento parcial da α -hélice e formação de dissulfeto, essa conformação é denominada *locally unfolded* (LU).

1.3 Redução das Prx: O Sistema Tiorredoxina

Para realizar um novo ciclo catalítico, o dissulfeto das Prx necessita ser reduzido. A Tiorredoxina (Trx) é o substrato da maioria das peroxirredoxinas e, além desta função, as Trx

estão envolvidas em diversos outros processos biológicos como crescimento celular, inibição de apoptose, ativação de transcrição e síntese de DNA. Trx foi inicialmente identificada em *E. coli* por Peter Reichard e colegas, em 1964, como o redutor da ribonucleotídeo redutase a qual é essencial para a síntese de DNA. Posteriormente também foi demonstrado seu envolvimento no ciclo de alguns bacteriófagos e em células eucarióticas é também um doador de elétrons para a ribonucleotídeo redutase como também da 3'-5'-fosfoadenosina fosfato redutase e metionina-sulfóxido redutase. Além de ser um antioxidante capaz de reduzir com baixa eficiência o H_2O_2 , entretanto, seu principal papel antioxidante ocorre por meio da redução das Prx (Pedrajas *et al.*, 2000). Membros da superfamília Trx estão envolvidos em reações tiól-dissulfeto e possuem em seus sítios ativos o motivo C-X-X-C. Neste motivo a cisteína reativa é localizada na região N-terminal e a natureza dos aminoácidos entre as duas cisteínas varia consideravelmente entre os membros da superfamília, influenciando o pK_a das cisteína e, conseqüentemente, as propriedades redox destas proteínas (Pedone *et al.*, 2010). Após a redução das Prx ou outro alvo biológico, as cisteínas de Trx são oxidadas a dissulfeto.

TrxR catalisa a redução do dissulfeto da Trx oxidada utilizando uma molécula de nicotinamida adenina dinucleotídeo fosfato (NADPH) via dois centros redox: um FAD e um motivo contendo duas cisteínas vicinais. Neste contexto, a flavina transfere equivalentes redutores da molécula de NADPH para a ponte dissulfeto de suas cisteínas e destas para as cisteínas oxidadas de Trx (Dai *et al.*, 1996; Waksman *et al.*, 1994) (Figura 4). NADPH, TrxR e Trx compreendem o sistema redutor denominado de sistema tiorredoxina (Holmgren e Lu, 2010).

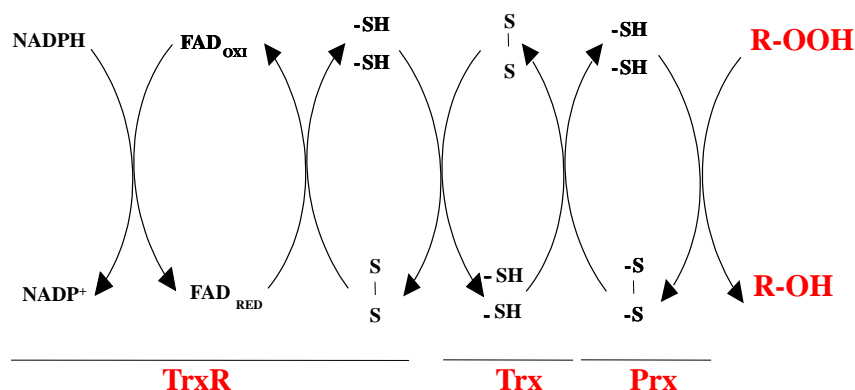


Figura 4. Relação redox das Prx com o sistema redutor tiorredoxina. Prx na forma reduzida reage com um peróxido, liberando H_2O , no caso da redução de H_2O_2 , ou um álcool, no caso de peróxidos orgânicos, tornando-se oxidada. Neste momento é formada uma ligação dissulfeto entre as duas cisteínas de Prx. Prx oxidada é reduzida por Trx que se oxida e, por sua vez, é reduzida pela proteína TrxR (Tiorredoxina redutase) com elétrons provenientes do NADPH.

As Prx possuem o mecanismo catalítico do tipo Bi Bi Ping Pong, desta forma, a enzima possui dois substratos (o hidropéroxido e a tiorredoxina reduzida), produz dois

produtos (H_2O ou um derivado álcool, no caso de peróxidos orgânicos, e a tiorredoxina oxidada) e a liberação de produto ocorre de forma intercalada à entrada de substrato no sítio ativo (Figura 5) (Baker *et al.*, 2001).

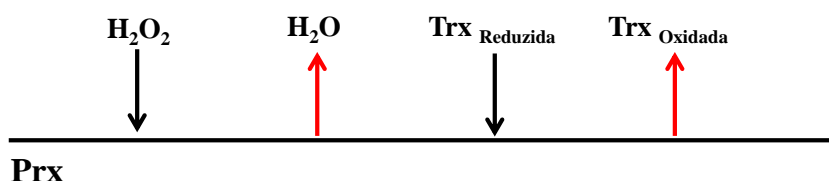


Figura 5. Representação do mecanismo catalítico do tipo Bi Bi Ping Pong das Prx. Prx inicialmente reage com um peróxido, no caso H_2O_2 , liberando água. Posteriormente reage com Trx recuperando sua forma reduzida.

1.4 Formação de complexos supramoleculares de peroxirredoxinas

Foi demonstrado que quando submetidas a choques térmicos ou ao estresse oxidativo as Prx 2-Cys típicas são capazes de se associar em estruturas de alto peso molecular (*High Molecular Weight* - HMW). Inicialmente foi demonstrado que estas espécies possuíam uma atividade de chaperona molecular que ocorre em concerto com a perda de sua atividade peroxidásica (Aran *et al.*, 2011; Jang *et al.*, 2004; Kumsta e Jakob, 2009). Como mencionado anteriormente, as Prx 2-Cys típicas são dímeros obrigatórios; entretanto, quando ocorre a transição de função peroxidásica \rightarrow chaperona, os dímeros ($\sim 40\text{kDa}$) se associam e formam decâmeros ou complexos com peso molecular mais elevado ($>1000\text{ kDa}$) (Aran *et al.*, 2011; Jang *et al.*, 2004; Kumsta e Jakob, 2009; Saccoccia *et al.*, 2012). Vale ressaltar que essa alteração estrutural só foi detectada para as Prx do grupo 2-Cys típicas (Jang *et al.*, 2004) (Jang e col., 2004). A figura 6 ilustra algumas estruturas quaternárias detectadas para as Prx.

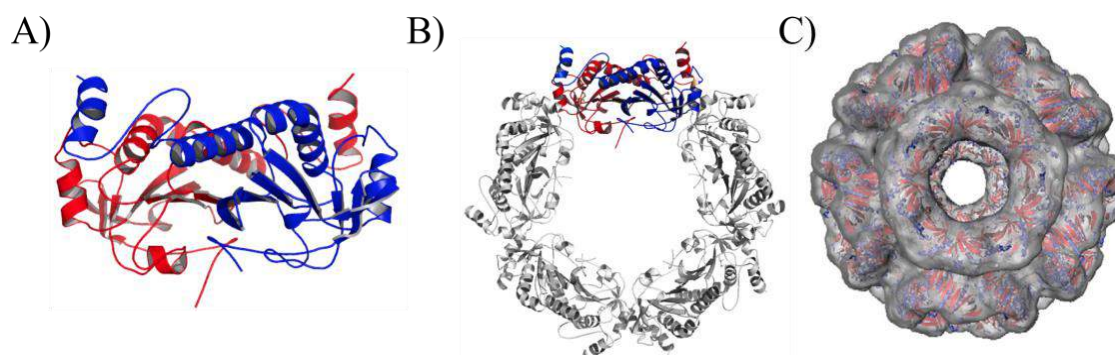


Figura 6. Diferentes estruturas quaternárias apresentadas por Prx 2-Cys típicas. A) Dímero de Tsa1 de levedura. Um monômero é colorizado em azul e o monômero adjacente em vermelho. B) União de cinco dímeros de Tsa1 formando uma estrutura de decâmero onde o dímero superior está colorizado em azul e vermelho e o restante dos dímeros estão representados em cinza. C) União de 12 estruturas decaméricas formando uma estrutura dodecaédrica detectada para Prx2 de humanos. Imagens A e B gerada por PyMOL (<http://www.pymol.org/>) e imagem C compilada de Meissner *et al.*, 2004.

O processo de oligomerização por choque térmico ainda não é bem compreendido, porém, o processo de resposta ao estresse oxidativo é reconhecidamente baseado em

alterações estruturais resultantes da superoxidação de C_P (Jang *et al.*, 2004; Lim *et al.*, 2008). Em situações de alta concentração de H₂O₂, as C_P sofrem superoxidação e são convertidas em cisteína ácido sulfínico (C_P-SO₂H) ou cisteína ácido sulfônico (C_P-SO₃H) que culminam na alteração estrutural das enzimas. No caso de superoxidação, as Prx não podem ser recicladas pelas tioredoxinas (Trx), mas de forma alternativa uma segunda enzima denominada sulfiredoxina (Srx) assume o papel na redução enzimática das Prx, sendo capaz de reduzir somente a forma cisteína ácido sulfínico (C_P-SO₂H) (Biteau, Labarre e Toledano, 2003). Contudo, até o presente momento, não se conhece redutor biológico para a forma C_P-SO₃H, porém dados demonstram que a formação de C_P-SO₃H é facilitada em condições *in vivo*, sugerindo a existência de fatores desconhecidos envolvidos nessa transição (Lim *et al.*, 2008).

Adicionalmente, diversos estudos apontam que a superoxidação das 2-Cys Prx também representa um mecanismo de defesa indireto através da Trx. Uma vez que as 2-Cys Prx estão entre as proteínas mais abundantes das células (Chemical *et al.*, 2003; Wood, Poole e Karplus, 2003) e há transdução de sinal envolvendo o H₂O₂ (Kumsta e Jakob, 2009; Toledano *et al.*, 2005; Vivancos *et al.*, 2006). De fato a redução das Prx implica no acúmulo de Trx na forma oxidada, o que é fundamental em diversos processos de transdução de sinal, os quais não ocorrem quando a Trx se encontra reduzida (Holmgren *et al.*, 2005; Perkins *et al.*, 2015). A oxidação direta da Trx por hidroperóxidos é lenta (Chae, H Z, Chung e Rhee, 1994) (Chae, Chung, Rhee, 1994) e, pelo fato das Prx serem altamente abundantes no ambiente celular, podendo representar 1% das proteínas solúveis na célula (Ghaemmaghami *et al.*, 2003), acredita-se que possuem grande relevância na oxidação das Trx *in vivo*.

Já foi demonstrado que a inativação da 2-Cys Prx de *Schizosaccharomyces pombe* permite que Trx permaneça reduzida e atue em mecanismos de reparo (Day *et al.*, 2012). Neste caso, em condições normais Trx1 se mantém reduzida possuindo como substratos além de Tpx1 (isoforma de Prx análoga a Tsa1 de *S. cerevisiae*) o fator de transcrição Pap1, que é responsável pelo controle da expressão de proteínas que combatem o estresse oxidativo e também é doadora de elétrons da proteína metionina sulfóxido redutase (Mxr1) na redução de resíduos de metionina (sulfóxido de metionina- Met-SOH) em proteínas danificadas por estresse. Em condições moderadas de estresse oxidativo Trx1 reduz preferencialmente Tpx1 promovendo assim uma adaptação da célula à condição de estresse oxidativo. Quando ocorre a exposição a altos níveis de hidroperóxidos, ocorre a superoxidação das C_P de Tpx1, o que permite que Trx1 seja substrato de Mxr1, permitindo o reparo de proteínas oxidadas, vital para a sobrevivência da célula (Day *et al.*, 2012) (Figura 7).

Outro exemplo se trata da ativação do NF- κ B pela Trx1, o qual precisa ter os resíduos de cisteína reduzidos para que ocorra a ligação da subunidade p50 à sequência de DNA alvo (Hayashi, Ueno e Okamoto, 1993; Matthews *et al.*, 1992). Em mamíferos, apenas Trx1 reduzida é capaz de se ligar a MAPKKK Ask1 (*Apoptose signaling kinase*), uma proteína envolvida na sinalização de apoptose, inibindo a sua atividade de quinase, enquanto a oxidação de Trx1 leva à dissociação física do complexo e, conseqüentemente, à ativação de Ask1 (Liu *et al.*, 2000).

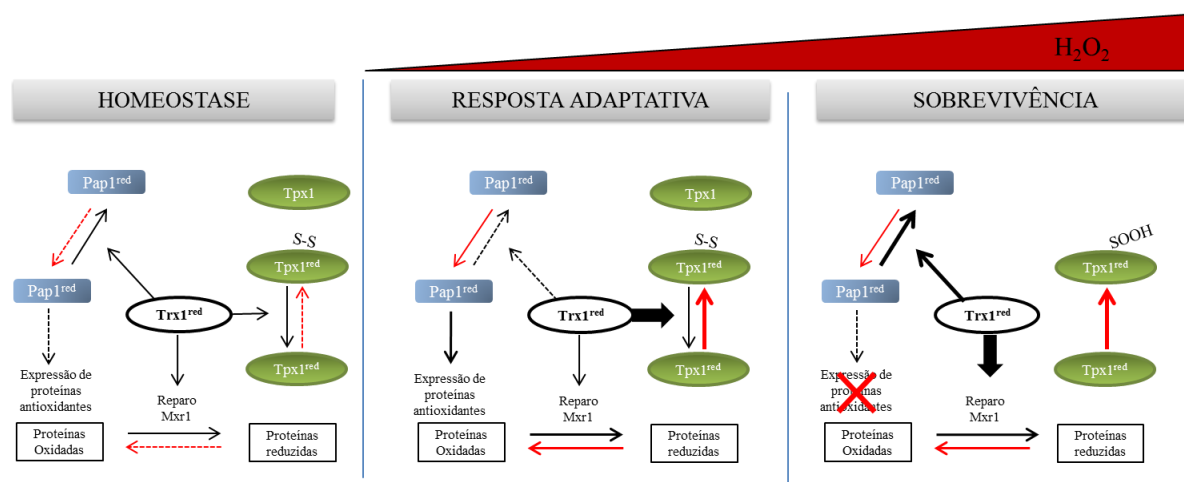


Figura 7: Respostas a H_2O_2 são iniciadas por mudanças no estado redox de Tpx1 e determinam a atividade de Trx1. Em condições homeostáticas normais Trx1 ($Trx1^{red}$) mantém como substrato proteínas que incluem Tpx1, Mxr1, e Pap1, em um estado reduzido. Quando as células são expostas a níveis moderados de peróxido de hidrogênio, Trx1 torna-se mais oxidada devido ao seu envolvimento na remoção catalítica de peróxido de hidrogênio por Tpx1. Assim, a redução mediada Trx1 de outros substratos é inibida. Isto permite a acumulação de Pap1 oxidada. Juntas, essas respostas adaptativas (atividade peroxidásica de Tpx1, expressão do gene e reparação de proteína) podem agir juntas para restaurar a homeostase. Em níveis elevados de peróxido de hidrogênio, há um aumento dos danos oxidativos a proteínas e a redução de Pap1 é inibida e com isso não ocorre à expressão de genes antioxidantes. Nestas condições de estresse oxidativo Tpx1 fica superoxidada permitindo que Trx reduza outros substratos, tais como Mxr1. As setas vermelhas indicam as reações em que o peróxido de hidrogênio participa. A espessura de cada seta dá uma indicação qualitativa das taxas de reação cada condição, setas tracejadas indicam reações mínimas. As formas predominantes Pap1 e Tpx1 no âmbito de cada estado são indicados por contorno em negrito (Adaptado de Day *et al.*, 2012).

Recentemente também foi demonstrada a presença de uma interação física por meio de um dissulfeto transitório entre Prx2 de humanos e STAT3 (*Signal transducer and activator of transcription 3*), proteína que está envolvida na ativação de fatores de crescimento. Neste trabalho foi observado que a oxidação de STAT3 por Prx2 leva à formação de oligômeros de STAT3, os quais possuem atividade transcricional reduzida e, para que a enzima possa voltar a sua atividade máxima, é necessária sua redução pelo sistema tioredoxina. Sendo assim, a superoxidação de Prx2 impede a formação do complexo STAT3-Prx2, impactando de forma direta e significativa as vias dependentes de STAT3 (Sobotta *et al.*, 2015). De forma geral, pode-se dizer que a inativação das Prx por superoxidação pode fazer com que ocorra um acréscimo na concentração local de peróxidos que poderiam reagir com outros alvos, além de

aumentar a quantidade de Trx reduzida, impactando nas vias de sinalização redox-dependentes (Day *et al.*, 2012; Wood, Poole e Karplus, 2003).

É interessante ressaltar que o trabalho pioneiro de Jang e colaboradores (2004) revelam que a superoxidação de Tsa1 e Tsa2, duas isoformas de Prx 2-Cys típicas de *S. cerevisiae*, assim como as enzimas Prx1 e Prx2 (homólogas de humanos) (Moon *et al.*, 2005), necessitam da ação das tiorredoxinas para que ocorra a superoxidação. Apesar de parecer contraditória, a necessidade da Trx ocorre por conta que a formação de um dissulfeto entre C_P e C_R , inviabiliza a superoxidação da cisteína peroxidásica em C_P-SO_2H ou C_P-SO_3H . Desta forma, a ação das Trx se torna imprescindível para que os dissulfetos das Prx sejam reduzidos, possibilitando nova exposição de C_P a hidroperóxidos, permitindo a formação de uma maior quantidade de espécies superoxidadas que podem se associar em estruturas de alto peso molecular (HMW) (Figura 8).

Um ponto importante a ser destacado com relação à superoxidação é o tempo de formação do dissulfeto, reações rápidas de formação de dissulfeto impedem a superoxidação. Já foi demonstrado que Prx3 de humanos é mais resistente a superoxidação que a isoforma Prx2, o que estaria fortemente relacionado com as constantes para formação de dissulfeto de Prx2 ($1.7 s^{-1}$) e Prx3 ($22 s^{-1}$) (Peskin *et al.*, 2013).

As diferenças encontradas nas taxas de formação de dissulfeto podem também estar relacionadas a presença de dois motivos hidrofóbicos (GGLG e YF), presentes em 2-Cys Prx típicas, localizados próximo a região C terminal os quais interagem entre si e interferem na dinâmica da formação de dissulfetos, no caso das diferenças de formação de dissulfeto entre Prx2 e Prx3 essa diferença é relacionada a outros aminoácidos presentes na região C-terminal (Cox, Pearson, Pullar, Jönsson, *et al.*, 2009; Lowther e Haynes, 2011; Hall *et al.*, 2009; Jang *et al.*, 2004).

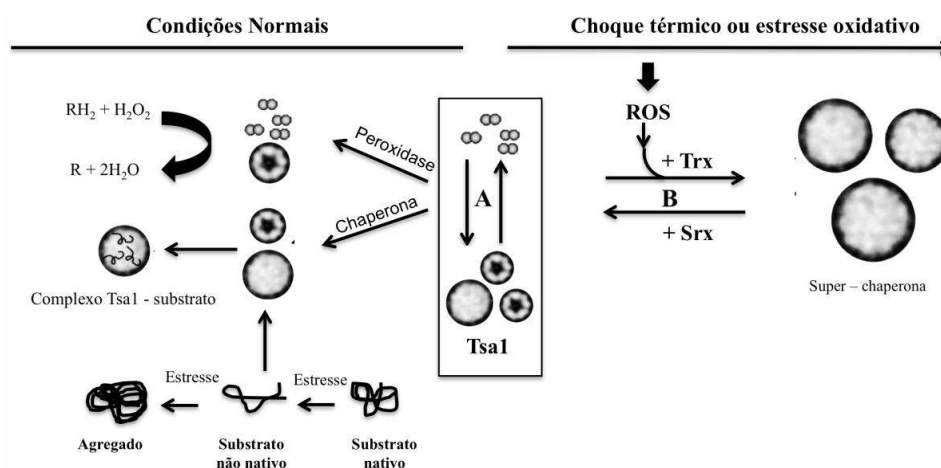


Figura 8. Modelo de formação de complexos HMW de Tsa1. Em baixos níveis de peróxidos hidroperóxidos (A) Tsa1 se apresenta na forma de dímero ou decâmero e atua na decomposição de peróxido. Em condições de

estresse oxidativo ou choque térmico **(B)** são formadas HMW com função de chaperona molecular e que estão envolvidas na sinalização redox. A formação destas estruturas HMW necessita da participação do sistema Trx, e as espécies superoxidadas (C_p-SO_2H) podem ser reduzidas pela enzima sulfiredoxina (Srx) (Adaptado de Jang e *et al.*, 2004).

Saccoccia e colaboradores (2012) demonstraram também a atividade de chaperona molecular em SmPrxI de *Schistosoma mansoni*. Neste trabalho os autores determinaram a estrutura tridimensional de SmPrxI em sua forma decamérica (Figura 9A) com função peroxidásica e também na forma de duplo decâmero ao qual os autores sugerem ser a responsável pela função de chaperona molecular (Figura 9B) (Saccoccia *et al.*, 2012). Neste mesmo trabalho é sugerido através de um modelo teórico que SmPrxI forma estruturas na forma de filamentos formados a partir da sobreposição de decâmeros estes responsáveis pela função de chaperona molecular. Recentemente Radjaina e colaboradores (2015) utilizando crio-microscopia eletrônica resolveram a estrutura da Prx3 humana na forma de filamento (resolução de 7Å), a qual também se acredita como a possível forma na qual a proteína atua como chaperona molecular (Saccoccia *et al.*, 2012) (Figura 9C).

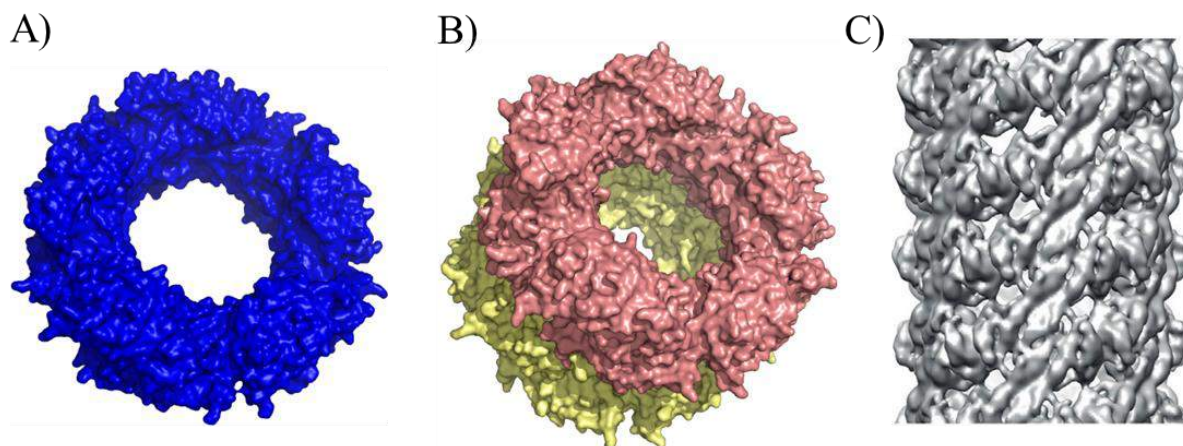


Figura 9: Estruturas de PrxI *Schistosoma mansoni* (SmPrxI) e Prx3 *Homo sapiens* (HsPrx3). (A) Estrutura decamérica de SmPrxI (código PDB 3ZTL) que apresenta atividade peroxidásica. (B) Estrutura de duplo decâmero na forma superoxidada (código PDB 3ZVJ). (C) Estrutura de alto peso molecular de HsPrx3 na forma de filamento, formada a partir da junção de decâmeros, estrutura resolvida por TEM. Imagens A e B geradas utilizando o programa PyMOL (www.pymol.org) e imagem C compilada de Radjaina e colaboradores (2015).

Com relação ao processo de superoxidação ainda é pouco investigado a capacidade de diferentes hidroperóxidos de promover esse processo. Trabalhos já apontaram que peróxidos de origem orgânica como é o caso de *t*-BOOH (*terc*-butil hidroperóxido) e CHP (hidroperóxido de cumeno) possuem uma maior capacidade de promover a superoxidação de Tpx de *E. coli* e Prx4 de rato (Baker e Poole, 2003; Ikeda *et al.*, 2011; Perkins *et al.*, 2013). Constantes cinéticas apontam que a capacidade de superoxidação pode estar relacionada a um

constitutiva (Kim, Kim e Rheet, 1989; Munhoz e Netto, 2004); **iii**) Diferença significativa no pK_a da cisteína envolvida na decomposição de peróxidos. Outro fato que corrobora para a suposição de não redundância entre Tsa1 e Tsa2 reside na diferença de pK_a da C_P destas proteínas. Enquanto que para Tsa1 o pK_a de C_P alcança 5.4, para Tsa2 reside em 6.3 (Ogusucu *et al.*, 2007). Cabe ressaltar que, uma vez que esta escala é logarítmica, isto significa que Tsa1 possuiria uma reatividade sobre hidroperóxidos $\sim 10 \times$ superior a de Tsa2. As razões de diferenças de pK_a entre estas proteínas muito provavelmente estão relacionados com o microambiente do sítio ativo. De forma geral, a alta reatividade da C_P está relacionada com o baixo pK_a de sua forma desprotonada (C_P-S^-), o qual é alcançado pelo ambiente proteico. Os tióis da maioria das proteínas possuem baixa reatividade por estarem protonados em pH fisiológico (Cys-SH), apresentando pK_a de 8.5, o mesmo da cisteína livre. No caso das Prx, a C_P está localizada próxima a um resíduo de arginina altamente conservado, o qual é um dos resíduos responsáveis pela sua desprotonação e estabilização na forma de tiolato (C_P-S^-) (Tairum *et al.*, 2012; Wood, Poole e Karplus, 2003). Desta forma, a diferença de reatividade de C_P entre Tsa1 e Tsa2 pode estar relacionada a aminoácidos constituintes do microambiente do sítio ativo, aspecto que será abordado a seguir.

Recentemente foi determinada por nosso grupo de pesquisa a estrutura cristalográfica de Tsa1^{C47S}, uma proteína carreando substituição da cisteína peroxidásica (C_P47) por uma serina, a qual mimetiza a forma reduzida da proteína (Tairum *et al.*, 2012) (Figura 11A). Uma vez que a forma reconhecida por Trx consiste naquela oxidada em dissulfeto, utilizamos as coordenadas de Prx1 (HBP23) de *Rattus norvegicus*, uma das poucas estruturas de Prx 2-Cys típica de eucariotos oxidada em dissulfeto determinada até o presente momento, para a construção de um modelo teórico de Tsa1 no estado oxidado (Figura 11B). O modelo obtido indica que existem grandes diferenças na topologia da proteína entre as formas oxidada e reduzida, incluindo a exposição na superfície da molécula dos dissulfetos formados entre C_P e C_R , o qual indica a face de interação com Trx.

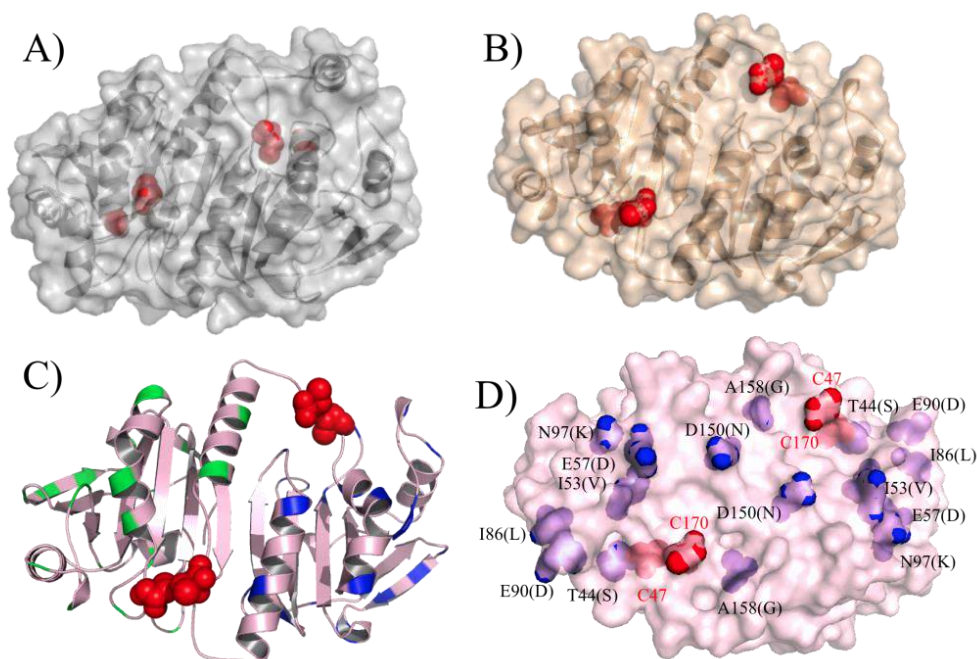


Figura 11. Análises comparativas das estruturas quaternárias de Tsa1 e Tsa2. **A)** Superfície molecular da estrutura cristalográfica de Tsa1 (dímero) de *S. cerevisiae* na forma reduzida (cinza). **B)** Superfície molecular do modelo de Tsa1 de *S. cerevisiae*, gerada utilizando o programa Modeller (salilab.org/modeller/) e as coordenadas da estrutura de *R. novorgicus* (código de pdb: 1QQ2) na forma oxidada (palha). No interior das superfícies moleculares estão representadas as estruturas secundárias em *cartoon* e as cisteínas reativas estão representadas por esferas vermelhas. A transparência das superfícies moleculares foi ajustada para 40% com o intuito de tornar observáveis as representações adicionais das moléculas (*cartoon* e esferas). Em **A** e **B** a orientação das moléculas é a mesma utilizada para a geração das figuras e mudanças estruturais dependentes do estado REDOX são responsáveis pelas diferenças topológicas apresentadas. **C)** Representação em *cartoon* do modelo do dímero de Tsa1 em sua forma oxidada em dissulfeto. A estrutura é representada em rosa e as regiões da estrutura destacadas em verde e azul representam os aminoácidos que diferem entre Tsa1 de Tsa2 nos diferentes monômeros. As representações na forma de esferas em vermelho representam as cisteínas. **D)** Representação da superfície molecular do dímero de Tsa1 (rosa) evidenciando os aminoácidos de superfície (azul) que diferem de Tsa2 na superfície de contato com as Trx. A identificação dos aminoácidos supostamente envolvidos em interações diferenciais com Trx1/Trx2 é dada pelo código de uma letra (preto) e denotam os resíduos em Tsa1. Entre parênteses estão representados os AAs presentes na mesma posição espacial em Tsa2. Os aminoácidos em vermelho representam as cisteínas. Os modelos gráficos foram gerados com o auxílio do programa Pymol (www.pymol.org/).

A estrutura cristalográfica de Tsa1 também foi utilizada como referência para a análise estrutural comparativa com Tsa2. A análise dos aminoácidos diferentes de ambas as enzimas revela que 27 dos 28 aminoácidos distintos estão posicionados na superfície da enzima e alguns deles situados próximos a C_P , reforçando a hipótese de que Tsa1 e Tsa2 possam interagir de modo diferenciado com outras biomoléculas. Adicionalmente, parece de grande relevância a interação de Tsa1 e Tsa2 com tioredoxina, uma vez que esta molécula possui papel fundamental para a transição de dímeros para estruturas HMW. A análise do modelo em dissulfeto aponta que diversos resíduos se encontram na face de interação com Trx (Figura 11C e 11D).

Levando em consideração aspectos estruturais e funcionais, um aminoácido merece destaque: T44(S) (presença de uma treonina em Tsa1 e uma serina em Tsa2. Estes

aminoácidos apresentarem características físico-químicas semelhantes, apresentam-se muito próximos a C_P e podem estar envolvidos nas diferenças no pK_a da cisteína peroxidásica e reatividade sobre substratos. Como já discutido anteriormente, Tsa1 apresenta maior afinidade por H_2O_2 do que por peróxidos orgânicos e Tsa2 não apresenta distinção de afinidade significativa entre os substratos. De fato, substituição equivalente efetuada em uma Prx de *Leishmania donovani* (T→S) resulta em considerável aumento da eficiência na decomposição de peróxido orgânico (Hofmann, Hecht e Flohé, 2002)(McHenry *et al.*, 2011; Sheinerman, Norel e Honig, 2000; Tairum *et al.*, 2012). Levando em consideração a interação de Tsa1 e Tsa2 com seu redutor biológico, a proteína Trx, ressalta-se que a levedura *S. cerevisiae* apresenta duas isoformas citosólicas de Trx, denominadas de Trx1 e Trx2, que apresentam 78% de identidade e 89% de similaridade em relação a suas estruturas primárias e apresentam quantidades de moléculas distintas por célula na fase *log* de crescimento (Trx2, 17200; Trx1, 8580) (Ghaemmaghami *et al.*, 2003). Assim como as Prx Tsa1 e Tsa2 de levedura, as Trx citosólicas de levedura são muitas vezes consideradas proteínas redundantes (Garrido e Grant, 2002). Entretanto, Oliveira e colaboradores (2010) demonstraram que a taxa de redução das Trx por TrxR1 apresentam parâmetros cinéticos levemente diferentes, sendo que Trx2 ($k_{cat}/K_M = 7.3 \times 10^7$) é mais eficientemente reduzida por TrxR1 quando comparada a Trx1 ($k_{cat}/K_M = 3.4 \times 10^7$). Adicionalmente, foi demonstrado que AA presentes na face de interação entre Trx1/Trx2 (face onde estão localizadas as cisteínas envolvidas em processos redox) e TrxR1 de levedura respondem por estas diferenças.

De forma geral, apesar dos numerosos estudos existentes com Prx e Trx de levedura, nenhuma investigação molecular detalhada sobre possíveis diferenças nas taxas de redução de Tsa1 e Tsa2 por Trx1 e Trx2 foi efetuada até o momento. Embora Tsa1 e Tsa2 apresentem somente diferenças sutis em suas estruturas, aspectos como diferenças na abundância e padrões de expressão sugerem papéis fisiológicos complementares e não redundantes. Diferenças no pK_a de C_P destas proteínas também contribuem para esta suposição, uma vez que uma maior afinidade sobre peróxidos poderia levar a uma maior superoxidação, resultando na formação de espécies de massa molecular acentuada com propriedades de chaperona molecular, que podem ter um importante papel na transdução de sinal. Neste contexto, a interação com as Trx é essencial para que em condições de alto estresse oxidativo o dissulfeto de formas diméricas de Prx seja reduzido, possibilitando a superoxidação de C_P e, conseqüentemente, sua organização em espécies superoxidadas de alto peso molecular. Cabe ressaltar que as estruturas tridimensionais de Tsa1, Trx1 e Trx2 já foram determinadas e

existem numerosos estudos envolvendo aspectos bioquímicos e estruturais destas proteínas. No entanto, estudos destas naturezas para Tsa2 são bastante escassos e, até o presente momento, a sua estrutura não foi determinada.

2 OBJETIVOS

Este trabalho teve como objetivos efetuar análises sistemáticas das relações moleculares de Tsa1 e Tsa2 com seus substratos redutores (Trx1 e Trx2) e seus substratos oxidantes (H_2O_2 , CHP e *t*-BOOH) com relação à atividade catalítica, susceptibilidade a superoxidação e formação de estruturas de alto peso molecular. Também foi nosso objetivo avaliar o efeito da substituição recíproca do resíduo da tríade catalítica Thr (Tsa1) e Ser (Tsa2).

3 JUSTIFICATIVA

Peroxirredoxinas 2-Cys típicas possuem alta atividade sobre peróxido de hidrogênio e hidroperóxidos orgânicos. *Saccharomyces cerevisiae* possui duas isoformas de 2-Cys Prx típicas que possuem alta identidade e similaridade e alguns autores as consideram redundantes. Por outro lado, trabalhos já demonstraram que estas proteínas possuem diferença de abundância intracelular e tem níveis de expressão aumentados quando a célula é exposta a altos níveis de hidroperóxidos. Além disso, foi demonstrado que C_P de Tsa1 apresenta um pK_a de 5.4 ao passo que Tsa2 possui um pK_a de 6.3, o que pode influenciar na reatividade destas proteínas. Diferenças nas taxas de redução de hidroperóxidos e redução do dissulfeto pelo sistema Trx podem influenciar consideravelmente o papel de proteínas tais como Tsa1 e Tsa2. Já foi demonstrado na literatura que estas proteínas estão intimamente ligadas a processos de sinalização redox, principalmente quando ocorre a inativação da proteína pela superoxidação. Tendo em vista esse contexto, análises comparativas de reatividade e superoxidação de Tsa1 e Tsa2 apresentam grande importância quando relacionadas à sinalização celular, já que hidroperóxidos muitas vezes funcionam como mediadores de sinalização. Peroxirredoxinas já foram relacionadas com inúmeros tipos de câncer e também ao processo de envelhecimento, o conhecimento aprofundado da sua bioquímica e estrutura pode contribuir significativamente para o desenvolvimento de novas terapias anticâncer e drogas específicas para combater organismos patogênicos.

4 METODOLOGIA

4.1 Reagentes

Os reagentes utilizados ao longo do trabalho como triptona, peptona, extrato de levedura, ágar, NaCl, etanol, isopropanol, ácido sulfúrico, ácido acético, PEG, glicose, H₂O₂, brometo de etídeo, acrilamida, bisacrilamida, persulfato de amônia, NADPH, TEMED dentre outros, foram adquiridos junto a Sigma e Merk. Os demais reagentes como enzimas de restrição, marcadores de peso molecular, agarose, Taq DNA polimerase, T4 DNA ligase e oligonucleotídeos foram fornecidos pela Thermo Fischer e GE Healthcare.

Meios de Cultura para microrganismos

Para bactérias

- **2XYT:** (1.6% triptona; 1% extrato de levedura; 0.5% NaCl, 0.2% NaOH 2M).

Linhagens de Bactérias para Expressão

- **E. coli Tuner (DE3):** *F ompT hsdS_B(r_B⁻m_B⁻)gal dcm lacYI(DE3).*

Plasmídeo de Expressão

- pProEX/*Tsa2* (Munhoz e Netto, 2004; Ogusucu *et al.*, 2007) gentilmente cedido pelo Dr. Luis E.S. Netto (IB,USP, SP).
- pET15b/*Tsa1* (Munhoz e Netto, 2004; Netto, LES *et al.*, 1996; Ogusucu *et al.*, 2007) gentilmente cedido pelo Dr. Luis E.S. Netto (IB,USP, SP).
- pET17/*Trx1*, pET17/*Trx2* (Munhoz e Netto, 2004; Oliveira *et al.*, 2010) gentilmente cedido pelo Dr. Luis E.S. Netto (IB,USP, SP).
- pPROEX/*TrxR1* (Munhoz e Netto, 2004; Oliveira *et al.*, 2010) gentilmente cedido pelo Dr. Luis E.S. Netto (IB,USP, SP).

4.2 Preparação e transformação de bactérias eletrocompetentes

Os procedimentos para a preparação e transformação de bactérias eletrocompetentes foram realizados de acordo Ausubel e col. (1998).

4.3 Mini preparação plasmidial

A metodologia utilizada para extração de todos os plasmídeos neste estudo foi à lise alcalina, descrita Ausubel e colaboradores (1998).

4.4 Mutagênese sítio dirigida

As mutações foram efetuadas utilizando o kit *Quick Change II* (Stratagene), seguindo as orientações do fabricante. A confirmação das mutações foi efetuada por meio de sequenciamento em sequenciador automático *ABI 3730 DNA Analyser* (Thermo Fisher) após reação de sequenciamento utilizando o kit *BigDye® Terminator v3.1 Cycle Sequencing*, seguindo as orientações do fabricante (Thermo Fisher). Os oligonucleotídeos utilizados para as mutações são apresentados na tabela 1 e foram adquiridos junto a *Thermo Fisher*. O mutante Tsa1^{T44S}, Tsa1^{T44A}, Tsa1^{ΔCT} estavam disponíveis, pois são provenientes de outro estudo realizado paralelamente (Resultados em Apêndice A). Para a realização das mutações sítio dirigidas de simples troca de aminoácidos foram utilizados os oligonucleotídeos apresentados na tabela a seguir.

Tabela 1. Oligonucleotídeos utilizados para a produção dos mutantes de Tsa2.

Oligonucleotídeos	Sequencia
Mutagênicos	
Tsa2 ^{S44T} F	5` ATTGGCTTTTACTTTTGTCTGTC 3`
Tsa2 ^{S44T} R	5` GACAGACAAAAGTAAAAGCCAAT 3`
Tsa2 ^{C47S} F	5` TTCATTTGTCAGTCCAACTGAGA 3`
Tsa2 ^{C47S} R	5` TCTCAGTTGGACTGACAAATGAA 3`
Oligonucleotídeos	Sequencia
T7 Forward	5` ATTTAGGTGACACTATAG 3`
T7 Reverse	5` TGCTAGTTATTGCTCAGGCGG 3`
M13F	5` CCCAGTCACGACGTTGTAAAACG 3`
M13R	5` AGCGGATAACAATTCACACAGG 3`

*Os nucleotídeos em vermelho representam os substituídos em relação a sequencia selvagem.

4.5 Expressão de proteínas em linhagens de bactérias

Linhagens de *E. coli* Tuner (DE3) contendo os plasmídeos de expressão foram cultivadas em 2 mL de meio 2XYT/Amp a 37 °C/16 hs/250 rpm em agitador orbital. Em

seguida, toda a cultura foi transferida para 0.5 L de meio 2XYT e cultivada até $OD_{600} = 0.6$. Nesse estágio, foi adicionado IPTG a concentração final de 0.5 mM. A indução foi realizada 30 °C/16 hs/250 rpm em agitador orbital, após esse período o cultivo foi centrifugado a 4 °C/20 min/5000 rpm e as células foram ressuspensas em tampão Tris 10 mM, pH 8.0, e condicionadas em ultrafreezer (-80.°C) até o momento do uso. As células foram rompidas por 3 ciclos de sonicação (30 s sonicando, 60 s descansando) com 35% de amplitude e foram tratadas com sulfato de estreptomicina para a remoção dos ácidos nucleicos ([] final = 1%). Posteriormente ocorreu uma nova centrifugação de 45 min/4 °C/12000 rpm, o precipitado foi descartado e foram coletados somente os extratos celulares livres de ácidos nucleicos. As proteínas contendo cauda de histidina (inseridas pelo plasmídeo de expressão) foram purificadas por cromatografia de afinidade em colunas Hi-Trap 5 mL (GE Healthcare) em gradiente de imidazol.

Para as proteínas Trx1 e Trx2 expressas utilizando plasmídeos pET17b foi realizada purificação através de fervura (*boiling method*), descrito por Porqué e colaboradores (1977). Quando necessário estas proteínas foram submetidas uma segunda etapa de purificação através de cromatografia de exclusão molecular em coluna Hitrap 16/600 Superdex 200 em ÄktaPrime (GE Healthcare).

4.6 Quantificação de proteínas

Após a purificação as proteínas foram submetidas à cromatografia de exclusão molecular para retirada do imidazol utilizado na purificação utilizando a coluna PD10 (GE Healthcare) e foram quantificadas pelo coeficiente de extinção molar, que pode ser acessado no seguinte endereço eletrônico: [http:// www.expasy.org/tools/protparam](http://www.expasy.org/tools/protparam). Tsa1: 24075 M⁻¹.cm⁻¹ (oxidada) e 23950 M⁻¹.cm⁻¹ (reduzida); Tsa1^{T44S}: 24075 M⁻¹.cm⁻¹ (oxidada) e 23950 M⁻¹.cm⁻¹ (reduzida); Tsa2: 27055 M⁻¹.cm⁻¹ (oxidada) e 26930 M⁻¹.cm⁻¹ (reduzida); Tsa2^{S44T}: 27055 M⁻¹.cm⁻¹ (oxidada) e 26930 M⁻¹.cm⁻¹ (reduzida); TrxR1: 27640 M⁻¹.cm⁻¹ (oxidada) e 27390 M⁻¹.cm⁻¹ (reduzida); Trx1 10095 M⁻¹.cm⁻¹ (oxidada) e 9970 M⁻¹.cm⁻¹ (reduzida); Trx2: 11585 M⁻¹.cm⁻¹ (oxidada) e 11460 M⁻¹.cm⁻¹ (reduzida).

4.7 Clivagem das caldas de poli Histidina (His-Tag)

A retirada da His-Tag quando necessária foi realizada utilizando 3C protease. Para tanto as proteínas após a purificação por IMAC, foram submetidas a uma gel filtração

utilizando coluna PD10 (GE Healthcare) e mantidas em tampão Tris 10 mM, pH=8.0. Foram adicionados as amostras 0.01 equivalentes moleres de 3C protease e a reação foi mantida a 4° C durante 16 horas. Após a reação de clivagem foi realizada uma nova etapa de cromatografia de afinidade a metal em colunas Hi-Trap 5mL, para retirada da His-Tag e da 3C protease.

4.8 Determinação das constantes cinéticas

As constantes cinéticas foram determinadas através do ensaio de consumo do NADPH ($\epsilon_{340\text{nm}} = 6200 \text{ M}^{-1} \text{ cm}^{-1}$) pelo sistema tiorredoxina conforme Cha e col. (1994). A reação foi realizada a 30 °C com volume final de 100 μl contendo NADPH a 150 μM , concentrações variáveis de hidroperóxidos, 1 μM de Prx, 2 μM Trx1 ou Trx2 e 0.3 μM de TrxR1, em tampão Hepes 50 mM pH 7.0 contendo 100 μM de DTPA e 1 mM de azida. Todos os reagentes exceto NADPH e o hidroperóxido foram misturados e mantidos a 30 °C por pelo menos 3 minutos para termoestabilização. Após esse período foi adicionado o NADPH e foram iniciadas as medidas de absorbância, após um período de estabilização da medida de 30 segundos foi adicionado o hidroperóxido. O tempo de medida total foi de 3 minutos sendo que a absorbância foi medida a cada 2 segundos. A determinação das constantes cinéticas foi determinada através da equação de cinética do estágio estacionário variando-se somente a concentração do hidroperóxido ou a concentração de Trx, para realização dos cálculos e construção dos gráficos foi utilizado o programa GraphPad Prisma.

4.9 Análise de inativação de Tsa1 e Tsa2 pelo substrato

A análise de inativação pelo substrato oxidante de Tsa1 e Tsa2 foi realizada utilizando ensaio de consumo do NADPH pelo sistema tiorredoxina. Foi monitorado o decaimento da absorbância ($\epsilon_{340\text{nm}} = 6200 \text{ M}^{-1} \text{ cm}^{-1}$) conforme descrito Chae e colegas (1994). A reação foi realizada a 30 °C com volume final de 100 μl contendo NADPH a 150 μM , concentrações variáveis de hidroperóxidos, 1 μM de Tsa1 ou Tsa2, 2 μM Trx 1 ou Trx2 e 0.3 μM de TrxR1, em tampão Hepes 50 mM pH 7.0 contendo 100 μM de DTPA e 1 mM de azida. O experimento foi realizado conforme descrito acima no item 4.8, somente diferindo pelo tempo de leitura que foi de 6 minutos. A construção dos gráficos foi realizada utilizando o programa GraphPad Prisma.

4.10 Análise de inativação por superoxidação utilizando SDS-PAGE em condições não redutoras

A análise de inativação por superoxidação foi realizada utilizando o sistema tiorredoxina. A reação foi realizada a 30 °C por 15 minutos em volume final de 50 µl contendo NADPH a 150 µM, concentrações variáveis de hidroperóxidos (0.01 – 10 mM), 9.3 µM de Prx, 1 µM Trx1 ou Trx2 e 0.3 µM de TrxR1, em tampão Hepes 50 mM pH 7.0 contendo 100 µM de DTPA e 1 mM de azida. Após, decorrido o tempo de reação, foi adicionado 100 mM de NEM a reação para promover a alquilação das cisteínas livres e evitar artefatos na detecção em gel. Para verificar o aparecimento de formas superoxidadas as amostras foram submetidas à SDS-PAGE sem a presença de redutores e a relação monômero/dímero foi analisada.

4.11 Obtenção de Tsa1 superoxidada

Para a obtenção da proteína Tsa1 superoxidada foi realizado um ensaio utilizando sistema Trx, contendo TrxR1 sem His-Tag. A reação foi realizada a 30 °C com volume final de 15 mL contendo NADPH a 150 µM, 10 mM de CHP, 9.3 µM de Prx, 1 µM Trx1 e 0.3 µM de TrxR1, em tampão Hepes 50 mM pH 7.0, 100 µM de DTPA e 1 mM de azida. Após decorrido 30 minutos de reação Tsa1 superoxidada foi purificadas por cromatografia de afinidade a metal em colunas Hi-Trap 1mL (GE Healthcare) em gradiente de imidazol. Como somente Tsa1 possuía His-Tag foi possível separa-la das outras proteínas e componentes da reação. Após a purificação a proteína foi submetida à cromatografia de exclusão molecular para retirada do imidazol utilizado na purificação e foi quantificada pelo coeficiente de extinção molar.

4.12 Determinação da taxa de superoxidação utilizando fluorescência intrínseca do triptofano

Tsa1 e Tsa2 foram reduzidos por 20 mM de DTT por 1 hora, o qual foi depois removido por dois procedimentos de gel filtração utilizando colunas PD10 (GE Healthcare). A eficiência da redução foi avaliada com o auxílio de DTNB acompanhando a formação de TNB ($\lambda=412$ nm) em tampão denaturante, e foi calculada a quantidade de cisteínas livres, sendo considerados aceitáveis valores acima de 90%. As enzimas em tampão 40 mM fosfato de sódio (pH 7.4) foram rapidamente misturadas com concentrações crescentes de H₂O₂ ou CHP a 30 °C em espectrofluorímetro *Applied*

Photophysics SX 17MV acoplado a *stopped flow* com tempo de mistura menor que 2 ms. Os comprimentos de onda utilizados foram 280 nm para excitação e 340 nm para emissão. A constante de superoxidação de Tsa1 por H₂O₂ e CHP calculada através do retorno da fluorescência intrínseca do triptofano após a reação com o hidroperóxido.

4.13 Cristalização de proteínas

Após a purificação de Tsa2 e do mutante Tsa2^{C47S} foram realizados ensaios iniciais de crescimento de cristais utilizando a infraestrutura do Laboratório Nacional de Luz Síncrotron (Campinas/Brasil) e do Centro de Investigaciones Biológicas (Madrid/Espanha). Foram realizadas triagens utilizando os kits Crystal Screen 1 e 2 (Hampton Research-96 condições), Matrix Maker (Emerald Biosystems) e Honeybee 963 (Genome Solutions), PACT, JCSG (Nextal/Qiagen-96 condições cada), SaltRx (Hampton Research-96 condições) e Precipitant Synergy (64 condições) – Emerald BioSystems. Foram realizados experimentos variando-se a concentração de proteínas (5, 7, 10 mg/mL) em tampão Tris-HCl 5 mM previamente tratadas com DTT (10 mM) ou H₂O₂ (1 mM). Condições iniciais de cristalização foram refinadas quanto a precipitantes, sais e pH. Além disso, foram abordados também procedimentos de sementeira de cristal e triagem de aditivos e detergentes.

4.14 Cromatografia de exclusão molecular (SEC)

O estado de oligomérico das proteínas e formação de HMW foi analisado por HPLC analítico utilizando sistema Jasco série vp. equipado com bomba *PU 2880 Plus*, forno de coluna CO 2065, detector *PDA MD 2018* e detectores de fluorescência FP 2020 Plus. Foram utilizadas colunas *Phenomenex BioSep-SEC-S3000* (7,8 × 300 mm, 5 cm, faixa de separação 5 a 700 kDa), equilibradas com Tris-Cl 100 mM pH 7.4 contendo 100 mM de NaCl. A eluição dos componentes foi monitorada por UV ($\lambda = 280$ nm) e por fluorescência ($\lambda_{\text{ex}}=280$ e $\lambda_{\text{em}}=330$ nm). Os padrões de massa molecular a serem usados foram: tiroglobulina (bovina) (670 kDa), γ -globulina (bovina) (158 kDa), ovalbumina (frango) (44 kDa), mioglobina (cavalo) (17 kDa) e Vitamina B₁₂ (14.2 kDa).

4.15 Espalhamento dinâmico de luz (DLS)

As medidas de espalhamento dinâmico de luz (DLS) de proteínas envolvidas neste projeto foram realizadas no Laboratório de Genética e Biologia Molecular, Antioxidantes e

Radicais Livres (IB-USP) utilizando o equipamento *DYNAPRO* (Protein Solutions). Para análises da estrutura das proteínas foram realizados ensaios com as proteínas reduzidas (5 mM de TCEP) ou oxidadas (1.2 equivalentes de H_2O_2). Para análise da formação de complexos HMW amostras de proteínas foram submetidas a ensaios de oxidação do NADPH utilizando altas concentrações de hidroperóxidos. A reação foi realizada a 4 °C *overnight* em volume final de 100 μ L contendo NADPH a 600 μ M, hidroperóxido (10 mM), 1 mg/mL (42.6 μ M) de Prx, 1 μ M Trx1 e 0.3 μ M de TrxR1, em tampão Hepes 50 mM pH 7.0 contendo 100 μ M de DTPA e 1 mM de azida. Antes dos experimentos todas as amostras foram centrifugadas a 14.000 g por 15 minutos para a decantação de proteínas desnaturadas e filtradas em microfiltros Millex[®] 0.22 μ m (Millipore). As medidas foram realizadas em volume final amostra de 20 μ L com intensidade do laser ajustada para 100% (880 nm). Para cada amostra foram efetuadas 200 leituras (10 subleituras/leitura) a 20°C e foi utilizado o modelo de esferas para o cálculo do raio de giro.

4.16 Avaliação da estrutura em meio líquido pelo método de espalhamento de luz em baixo ângulo (SAXS)

Amostras em tampão 10 mM Tris-HCl pH 8 foram centrifugadas por 10 min/ 14.000 rpm/ 4 °C e em seguida, filtradas para remoção de agregados. Os dados de SAXS foram coletados na linha de luz Linha B21 do *Diamond Light Source* (Oxfordshire, Inglaterra). O λ utilizado foi de 1.48 Å e a distância inicial do detector *Pilatus* (Dectris) utilizado para registrar os padrões de dispersão foi de 165 milímetros. Linhas de base tampão foram coletadas em condições idênticas para garantir uma correção do solvente precisa. O cálculo da P_r e D_{max} foi realizado utilizando o programa PRIMUSqt (Konarev *et al.*, 2003), modelos foram gerados utilizando o programa DAMMIF (Franke e Svergun, 2009) e as coordenadas cristalográficas foram sobrepostas utilizando o programa Supcomb (Kozin e Svergun, 2001a).

4.17 Análise de formação de estruturas de alto peso molecular (HMW) de Tsa1 e Tsa2 por microscopia eletrônica de transmissão (TEM)

Análise da formação de complexos HMW foi por realizada por TEM utilizando amostras de Prx após a reação com sistema Trx utilizando CHP como substrato, metodologia apresentada no item 4.10. Amostras foram centrifugadas por 30 min/14000 rpm/4 °C e filtradas microfiltros Millex[®] 0.22 μ m (Millipore), diluídas em tampão Hepes 50 mM pH 7.4

quando necessário. Grades de cobre Ultrathin Carbon Film / Holey Carbon 400 mesh/ (TE Pella) foram carregadas negativamente a 15 mA por 25 seg em aparelho Easy Glow (Ted Pella). Para obtenção de imagens de boa qualidade as amostras foram diluídas quando necessário. Um volume de 3 μ L de amostra foi aplicado sobre a grade, após 3 min o excesso de líquido foi removido com auxílio de papel filtro. Para a retirada do sal presente da amostra foram adicionados 3 μ l de água sobre a grade sendo esta seca com papel filtro, este processo foi repetido por 3 vezes. Posteriormente foram aplicados 3 μ L de acetato de uranila 2%, após 30 seg seu excesso foi removido com papel filtro, este processo foi realizado 2 vezes. A grade então foi seca a temperatura ambiente e acondicionada a temperatura ambiente. As imagens de TEM foram adquiridas 1-3 μ M de foco, 15-18000 \times de magnificação utilizando microscópio eletrônico de transmissão Jeol JEM-2100 operado a 200 kV e equipado com câmera F-416 CMOS (Tietz Video and Image Processing Systems).

5 RESULTADOS

Para facilitar o entendimento desta tese os resultados serão apresentados em cinco capítulos. No **Capítulo 1** são demonstrados dados referentes à reatividade de Tsa1 e Tsa2 sobre seus substratos oxidantes H_2O_2 , *t*-BOOH e CHP e sobre seus substratos redutores Trx1 e Trx2. O **Capítulo 2** demonstrara resultados referentes à superoxidação da C_P de Tsa1 e Tsa2, influenciada por diferentes hidroperóxidos e são caracterizados mutantes de aminoácidos que influenciam a resposta diferencial destas proteínas a este processo. No **Capítulo 3** são demonstrados resultados referentes a influencia da superoxidação na formação de estruturas de alto peso molecular de Tsa1 e Tsa2. O **Capítulo 4** demonstra resultados de cristalização de Tsa2 e um modelo estrutural obtido através de espalhamento de raio X de baixo ângulo (SAXS) . O **Capítulo 5** resume o artigo apresentado no Apêndice A onde é demonstrada a importância da Th44 na atividade e manutenção da estrutura de Tsa1 e também algumas abordagens adicionais referentes ao mecanismo de catálise das Prx.

5.1 CAPÍTULO 1 - Reatividade e superoxidação de Tsa1 e Tsa2

5.1.1 Determinação de constantes cinéticas de Tsa1 e Tsa2 sobre substratos oxidantes

As proteínas Tsa1 e Tsa2 possuem alta reatividade sobre H_2O_2 e peróxidos orgânicos e podem ser eficientemente reduzidas por Trx1 e Trx2 (Ogusucu *et al.*, 2007; Watzlawik *et al.*, 2006). Com o objetivo de verificar diferenças nas taxas de redução de Tsa1 e Tsa2 sobre hidroperóxidos foi utilizado o ensaio de NADPH. Os parâmetros cinéticos foram calculados utilizando a equação de estado estacionário, variando somente a concentração do hidroperóxido e mantendo a concentração de Trx fixa ou variando-se a concentração de Trx e mantendo fixa a concentração de hidroperóxido. Na determinação das constantes cinéticas de redução de hidroperóxidos foram utilizadas concentrações saturantes de Trx (2 μ M) de modo que o passo de redução da Prx não fosse um limitante para a reação. Nos ensaios onde foi variado o substrato redutor (Trx) foi utilizada uma concentração saturante de H_2O_2 de modo que o passo oxidação da Prx também não fosse um limitante da reação.

As taxas de redução de Tsa1 e Tsa2 sobre seus substratos oxidantes foram determinadas utilizando concentrações crescentes de H_2O_2 , CHP e *t*-BOOH e concentração fixa de Trx1 e de Trx2. Nestes experimentos foram utilizadas concentrações saturantes de Trx (2 μ M), concentração na qual o passo de redução da Prx não é limitante.

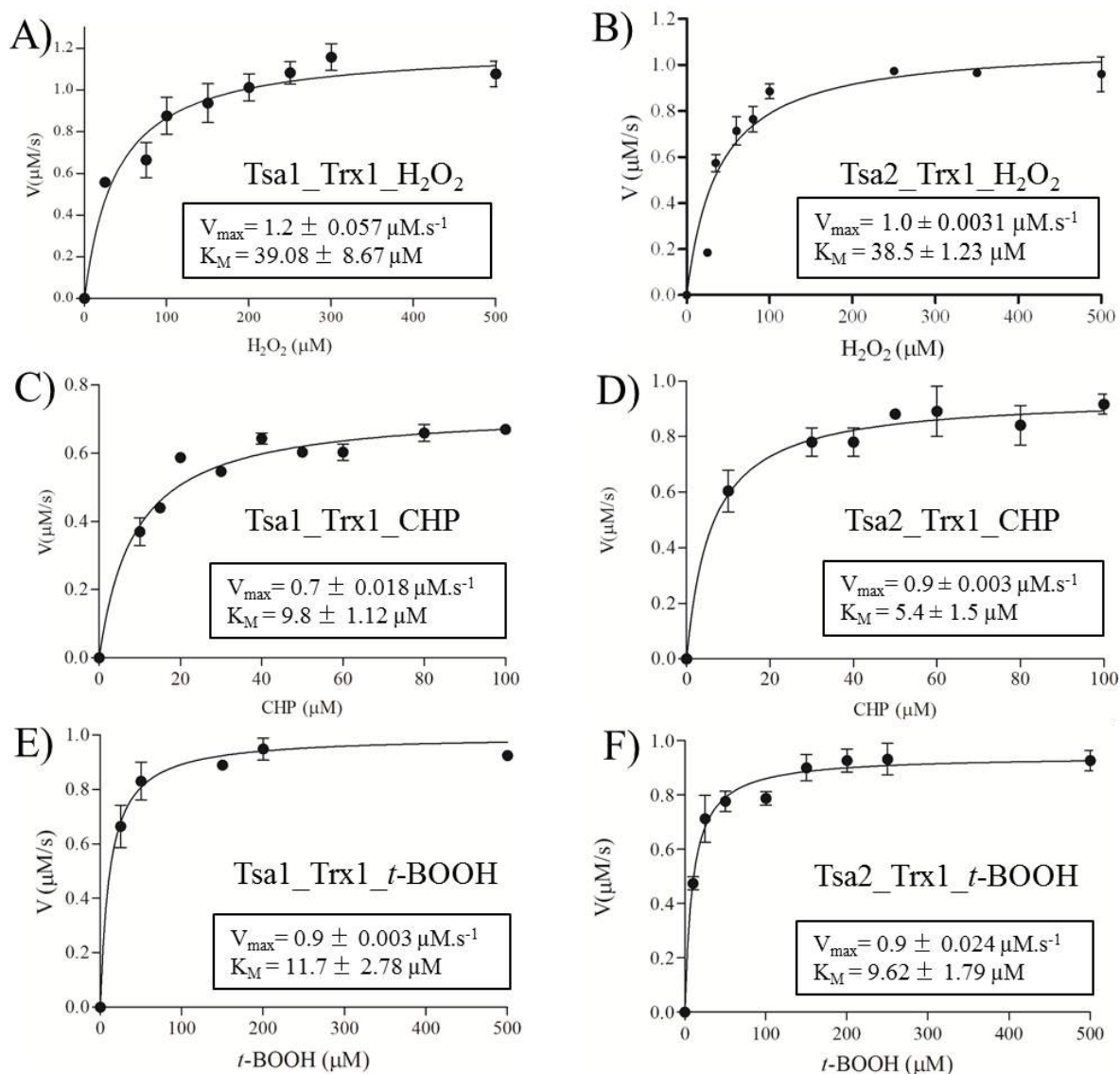


Figura 12. Efeito da concentração de hidroperóxidos na atividade peroxidásica de Tsa1 e Tsa2 ligada a oxidação de NADPH via Trx1 e TrxR1. A oxidação do NADPH foi monitorada a 30 °C pela diminuição da absorbância a 340 nm. A reação (100 μl) foi realizada em tampão 50 mM HEPES-NaOH pH 7.4 contendo 1 mM de azida, 100 μM de DTPA na presença de 150 μM de NADPH, 1 μM de Tsa1 ou Tsa2, 2 μM de Trx1, 0.3 μM de TrxR1 e diferentes concentrações de hidroperóxidos. A reação foi iniciada pela adição do hidroperóxido. As curvas representam a velocidade de oxidação do NADPH em diferentes concentrações de H₂O₂, CHP e t-BOOH.

Utilizando Trx1 como substrato redutor foram obtidos parâmetros cinéticos semelhantes entre Tsa1 e Tsa2 quando o substrato utilizado foi o H₂O₂ e uma variação maior foi verificada quando foi utilizado como substrato CHP e t-BOOH (Figura 12). Os valores de V_{max} de Tsa1 foram $1.2 \mu\text{M}\cdot\text{s}^{-1}$ para H₂O₂, $0.7 \mu\text{M}\cdot\text{s}^{-1}$ para CHP e $0.9 \mu\text{M}\cdot\text{s}^{-1}$ para t-BOOH, os valores de K_M foram de $39.08 \mu\text{M}$ para H₂O₂, $9.8 \mu\text{M}$ para CHP e $11.7 \mu\text{M}$ para t-BOOH. Em conjunto os resultados indicam uma maior afinidade de Tsa1 por peróxidos orgânicos, resultado similar foi observado para Tsa2. Para hidroperóxidos orgânicos foi observada uma diminuição significativa do K_M tanto para CHP ($5.4 \mu\text{M}$) quanto para t-BOOH ($9.62 \mu\text{M}$)

quando comparado com H_2O_2 (38.5 μM) (Figura 12, Tabela 2). Entretanto, enquanto a eficiência catalítica para todas as condições testadas foi na ordem de $\sim 10^4 \text{ M}^{-1}\text{s}^{-1}$, chama a atenção que para Tsa2 quando foi utilizado CHP foi determinado $\sim 10^5 \text{ M}^{-1}\text{s}^{-1}$, indicando que este é um substrato oxidante melhor para Tsa2. Quando o substrato redutor foi Trx2 assim como o observado para Trx1, apenas diferenças sutis foram verificadas nas taxas de redução (Figura 13). Novamente, a eficiência se mostrou mais acentuada para CHP (Figura 12, Tabela 2).

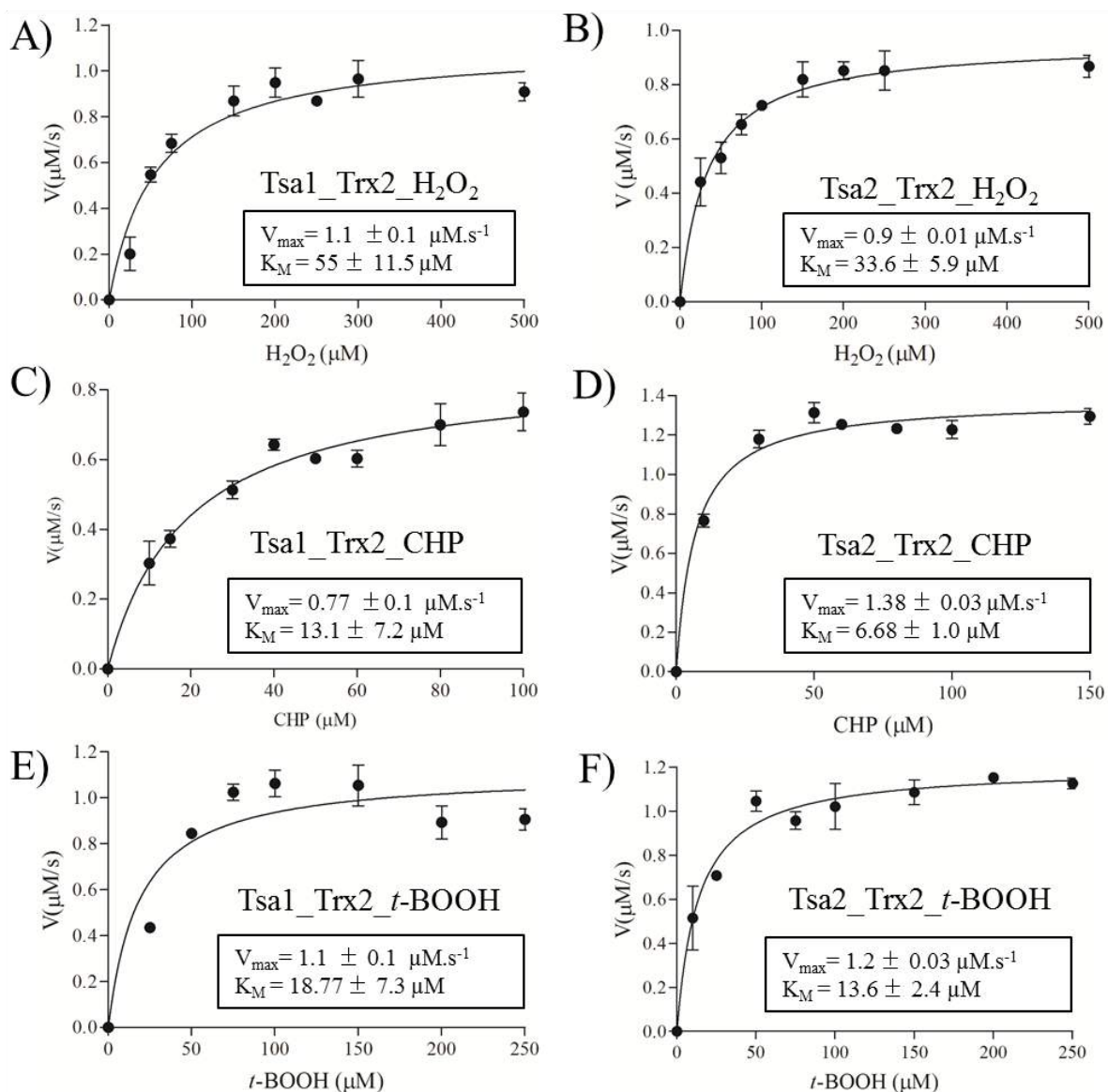


Figura 13. Efeito da concentração de diferentes hidroperóxidos na atividade peroxidásica de Tsa1 e Tsa2 utilizando Trx2. A oxidação do NADPH foi monitorada a 30 °C pela diminuição da absorbância a 340 nm. A reação (100 μl) foi realizada em tampão 50 mM HEPES-NaOH pH 7.4 contendo 1 mM de azida, 100 μM de DTPA na presença de 150 μM de NADPH, 1 μM de Tsa1 ou Tsa2, 2 μM de Trx2, 0.3 μM de TrxR1 e diferentes concentrações de hidroperóxidos. A reação foi iniciada pela adição do hidroperóxido. As curvas representam a velocidade de oxidação do NADPH em diferentes concentrações de H_2O_2 , CHP e *t*-BOOH.

Cabe ressaltar que as constantes de Tsa1 e Tsa2 para decomposição de H₂O₂ ou peróxidos orgânicos obtidas são inferiores as determinadas metodologias que não envolvem reações intermediárias de troca de elétrons entre as proteínas do sistema Trx envolvendo diversas reações de oxido redução (Figura 4). De fato, através de ensaio de competição com HRP foram determinadas constantes na ordem $\sim 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Ogusucu *et al.*, 2007; Tairum *et al.*, 2012; Toledo *et al.*, 2011), similar ao determinado para outras Prx. As constantes de redução de Trx1 e Trx2 por TrxR1 revelaram constantes de $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Oliveira *et al.*, 2010).

Estes resultados sugerem reações intermediárias lentas, seja a formação de dissulfeto de Tsa1/Tsa2 ou sua redução por Trx. Estas etapas devem limitar as constantes de decomposição de hidroperóxido no ensaio acoplado utilizando o sistema Trx. Corroborando para esta hipótese, demonstramos por meio de ensaios fluorométricos que a constante de pseudo-primeira ordem de Tsa1 e Tsa2 para redução de H₂O₂ e CHP reside na ordem de $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Apêndice A- Manuscrito em fase final de elaboração que será submetido ao periódico *Nature chemical biology*). Para avaliar as taxas de redução de Tsa1 e Tsa2 foram empregadas análises cinéticas que serão apresentadas nas próximas seções.

5.1.2 Determinação de constantes cinéticas de Tsa1 e Tsa2 utilizando Trx1 ou Trx2

Para determinar as constantes de reação de Tsa1 e Tsa2 com Trx1 e Trx2 foram realizados experimentos variando-se a concentração de Trx e utilizando H₂O₂ como substrato oxidante em altas concentrações (500 μM) de forma que a reação da Prx com o hidroperóxido não se apresenta como um passo limitante da reação (Figura 14). Foram realizados experimentos somente com H₂O₂, pois a utilização em altas concentrações de CHP e *t*-BOOH causa inibição da reação, tema este que será abordado no próximo capítulo.

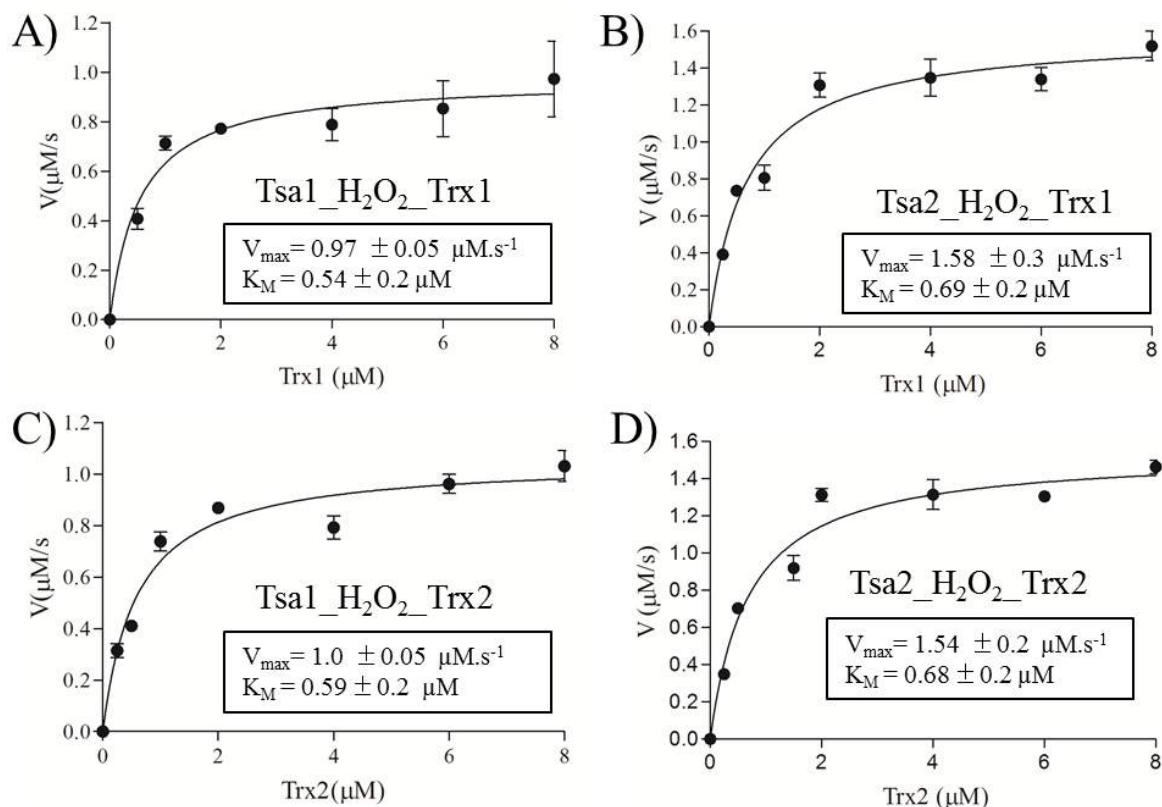


Figura 14. Efeito da concentração de tioredoxina na atividade peroxidásica de Tsa1 e Tsa2 ligada a oxidação de NADPH via H_2O_2 e TrxR1. A oxidação do NADPH foi monitorada a 30 °C pela diminuição da absorbância a 340 nm. A reação (100 μl) foi realizada em tampão 50 mM Hepes-NaOH pH 7.4 contendo 1 mM de azida, 100 μl de DTPA na presença de 150 μM de NADPH, 1 μM de Tsa1 ou Tsa2, 0.3 μM de TrxR1, 500 μM de H_2O_2 e diferentes concentrações de tioredoxina. A reação foi iniciada pela adição do hidroperóxido. As curvas representam a velocidade de oxidação do NADPH em diferentes concentrações de Trx1 e Trx2.

Experimentos para determinação das constantes de Tsa1 e Tsa2 com seus substratos redutores Trx1 e Trx2 revelaram resultados contrastantes. Enquanto que para Tsa1 ambas as Trx apresentam resultados muito semelhantes ($V_{\max_Trx1} = 0.97$ e $V_{\max_Trx2} = 1.0$ $\mu\text{M}/\text{s}$; $K_{M_Trx1} = 0.54$ e $K_{M_Trx2} = 0.59$) Tsa2 apresentou parâmetros superiores ($V_{\max_Trx1} = 1.58$ e $V_{\max_Trx2} = 1.54$ $\mu\text{M}/\text{s}$; $K_{M_Trx1} = 0.69$ e $K_{M_Trx2} = 0.68$ $\mu\text{M}/\text{s}$). Neste contexto enquanto a eficiência catalítica para Tsa1 foi de $\sim 6 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ utilizando Trx1 e Trx2, para Tsa2 as constantes foram de $\sim 2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, ou seja uma diferença significativa nas taxas de redução. Os resultados podem sugerir duas possibilidades não excluídas: 1) Uma menor eficiência de redução de Tsa1 por Trx1 ou Trx2 ou 2) uma vez que as enzimas são passíveis de superoxidação de C_P estes resultados poderiam indicar que C_P de Tsa1 é mais suscetível a superoxidação o que mascararia as taxas de redução em razão de que uma parcela das proteínas presentes na reação seriam superoxidadas a cada ciclo de redução. Os resultados dos experimentos de cinética com sistema Trx são apresentados de maneira comparativa na tabela 2.

5.1.3 Determinação das constantes de redução de Tsa1 e Tsa2 por Trx1 utilizando fluorescência intrínseca do triptofano

Visando aprofundar no entendimento das relações de Tsa1/Tsa2 e Trx1/Trx2 foram realizadas abordagens da redução acompanhando alterações da fluorescência intrínseca de resíduos de Trp. Este trabalho contou com a participação do aluno Felipe Zurlo (Ciências Biológicas – Unesp/CLP), sendo tema do seu trabalho de conclusão de curso.

Inicialmente, foram realizadas varreduras de fluorescência para se verificar alterações de fluorescência entre os estados reduzidos e oxidados de Tsa1/Tsa2 e Trx1/Trx2. Os resultados revelaram que Tsa1 e Tsa2 possuem diferença significativa na emissão de fluorescência do Trp no estado reduzido e oxidado (Figuras 15A e 15B). Por outro lado a avaliação da fluorescência de Trx1 revelou que apesar de possuir um resíduo de Trp (resíduo 29) próximo aos resíduos de Cys do sítio ativo (30 e 33) não foi detectada diferenças no perfil de fluorescência da proteína (Figura 15C). No caso de Trx2 foi possível detectar que existe um pequeno aumento da fluorescência quando a enzima esta no estado reduzido, no entanto a diferença é ínfima e pode ser desconsiderada (Figura 15D).

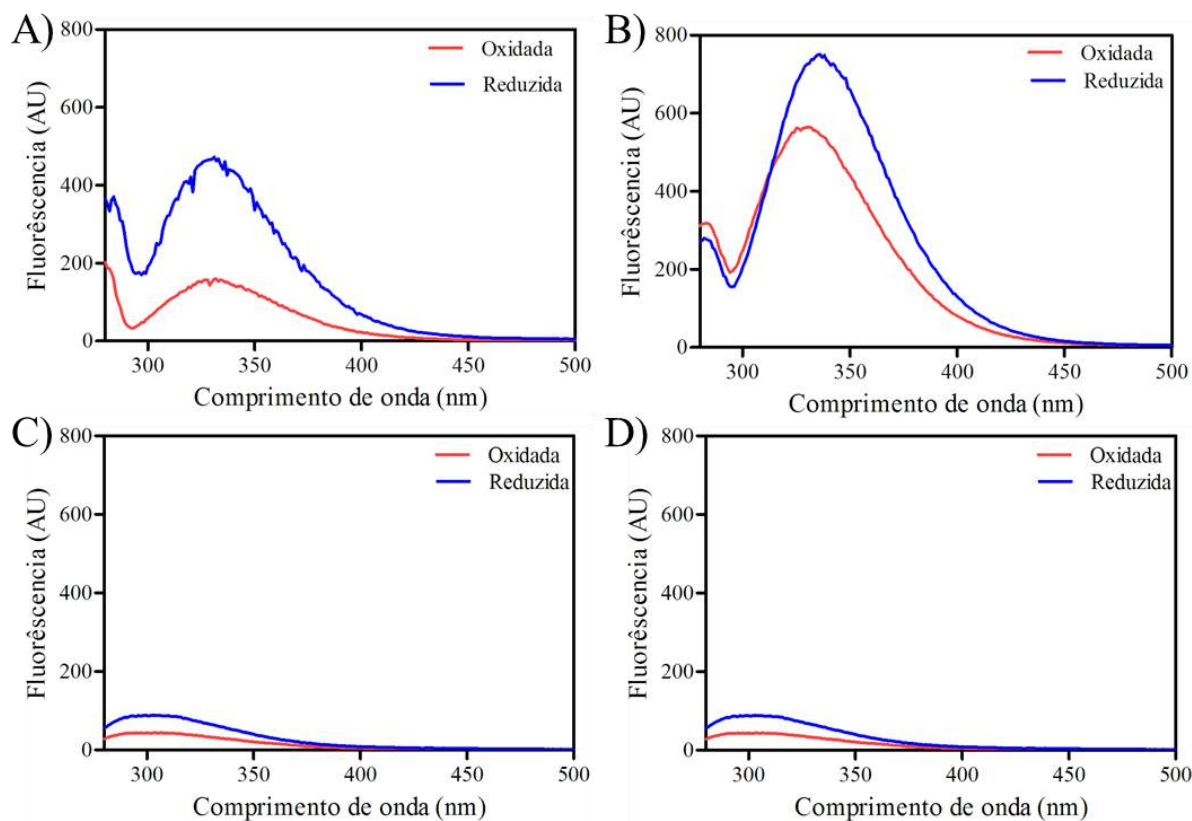


Figura 15. Diferença da fluorescência de Tsa1, Tsa2, Trx1 e Trx2. Amostras das proteínas Tsa1 (A), Tsa2 (B), Trx1 (C) e Trx2 (D) foram previamente reduzidas com DTT (10 mM) ou oxidadas (1.2 equivalentes de H_2O_2) e a emissão de fluorescência foi monitorada (280 a 500 nm) após excitação a 280 nm. Linha vermelha representa a proteína reduzida e a linha azul oxidada.

Apesar de ser curiosa a fluorescência ínfima ou inexistente das Trx de levedura, este fenômeno já é conhecido para outras proteínas e pode ser explicado por fatores como ligações específicas, solvente e fatores ambientais (Cowgill e Lang, 1970). Neste contexto, os resultados revelam que é possível determinar as taxas de redução de Tsa1 e Tsa2 acompanhando o aumento da fluorescência no processo de redução por Trx1/Trx2.

Os experimentos para estimar as taxas de redução de Tsa1 e Tsa2 pela Trx1 foram realizados no laboratório de Bioquímica e Radicais Livres coordenado pela Profa. Dra. Ohara Augusto (IQ/USP) utilizando espectrofluorímetro com *stopped-flow*. Para a determinação das taxas de redução, Tsa1 e Tsa2 foram previamente oxidadas com 1.2 equivalentes de H_2O_2 e foram misturadas a Trx1 previamente reduzida com 20 mM de DTT e o aumento da fluorescência foi monitorado (excitação a 280nm e emissão 340nm) (Figura 16). Para a determinação das constantes de pseudo-primeira ordem foi utilizado uma concentração molar $8 \times$ maior de Trx1 para Tsa1 e Tsa2 ($8 : 1 \mu M$). Através dos experimentos a constante pseudo-primeira ordem da redução de Tsa1 por Trx1 foi determinada como $1.8 \times 10^6 M^{-1}s^{-1}$ e a constante de redução de Tsa2 por Trx1 como $4.0 \times 10^6 M^{-1}s^{-1}$. Os dados obtidos nestes ensaios apresentam-se semelhantes aos obtidos utilizando o ensaio de NADPH, onde se obteve uma constante de redução por Trx1 de $5.67 \times 10^5 M^{-1}s^{-1}$ para Tsa1 e $2.2 \times 10^6 M^{-1}s^{-1}$ para Tsa2. Neste contexto os dados revelam que a redução de Tsa1 e Tsa2 ocorre de forma extremamente eficiente.

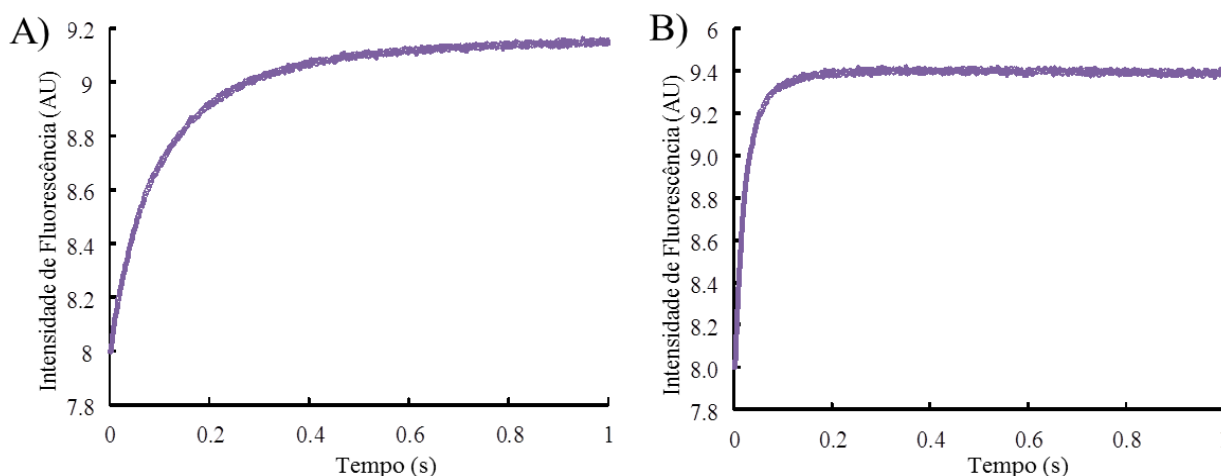


Figura 16. Mudança de fluorescência de Tsa1 e Tsa2 pela adição concentrações crescentes de Trx1 reduzida. Tsa1 (A) e Tsa2 (B) previamente oxidadas com 1.2 equivalentes de H_2O_2 e Trx1 previamente reduzida por 30 min com 20 mM de DTT (o excesso de DTT foi retirado através de gel filtração) foram reagidas com Trx1 em excesso (8:1 equivalentes) e os dados de fluorescência intrínseca do triptofano (excitação 280 nm e emissão 340 nm) foram adquiridos em espectrofluorímetro com *stopped-flow*.

Cabe ressaltar que apesar de algumas constantes de redução já terem sido calculadas para algumas 2-Cys Prx, os trabalhos foram realizados com proteínas mutantes (Parsonage *et*

al., 2005) e revelaram taxas de redução bastante significativas ($\sim 10^7 \text{ M}^{-1}\text{s}^{-1}$). As taxas de redução da metionina sulfóxido redutase (MSR) pela Trx de *E. coli* foi determinada pela mesma metodologia e microcalorimetria e os autores concluíram que o passo limitante da velocidade para a regeneração da forma reduzida de MSR por Trx foi a dissociação do complexo entre Trx oxidada e MSR reduzida (Antoine, Boschi-Muller e Branlant, 2003). Adicionalmente, já foi demonstrado que a formação do dissulfeto em 2-Cys Prx pode ser um processo lento com taxas que variam de 75 s^{-1} (AhpC de *Salmonella typhimurium*) a 1.7 s^{-1} (Prx2 de *Homo sapiens*) (Parsonage *et al.*, 2015; Peskin *et al.*, 2013).

Neste contexto, uma vez que as constantes para decomposição de H_2O_2 e CHP e a redução de Trx1/Trx2 por TrxR1 residem na ordem de $10^7 \text{ M}^{-1}\text{s}^{-1}$ (Oliveira *et al.*, 2010) e as constantes de redução de Tsa1 e Tsa2 por Trx1 são de $10^6 \text{ M}^{-1}\text{s}^{-1}$, é pertinente supor que as taxas determinadas para decomposição de hidroperóxidos por cinética de estado estacionário utilizando o sistema Trx ($10^{4-5} \text{ M}^{-1}\text{s}^{-1}$) possam estar sob influência de fenômenos não excludentes como: 1) lentidão da formação de dissulfeto de Tsa1 e Tsa2 no processo de condensação; 2) dissociação do complexo entre Trx oxidada e Tsa1 reduzida e 3) superoxidação de parcela de Tsa1 e Tsa2 nas reações. A hipótese de superoxidação foi avaliada e os dados são apresentados a seguir.

Tabela2. Determinação dos parâmetros cinéticos de Tsa1 e Tsa2 para substratos oxidantes (H₂O₂, *t*-BOOH e CHP) e para os substratos redutores Trx1 e Trx2

	Parâmetros cinéticos de Tsa1							
	Trx1			Trx2			H ₂ O ₂	
	H ₂ O ₂	CHP	<i>t</i> -BOOH	H ₂ O ₂	CHP	<i>t</i> -BOOH	Trx1	Trx2
k_{cat} (s ⁻¹)	1.2 ± 0.05	0.7 ± 0.01	0.9 ± 0.02	1.1 ± 0.1	0.77 ± 0.1	1.1 ± 0.07	0.97 ± 0.05	1.0 ± 0.05
K_M (μM)	39.0 ± 8.67	9.8 ± 1.12	11.7 ± 2.7	55.5 ± 11.5	13.1 ± 7.2	18.7 ± 7.3	0.54 ± 0.2	0.59 ± 0.2
V_{max} (μM/s)	1.2 ± 0.05	0.7 ± 0.01	0.9 ± 0.02	1.1 ± 0.1	0.77 ± 0.1	1.1 ± 0.07	0.97 ± 0.05	1.0 ± 0.05
k_{cat}/K_M (M ⁻¹ s ⁻¹)	3.8 ± 1.1 × 10 ⁴	6.8 ± 0.5 × 10 ⁴	8.5 ± 2.6 × 10 ⁴	2 ± 3 × 10 ⁴	5.8 ± 2.0 × 10 ⁴	5.9 ± 3 × 10 ⁴	5.67 ± 2 × 10 ⁵	5.9 ± 2 × 10 ⁵
	Parâmetros cinéticos de Tsa2							
	Trx1			Trx2			H ₂ O ₂	
	H ₂ O ₂	CHP	<i>t</i> -BOOH	H ₂ O ₂	CHP	<i>t</i> -BOOH	Trx1	Trx2
k_{cat} (s ⁻¹)	1.0 ± 0.003	0.9 ± 0.030	0.9 ± 0.024	0.95 ± 0.03	1.38 ± 0.03	1.2 ± 0.03	1.58 ± 0.3	1.54 ± 0.2
K_M (μM)	38.5 ± 1.23	5.4 ± 1.50	9.62 ± 1.79	33.67 ± 5.9	6.68 ± 7.2	13.6 ± 2.4	0.69 ± 0.2	0.68 ± 0.2
V_{max} (μM/s)	1.0 ± 0.003	0.9 ± 0.0030	0.9 ± 0.024	0.95 ± 0.03	1.38 ± 0.03	1.2 ± 0.03	1.58 ± 0.3	1.54 ± 0.2
k_{cat}/K_M (M ⁻¹ s ⁻¹)	2.8 ± 0.7 × 10 ⁴	1.7 ± 0.9 × 10 ⁵	9.8 ± 2 × 10 ⁴	2.8 ± 1 × 10 ⁴	2.0 ± 0.5 × 10 ⁵	8.7 ± 1.8 × 10 ⁴	2.2 ± 1.7 × 10 ⁶	2.2 ± 0.3 × 10 ⁶

* Condições para determinação dos parâmetros cinéticos:

Substratos oxidantes: Tsa2 (1 μM), Trx1 ou Trx2 2 (μM), TrxR1 (0.3 μM), NADPH (150 μM) e concentrações variáveis de hidroperóxidos.

Substratos redutores: Tsa2 (1 μM), TrxR1 (0.3 μM), NADPH (150 μM), H₂O₂ (500 μM) e concentrações variáveis de Trx1 ou Trx2.

5.2 CAPÍTULO 2 - Susceptibilidade de Tsa1 e Tsa2 a superoxidação por diferentes peróxidos

5.2.1 Avaliação da susceptibilidade a superoxidação de Tsa1 e Tsa2 utilizando ensaio de NADPH e SDS-PAGE

Para se avaliar comparativamente a superoxidação de Tsa1 e Tsa2 na presença de altas doses de hidroperóxidos foram realizados ensaios de oxidação do NADPH utilizando o sistema Trx. Foram realizados experimentos utilizando como substrato redutor Trx1 ou Trx2 e como substrato oxidante altas concentrações de H_2O_2 , CHP e *t*-BOOH (250 μ M, 500 μ M, 1 mM, 5 mM e 10 mM). Neste contexto, a inibição do consumo de NADPH indica de forma indireta a formação de espécies superoxidadas em C_P-SO_2H ou C_P-SO_3H , o que impossibilita a redução por Trx e conseqüentemente o consumo de NADPH (Ikeda *et al.*, 2011; Nelson *et al.*, 2013). A figura 17 demonstra os resultados destes experimentos para as proteínas Tsa1 e Tsa2 utilizando Trx1 como substrato redutor e H_2O_2 , CHP e *t*-BOOH como substratos oxidantes.

Analisando o resultado desse experimento pode-se observar que quando o substrato utilizado é a H_2O_2 a reação da proteína Tsa1 (Figura 17A) é inibida com concentrações superiores a 500 μ M de substrato, ao passo que a reação de Tsa2 apresenta uma inibição quase imperceptível (Figura 17B). Já quando o CHP é utilizado como substrato à reação com Tsa1 é inibida na concentração mínima testada (250 μ M), sendo que a enzima apresenta-se praticamente inativa (Figura 17C), enquanto que nas mesmas condições Tsa2 apresentou uma inibição muito menor (Figura 17D). Quando se utilizou o *t*-BOOH como substrato oxidante verificou-se uma inibição intermediária (Figura 17E e 17F), sendo que utilizando este substrato a reação de Tsa2 também apresentou uma menor inibição.

Foram realizados também experimentos semelhantes utilizando Trx2 como substrato redutor, os resultados demonstraram diferenças pouco significativas quando comparados à utilização de Trx1 (Figura 18). Tsa1 novamente apresentou uma maior inibição quando comparada a Tsa2 e a inibição apresenta aumento significativo quando o substrato utilizado é um peróxido orgânico (CHP ou *t*-BOOH). Os resultados obtidos nestes experimentos revelam que ocorre uma superoxidação diferencial entre Tsa1 e Tsa2 e que peróxidos orgânicos promovem esse processo mais eficientemente que o H_2O_2 .

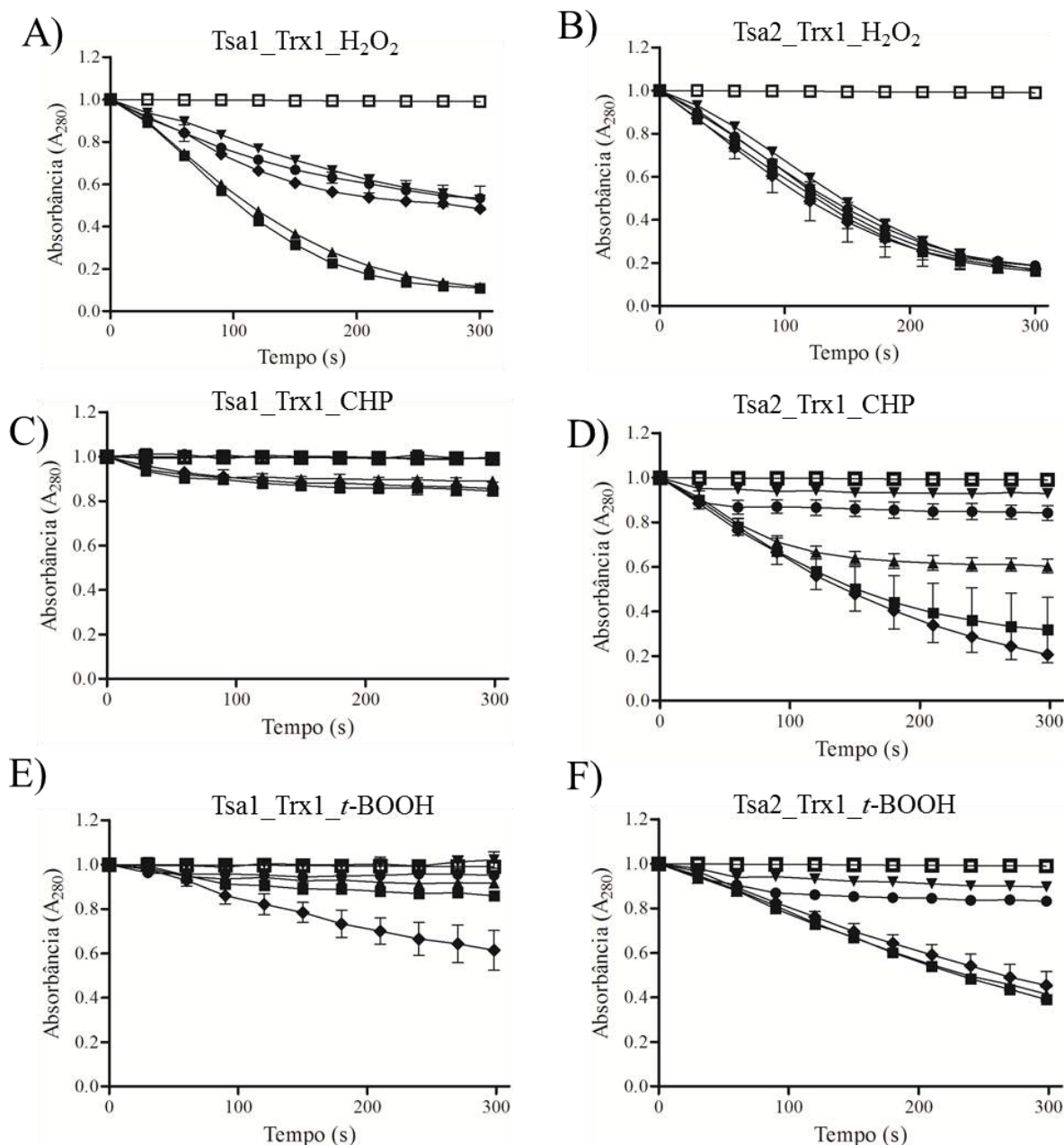


Figura 17. Susceptibilidade a superoxidação de Tsa1 e Tsa2. A atividade peroxidásica de Tsa1 (A, C e E) e Tsa2 (B, D e F) foi monitorada através da oxidação do NADPH (340 nm) com concentrações crescentes de hidroperóxidos: 0 μM , (\square), 250 μM (\diamond), 500 μM (\blacksquare), 1 mM (\blacktriangle), 5 mM (\bullet), 10 mM (\blacktriangledown). A reação (100 μl) foi realizada em tampão 50 mM HEPES-NaOH contendo 1 mM de azida, 100 μM de DTPA na presença de 150 μM de NADPH, 1 μM de Tsa1 ou Tsa2, 2 μM de Trx1, 0.3 μM de TrxR1 e diferentes concentrações de hidroperóxidos. A reação foi iniciada pela adição do hidroperóxido. As curvas representam a oxidação do NADPH em diferentes concentrações de H₂O₂ (A e B), CHP (C e D) e *t*-BOOH (E e F).

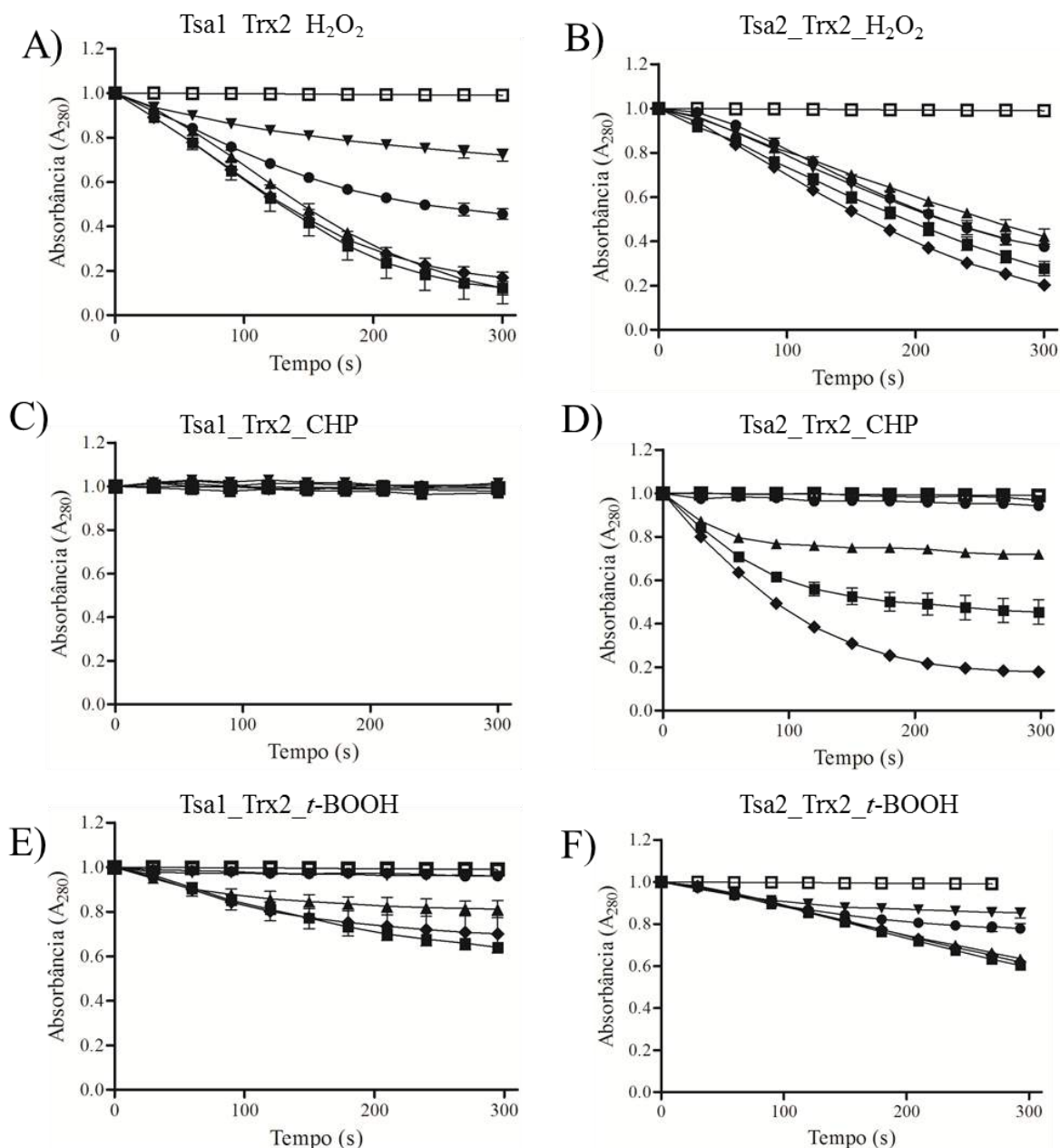


Figura 18. Susceptibilidade a superoxidação de Tsa1 e Tsa2. A atividade peroxidásica de Tsa1 (A, C e E) e Tsa2 (B, D e F) foi monitorada através da oxidação do NADPH (340 nm) com concentrações crescentes de hidroperóxidos: 0 μ M (\square), 250 μ M (\blacklozenge), 500 μ M (\blacksquare), 1 mM (\blacktriangle), 5 mM (\bullet), 10 mM (\blacktriangledown). A reação (100 μ l) foi realizada em tampão 50 mM HEPES-NaOH contendo 1 mM de azida, 100 μ M de DTPA na presença de 150 μ M de NADPH, 1 μ M de Tsa1 ou Tsa2, 2 μ M de Trx2, 0,3 μ M de TrxR1 e diferentes concentrações de hidroperóxidos. A reação foi iniciada pela adição do hidroperóxido. As curvas representam a oxidação do NADPH em diferentes concentrações de H₂O₂ (A e B), CHP (C e D) e *t*-BOOH (E e F).

A superoxidação da C_P pode ser também observada por SDS-PAGE através do monitoramento da forma dimérica e monomérica das 2-Cys Prx (Nelson *et al.*, 2013; Perkins *et al.*, 2013). Em reações com concentrações baixas de hidroperóxidos e presença de sistema redutor ocorre o predomínio da forma reduzida da proteína, o que pode ser observado em SDS PAGE como monômeros de ~23 kDa (Figura 19), quando há um aumento na concentração de

hidroperóxidos ocorre um predomínio de formas diméricas ($C_P S - S C_R$), pois há formação de dímeros mantidos por ligação dissulfeto entre C_P e C_R de diferentes monômeros (Figura 19). Por fim, quando ocorre a superoxidação moderada (formação de espécies $C_P SO_2H$ ou $C_P SO_3H$) podem ser observadas duas diferentes formas em SDS-PAGE, dimérica e monomérica. Na forma dimérica ocorre a superoxidação de apenas uma subunidade do dímero, sendo que a outra permanece em dissulfeto, esse estado aumenta a resistência da migração no SDS-PAGE fazendo com que a proteína apareça levemente superior a forma oxidada. Já a forma monomérica apresenta superoxidação de ambas as C_P do dímero, o que acarreta na sua dissociação em SDS-PAGE. Esta forma é esperada em altas concentrações de hidroperóxidos (Figura 19).

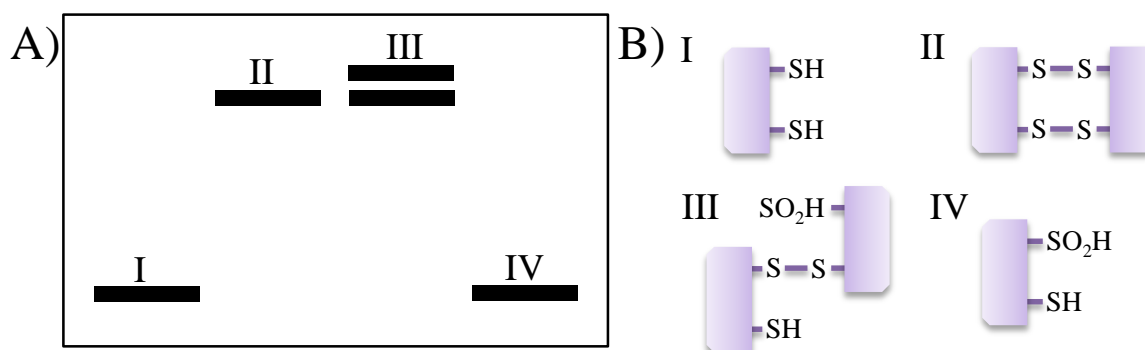


Figura 19. Diagrama exemplificando o resultado em SDS-PAGE de formas reduzidas, oxidadas e superoxidadas e formação de dissulfetos de Prx 2-Cys típica. Em A) e B) as representações são: I) 2-Cys Prx na forma reduzida, detectada como monômero. II) Representação da forma oxidada, detectada como dímero. III) Forma dimérica oxidada em dissulfeto (banda inferior) e dimérica com uma C_P superoxidada (superior). IV) Forma monomérica com ambas as C_P superoxidadas.

Para verificar através de SDS-PAGE o nível de superoxidação de Tsa1 e Tsa2 foram realizados ensaios de oxidação de NADPH semelhante aos ensaios de superoxidação descritos acima, no entanto a concentração de Tsa1 e Tsa2 utilizada foi de $9.3 \mu M$ para possibilitar a detecção em SDS-PAGE. Os experimentos foram realizados com concentrações crescentes de hidroperóxidos ($10 \mu M$ a $10 mM$), sendo que após 30 minutos de reação, foram adicionados a cada reação $100 mM$ de NEM, ao qual é um agente alquilante que se liga a cisteínas livres impedindo a formação de dissulfetos artefatuais.

Para reações utilizando H_2O_2 as proteínas Tsa1 e Tsa2 apresentaram comportamentos bastante similares quando comparadas (Figura 20A e 20B), Tsa1 apresentou formas reduzidas nas reações que continham até $75 \mu M$ de H_2O_2 , indicando que até essa concentração de substrato oxidante a enzima foi capaz de degradar sem a ocorrência de superoxidação de C_P . Em concentrações entre $125 \mu M$ e $1 mM$ ocorreu a predominância de dímeros, o qual representa a proteína oxidada em dissulfeto, já na concentração de $1 mM$ já é possível

verificar o surgimento de uma banda correspondente ao monômero, a qual representa a forma superoxidada, sendo possível verificar um aumento gradativo da intensidade desta banda quando a concentração de H_2O_2 é aumentada. Tsa2 apresentou formas reduzidas até concentração de $50 \mu M$ e espécies superoxidadas são visualizadas a partir da concentração de $1 mM$ de H_2O_2 . Ensaio utilizando CHP como substrato oxidante demonstraram que Tsa1 sofre uma superoxidação muito acentuada não sendo possível verificar a concentração exata onde inicia a superoxidação, desde a concentração mais baixa de CHP já é possível verificar bandas diméricas, as quais possivelmente representam dímeros contendo apenas uma ligação dissulfeto e uma cisteína superoxidada (Figura 20C).

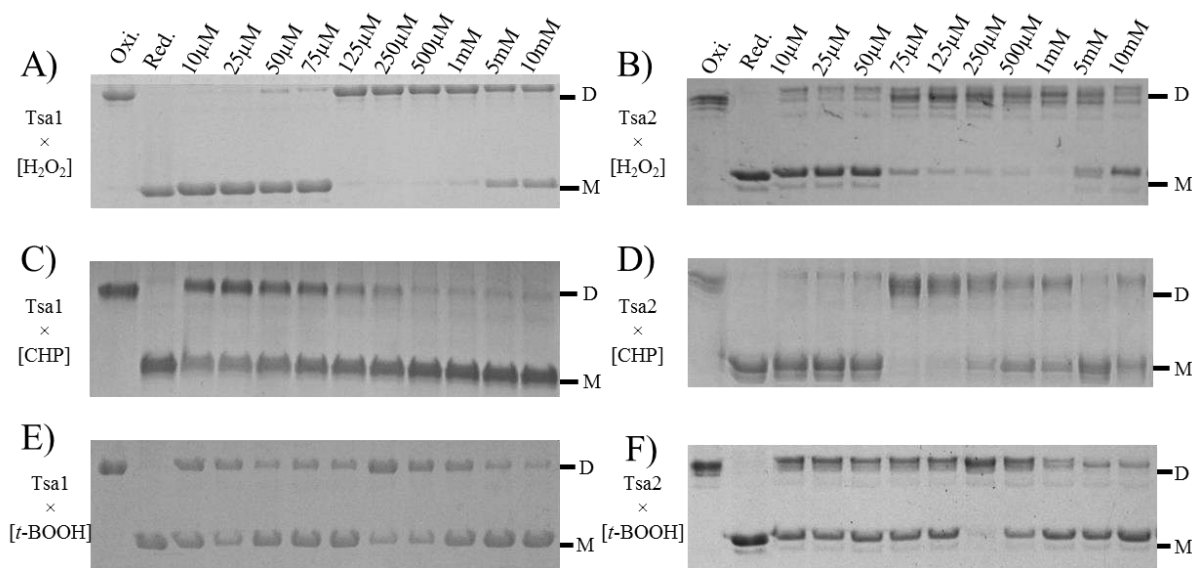


Figura 20. Ensaio de superoxidação de Tsa1 e Tsa2 utilizando sistema Trx (Trx1) na presença de concentrações crescentes de hidroperóxidos. O estado oligomérico de Tsa1 (A, C e E) e Tsa2 (B, D e F) foi monitorado através de SDS-PAGE em condições não redutoras. As reações foram realizadas em tampão $50 mM$ Hesper-NaOH pH 7.4 contendo $100 \mu M$ de DTPA, $1 mM$ de azida, $150 \mu M$ de NADPH, $9.3 \mu M$ de Tsa1, $1 \mu M$ de Trx1, $0.3 \mu M$ de TrxR1 e concentrações crescentes de hidroperóxidos ($10 \mu M - 10 mM$). A e B: H_2O_2 ; C e D: CHP e E e F: $t-BOOH$. As reações foram iniciadas pela adição do hidroperóxido, mantidas a $30^\circ C$ por 30 minutos e alquiladas com $100 mM$ de NEM por 30 min. Em seguida as amostras foram aplicadas em SDS-PAGE em condições não redutoras. As legendas do lado direito das figuras denotam a enzima e o peróxido utilizado e do lado direito: M = monômero, D = dímero.

O experimento realizado com a proteína Tsa2 utilizando CHP como substrato oxidante demonstrou que Tsa2, possui maior resistência a superoxidação por CHP, sendo possível verificar formas superoxidadas a partir da concentração de $250 \mu M$ (Figura 20D). Com relação à utilização com $t-BOOH$, ambas as proteínas apresentaram alto nível de superoxidação, Tsa1 apresentou superoxidação com $250 \mu M$ e Tsa2 a partir de $500 \mu M$ (Figura 20E e 20F).

Para verificar se o nível de superoxidação pode ser alterado pelo substrato redutor experimentos semelhantes foram realizados utilizando Trx2 e concentrações crescentes de

H_2O_2 , CHP e *t*-BOOH como substratos oxidantes. Os resultados foram equivalentes aos obtidos utilizando Trx1 como substrato redutor (Figura 21).

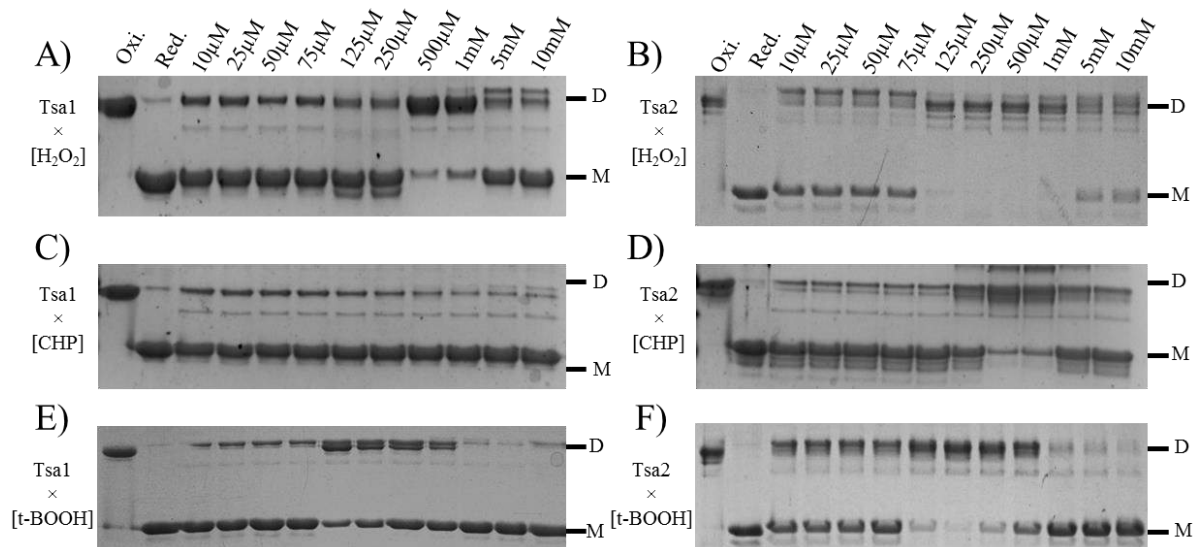


Figura 21. Ensaio de superoxidação de Tsa1 e Tsa2 utilizando sistema Trx (Trx2) na presença de concentrações crescentes de hidroperóxidos. O estado oligomérico de Tsa1 (A, C e E) e Tsa2 (B, D e F) foi monitorado através de SDS-PAGE em condições não redutoras. As reações foram realizadas em tampão 50 Hepes-NaOH pH 7.4 contendo 100 μ M de DTPA, 1 mM de azida, 150 μ M de NADPH, 9.3 μ M de Tsa1, 1 μ M de Trx2, 0.3 μ M de TrxR1 e concentrações variáveis de hidroperóxidos (10 μ M – 10 mM) A e B: H_2O_2 ; C e D: CHP e E e F: *t*-BOOH. por 30 min. Em seguida as amostras foram aplicadas em SDS-PAGE em condições não redutoras. As legendas do lado direito das figuras denotam a enzima e o peróxido utilizado e do lado direito: M = monômero, D = dímero.

Para confirmar a superoxidação das proteínas foram realizados experimentos de *western blot* com anticorpo para a forma Prx-SO_{2/3}, entretanto esse anticorpo não apresentou especificidade para as espécies superoxidadas, sendo reativo também com a forma reduzida e oxidada da proteína (dados não apresentados).

Os experimentos de cinética enzimática utilizando H_2O_2 e peróxidos orgânicos juntamente com os experimentos de superoxidação demonstram que hidroperóxidos orgânicos tais como CHP e *t*-BOOH são capazes de promover mais eficientemente a superoxidação quando comparados ao H_2O_2 . Outra observação importante verificada nestes experimentos é que Tsa2 é mais resistente a superoxidação que Tsa1, de fato este resultado demonstra uma diferença bastante significativa entre essas proteínas. Por outro lado o passo de redução de Tsa1 e Tsa2 não apresentou diferenças significativas quando os ensaios foram realizados com Trx1 ou Trx2, confirmando os dados cinéticos.

5.2.2 Avaliação da superoxidação da C_P por meio de ensaios envolvendo alterações de fluorescência do triptofano

Como mencionado anteriormente, as constantes de pseudo-primeira ordem para as proteínas Tsa1 e Tsa2 para H₂O₂ e CHP foram determinadas por experimentos fluorométricos. Um fenômeno importante observado foi que logo após a queda de fluorescência causada pela oxidação da C_P ocorreu um aumento na emissão de fluorescência para Tsa1 (Figura 22A) que não foi observada para Tsa2 (Figura 22B). Cabe ressaltar que a razão de hidroperóxido para proteína utilizada foi de 10:1 equivalentes molares. Experimentos adicionais utilizando diferentes concentrações de hidroperóxidos revelaram que esta segunda fase da reação é dependente da concentração de hidroperóxido (Figura 22C), entretanto quando Tsa2 foi submetida às mesmas condições esta segunda fase não foi detectada mesmo em concentrações muito superiores de hidroperóxidos (50 equivalentes).

Neste contexto, uma das possibilidades para a segunda fase apresentada por Tsa1 poderia ser atribuída a superoxidação de C_P, uma vez que análises de estruturas criptográficas revelam que 2-Cys Prx no estado reduzido ou superoxidado apresentam conformação similar (Figura 22D), mas muito diferente da forma oxidada. Para avaliar se o retorno da fluorescência se tratava de superoxidação foram realizados ensaios fluorométricos de Tsa1 reduzida, oxidada com baixas doses de hidroperóxidos (1.2 eq.) ou superoxidada com o auxílio do sistema tiorredoxina. Para confirmar que o tratamento levou a superoxidação de Tsa1 foi realizado um ensaio comparativo em SDS-PAGE não redutor onde foram comparadas Tsa1 reduzida, oxidada, superoxidada, superoxidada após a adição de 10 mM de DTT e superoxidada após a adição de 1.2 equivalentes de H₂O₂. O resultado indica que mesmo após a adição de H₂O₂ a proteína manteve-se em monômero o que indica fortemente que se tratar da forma contendo C_P superoxidada (dados não apresentados).

Após a comprovação da superoxidação foi realizado um ensaio comparativo de fluorescência, onde foram comparadas a proteína Tsa1 previamente reduzida, oxidada e superoxidada como se pode observar na figura 22E ocorre um aumento de fluorescência quando a proteína se encontra superoxidada, o indica que o retorno de fluorescência se trata da formação de C_PSO_{2/3}. De fato, Reyes e colaboradores (2011) observam um fenômeno semelhante na 1-Cys Prx AhpE de *Mycobacterium tuberculosis*, e utilizaram esse retorno para determinar as constantes de superoxidação desta enzima. Tendo em vista os resultados obtidos e os dados presentes literatura, consideramos que o retorno da fluorescência representa superoxidação de C_P de Tsa1. Assim foram determinadas as constantes de pseudo-

primeira ordem para superoxidação de Tsa1 por H_2O_2 e CHP (Figura 22F), sendo obtida uma constante de $6.43 \times 10^5 \text{ M}^{-1}\cdot\text{s}^{-1}$ para H_2O_2 e $8.12 \times 10^5 \text{ M}^{-1}\cdot\text{s}^{-1}$ para CHP. De fato, apesar destas taxas de superoxidação se apresentarem bastante elevadas elas são muito similares ao determinado previamente para AhpE ($10^5 \text{ M}^{-1}\cdot\text{s}^{-1}$) (Reyes *et al.*, 2011). Neste contexto, os dados obtidos por ensaios fluorométricos utilizando baixas doses de hidroperóxidos estão de acordo com os observados por cinética de estado estacionário utilizando o sistema Trx que indicam uma maior suscetibilidade a superoxidação C_P de Tsa1 quando comparada a Tsa2 (Figuras 17, 18, 20 e 21).

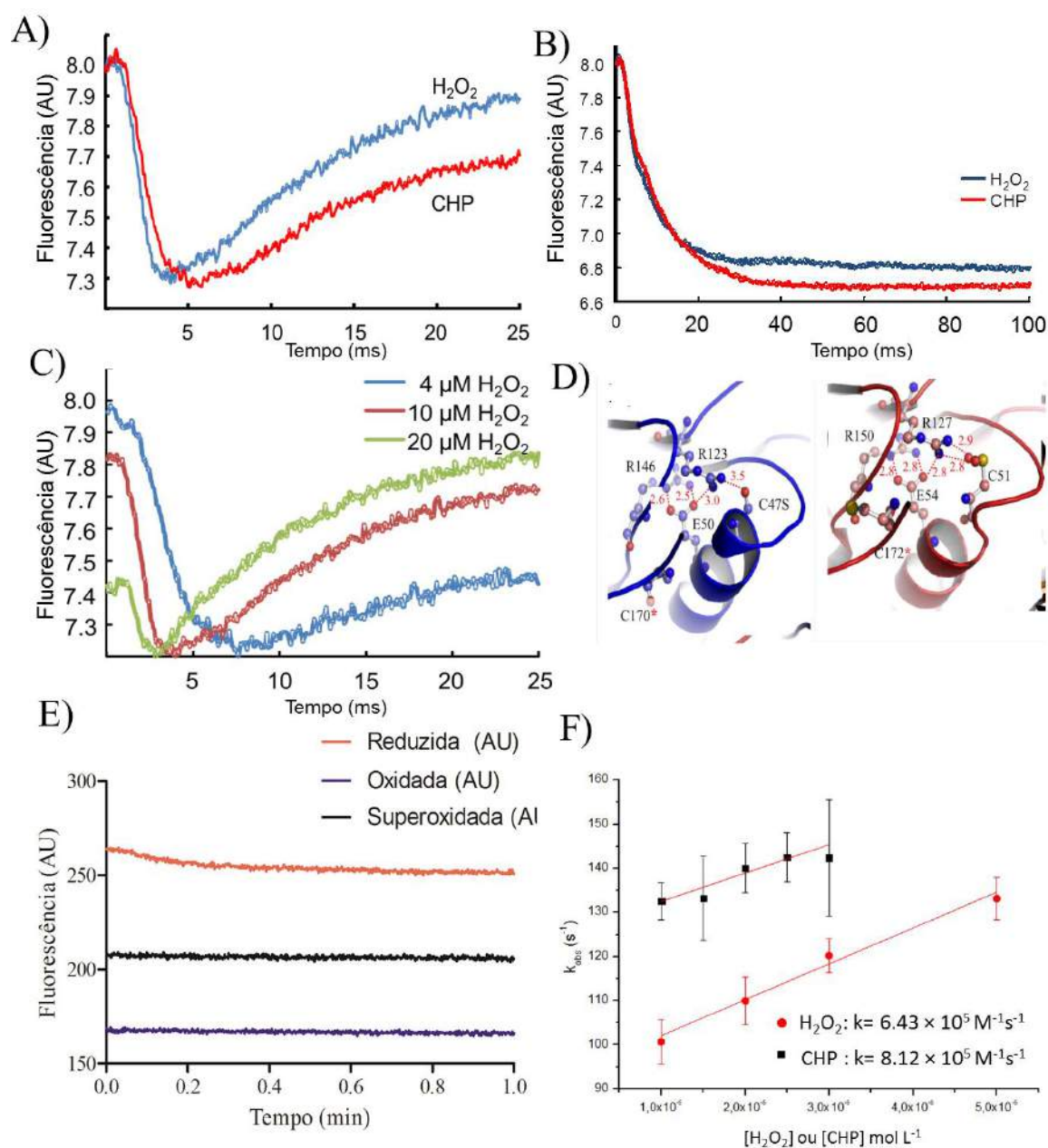


Figura 22. Análises de superoxidação de Tsa1 e Tsa2 através de fluorimetria. A) Espectro de fluorescência de Tsa1 ($1\mu\text{M}$) previamente reduzida com 20 mM de DTT (excesso removido através gel filtração) e reagida com H_2O_2 (linha azul) ou CHP (linha vermelha) ($10\mu\text{M}$). Nota-se o aumento de fluorescência após a fase de redução do peróxido. B) Espectro de fluorescência de Tsa2 ($1\mu\text{M}$) foi reagida com H_2O_2 (linha azul) ou CHP

(linha vermelha) (10 μ M). C) Emissão de fluorescência de Tsa1 em doses crescentes de peróxido de hidrogênio (4, 10 e 20 μ M), demonstrando a dose dependência do aumento de fluorescência. D) Estruturas cristalográficas de Tsa1^{C47S} (código de pdb: 3SBC) que mimetiza a forma reduzida (azul) e Prx2 de *H. sapiens* com C_P superoxidada em C_P-SO₂H (1QMV). E) Espectro de emissão de fluorescência da forma reduzida, oxidada e superoxidada de Tsa1 ($\lambda_{\text{ex}} = 280$ e $\lambda_{\text{em}} = 340$ nm. F) Valores de k_{obs} foram plotados em relação à concentração de H₂O₂ e CHP utilizadas e demonstram que a inclinação da reta é dependente da concentração de hidroperóxido.

Curiosamente, análises efetuadas por SDS-PAGE, utilizando tratamentos com altas doses de hidroperóxidos não revelam a formação de espécies superoxidadas de Tsa1 ou Tsa2 mesmo com altas doses (100 eq.) de hidroperóxidos (Figura 23) o que pode indicar que a superoxidação detectada por experimentos fluorométricos representa somente uma pequena parcela da população de enzimas presentes na reação. De fato, mesmo em altas doses de hidroperóxido não foi possível à detecção em SDS-PAGE de espécies superoxidadas quando além de altas doses de hidroperóxidos foi adicionado o sistema tiorredoxina, sugerindo que esta age como um catalisador para a superoxidação das Prx.

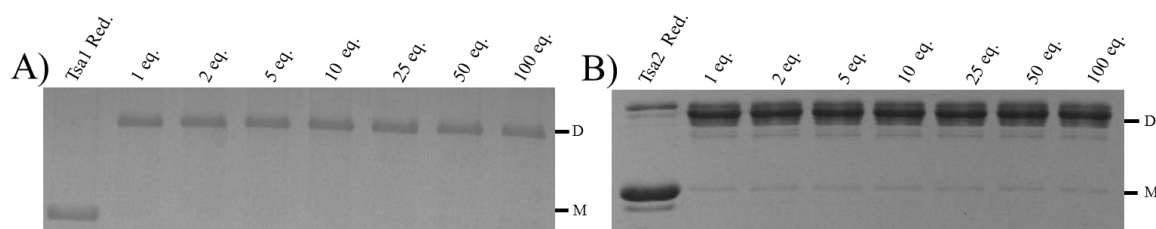


Figura 23. Ensaio de susceptibilidade a superoxidação de Tsa1 e Tsa2 com CHP sem a presença do sistema Trx. Tsa1 e Tsa2 foram previamente reduzidas com 20 mM de DTT (excesso de DTT retirado por gel filtração), e foram adicionadas concentrações crescentes de CHP (1-100 equivalentes). Em seguida as amostras foram aplicadas em SDS-PAGE em condições não redutoras. As legendas superiores denotam a enzima e as concentrações equimolares de CHP adicionadas a reação, ao lado direito: M = monômero, D = dímero.

5.2.3 Influência a substituição Thr/Ser no processo de superoxidação

Tsa1 e Tsa2 possuem uma grande semelhança estrutural e algumas diferenças funcionais, a que mais merece destaque está relacionada à maior resistência de Tsa2 a superoxidação. Análises da região do sítio ativo revelam que a diferença mais marcante reside na substituição de uma Thr em Tsa1 por uma Ser em Tsa2. De fato a análise da região contendo a tríade catalítica da estrutura cristalográfica de Tsa1 (Figura 24A) com um modelo teórico de Tsa2 (Figura 24B) revela que a única diferença encontrada na rede de interações polares efetuada pelos resíduos da tríade catalítica reside em uma interação do tipo CH-- π realizado pelo C γ de Thr o qual é inexistente para Tsa2.

Neste contexto, para uma análise comparativa da influência da substituição Thr/Ser foi gerado o mutante Tsa2^{S44T} bem como foi utilizado o mutante Tsa1^{T44S} obtido previamente em outra investigação do nosso grupo (Apêndice A).

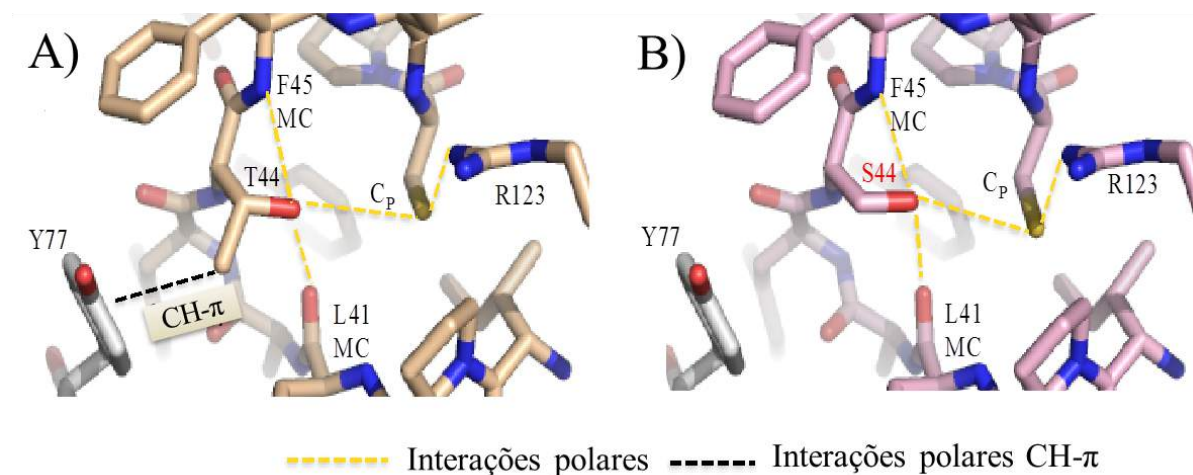


Figura 24. Diferenças de interações dos aminoácidos da tríade catalítica entre Tsa1 e Tsa2. (A) Estrutura cristalográfica de Tsa1 (1SBC) e (B) Modelo teórico de Tsa2 mostrando a rede de interações polares entre aminoácidos da tríade catalítica (C_p, Thr/Ser e Arg). As estruturas estão representadas em *sticks* e coloridas em CPK (N=azul, O= vermelho e S=laranja claro). Os carbonos em Tsa1 estão representados em bege e de Tsa2 em rosa.

Inicialmente foram realizados ensaios de oxidação do NADPH em concentrações crescentes de H₂O₂ e CHP. Para efeitos comparativos os resultados apresentados anteriormente para as proteínas selvagens serão apresentados novamente. Os resultados revelaram que a substituição da Thr da tríade catalítica em Tsa1, mutante Tsa1^{T44S}, levou a uma maior resistência à superoxidação (Figuras 25C e 25D) quando comparado com a proteína Tsa1 selvagem (Figuras 25A e 25B), tanto para H₂O₂ quanto para CHP. De fato esta única substituição de aminoácido deixou o comportamento de Tsa1 muito similar ao observado para Tsa2 selvagem (Figuras 25E e 56F). Por outro lado o mutante Tsa2^{S44T} apresentou maior susceptibilidade a superoxidação (Figura 25G e 25H), resultados muito semelhantes aos observados para a proteína Tsa1 (Figura 25A e 25B).

Para uma análise mais elaborada da susceptibilidade a superoxidação dos mutantes Tsa1^{T44S} e Tsa2^{S44T} também foram realizados ensaios utilizando SDS-PAGE. Novamente, para termos comparativos os resultados das proteínas selvagens são reapresentados para maior clareza. Como pode ser observado na figura 26 o mutante Tsa1^{T44S} apresenta maior resistência a superoxidação quando comparado ao selvagem, na reação com H₂O₂ bandas monoméricas superoxidadas são visualizadas a partir da concentração de 5 mM (Figura 26C). Já em reações com CHP bandas superoxidadas são visualizadas a partir de concentrações de 250 μ M (Figura 26D). Novamente os dados são bastante contrastantes com os obtidos para a proteína selvagem (Figura 26A e 26B) e remetem aos dados obtidos para Tsa2 selvagem (Figura 26E e 26F) o que indica que esta substituição no sitio ativo deixa a enzima muito mais resistente a superoxidação da C_p. Por outro lado o mutante Tsa2^{S44T} apresentou um aumento da

suscetibilidade a superoxidação uma vez que espécies superoxidadas são observadas em menores concentrações de hidroperóxidos (Figura 26G e 26H). Novamente quando o CHP (Figura 26) é utilizado como substrato, a superoxidação é extremamente evidente mesmo em doses baixas de CHP de forma muito semelhante ao observado para a proteína Tsa1 (Figura 26B).

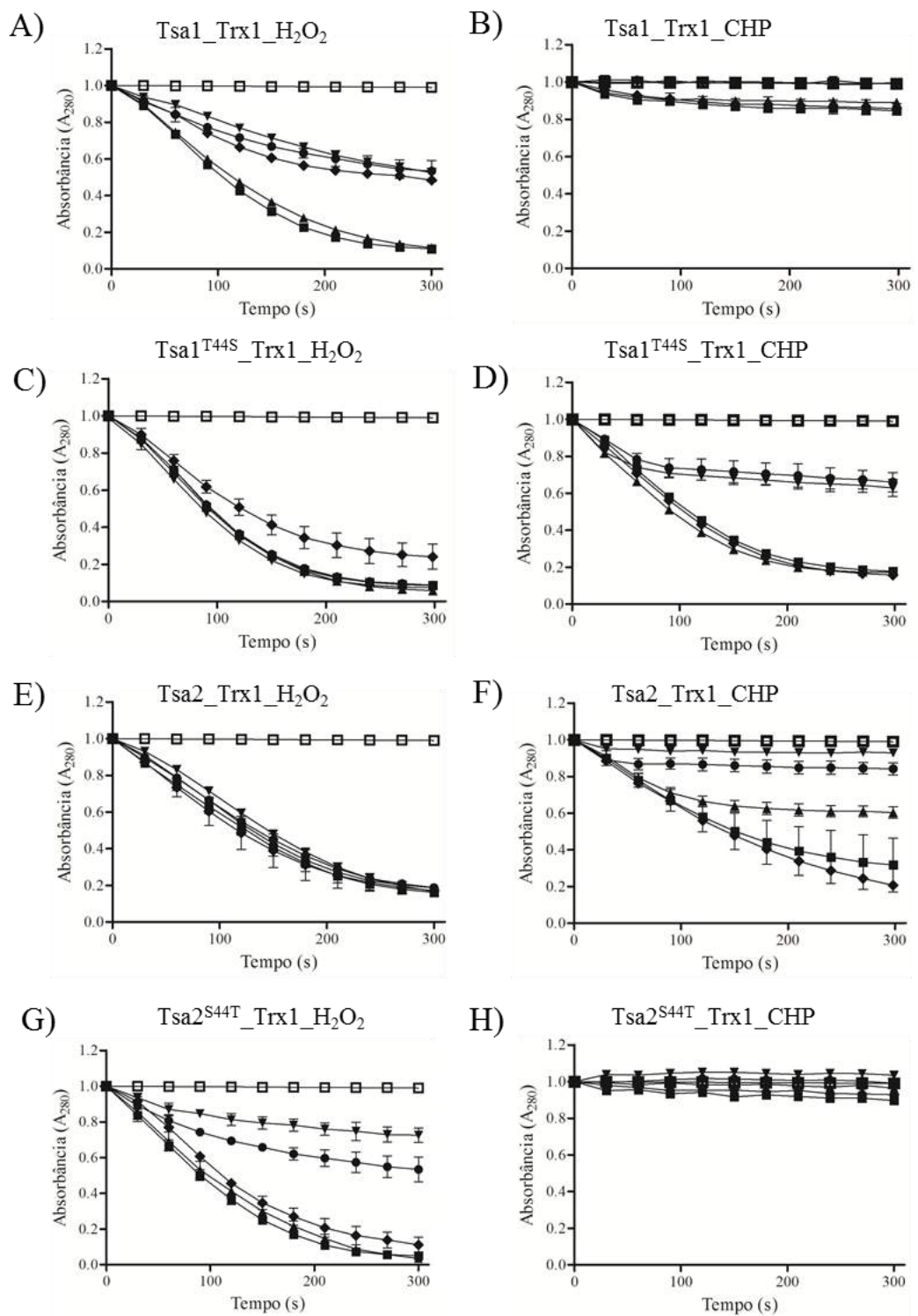


Figura 25. Suscetibilidade a superoxidação de Tsa1, Tsa2, Tsa1^{T44S}, Tsa2^{S44T}. A atividade peroxidásica de Tsa1 (A e B), Tsa1^{T44S} (C e D), Tsa2 (E e F) e Tsa2^{S44T} (G e H) foi monitorada através da oxidação do NADPH

(340nm) com concentrações crescentes de hidroperóxidos: 0 μM , (\square), 250 μM (\blacklozenge), 500 μM (\blacksquare), 1 mM (\blacktriangle), 5 mM (\bullet), 10 mM (\blacktriangledown). A reação (100 μl) foi realizada em tampão 50 mM Hepes-NaOH pH 7.4 contendo 1 mM de azida, 100 μM de DTPA na presença de 150 μM de NADPH, 1 μM de Tsa1, Tsa2, Tsa1^{T44S} ou Tsa2^{S44T}, 2 μM de Trx1, 0.3 μM de TrxR1 e diferentes concentrações de hidroperóxidos. A reação foi iniciada pela adição do hidroperóxido. As curvas representam a oxidação do NADPH em diferentes concentrações de H₂O₂ (A, C, E e G) e CHP (B, D, F e H).

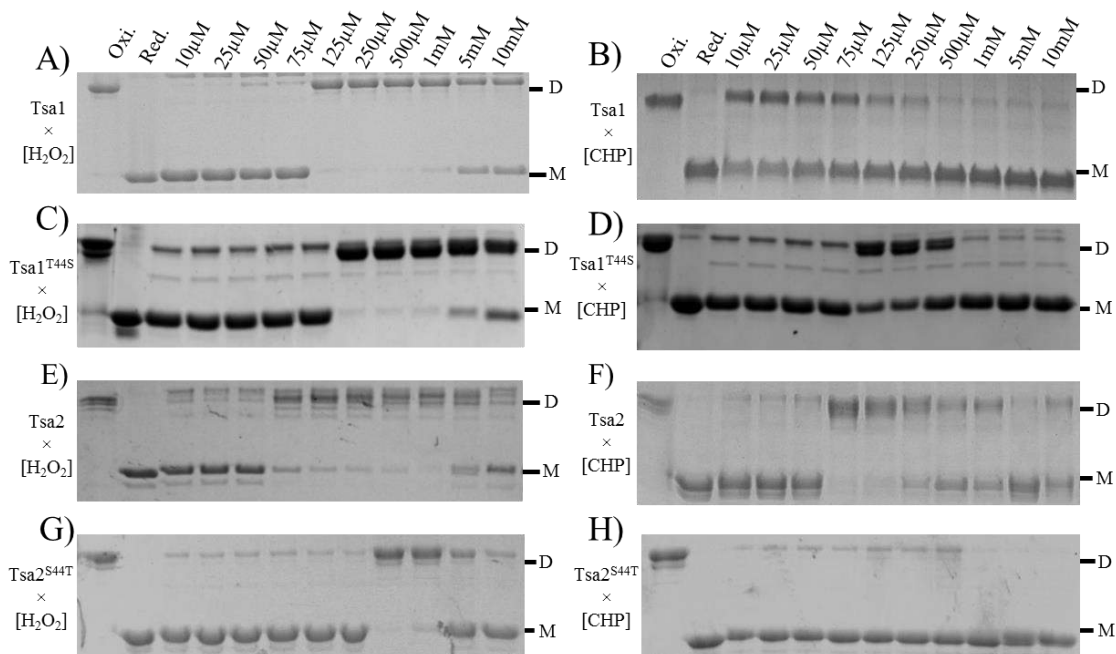


Figura 26. Ensaio de superoxidação de Tsa1, Tsa1^{T44S}, Tsa2 e Tsa2^{S44T} utilizando sistema Trx na presença de concentrações crescentes de hidroperóxidos. O estado oligomérico de Tsa1 (A, B), Tsa1^{T44S} (C e D), Tsa2 (E e F) e Tsa2^{S44T} (G e H) foi monitorado através de SDS-PAGE em condições não redutoras. As reações foram realizadas em tampão 50 mM Hepes-NaOH pH 7.4 contendo 100 μM de DTPA, 1 mM de azida, 150 μM de NADPH, 9.3 μM de Prx, 1 μM de Trx1, 0.3 μM de TrxR1 e concentrações variáveis de H₂O₂ (A, C, E e G) e CHP (B, D, F e H). As reações foram iniciadas pela adição do hidroperóxido, mantidas a 30 °C por 30 minutos e alquiladas com 100 mM de NEM. Após a reação as amostras foram aplicadas em SDS-PAGE em condições não redutoras. M = monômero, D = dímero.

Os resultados obtidos revelam de forma bastante clara que o resíduo da tríade catalítica o qual é substituído entre Tsa1 (Thr) e Tsa2 (Ser) apresenta um papel de grande importância para a suscetibilidade ou resistência a superoxidação. De fato análises de sequências de 2-Cys Prx típicas revelam que substituições naturais da Thr da tríade catalítica não é comum em organismos eucariotos, sendo encontrada somente em uma espécie de formiga (*Harpegnathos saltator*). Entretanto, esta substituição é encontrada em diversas 2-Cys Prx típicas de bactérias (AhpC) (Figura 27) o que pode indicar que esta robustez à superoxidação, em especial a peróxidos orgânicos pode estar relacionado a uma maior resistência a processos de peroxidação lipídica nos quais estes organismos podem estar envolvidos.

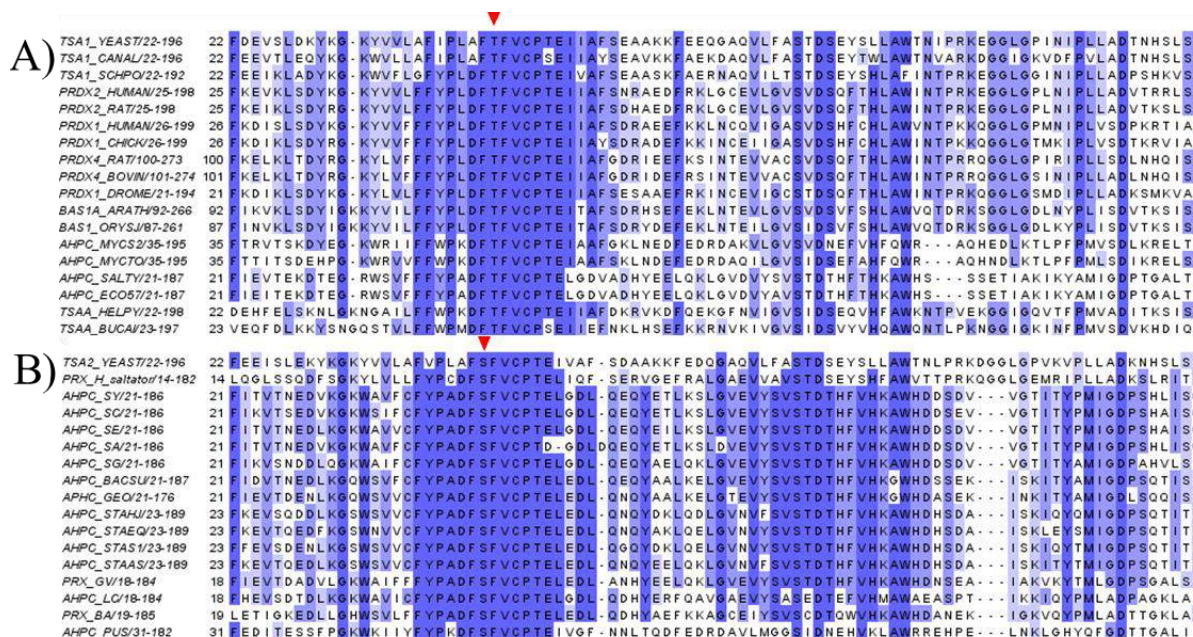


Figura 27. Alinhamento de estruturas de 2-Cys Prx típicas que contém Thr/Ser no sítio ativo e aminoácidos aromáticos na posição de interface decamérica. As sequências foram alinhadas utilizando o programa ClustalX (figura gerada pelo Jalview). Os aminoácidos destacados pelo retângulo vermelho destacam a conservação da Thr/Ser e de aminoácidos aromáticos na posição similar a Tyr77. As abreviaturas representadas em itálico o representam as espécies e são as seguintes: *Saccharomyces cerevisiae* = TSA1_YEAST (P34760.3); *Candida albicans* = TSA1_CANAL (Q9Y7F0.1); *Schizosaccharomyces pombe* = TSA1_SCHPO (O74887.1); *Rattus norvegicus* = PRDX2_RAT (P35704.3); *Homo sapiens* = PRDX1_HUMAN (Q06830.1); PRDX2_HUMAN (P32119.5) e PRDX4_HUMAN (Q13162.1); *Gallus gallus* = PRDX1_CHICK (001258861.1); *Rattus norvegicus* = PRDX4_RAT (Q9Z0V5.1); *Bos Taurus* = PRDX4_BOVIN (Q9BGI2.1); *Drosophila melanogaster* = PRDX1_DROME (Q9V3P0); *Arabidopsis thaliana* = BAS1A_ARATH (Q96291.2); *Oryza sativa japonica* = BAS1_ORYSJ (Q6ER94); *Mycobacterium smegmatis* = AHPC_MYCS2 (A0R1V9); *Mycobacterium tuberculosis* = AHPC_MYCTO (P9WQB6.1); *Salmonella enterica* = AHPC_SALTY (P0A251.2); *Escherichia coli* = AHPC_ECO57 (P0AE10.2); *Helicobacter pylori* = TSAA_HELPY (P21762.3); *Acyrtosiphon pisum* = TSAA_BUCAI (P57279.1); *Saccharomyces cerevisiae* = TSA2_YEAST (S000002861); *Harpegnathos saltator* = PRX_H_saltator (E2C951); *Streptococcus pyogenes* = AHPC_SY (WP_011185039.1); *Streptococcus castoreu* = AHPC_SC (WP_027970180.1); *Streptococcus equi* = AHPC_SE (WP_012678828.1); *Streptococcus agalactiae* = AHPC_SA (WP_025196649.1); *Streptococcus gallolyticus* = AHPC_SG (WP_009854906.1); *Bacillus subtilis* = AHPC_BACSU (P80239.2); *Geomicrobium sp.* = AHPC_GEO (GAK13620.1); *Staphylococcus haemolyticus* = AHPC_STAHJ (Q4L376.1); *Staphylococcus epidermidis* = AHPC_STAEQ (Q5HRY1.1); *Staphylococcus saprophyticus* = AHPC_STAS1 (Q49UT8.1); *Staphylococcus aureus* = AHPC_STAAS (Q6GC91.1); *Gardnerella vaginalis* = PRX_GV (WP_016637621.1); *Lactobacillus casei* = AHPC_LC (WP_029507391.1); *Bifidobacterium animalis* = PRX_BA (WP_012754385.1); *Pusillimonas sp* = AHPC_PUS (013742821.1).

5.3 CAPÍTULO 3 - Influência da superoxidação na formação de estruturas de alto peso molecular em Tsa1 e Tsa2

Trabalhos da literatura demonstram que 2-Cys Prx são capazes de formar estruturas de alto peso molecular (HMW) (Angelucci *et al.*, 2013; Jang *et al.*, 2004; Meissner *et al.*, 2007; Saccoccia *et al.*, 2012). Estas estruturas podem apresentar função de chaperona molecular e aparentemente estão envolvidas na transdução de sinal, sua função é dependente da superoxidação da C_P, pH e choque térmico (Jang *et al.*, 2004; Radjainia *et al.*, 2015; Saccoccia *et al.*, 2012). Jang e colaboradores demonstraram em um trabalho pioneiro a formação HMW da proteína Tsa1 com função de chaperona molecular dependente de superoxidação ou choque térmico. Neste mesmo trabalho os autores relatam que Tsa2 é capaz de formar de formar HMW semelhantes a Tsa1, mas os resultados não são apresentados no artigo. Tendo em vistas os resultados obtidos neste trabalho, a formação de complexos HMW de Tsa1 e Tsa2 foram analisadas em detalhes.

Inicialmente, com o objetivo de verificar possíveis diferenças estruturais entre as proteínas Tsa1 e Tsa2 foram realizados experimentos de SEC e DLS com as proteínas na forma reduzida (5 mM TCEP) e oxidada (1.2 equivalentes de H₂O₂) em concentração de 1 mg/mL (43.6 µM). Experimentos utilizando SEC demonstraram que a proteína Tsa1 na forma reduzida apresenta um pico de eluição, o qual representa o peso molecular aproximado de um decâmero, já na forma oxidada ocorre também à predominância de decâmeros, mas é observada uma parcela proteica na forma dimérica (Figura 28).

Experimentos de SEC com a proteína Tsa2 demonstraram que na concentração testada, independente, do estado redox a proteína se apresenta na forma decamérica (Figura 28), diferentemente de Tsa1, que quando oxidada possui as duas formas: decamérica e dimérica. Outra importante observação demonstrada através dos experimentos de SEC está relacionada ao tempo de retenção das moléculas na coluna. Tsa1 apresentou eluição do pico de maior intensidade com 7.8 minutos, enquanto Tsa2 apresentou a eluição com 7.4. Como este pico representa estruturas decaméricas é verificada uma diferença estrutural interessante a qual pode representar diferentes níveis de relaxação da molécula.

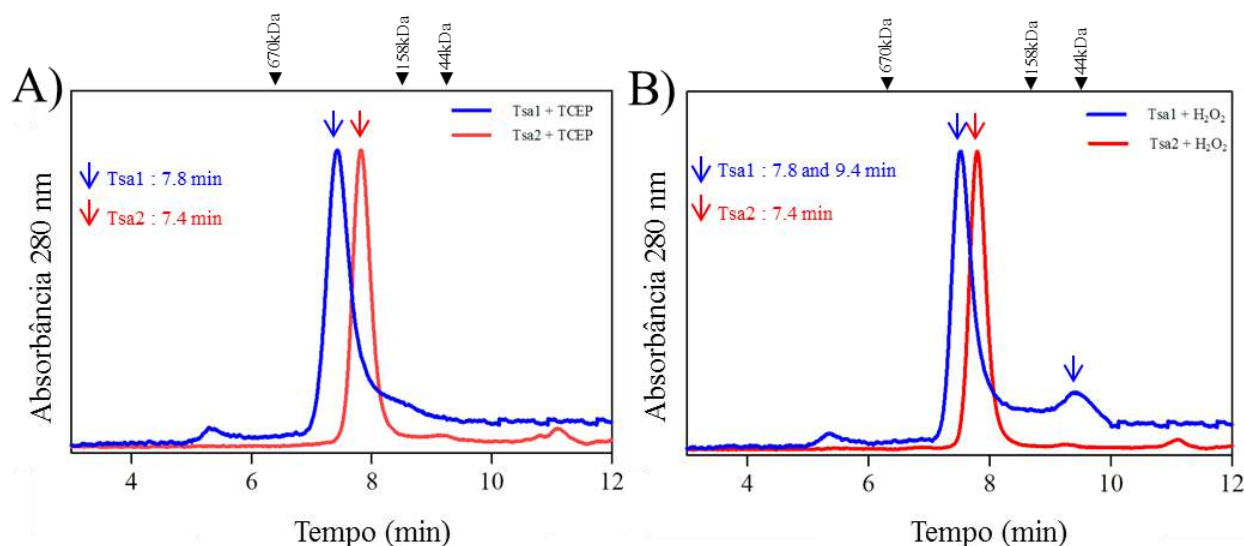


Figura 28. Cromatografia de exclusão molecular (SEC) de Tsa1 e Tsa2 nas formas reduzidas e oxidadas. Tsa1 e Tsa2 nas concentrações de 1mg/mL foram aplicadas em coluna Phenomenex BioSep-SEC-S 3000, a eluição dos componentes foi monitorada através da absorbância a 280 nm. Amostras de Tsa1 e Tsa2 foram reduzidas com 5 mM de TCEP (A) ou oxidadas com 1.2 equivalentes de H_2O_2 (B). Os padrões de peso molecular são representados na parte superior dos cromatogramas: tiroglobulina (bovina) (670 kDa), γ -globulina (bovina) (158 kDa), ovalbumina (frango) (44 kDa). Representações gráficas foram geradas utilizando GraphPad Prisma 4.0. Para motivo de comparação dos tempos de retenção, todos os cromatogramas estão normalizados.

Para comprovar os resultados obtidos foram realizados experimentos DLS nas mesmas condições utilizadas no SEC (Tabela 3). Para esses experimentos, Tsa1 na forma reduzida apresentou raio hidrodinâmico (R_h) de 6.835 nm (polidispersidade = 24.2% de massa = 99.8%) e na forma oxidada apresentou um R_h = 5.855 nm (polidispersidade = 9.8 % e massa = 53.8%). Os dados de DLS da proteína Tsa1 demonstraram que na forma reduzida a proteína se apresenta na forma decamérica como demonstrado nos experimentos de SEC. Já os dados da forma oxidada demonstram que a proteína apresenta-se na forma decamérica, mas 46.1% da massa apresenta-se em uma segunda forma (R_h = 0.864 e polidispersidade = 10.4%), que provavelmente representa as estruturas diméricas. A proteína Tsa2 apresentou na forma reduzida R_h de 6.832 (polidispersidade = 11.9% e % de massa = 97.9) e para forma oxidada R_h de 5.506 nm (polidispersidade = 11.9% e % de massa = 100), esses dados demonstram que independente do estado redox Tsa2 apresenta-se decamérica nas condições testadas.

Tabela 3. Espalhamento dinâmico de luz (DLS) de Tsa1 e Tsa2 previamente reduzida com 50mM de TCEP ou oxidada com 1.2 equivalentes de H_2O_2 .

	Tsa1		Tsa2	
	Reduzida	Oxidada	Reduzida	Oxidada
R_h (nm)	6.8	5.8/0.8	6.8	5.5
Mass (%)	99.8	53.8/46.1	97.9	100
Pd (%)	24.2	9.8/10.4	11.9	11.9

Como pode ser demonstrado pelos dados obtidos não foram detectados complexos de alto peso molecular nas enzimas oxidadas e reduzidas. Para analisar a formação de HMW foram então realizados ensaios de superoxidação em reações utilizando o sistema Trx em concentrações altas de H_2O_2 e CHP. Primeiramente a formação de complexos HMW de Tsa1, Tsa2 e mutantes relacionados à superoxidação foram analisados através de SEC. Para obtenção de resultados satisfatórios, foi necessária uma etapa de padronização dos resultados, sendo possível a obtenção de HMW com a utilização de altas concentrações de proteína (1mg/mL ou 43.6 μ M) e hidroperóxido (50 mM).

Os resultados dos experimentos demonstraram a formação de HMW para Tsa1 e Tsa2, semelhante aos complexos demonstrados por Jang e colaboradores (2004). Para a proteína Tsa1 são formadas diferentes espécies, que variam de 40 kDa (dímeros), espécies de com cerca de 200 kDa, compatível com formas decaméricas e também espécies HMW com mais de 1000 kDa. Cabe ressaltar, que um grande número de espécies intermediárias também são detectadas (Figura 29A e 29B). Quando o substrato da reação é o H_2O_2 é verificada uma predominância de estruturas decaméricas, mas também são detectadas estruturas de maior peso molecular (Figura 29A, linha vermelha). Já quando o substrato utilizado é o CHP (Figura 29A, linha azul), existe uma diminuição na formação de estruturas com cerca de 200 kDa (decâmero) e ocorre um aumento significativo na formação de HMW.

Para a proteína Tsa2 também foi verificada a formação de HMW, quando o H_2O_2 foi utilizado como substrato, porém em quantidades muito mais modestas quando comparado a Tsa1 (Figura 29B, linha vermelha). Por outro lado quando o substrato da reação foi o CHP também foi verificada a formação de complexos HMW (> 1000 kDa) (Figura 29B, linha azul). Adicionalmente, também é possível destacar que para Tsa2 ocorre a formação de uma quantidade muito menor de estruturas de massas moleculares intermediárias, prevalecendo estruturas de ~200 kDa e estruturas com mais de 1000 kDa.

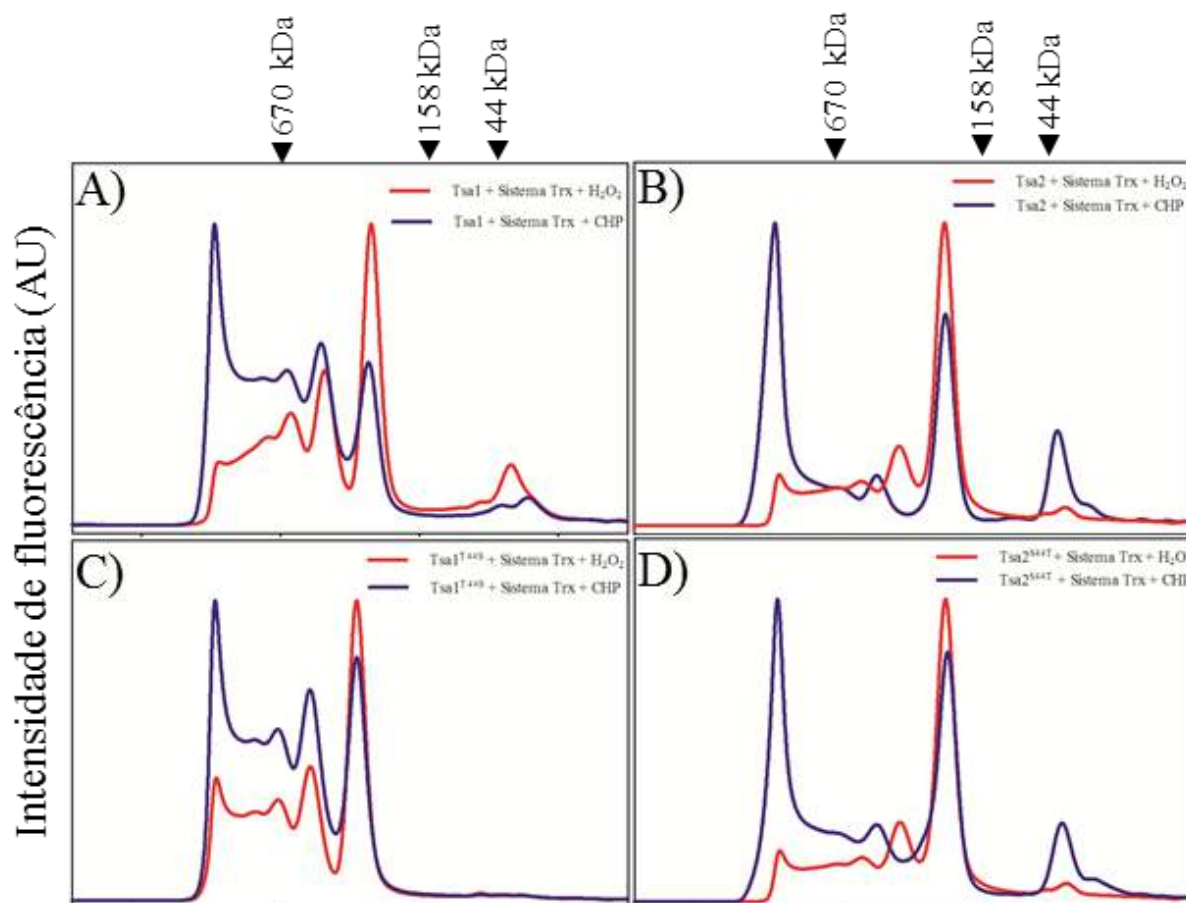


Figura 29. Análise da formação de HMW por SEC. Ensaios contendo Tsa1 (A), Tsa2 (B), Tsa1^{T44S} (C) e Tsa2^{S44T} (D) foram realizados *overnight* em tampão HEPES-NaOH pH 7.0 contendo 100 μ M de DTPA, 1 mM de azida, 1 mM de NADPH, 1 mg/mL (42.4 μ M) de Prx, 1 μ M de Trx1, 0.3 μ M de TrxR1 e concentrações 50 mM de H₂O₂ (linha vermelha) ou 50 mM de CHP (linha azul). Os padrões de peso molecular são representados pelas setas pretas na parte superior dos cromatogramas: tiroglobulina (bovina) (670 kDa), γ -globulina (bovina) (158 kDa) e ovalbumina (frango) (44 kDa). Representações gráficas foram geradas utilizando GraphPad Prisma 4.0. Para motivo de comparação dos tempos de retenção, todos os cromatogramas estão normalizados.

A análise dos mutantes Tsa1^{T44S} e Tsa2^{S44T} por outro lado, revela que neste caso os mutantes apresentaram formação de HMW muito semelhantes aos formadas pelas proteínas selvagens (Figura 29C e 29D). Neste contexto, os resultados indicam que apesar do resíduo Thr/Ser da tríade catalítica estar envolvido em maior (Thr) ou menor (Ser) suscetibilidade a superoxidação de C_P, como demonstrado pelos resultados obtidos nos experimentos espectrofluorométricos, de oxidação de NADPH e análises de superoxidação por SDS-PAGE, os dados sugerem que outros fatores estruturais adicionais devem estar relacionados a formação de complexos HMW.

Para uma análise mais aprofundada da formação de complexos HMW foram realizadas análises utilizando microscopia eletrônica de transmissão (TEM). Para as análises de TEM foram realizados ensaios de superoxidação com sistema Trx em altas concentrações de CHP. Em todas as análises de TEM foram testadas diferentes diluições de amostras, até serem

obtidas as melhores imagens (Figura 30). Para a proteína Tsa1 foram observados decâmeros e estruturas esféricas de tamanhos variados, resultados muito semelhantes aos observados por Jang e colaboradores (2004) (Figura 30A). A proteína Tsa2 também apresentou decâmeros e estruturas esféricas com tamanhos variados, mas foi verificada a presença de decâmeros empilhados (Figura 30B), estruturas estas não observadas no trabalho de Jang e colaboradores (2004).

Os resultados dos mutantes Tsa1^{T44S} e Tsa2^{S44T}, revelam que Tsa1^{T44S} apresentou estruturas decaméricas e empilhamento de decâmeros e o mutante Tsa2^{S44T} apresentou decâmeros, esferas de tamanhos variados e empilhamento de decâmeros (Figura 30C e 30D).

A formação de diferentes HMW tem sido demonstrada na literatura, Jang e colaboradores (2004) foram os primeiros a demonstrar a formação destas estruturas e as relaciona a atividade de chaperona molecular. Neste trabalho os autores relatam a existência de estruturas semelhantes em Tsa2. A análise dos resultados de TEM obtidos neste trabalho demonstram a formação de estruturas muito semelhantes às observadas por Jang e colaboradores após reações de superoxidação com CHP, inclusive no que se refere ao um tamanho variado das esferas. Por outro lado, foi verificada a formação de estruturas tubulares, ocasionada pelo empilhamento de decâmeros, as quais não foram visualizadas no trabalho pioneiro. Outro fato bastante interessante está relacionado à ocorrência de estruturas diferenciais, Tsa1 apresentou a formação de HMW em forma de esferas e em todo o conjunto de dados e não foi visualizada nenhuma estrutura formada pelo empilhamento de decâmeros. Tsa2 por sua vez, apresentou uma acentuada formação de HMW, sendo verificada a ocorrência de estruturas esféricas e tubulares de tamanhos variados. Já o mutante Tsa1^{T44S} apresentou somente estruturas tubulares, não sendo verificadas estruturas esféricas e finalmente o mutante Tsa2^{S44T} apresentou ocorrência de ambas estruturas, mas a maior parte formada a partir de empilhamento de decâmeros.

De fato decâmeros empilhados já foram observadas em outras 2-Cys Prx, mas não para Tsa1 (Aran *et al.*, 2011; Jang *et al.*, 2004; Kumsta e Jakob, 2009; Saccoccia *et al.*, 2012). Neste contexto essas análises revelam mais uma diferença marcante entre estas duas proteínas, apesar de diferenças funcionais relacionadas a estas estruturas de alto peso molecular, ainda não serem compreendidas.

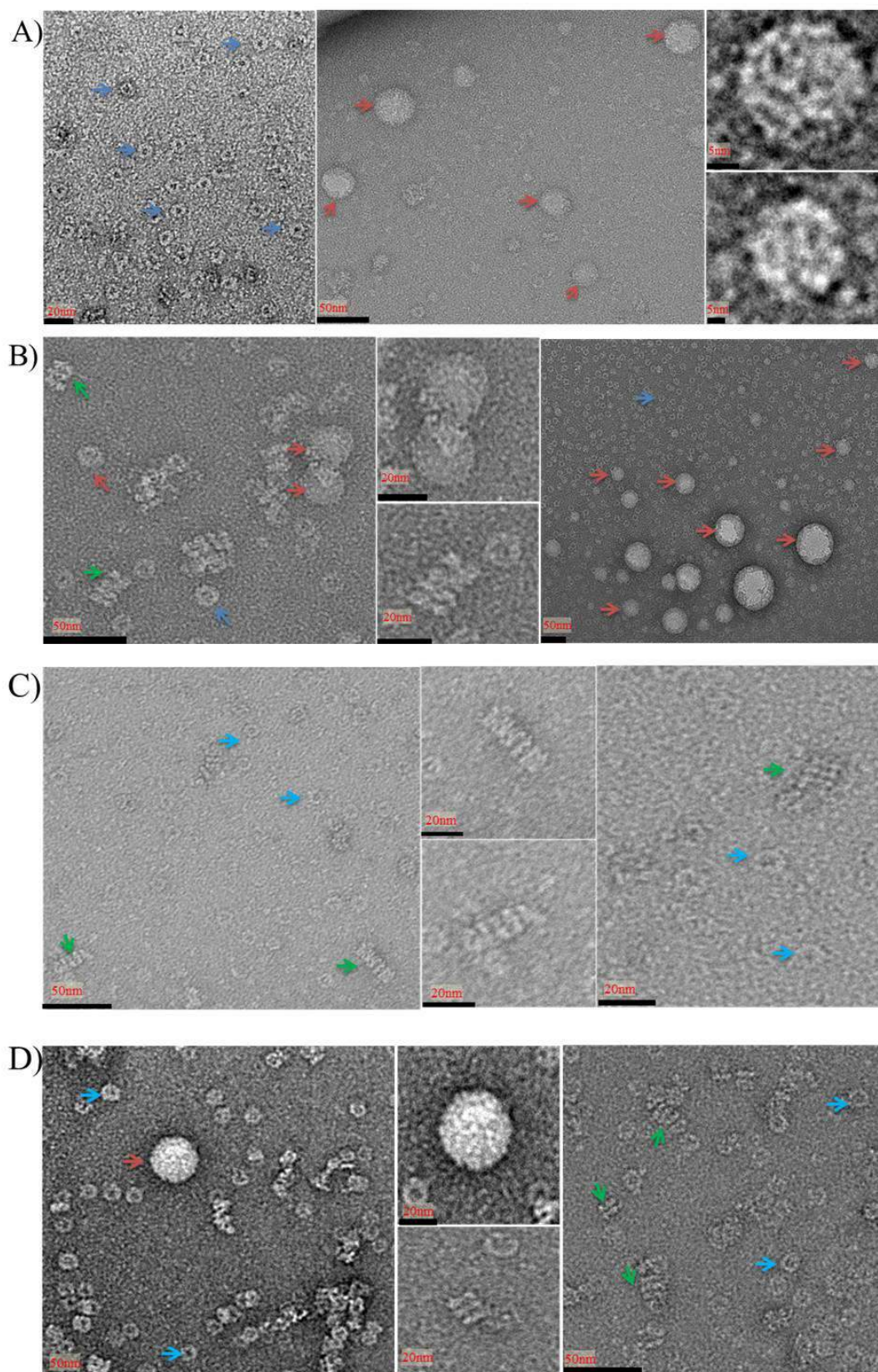


Figura 30. Resultado de TEM de amostras de Tsa1 e Tsa2, Tsa1^{T44S} e Tsa2^{S44T} superoxidadas. Análise de HMW de Tsa1 (A) e Tsa2 (B) Tsa1^{T44S} (C) e Tsa2^{S44T} (D) após reação com sistema Trx em altas concentrações de CHP. Ensaio para formação de espécies superoxidadas de Tsa1, foram realizados *overnight* a 4°C em tampão HEPES-NaOH 50 mM pH 7.4 contendo 100 µM de DTPA, 1 mM de azida, 600 µM de NADPH, 9.3 µM de Prx, 1 µM de Trx1, 0.3 µM de TrxR1 e concentração 10 mM de CHP. Após a reação as amostras foram filtradas em filtro Millex 0.22 µM (Millipore) e diluídas em concentrações variáveis até serem obtidas as melhores imagens.

As imagens de TEM foram adquiridas utilizando microscópio eletrônico de transmissão Jeol JEM-2100 operado a 200 kV e equipado com câmera F-416 CMOS (Tietz Video and Image Processing Systems). A escala em cada imagem. Setas azuis representam estruturas decaméricas, setas vermelhas estruturas esféricas de alto peso molecular e setas verdes representam a decâmeros empilhados.

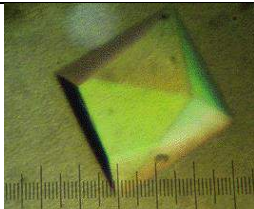
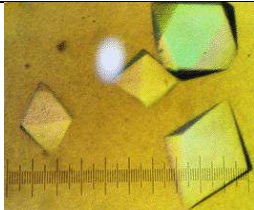
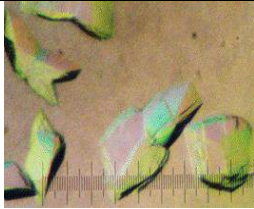
5.4 CAPÍTULO 4 - Cristalização de Tsa2 e Obtenção de Modelo por SAXS

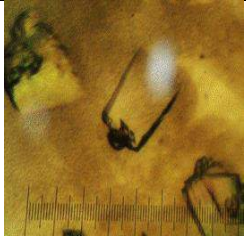
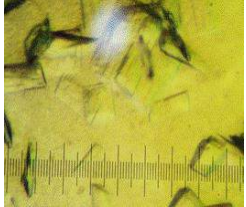
5.4.1 Cristalização de Tsa2 e Tsa2^{C47S}

Como pode ser observado ao longo da tese, encontramos diferenças funcionais e estruturais significativas entre Tsa1 e Tsa2. Uma vez que peculiaridades nas estruturas destas proteínas devem responder pelas diferenças observadas e a estrutura cristalográfica de Tsa1 foi determinada por nosso grupo de pesquisa (Oliveira, de *et al.*, 2007; Tairum *et al.*, 2012), também foi objetivo deste doutoramento a determinação da estrutura de Tsa2.

Inicialmente foram realizados experimentos de triagem de condições de cristalização da proteína reduzida por DTT ou oxidada com H₂O₂ ou CHP (seção Materiais e Métodos) e após a obtenção dos microcristais foram realizados experimentos de refinamento das condições de cristalização mais promissoras, variando a concentração de precipitante, sais e pH. Várias tentativas de obter cristais passíveis de difração foram realizadas, mas infelizmente não foram obtidos cristais com difração suficiente para resolver a estrutura de Tsa2. Alguns cristais obtidos são apresentados na Tabela 4. De todos os cristais obtidos poucos apresentaram difração, sendo a melhor difração obtida a 3.7 Å (Figura 31).

Tabela 4. Refinamento de cristalização da condição 0.1 M Acetato de sódio trihidratado pH 4.0 e 8% PEG 8000.

	Condição	Tratamento	Cristal
Tampão	0.1 M Acetato de sódio trihidratado pH 4.0		
Precipitante	6% de PEG 4000	1 mM H ₂ O ₂	
Sal	-		
Tampão	0.1 M Acetato de sódio trihidratado pH 5.6		
Precipitante	10% de PEG 4000	1 mM H ₂ O ₂	
Sal	-		
Tampão	0.1M Acetato de sódio trihidratado pH 3.6		
Precipitante	12% PEG 4000	1 mM H ₂ O ₂	
Sal	-		

Tampão	0.1 M Acetato de sódio trihidratado pH 4.3		
Precipitante	6% de PEG 4000	1 mM H ₂ O ₂	
Sal	-	-	
Tampão	0.1 M Acetato de sódio trihidratado pH 3.0		
Precipitante	6% de PEG 4000	1 mM H ₂ O ₂	
Sal	-	-	

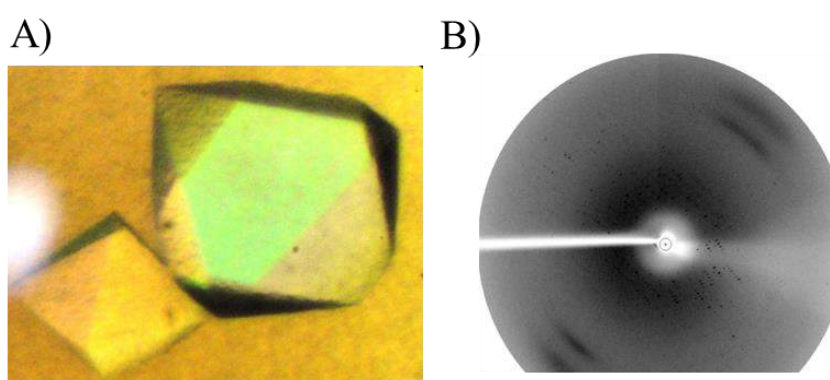


Figura 31. Cristais e difração dos cristais de Tsa2. **A)** Cristais de Tsa2 obtidos através do método de gota pendurada nas condições de acetato de sódio trihidratado 0.1 M pH 5.6 e 10% de PEG 4000. **B)** Padrão de dispersão registrado pela placa de imagem MARCCD. Os cristais foram crioprottegidos com glicerol (20%) e submetidos à difração de raio-X da linha de luz DB03-MX1. A melhor difração obtida foi a 3.7Å.

Uma vez que todas as tentativas de obtenção de cristais passíveis de difração da proteína Tsa2 selvagem não geraram resultados satisfatórios, foram efetuadas uma série de triagens utilizando o mutante Tsa2^{C47S}, o qual carrega a substituição da C_P por uma serina. Cabe ressaltar que esta abordagem foi adotada anteriormente com Tsa1, no qual apesar de inúmeras abordagens a proteína selvagem não formou cristais passíveis de difração, e com a substituição de C_P por Ser foi possível a determinação da estrutura (Oliveira, de *et al.*, 2007; Tairum *et al.*, 2012).



Como efetuado para a proteína Tsa2 selvagem, as condições em que apareceram microcristais, foram refinadas (concentração de proteína, sal, precipitante e pH), entretanto todos os cristais obtidos se apresentaram muito frágeis, dificultado muito os experimentos de difração, e difratando em baixa resolução (>7Å). Ainda com intuito de resolver a estrutura da proteína Tsa2 foi realizado um estágio no Laboratório de Biologia Estrutural de Proteínas no Centro de Investigaciones Biológicas (Madrid/Espanha) sob supervisão do Dr. Francisco Javier Medrano Martin.


Para a realização dos experimentos inicialmente foram realizadas otimizações nos protocolos de expressão e purificação das proteínas para a obtenção de altas concentrações proteicas com alto nível de pureza de uma forma rápida. Diferentemente dos experimentos realizados anteriormente foi optado pela retirada da *HIS-Tag* das proteínas Tsa2 e Tsa2^{C47S}. A retirada da *HIS-Tag* destas proteínas foi realizada utilizando a protease 3C (*Human rhinovirus 3C protease*) a qual reconhece o sítio Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro que está localizado entre a *HIS-Tag* e o códon de iniciação da proteína (Met). A possibilidade dessa digestão ocorreu, pois as proteínas foram expressas utilizando o plasmídeo pEPROEX-HT o qual insere o sítio reconhecido pela 3C protease.

Apesar dos cristais obtidos não apresentarem um padrão considerado ideal para experimentos de difração foram realizados refinamentos de todas as condições obtidas, sendo variados nos refinamentos o pH e a concentração de precipitante. Em condições que apresentavam duas soluções precipitantes foram testadas diferentes combinações destas soluções. Os melhores cristais obtidos são apresentados na tabela 5. Apesar de todas as proteínas testadas terem cristalizado em no mínimo uma condição testada, só foram obtidos cristais com boa formação para a proteína Tsa2.

Experimentos de difração de cristais foram realizados na linha BL13 do ALBA Synchrotron (Barcelona/Espanha). Infelizmente os cristais obtidos para a proteína Tsa2 não apresentaram difração, possivelmente por apresentarem uma alta desorganização interna, que não permite a difração.

Tabela 5. Refinamento das condições de cristalização das proteína Tsa2 realizadas no Laboratório de Biologia Estrutural de Proteínas no Centro de Investigaciones Biológicas (Madrid/Espanha)

	Condição	Proteína e Tratamento	Cristal
Tampão	0.1 M Fosfato de sódio e potássio pH6.2		
Precipitante	10% PEG 800	Tsa2	
Sal	0.2 M de NaCl	10 mM H ₂ O ₂	
Tampão	-		
Precipitante	15% PEG 8000	Tsa2	
Sal	0.5 M Sulfato de lítio	10 mM H ₂ O ₂	

Tampão			
Precipitante	15% PEG 8000	Tsa2	
Sal	0.5 M Sulfato de lítio	10 mM DTT	

5.4.2 Análise estrutural de Tsa2 utilizando SAXS

Uma vez que não foi possível estabelecer condições para cristalização e determinar a estrutura de Tsa2, optamos por fazer a análise estrutural de Tsa2, reduzida e oxidada, através de experimentos de SAXS utilizando a Linha B21 do *Diamond Light Source*, Oxfordshire, Inglaterra. Infelizmente, só foi possível obter o modelo estrutural da proteína Tsa2 na forma reduzida, os dados das outras amostras apresentaram padrões de qualidade baixos não permitindo a obtenção de modelos confiáveis. Para a obtenção do modelo, a proteína Tsa2 foi previamente reduzida com 10 mM de DTT e foram realizados experimentos com diferentes concentrações proteicas e os melhores resultados foram obtidos com concentração de 10 mg/mL. Após a coleta dos dados de SAXS a subtração do tampão foi realizada utilizando o programa ScÅtter (<http://www.bioisis.net/>).

A análise dos dados demonstra que Tsa2 apresenta estrutura muito semelhante a Tsa1, a análise comparativa dos dados experimentais de SAXS e a da curva teórica gerada com base na estrutura de Tsa1 (PDB 3SBC) pelo servidor FOXS (<http://modbase.compbio.ucsf.edu/foxs/index.html>) demonstra essa semelhança (Figura 32A). A análise de Kratky demonstrou que a proteína se apresentava bem enovelada no momento dos experimentos, sem agregados e regiões desenoveladas (Figura 32B) (Putnam *et al.*, 2007). O cálculo da P_r e D_{max} foi realizado utilizando o programa PRIMUSqt (Konarev *et al.*, 2003) e os modelos foram gerados utilizando o programa DAMMIF *online* (Franke e Svergun, 2009).

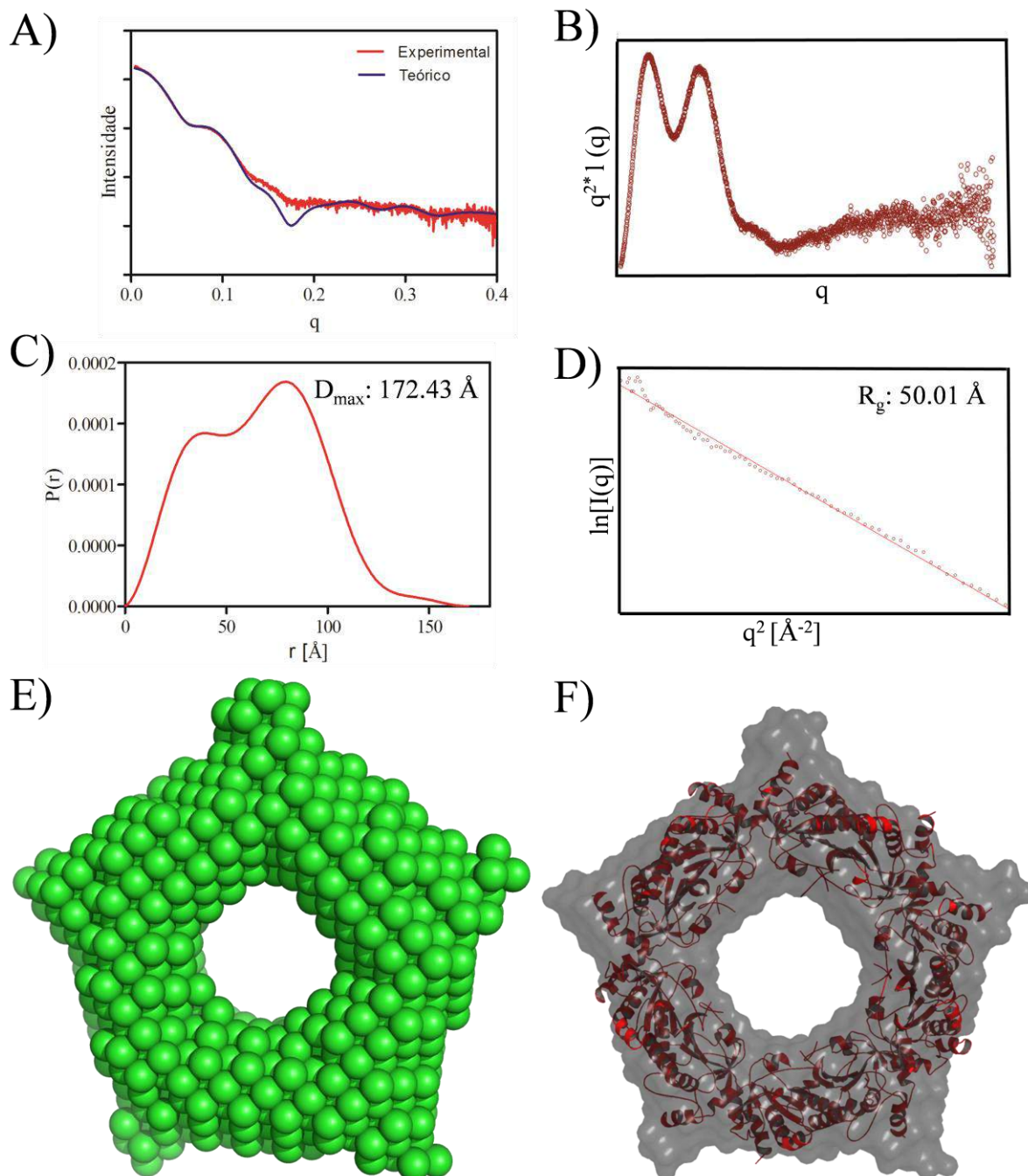


Figura 32. Modelo estrutural de Tsa2 obtido através de SAXS. A) Comparação dos dados experimentais de Tsa2 com a curva teórica de Tsa1. A comparação dos dados foi realizada utilizando o servidor FOXS (<http://modbase.compbio.ucsf.edu/foxs/index.html>) (Schneidman-Duhovny, Hammel e Sali, 2010; Schneidman-Duhovny *et al.*, 2013). B) A análise de Kratky foi realizada utilizando o programa Primusqt (Konarev *et al.*, 2003). C) Análise da Pr do modelo de Tsa2, realizado utilizando o programa Primusqt (Konarev *et al.*, 2003). D) Análise de Guinier de Tsa2 realizada através do programa Primusqt (Konarev *et al.*, 2003). E) Modelo de Tsa2 obtido através programa DAMMIF *online* (Franke e Svergun, 2009). F) Alinhamento do modelo de SAXS de Tsa2 com a estrutura de Tsa1 (PDB:3SBC), alinhamento realizado com o programa SUPCOMB (Kozin e Svergun, 2001b). As imagens das figuras E e F foram geradas através do programa PyMOL.

A tabela 6 demonstra os valores experimentais de SAXS de Tsa2 em comparações a valores teóricos obtidos a partir da estrutura de Tsa1 (PDB: 3SBC).

Tabela 6. Valores Experimentais do Modelo de Tsa2

	Tsa2 (SAXS)	Tsa1 (3SBC)
R_g (Å)	51.28	-
R_g (Å) real	51.25	49.83
D_{max} (Å)	172.43	137
V_{Porod} (Å³)	320231	-
Massa (kDa)	200.14	216

* Comparação dos dados de SAXS de Tsa2 com dados teóricos baseados estrutura da proteína Tsa1.

O modelo da proteína Tsa2 foi obtido através do programa DAMMIF *online* (<http://www.embl-hamburg.de/biosaxs/atsas-online/DAMMIF>), para a obtenção do modelo final foram gerados 20 modelos, para facilitar a obtenção dos modelos, foi imposto o grupo espacial P5 e simetria oblata. O NSD obtido foi de 0.663 com uma variação de 0.451, sendo utilizando para a geração do modelo final 19 modelos (Figura 32E). Para uma análise comparativa o modelo obtido foi alinhado com a estrutura de Tsa1 utilizando o programa SUPCOMB (Kozin e Svergun, 2001b), o resultado do alinhamento é apresentado na figura 32F e demonstra grande similaridade com a estrutura de Tsa1.

5.5 CAPÍTULO 5 - Importância da Thr44 na atividade e estabilização do decâmero de Tsa1

Adicionalmente a investigação conduzida, também participei da investigação do papel da Thr⁴⁴ e da Arg¹²³ de Tsa1 na reatividade sobre hidroperóxidos e manutenção do decâmero (Apêndice A - Manuscrito em fase final de elaboração que será submetido ao periódico *Nature Chemistry Biology*). Neste trabalho uma análise aprofundada da estrutura demonstrou que o C γ da Thr⁴⁴ atua como um doador de hidrogênio para o sistema π da Tyr⁷⁷ (Apêndice A, Figura 2). Este tipo de ligação é considerada uma ligação de hidrogênio não clássica, denominada de CH- π e vem apresentando um papel muito importante na estrutura e função de uma série de proteínas (Brandl *et al.*, 2001). De fato, a distância entre o C γ da Thr⁴⁴ e o centroide da Tyr⁷⁷ (3.30 - 3.68 Å) e o ângulo da ligação (113.23 - 134.40°) ficam dentro dos parâmetros teóricos determinados para esse tipo de interação (Apêndice A, Figura 3A) (Brandl *et al.*, 2001).

A avaliação de diferentes estruturas de 2-Cys Prx nos estados FF e LU demonstra que essa ligação é conservada (Apêndice A, Figura 3), sendo que em alguns casos a Tyr⁷⁷ é substituída por uma Phe, que também é capaz de realizar uma ligação CH- π com a Thr (Apêndice A, Figura S1). Vale ressaltar que quando as enzimas apresenta-se no estado LU, ocorrem modificações significativas no posicionamento do C γ da Thr⁴⁴ o que nos levou a postular que está mudança estaria relacionada com a transição dímero \rightarrow decâmero (Apêndice A, Figura 3). De acordo com as análises realizadas, a Thr⁴⁴ possui envolvimento na estabilização do decâmero através da CH- π com Tyr⁷⁷ e atividade através do átomo de O γ que possui interações polares com o S γ da C_P, que é estabilizada através de interações polares com o grupo guanidina da Arg¹²³. Tendo em vista as análises realizadas postulamos que a substituição da Thr⁴⁴ por outros aminoácidos pode desestabilizar a ligação CH- π afetando a estabilidade do decâmero e também a atividade da enzima. Para investigar estes aspectos, foram geradas substituições de Thr⁴⁴ por Ala, Ser, Val e Arg¹²³ por Lys e Gly e análises estruturais utilizando cromatografia de exclusão molecular (SEC) foram realizadas (Apêndice A, Figura 4). Em condições redutoras Tsa1, Tsa1^{T44A} e Tsa1^{T44S} apresentam estruturas decaméricas (Apêndice A, Figura 4A, 4B e 4C, linhas sólidas), já Tsa1^{T44V} apresenta uma mistura de dímeros e decâmeros, sendo que ocorre uma predominância de estruturas diméricas (Apêndice A, Figura 4D, linhas sólidas). Na forma oxidada (dissulfeto) ocorre um aumento da formação de estruturas diméricas (Apêndice A, Figura 4, linhas pontilhadas) com exceção da proteína Tsa1^{T44S} que apresentou apenas estruturas decaméricas, e Tsa1^{T44V} que

apresentou majoritariamente estruturas diméricas. Com relação às mutações na Arg¹²³ as mudanças estruturais foram bem menos significantes, e se apresentam com estrutura quaternária similar a Tsa1 selvagem (Apêndice A, Figura 4E e 4F).

Ensaio de atividade por meio de cinética de estado estacionário, utilizando ensaio de NADPH e determinação de constantes de pseudo-primeira ordem, revelaram que enquanto Tsa1^{T44S} apresentou parâmetros cinéticos similares a da enzima selvagem e Tsa1^{T44A} uma moderada redução na atividade, Tsa1^{T44V} apresentou atividade peroxidásica altamente comprometida (Apêndice A, Figura 5, Tabela 1). No caso dos mutantes Tsa1^{R123G} e Tsa1^{R123K} foi verificada apenas uma atividade residual, dados esses já demonstrados na literatura para outras 2-Cys Prx típicas (Flohé *et al.*, 2002; Montemartini *et al.*, 1999; Portillo-Ledesma *et al.*, 2014).

Para um maior entendimento da importância da ligação CH- π foi gerado o mutante Tsa1^{Y77A} e em seguida avaliamos a atividade e o grau oligomérico deste mutante bem como de Tsa2 o qual apresenta a substituição natural de Thr⁴⁴ por uma Ser. Análises de SEC demonstram que Tsa2 migra exclusivamente em decâmeros, independente do estado redox da enzima, resultado semelhante ao observado no mutante Tsa1^{T44S} (Apêndice A, Figura 9A). Em contraste Tsa1^{Y77A} apresentou somente estrutura dimérica, demonstrando que a interação CH- π apresenta grande importância na manutenção da estrutura decamérica (Apêndice A, Figura 9B). De fato, análises de modelos contendo as substituições de Thr estudadas no trabalho indicam que as substituições devem influenciar de forma significativa a rede de interações polares existente entre os resíduos da tríade catalítica (Figura 33).

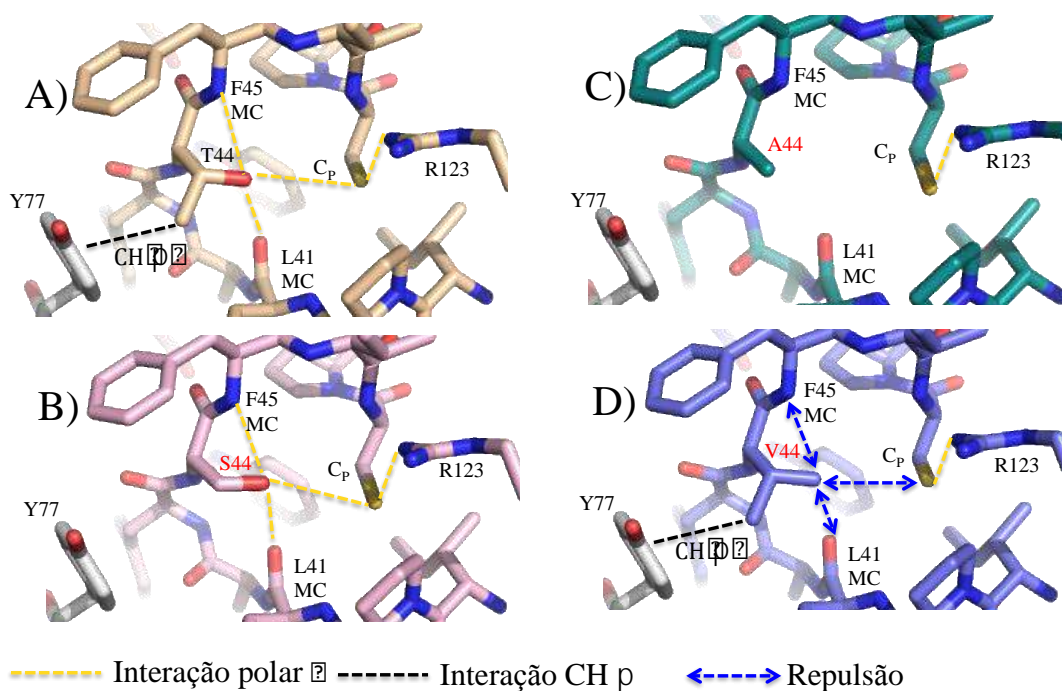


Figura 33. Efeitos teóricos da substituição de Thr sobre interações moleculares no sítio ativo de Tsa1. (A) Tsa1 representado *sticks* evidenciando as interações polares (linha tracejada amarela) e CH π (linha tracejada amarela). (B) Tsa1^{T44A}; (C) Tsa1^{T44S} e (D) Tsa1^{T44V}, a linha contendo a dupla seta em azul denota repulsão. Os modelos teóricos foram criados utilizando o programa WINCOOT, utilizando a estrutura de Tsa1 (3SBC), a substituição foi realizada mantendo a mesma posição da Thr.

Conjuntamente, nossos resultados demonstraram de forma sistemática a importância da ligação CH- π entre a Thr da tríade catalítica e a Tyr do dímero adjacente para a manutenção do decâmero e atividade peroxidásica máxima de Tsa1. Os resultados compõem um manuscrito em fase final de elaboração que será submetido ao *Nature Chemical Biology* (Apêndice A).

Como pode ser constatado na figura 33, assunto que não foi abordado no manuscrito, o resíduo seja de Thr ou Ser é capaz de estabelecer ligações polares com o grupo amina de Phe⁴⁵ e o grupo carboxila da Leu⁴¹. Cabe ressaltar que estas interações foram detectadas em todos os sítios ativos analisados de Tsa1, o que nos parece importante uma vez que até o presente momento estas interações não foram levadas em consideração na literatura. Cabe ressaltar que a análise de enzimas 2-Cys Prx típicas no estado oxidado em dissulfeto (LU) revela que estas interações são desfeitas.

Para avaliar a importância destas interações foram analisadas todas as estruturas em FF (estado reduzido) presentes no banco de dados pdb. De fato como pode ser observado na figura 34 estas interações são conservadas entre as estruturas. A análise das distâncias do O γ de Thr⁴⁴ para O da cadeia principal do resíduo de Leu (substituído por Ala em enzimas de procaríotos) é invariavelmente abaixo de 3Å, enquanto que para o N da Phe, conservado entre

eucariotos e procariotos, varia de 3-4Å, que representa sempre uma distância similar ao S_{λ} da cisteína peroxidásica. Para uma melhor compreensão calculamos as distâncias médias das interações polares entre os resíduos (Tabela 7). Neste contexto, estas análises incluem um pouco mais de complexidade e propõe a participação do O_{γ} da Thr da tríade catalítica na estabilização do tiolato de C_p .

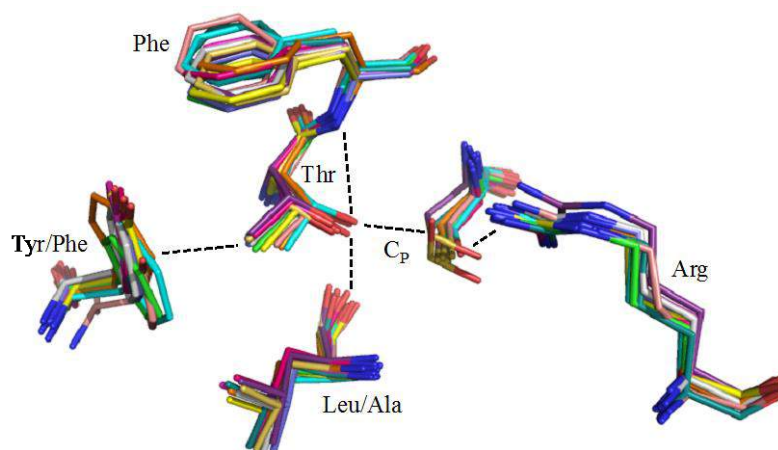


Figura 34. Sobreposição de estruturas de 2-Cys Prx típicas no estado FF revelando a rede de interações polares entre aminoácidos do sítio ativo. Estruturas de 2-Cys Prx típicas no estado FF e coloridas CPK: O: vermelho; S: Laranja; N: Azul. A cor dos carbonos diferem as estruturas analisadas: verde (4MA9); azul claro (1N8J); cereja (4K1F); amarelo (2I81); rosa claro (3ZTL);cinza (3QMP); azul claro (3TKR); laranja (2Z9S); azul celeste (1QMV); azul ardósia (3SBC); caqui (3TKP) e ametista (3TKS).

Tabela 7. Distancias médias da interações entre o O_{λ} de Thr⁴⁴ para O de da cadeia principal do resíduo de Leu /Ala e para o N da Phe.

Coordenada	AA	Média (Å)	Coordenada	AA	Média (Å)
4MA9	A40 (O-MC)	2.690	3TKR	L ⁸¹ (O-MC)	2.648
	F44 (N-MC)	3.348		F ⁸⁵ (N-MC)	3.113
	C46 (Sg)	3.206		C ⁸⁷ (Sg)	3.412
1N8J (A-J)	A ⁴⁰ (O-MC)	2.612	2Z9S	L ⁴⁶ (O-MC)	2.835
	F ⁴⁴ (N-MC)	3.321		F ⁴⁴ (N-MC)	3.212
	S ⁴⁶ (Og)	3.487		S ⁴⁶ (Og)	3.340
1N8J(K-T)	A ⁴⁰ (O-MC)	2.583	1QMV	L ⁴⁵ (O-MC)	2.669
	F ⁴⁴ (N-MC)	3.351		F ⁴⁹ (N-MC)	3.181
	S ⁴⁶ (Og)	3.455		C ⁵¹ (Sg)	3.014
4K1F	L ⁴⁶ (O-MC)	2.726	3SBC	L ⁴¹ (O-MC)	2.641
	F ⁵⁰ (N-MC)	3.062		F ⁴⁵ (N-MC)	3.135
	C ⁵² (Sg)	3.298		S ⁴⁷ (Og)	3.535
2I81	L ⁴⁴ (O-MC)	2.760	3TKP	L ⁸¹ (O-MC)	2.578
	F ⁴⁸ (N-MC)	3.422		F ⁸⁵ (N-MC)	3.122
	C ⁵⁰ (Sg)	3.320		C ⁸⁷ (Sg)	3.446

3ZTL	A ⁴² (O-MC)	2.665	3TKS	L ⁸¹ (O-MC)	2.594
	F ⁴⁶ (N-MC)	3.680		F ⁸⁵ (N-MC)	3.044
	C ⁴⁸ (Sg)	3.203		C ⁸⁷ (Sg)	3.106
3QPM	L ¹⁰⁷ (O-MC)	2.712			
	F ¹¹¹ (N-MC)	3.132			
	C ¹¹³ (Sg)	3.396			

Os resultados das análises sugerem que o resíduo de Thr não deve estar somente relacionado com a manutenção de C_P em S⁻, mas que estes resíduos poderiam estar relacionados com o direcionamento do hidroperóxido para o tiolato, para a formação do complexo de transição e proporcionar o mecanismo de substituição nucleofílica do tipo SN2 conforme proposto por Hall e colaboradores (2010). Neste contexto, as interações polares efetuadas entre os átomos da cadeia principal de Leu e Phe inicialmente manteriam e criariam um ambiente carregado positivamente. Essa ambiente permitiria a entrada do hidroperóxido, em um segundo momento o oxigênio distal da molécula do hidroperóxido seria estabilizado por interações polares com hidrogênios oriundos do grupo amino de Phe⁴⁵ e do O γ da Thr44. O oxigênio proximal do hidroperóxido seria positivado por um hidrogênio do grupo amino da Arg123, o que permitiria o ataque do tiolato sobre a ligação O-O do hidroperóxido, com a formação de cisteína ácido sulfênico (C_P-SOH) e liberação do grupo abandonador (OH⁻). Esse processo desencadearia o desenovelamento da alfa hélice e transição do estado FF→LU, com consequente disrupção do decâmero (Figura 35).

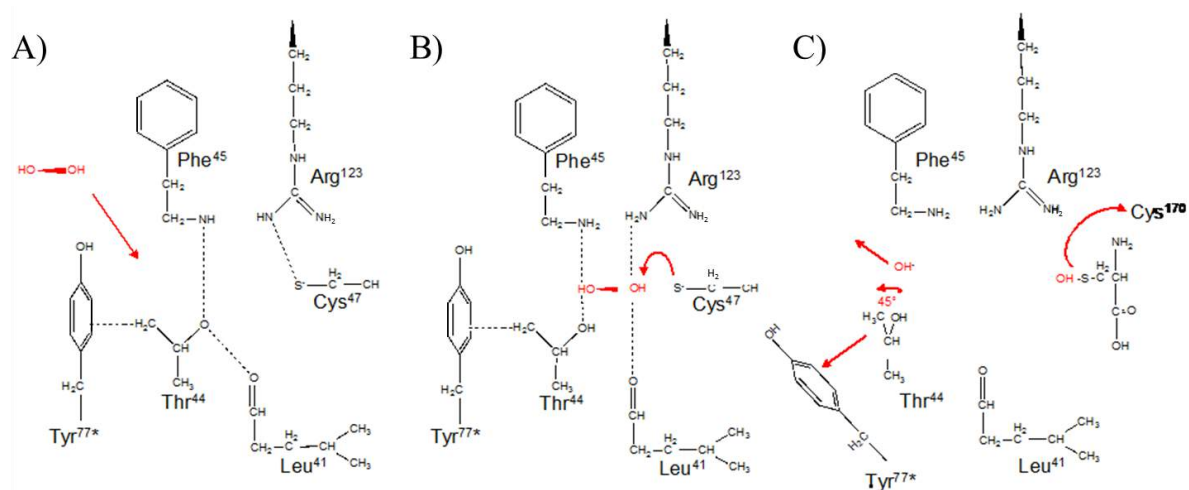


Figura 35. Mecanismos proposto para a estabilização do hidroperóxido e formação do complexo de transição de 2-Cys Prx típicas. A) Leu e Phe inicialmente mantem e criam um ambiente carregado positivamente para permitir a entrada do hidroperóxido. **B)** Em um segundo momento o oxigênio distal da molécula do hidroperóxido seria estabilizado por interações polares com hidrogênios oriundos do grupo amino de Phe⁴⁵ e do O γ da Thr44, enquanto que o oxigênio proximal do hidroperóxido seria positivado por um hidrogênio do grupo amino da Arg123. **C)** Ocorre ataque do tiolato sobre a ligação O-O do hidroperóxido, com a formação de cisteína ácido sulfênico (C_P-SOH) e liberação do grupo abandonador (OH⁻) que desencadearia o desenovelamento da alfa hélice e transição do estado FF→LU.

Entretanto, este mecanismo precisa ser investigado de forma mais consistente. De fato, a utilização de mutantes esbarra no fato de que as interações propostas de Phe e Leu são efetuadas por átomos da cadeia principal. Uma abordagem seria a substituição de Phe45 e a Leu41 por uma Pro, com o intuito de afetar o posicionamento da cadeia principal destes aminoácidos. No caso da Leu uma substituição adequada poderia ser por Asp, pois a cadeia lateral da Leu estabelece interações de caráter apolar com aminoácidos vicinais e uma alteração desta natureza deve também perturbar a posição de sua cadeia principal.

6 CONSIDERAÇÕES FINAIS

Este trabalho teve por objetivo uma análise detalhada da redundância de duas 2-Cys Prx de *S. cerevisiae*, Tsa1 e Tsa2, a qual é proposta por alguns autores (Chae, Kim e Gene, 1993; Netto *et al.*, 2007; Ross *et al.*, 2000). Portanto, neste trabalho foram realizadas análises comparativas das proteínas Tsa1 e Tsa2 com relação à atividade sobre seus substratos redutores e oxidantes e relações com a superoxidação de C_P levando em conta ambos os substratos. Estes aspectos também foram relacionados com transições estruturais que levam a formação de complexos de alto peso molecular (HMW) em 2-Cys Prx como consequência da superoxidação de C_P.

Através de experimentos envolvendo consumo de NADPH demonstramos que Tsa1 e Tsa2 possuem uma alta atividade tanto para H₂O₂ quanto para peróxidos orgânicos (CHP e *t*-BOOH) e ambas são reduzidas com a mesma eficiência tanto por Trx1 quanto por Trx2. Cabe ressaltar que este tipo de abordagem não havia sido efetuado de forma comparativa apesar destas proteínas serem largamente estudadas (Park *et al.*, 2000; Watzlawik *et al.*, 2006). Também constatamos que altas concentrações de peróxido orgânico, em especial o CHP, quando comparado com H₂O₂ leva a uma acentuada inibição no consumo de NADPH o que sugeriu fortemente a superoxidação da C_P a C_PSO_{2/3}. Para investigar este aspecto foram efetuados ensaios de consumo de NADPH com quantidades variáveis de peróxidos e a detecção das formas oligoméricas foi efetuada por meio de SDS-PAGE, conforme metodologia descrita anteriormente (Nelson *et al.*, 2013; Perkins *et al.*, 2013). Os resultados demonstraram que a inibição observada nos ensaios de consumo de NADPH é causado pela superoxidação de C_P a C_PSO_{2/3}, e também confirmaram que Tsa2 apresenta-se muito mais resistente a superoxidação quando comparada a Tsa1.

Adicionalmente, foram efetuadas análises cinéticas de fluorescência, método pelo qual foi possível detectar a superoxidação de outra classe de peroxirredoxina (AhpE) de *Mycobacterium tuberculosis* (Reyes *et al.*, 2011), que revelaram que mesmo pequenas doses de hidroperóxidos são capazes de superoxidar Tsa1. Entretanto, nas condições testadas não foi possível detectar a superoxidação de Tsa2, o que confirma os resultados obtidos por oxidação de NADPH e observados também em SDS PAGE.

Entretanto, os dados dos ensaios fluorométricos foram bastante contrastantes com os obtidos por cinética de estado estacionário. De fato nos experimentos envolvendo fluorometria foram utilizadas doses muito menores de hidroperóxidos (máximo de 10 equivalentes molares) que nos ensaios de cinética estacionária de oxidação de NADPH

utilizando o sistema Trx, onde a indícios de superoxidação de Tsa1 só foi detectada em altas concentrações de hidroperóxidos (50-500 eq.). Neste contexto, os dados de fluorescência devem indicar que somente uma pequena parcela das proteínas deveria ser responsável pelo aumento de fluorescência detectado. Para investigar este aspecto, primeiramente foram feitas análises de SDS PAGE de Tsa1 e Tsa2 tratadas com altas quantidades de hidroperóxidos (100 eq), entretanto não foi possível observar qualquer indicio de superoxidação.

As diferenças de suscetibilidade/resistência foram então investigadas em função do ambiente do sitio ativo. De fato, a diferença mais marcante reside na substituição de um resíduo de Thr de Tsa1 por uma Ser em Tsa2, que podem realizar interações polares similares, mas não idênticas no sitio ativo. Para investigar a influencia desta substituição foram utilizados mutantes carreando substituições reciprocas (Tsa1^{T44S} e Tsa2^{S44T}). Os resultados revelaram que esta única substituição foi capaz de conferir alta resistência a superoxidação por CHP para Tsa1^{T44S} e aumentar a sensibilidade de forma significativa para Tsa2^{S44T}, demonstrando a importância deste aminoácido para a resistência e suscetibilidade ao processo de superoxidação.

Análises da sequencia de aminoácidos de diversas 2-Cys Prx revelaram que esta substituição de Thr-Ser só é compartilhada por um único organismo eucariótico (*Harpegnathos saltator*) sendo que as demais são só detectadas para Prx 2-Cys de bactérias, o que deve indicar uma maior necessidade de enzimas mais resistentes a superoxidação nestes organismos.

Por fim também investigamos a formação de espécies HMW em resposta a processos hiperoxidativos por cromatografia de exclusão molecular e microscopia eletrônica de transmissão. Os resultados de SEC revelaram que as espécies HMW formadas entre Tsa1 e Tsa2, não são idênticas uma vez que Tsa1 possui diversas espécies intermediarias entre decâmeros e complexos HMW. Entretanto, por esta abordagem não foi possível detectar diferenças significativas no perfil cromatográfico de Tsa1^{T44S} e Tsa2^{S44T} quando comparadas com as proteínas selvagens. Por outro lado, quando as enzimas foram avaliadas por TEM foi possível demonstrar que enquanto Tsa1 forma estruturas HMW esféricas de tamanhos distintos, Tsa2 não apresenta somente estruturas esféricas, mas também empilhamento de estruturas toróides decaméricas. Em contraste com os resultados de SEC foi possível observar por TEM que a substituição reciproca do resíduo de Thr/Ser, surtiu efeitos na estrutura de complexos de alto peso molecular, uma vez que foram detectadas estruturas esféricas para Tsa2^{S44T} e em Tsa1^{T44S} foram detectadas estruturas toróides decaméricas.

Conjuntamente nossos resultados revelam que Tsa1 e Tsa2 diferem fortemente no que concerne a suscetibilidade a superoxidação de C_p , a qual é fortemente dependente de Trx, e também na formam estruturas HMW, distintas. Sendo que em ambos os processos a presença do resíduo de Thr/Ser está fortemente envolvida. Portanto, nossos resultados demonstram de maneira clara que Tsa1 e Tsa2 não são proteínas redundantes e que apesar das semelhanças na estrutura primária elas podem apresentar funções distintas ou complementares na célula.

7 PERSPECTIVAS

1. Determinação das constantes de superoxidação para H₂O₂ e CHP utilizando fluorescência intrínseca do triptofano.

Como demonstrado nesta tese a análise da fluorescência intrínseca do triptofano apresenta-se como uma importante metodologia para determinação de constantes de superoxidação. Neste contexto nos parece de grande importância a determinação das constantes de superoxidação de C_P de Tsa2, Tsa1^{T44S} e Tsa2^{S44T}. Esses resultados são fundamentais para a finalização do manuscrito que compreenderá os resultados obtidos nesta tese.

2. Determinação da estrutura de HMW de Tsa1 e Tsa2 utilizando Crio-Microscopia de eletrônica de transmissão

O entendimento das transições estruturais para formação de HMW ainda é muito pouco compreendido, foi demonstrado aqui que o processo de superoxidação é fundamental para a transição de LMW para HMW. Um ponto que inviabilizou a determinação de estruturas HMW por Crio-TEM foi a grande heterogeneidade das amostras, sendo visualizados em nossos experimentos de *negative stain* diversas formas variando de dímeros a estruturas de ~1000 kDa. Tendo em vista essa heterogeneidade é muito difícil a obtenção de uma reconstrução molecular, por outro lado trabalhos presentes na literatura que realizaram reconstruções de HMW de peroxirredoxinas estabilizaram estes complexos através da acidificação do meio onde a proteína está contida. Segundo esses autores, condições ácidas mimetizam a superoxidação da C_P de forma homogênea proporcionando a obtenção de estruturas perfeitas análises por Crio-TEM (Radjainia *et al.*, 2015; Saccoccia *et al.*, 2012).

3. Determinação da estrutura de Tsa2

Apesar das diversas abordagens utilizadas para obter a estrutura de Tsa2, não foi possível a obtenção de cristais passíveis de difração. Apesar das dificuldades encontradas para obtenção desta estrutura novas abordagens ainda são válidas pois a estrutura da proteína Tsa2 poderia explicar em detalhes as diferenças com Tsa1 relacionadas a estrutura e reatividade. Assim novos experimentos de cristalização utilizando diferentes abordagens tais como proteólise limitada e etapas adicionais de cromatografia para preparo de amostras podem viabilizar a obtenção de cristais passíveis de difração.

4- Determinação das constantes de reatividade de Tsa1 e Tsa2 com peróxidos orgânicos naturais

Um ponto importante a ser destacado é que os hidroperóxidos orgânicos *t*-BOOH e CHP são hidroperóxidos sintéticos, não sendo encontrados naturalmente nos orgânicos. Assim seria de grande importância a determinação de constantes de reatividade de Tsa1 e Tsa2 com hidroperóxidos orgânicos naturais tais como peróxido de ácido oleico, ácido linoleico e colesterol, dentre outros. Além disso, a análise da susceptibilidade a superoxidação por esses hidroperóxidos é de grande importância, pois pode contribuir para o entendimento em detalhes deste fenômeno *in vivo*.

5- Avaliação do papel da Phe45 e Leu41 na atividade de Tsa1

As análises estruturais de estruturas de diferentes Prx revelaram que a Thr44 possui interações polares com Phe45 e a Leu41. Essa interações aparentemente apresentam grande importância para a atividade das 2-Cys Prx, assim estudos utilizando mutagênese sítio dirigida apresentam-se fundamentais para determinar a real importância dessas interações e podem contribuir para o entendimento da formação do complexo de transição.

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APÊNDICE A

Tairum, C.A.; Santos, M. C.; **Breyer, C. A.**; Geyer, R, R; Nieves, C. J.; Portillo-Ledesma, S.; Ferrer-Sueta, J.; Toledo, J.C. ;Toyama, M. H.; Augusto, O.; Netto, L. E. S.; Oliveira, M. A. **THE THREONINE RESIDUE OF PEROXIREDOXIN CATALYTIC TRIAD STABILIZES ENZYME DECAMERIC STRUCTURE.** Manuscrito em fase final de elaboração que será submetido ao periódico *Nature Chemical Biology*.

THE THREONINE RESIDUE OF PEROXIREDOXIN CATALYTIC TRIAD STABILIZES ENZYME DECAMERIC STRUCTURE

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Abstract: Typical 2-Cys Peroxiredoxins (2-Cys Prxs) are proteins which use a reactive cysteine (Cys_p) to reduce hydroperoxides at extraordinary rates. Cys_p, together with a Thr (or Ser) and an Arg, compose the catalytic triad. During catalysis, 2-Cys Prx switch between decamer and dimers in a redox dependent manner. Analysis of yeast Tsa1 structure revealed a highly conserved non-conventional hydrogen bond (CH- π) between Thr44 of a dimer with Tyr77 of adjacent dimer. Investigation of Tsa1 proteins mutated at Thr44 indicated that Thr44-Tyr77 bond stabilizes decamer structure. As a general trend, mutations favoring the dimer structure also provoked decreased reactivity, suggesting that Thr44 links oligomeric state with peroxidase activity of Tsa1. Moreover, Tsa1^{Y77A} mutant appeared as dimer and displayed reduced peroxidase activity, further indicating that Thr44-Tyr77 interaction is relevant for decamer stabilization. The results indicated that the conserved Thr residue displays roles in both decamerization and reactivity towards hydroperoxide of 2-Cys Prx.

Introduction

Typical 2-Cys peroxiredoxins (2-Cys Prx) from the Prx1/AhpC sub-group comprise a large class of thiol-specific antioxidant enzymes able to reduce hydroperoxides with high efficiency ($10^6 - 10^8 \text{ M}^{-1} \text{ s}^{-1}$) and specificity¹⁻⁵. In the catalysis, the typical 2-Cys Prx use one conserved cysteine residue, called peroxidatic cysteine (Cys_P), which is responsible for the reduction of hydroperoxide, forming a sulfenic acid in the Cys_P ($\text{Cys}_P\text{-SOH}$). The basic units of these enzymes are homodimers (α_2) and during the catalytic cycle a second Cys residue, the so-called resolving cysteine (Cys_R) reacts with the Cys_P in sulfenic acid form of the adjacent monomer to form an intermolecular disulfide bond (reviewed in 6,7). A partial unfolding of the α helix containing Cys_P is required for the inter-molecular disulfide formation and thioredoxin (Trx) is commonly the electron donor to reduce the disulfide^{6,7}. Therefore, during catalysis, Prx1/AhpC enzymes assume two structural states: Fully Folded (FF) and locally unfolded (LU). Alternatively, $\text{Cys}_P\text{-SOH}$ can further react with additional hydroperoxide molecules giving rise to overoxidized states such as sulfinic ($\text{Cys}_P\text{-SO}_2\text{H}$) and sulfonic ($\text{Cys}_P\text{-SO}_3\text{H}$) acids (Figure 1). These overoxidized forms are not reducible by Trx. In eukaryotic organisms, $\text{Cys}_P\text{-SO}_2\text{H}$ can be reduced back to $\text{Cys}_P\text{-SOH}$ by a sulfiredoxin catalyzed reaction in a process dependent on ATP⁶.

In the FF state, Cys_P is located in the first turn of an α -helix, surrounded by the Thr (or Ser, in some cases) and the Arg^{7,8}. The three residues compose the so called Prx catalytic triad, which is implicated in the reactivity and specificity of Prx towards hydroperoxides^{6,7}. Transient interactions in the active site of Prxs are thought to play crucial roles, by stabilizing the transition state of a S_N2 reaction. In the transition state, the nucleophilicity of the thiolate and the electrophilicity of the hydroperoxide are both increased^{9,10}. Some studies revealed the importance of Arg residue to Prx reactivity, since the substitution of the residue profoundly

affects Cys_{SP} reactivity¹¹⁻¹⁵. In contrast, studies involving the substitution of the conserved Thr residue are very scarce^{14,15}.

Another intriguing feature of Prx1/AhpC enzymes is their ability to switch among distinct quaternary structures, which is affected by redox state among other factors^{6,7,13-19}. Prx1/AhpC enzymes are α_2 homodimers that can associate in $(\alpha_2)_5$ decamers, *i.e.* pentamers of dimers. The factors governing the stabilities and possible significance of these distinct quaternary structures are not completely understood, but intermolecular disulfide formation favors dimer configuration. Noteworthy, in AhpC from *Salmonella typhimurium* (StAhpC), decamerization was associated with an increase in the peroxidase activity¹. Therefore, reduced or overoxidized Prx1/AhpC enzymes appear preferentially as decamers (or even higher molecular weight complexes), whereas intermolecular disulfide destabilize decamers into dimers^{1,18-21}.

Saccharomyces cerevisiae presents two very similar cytosolic 2-Cys Prx enzymes, named Thiol specific antioxidant (Tsa1 and Tsa2 - 96% of similarity and 86% of identity)^{22,23}. One of the few differences in the primary structure between these two enzymes is the fact that Tsa1 possesses a Thr residue in the catalytic triad (Thr44), whereas Tsa2 presents a serine residue (Ser⁴⁴). We had observed before that Tsa1 and Tsa2 reactivity towards hydrogen peroxide is very similar ($2.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ to Tsa1 and $1.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ to Tsa2), but their Cys_{SP} pK_a values differ significantly².

In this work, analysis of all the available 2-Cys Prx structures showed that the catalytic Thr (Thr44 in yeast Tsa1) is located at the interface between two dimers, making a non-conventional hydrogen bond with the π system of a Tyr/Phe residue (Tyr77 in yeast Tsa1) (CH- π bond) of the adjacent dimer. Replacements of Thr44 to distinct residues altered the quaternary structure and the activity of the enzyme. Together, our results indicate that Thr44

displays roles in the stabilization of Tsa1 decameric structure and also in hydroperoxide reduction.

Results

Structural characterization of Tsa1 mutants - Reversible transition between dimer and decamer is a remarkable feature of 2-Cys Prx with consequences in their activity^{1,6,7,13,19,21}. Tsa1 crystallographic structure²⁴ was re-analyzed (Figures 2A and 2B). In reduced form, the (α_2)₅ decamer is stabilized by several non-covalent interactions at the dimer-dimer interface (Figure 2C). Among these interactions, the C γ atom of Thr44 acts as hydrogen-bond donor to the π -system of Tyr77 (Figure 2D). Such CH- π bond is a non-conventional hydrogen bond that plays important contributions in protein structure and function (reviewed in 25,26). In fact, all five dimer-dimer interfaces of the Tsa1 structure have two CH- π hydrogen bonds between Thr44 and Tyr77 residues (Figure 3A). The presence of CH- π hydrogen bonds is supported by the fact that the angles (113.23 - 134.40°) and distances (3.30 - 3.68 Å) measured are consistent with such kind of interaction^{25,26}. The described CH- π hydrogen bond involves residues that are part of the so-called region I (Thr44) and region II (Tyr77), which are complementary at the dimer-dimer interface¹⁹.

Remarkably, CH- π hydrogen bonds between the catalytic Thr and Tyr of the adjacent dimer are fully conserved among all available structures of 2-Cys Prx (Prx1/AhpC) in the FF state (Figures 3 A-C, 3E). In some cases, Tyr (Tyr77 in yeast Tsa1) is substituted by a Phe residue, which also has an aromatic side chain and therefore can accept H bonds through electrons of its π -system (Figures 3 A-C, 3E; See Supplementary Fig. 1). In the LU state, the orientation between Thr44 and Tyr77 residues changes, and consequently the CH- π hydrogen bond is disrupted (Figures 3D,F).

According to our analysis, the catalytic Thr participates not only in catalysis^{9,10}, but also displays a structural role. While Thr44 C γ atom is involved in the interaction with the

neighbor dimer, the O γ atom has a polar interaction with the S γ atom of Cys_{SP}, which is also stabilized by polar contacts with the guanidine group of Arg123 (Figures 2D, 3B). Therefore, we hypothesized that substitution of Thr44 by other residues would affect decamer stability by disturbing the two CH- π hydrogen bonds per interface, which might also destabilize other neighbor non covalent bonds at the dimer-dimer interface that are important for decamer stabilization. Noteworthy, the catalytic triad of Tsa1 is located near the dimer-dimer interface delimited by Tyr77 (see Supplementary Fig. 2), with Thr44 providing physical interaction between the catalytic center and the dimer-dimer interface.

To experimentally evaluate the effects of Thr44 substitutions on the Tsa1 quaternary structure, we employed size exclusion chromatography (SEC). Samples were reduced with 5 mM tris(2-carboxyethyl)phosphine (TCEP) or previously reduced with TCEP (5 mM) and then oxidized with H₂O₂ (1.2 eq.). Under reducing conditions, Tsa1^{WT}, Tsa1^{T44A} and Tsa1^{T44S} eluted as decamers (Figures 4A-C, solid lines), whereas Tsa1^{T44V} eluted as a mixture of dimers and decamers, with dimeric forms being clearly predominant (Figure 4D, solid line). As expected, the oxidized (disulfide) samples favored dimerization⁷ as compared with the reduced forms (Figure 4, dashed lines). The exception was Tsa1^{T44S} that migrated exclusively as decamers in both oxidized and reduced forms (Figure 4C, dashed line). In contrast, Tsa1^{T44V} migrated mainly as dimer (Figure 4D, dashed line). With intermediate behavior, equivalent amounts of dimers and decamers of Tsa1^{WT} and Tsa1^{T44A} were detected in oxidizing conditions (Figures 4A,B, dashed lines). In summary, Thr mutations provoked dramatic effects on quaternary structure, with T44S substitution favoring decamerization and T44V favoring dimerization. In the case of the Arg123 mutations, changes in SEC profiles were not so drastic. Tsa1^{R123K} eluted predominantly as a decamer (Figure 4F) and Tsa1^{R123G} migrated mainly as dimer in oxidizing conditions (Figure 4E).

Finally, to check if the Thr44 or Arg123 amino acids substitutions provoked major perturbations on the overall Tsa1 structure, circular dichroism (CD) analysis were carried out. All CD spectra indicated that the amino acid substitutions did not provoke perturbations in the content of secondary structures (see Supplementary Fig. 3). The CD spectra for all enzymes were consistent with α helix content of about 33% and about 20% of β sheet, which is agreement with Tsa1 crystallographic data²⁴.

Kinetic characterization of Tsa1 mutants for Thr44 and Arg123 - Next, we examined the effects of the mutations on Tsa1 reactivity towards hydroperoxides using the Trx system (Thioredoxin 1, Thioredoxin reductase 1 [TrxR1] and NADPH) coupled assay. Tsa1^{WT} and Tsa1^{T44S} presented similar catalytic efficiencies towards hydrogen peroxide (Figures 5A,C; Table 1). Tsa1^{T44A} presented a slight decrease in activity (Figure 5B; Table 1). In contrast, Tsa1^{T44V} presented a very high K_M (519.9 μ M) and the catalytic efficiency for hydrogen peroxide decreased two orders of magnitude (Figure 5D; Table 1). In human PrxV, T44V mutation also decreased the second order rate constant of the reaction with hydrogen peroxide by a factor of one thousand¹⁵. All kinetic parameters were determined under conditions where thioredoxin (at 2 μ M) was not limiting the catalysis (see Supplementary Fig. 4).

In the case of cumene hydroperoxide (CHP) reduction, Tsa1^{WT} and Tsa1^{T44S} also presented similar kinetic parameters (Insets Figures 5A,C; Table 1). In contrast to hydrogen peroxide reduction, Tsa1^{T44A} presented very high K_M (334.3 μ M) for CHP and consequently a four-fold decrease in the catalytic efficiency (Inset Figure 5B; Table 1). In the case of Tsa1^{T44V} mutant, an even more drastic drop in the reduction of CHP was observed (Inset Figure 6D), precluding the determination of the kinetic parameters. Similar results were observed for *L. donovani* TXNPx¹⁴. Therefore, mutations eliminating the hydroxyl group from the side chain impair more CHP reduction than hydrogen peroxide reduction. The

factors underlying this phenomenon are not clear, and may be related with the distinct effects of amino acid side chains on stabilization of the respective transition states with hydrogen peroxide and CHP; and/or with the release of the corresponding leaving groups.

We also evaluated the effects of Thr mutations by non-reducing SDS-PAGE. Reduced proteins were oxidized with three equivalents of hydrogen peroxide or CHP and the disappearance of monomers were followed. Consistent with the thioredoxin-coupled assay, among the three mutants, only Tsa^{T44V} presented a significant delay in disulfide formation for both hydrogen peroxide and CHP (see Supplementary Fig. 5 and 6). To investigate the effects of Thr44 substitutions on Tsa1 reduction by 1,4 dithiotreitol (DTT) or by Trx1, pre-oxidized enzymes were treated with different amounts of DTT (1-100 eq) or Trx (1-25 eq) at short intervals. Reduction of wild type and mutant proteins proceeded similarly, indicating that the residues at position 44 had a minor role in the reduction step (see Supplementary Fig. 7 and 8).

In the case of Arg123 mutants, in agreement with previous reports^{11,12,14,15}, Tsa1^{R123G} and Tsa1^{R123K} displayed only residual thioredoxin peroxidase activities (Figure 6A). As an independent and confirmatory assay, we evaluated disulfide formation in Tsa1^{R123G} and Tsa1^{R123K} from pre-reduced samples treated with hydrogen peroxide in non-reducing SDS-PAGE. Disulfide bond was rapidly formed in Tsa1^{WT} (10 seconds or less; Figure 6B). In contrast, replacement of Arg123 resulted in a substantial delay of disulfide formation, since monomeric bands were still detected even after 10 min of reaction with H₂O₂ (Figures 6C, D). Thus, both substitutions of Arg123 resulted in great drop of Tsa1 reactivity towards hydroperoxides.

Determination of apparent second order rate constants of the reaction of Tsa1 proteins with hydroperoxides - The interpretation of the results obtained by the Trx coupled assay is not

always straightforward, among other reasons because the overall catalytic cycle of 2-Cys Prx enzymes is complex involving three reactions (hydroperoxide reduction; condensation; disulfide reduction) and conformational changes (LU-FF transitions; dimer–decamer switches)⁷. Therefore, direct rate constants between hydroperoxides and reduced Tsa1 proteins were determined taking advantage of the change in their intrinsic fluorescence upon oxidation (Figure 7A). Such an overall decay was shown to be proportional to substoichiometric concentrations of hydrogen peroxide but levelled-off with stoichiometric or higher hydroperoxide concentration (Figure 7B), indicating Cys_p oxidation. Under pseudo first-order conditions (excess of peroxide), a two phase kinetics was observed for wild type Tsa1 (Figure 8A). In the first phase, the drop in fluorescence was very rapid (first 5 ms), justifying the apparent lag time, which coincides with the mixing time of the instrument (approximately 2 ms). The second and slower phase resulted in a fluorescence increase, and its meaning remains unclear at this point.

Recently, a similar kinetic profile was described for a StAhpC²⁷. To be able to fit the experimental data, the authors proposed a typical Michaelis-Menten model. In the first step, hydrogen peroxide would reversibly bind into StAhpC catalytic site, forming a Michaelis-Menten type of complex, followed by Cys_p peroxidation (which was associated to the fast fluorescence decay) and resolution (which was associated to second fluorescence increase phase). In our case and our experimental conditions, however, the raising in fluorescence appears to be dependent on hydroperoxide concentration (Figures 7B,C), suggesting unfavorable peroxide and Prx equilibrium or that overoxidation might contribute to it, although neither hypothesis have been pursued so far. Nevertheless, the initial rapid fluorescence decay increases linearly with both Prx and peroxide concentrations and is accompanied by loss of Tsa1 peroxidase activity, indicating that this phase is associated with Cys_p oxidation. To test this assumption, we determined what would be the rate constant of

Cys_p oxidation under pseudo first-order conditions, fitting the fast decay in the fluorescence to a single first-order exponential equation for the wild type enzyme, since we have previously determined this rate constant by an independent competitive kinetic approach². In this way, the rate constant for the Tsa1^{WT} oxidation by hydrogen peroxide was $5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, which agrees well with the value previously determined by the competitive assay². In addition, the second order rate constant for the Tsa1^{WT} and Tsa1^{T44S} oxidation by hydrogen peroxide and by CHP were similar and in the $10^7 \text{ M}^{-1} \text{ s}^{-1}$ range (Table 2), again in agreement to previous determinations². These convergences stimulated us to apply the same approach for the remaining Tsa1 mutants. For Tsa1^{T44A} and Tsa1^{T44V}, a decrease in reactivity was observed for both enzymes, and once again the valine mutant was the less reactive (Table 2). Nevertheless, the Tsa1^{T44A} was still quite reactive: one thousand times fold faster than free Cys²⁸, while the corresponding rate constants for the oxidation of Tsa1^{T44V} could not be determined due to its very low reactivity.

Therefore, Tsa1 from *S. cerevisiae* and StAhpC display different properties related to the dependence on hydrogen peroxide in the second phase with fluorescence increase. This difference might be related with the positions of the respective Trp residues. Noteworthy, Trp residues close to the resolving Cys in the two 2-Cys Prx are strikingly different (Figures 7D-E). Another factor that might contribute to this different fluorimetric behavior is that Tsa1 is sensitive to overoxidation, whereas StAhpC is not²⁹.

Values for catalytic efficiencies (k_{cat}/K_M) obtained were consistently three orders of magnitude lower than direct rate constant measures by the intrinsic fluorescence. Probably, some other reaction or structural rearrangements is limiting the overall turnover under catalytic conditions (Figure 1). Nevertheless, the same trend was observed whatever the approach (Trx coupled or fluorimetric assay) employed. Mutation of Thr44 by Val decreased the ability of Tsa1 to reduce hydroperoxide by several orders of magnitude, whereas T44A

induced a less pronounced effect. These results indicate the importance of the polar hydroxyl group in the catalysis. Nevertheless, since mutants Tsa1^{R123G} and Tsa1^{R123K} displayed only residual activity, Arg123 appears to play the major role in Tsa1 reactivity.

Relationship between quaternary structures and catalytic activity of 2-Cys Prx – The results presented so far indicated that Thr44 plays roles on both decamer stabilization and peroxidase activity. To gain further insights on the roles of the non-conventional Thr44 - Tyr77 CH- π hydrogen bond, Tsa1^{Y77A} mutant was also produced. Furthermore, Tsa1^{S78D} mutant was generated based on the information that in StAhpC and in HsPrx4, similar substitution provoked dimer destabilization^{1,20}. Thr77 in StAhpC (Ser78 in Tsa1) is located in the decameric interface of the enzyme but does not have direct interactions with the active site residues, including Cys_{sp}. Substitution of Thr77 by an aspartic acid in StAhpC precluded decamer formation¹. Yeast Tsa2 (86% of amino acid identity with Tsa1) was also analyzed, since this enzyme naturally presents a Ser in the position equivalent of Thr44 in Tsa1².

Similar to the Tsa1^{T44S} mutant, Tsa2 migrated exclusively as decamer independently of redox state of the enzyme (Figure 8A). In contrast, Tsa1^{Y77A} always eluted as dimer (Figure 8B). Once again these results indicated that the Thr44-Tyr77 bond favors dimer-dimer interaction and that Ser in place of Thr stabilize Tsa1/Tsa2 in the decameric state. The peroxidase activity of Tsa1^{Y77A} was highly affected, which is in agreement with the results obtained with Tsa1^{T44V} (Figure 5D). In contrast, Tsa2 efficiently reduced both CHP and hydrogen peroxide (Figures 5B; 8D,E; Table 2). As predicted, Tsa1^{S78D} also eluted as dimers, independently of the redox state (Figure 8F) and the peroxidase activity was only residual in the Trx coupled assay (Figure 8G). Actually, S78D mutation provoked a more drastic result than the corresponding experiment with StAhpC¹. As an independent and confirmatory assay, non-reducing SDS PAGE revealed that rates of disulfide formation in Tsa1^{Y77A} and Tsa1^{S78D}

were very slow (Figure 8H,I). Finally, the second order rate constants for Tsa2 oxidation by hydrogen peroxide and CHP were similar to the corresponding rate constants for Tsa1 (Table 2; see Supplementary Fig. 9). In contrast, the reactivities of Tsa1^{Y77A} and Tsa1^{S78D} were extremely low, precluding the determination of the rate constants values by available methods (Table 2).

Discussion

Among Prx enzymes, the 2-Cys Prx (AhpC-Prx1) sub-group displays the remarkable capability to switch between dimers and decamers, which is part of the catalytic cycle⁷. Dimers and decamers are apparently in a dynamic process, in which redox state, pH and protein concentration, among other factors, shift the equilibrium to the dimer or to the decamer^{16,19,30,31}. In the reduced state, 2-Cys Prx enzymes are preferentially in the FF state as decamers, in an optimal conformation for hydroperoxide reduction⁷. After oxidation to sulfenic acid, a locally unfolding process must take place to allow approximation of the two Cys residues. This locally unfolding process destabilizes the decamer, favoring dimers⁷. Therefore, a weakly associated decamer (in the LU state) has been proposed as an intermediate between the FF decamer and the LU dimer¹⁹ and other intermediate oligomeric structures have also been frequently observed^{13,17}. Here, we present evidences that the highly conserved Thr residues in the active site (Thr44 in Tsa1) play also a structural role in the stabilization of Tsa1 as a FF decamer.

Through a comparative structural analysis of 2-Cys Prx enzymes, it became clear that although most residues in the dimer-dimer interface retain similar conformation between FF and LU states, the conserved Thr residue (Thr44 in Tsa1) rotates approximately 45° upon oxidation (Figure 3, compare panels C, E with D and F). This rotation is conserved in all of the analyzed structures and is associated with the disruption of the CH- π bond in the LU state.

Since there are two CH- π bond per dimer-dimer interface, it is possible that the linkage contributes to a higher decamer stability of the FF state as compared with the LU state^{7,19}. It should be taken into account that under the high protein concentrations required for crystallization, 2-Cys Prx enzymes tend to be stabilized as decamers, even in LU form. Indeed, it has been proposed that the LU decamers (“metastable oligomerization intermediate”) are less stable than the FF decamers^{7,19}. Disruption of two CH- π bond per dimer-dimer probably contributes to the FF to LU transition.

Other mutations in the dimer-dimer interface (Thr77 residue of StAhpC and Thr¹¹⁸ in Prx4) were reported to provoke major changes in the stability of dimers and decamers^{1,20}. However, crystal structures of these proteins did not reveal major alterations in the dimer-dimer interface. In most of these structures, 2-Cys Prx enzymes are in the LU state with an intermolecular disulfide bond and the active site Thr (Thr⁴³ in StAhpC) is rotated approximately 45°, which we describe above as a common feature for these enzymes in the LU state. Our structural analysis of StAhpC and Prx4 mutants indicated that subtle modifications in the dimer-dimer interface can provoke major changes in the dimer-decamer transitions at lower protein concentrations.

Therefore, mutations of residues in the dimer-dimer interface are not expected to result in major structural modifications. Nevertheless, some hypothesis for the structural roles of residues equivalent to Thr44 in Tsa1 can be raised based on our structural analysis and experimental data. Comparing wild type and mutant proteins, considering the amino acid at position 44 with similar polarity, we observed that residues with smaller side chains tend to stabilize the decamer. Indeed, Tsa1^{T44S} was only observed in the decameric form, whereas Tsa1^{WT} is found in both dimeric and decameric states. Tsa1^{T44A} was found in both dimeric and decameric states, whereas Tsa1^{T44V} was found mostly as dimer under both reducing and oxidizing conditions (Figure 4). These observations suggested a steric factor underlying the

decamer stability. In fact, the microenvironment around the active site Thr is crowded (Figure 9A-B) and re-accommodation of neighbor residues is likely to be required to allow a 45° rotation. Possibly, smaller side chains might allow softer FF–LU transitions, without major perturbations at the dimer-dimer interface. The extra C γ in the Thr side chain (in comparison with Ser side chain) may create steric shock with neighbor residues, disfavoring the decameric state during the transition from FF to LU. Indeed, it is clear that the microenvironment around the active site Ser in AhpC from *A. xylanus* (Figure 9C) is less crowded than Thr residues in similar LU structures. Prove of this concept will depend on the availability of the structure of a 2-Cys Prx in FF state with a Ser in the active site.

Apparently, not only the size, but also the hydroxyl group in the side chain stabilizes the decamer. Indeed, Tsa1^{WT} (with a Thr in position 44) is found in both dimeric and decameric states, whereas Tsa1^{T44V} was found mostly as dimer. Tsa1^{T44S} was only observed in the decameric form, whereas Tsa1^{T44A} was found in both dimeric and decameric states. The O atom in the side chain of Thr residues may perform distinct polar interactions in the FF and LU states possibly helping to stabilize 2-Cys Prx in the decameric form (Figure 9D-K). The few available structures of 2-Cys Prx in the dimeric form show the active site Thr of Prx1^{C83S} from *R. novorgicus* participating in distinct polar interactions¹⁸ than in the FF and LU decamers.

Remarkably, Ser is a residue that has the two properties that appear to be important to stabilize the decamer, the small size and the hydroxyl group. Accordingly, Tsa2 is one 2-Cys Prx that naturally have a Ser residue occupying position 44 and was also found mainly in the decameric state (Figure 8).

The results described here are in line with previous studies showing that decamers display higher peroxidase activity than dimers¹. However, the implications of Thr44 mutations in Tsa1 are complex because the residue is also a member of the catalytic triad⁸. The hydroxyl

group of the conserved Thr plays a role in the catalytic properties of the enzyme by polar interaction with the hydroperoxide substrate^{9,10}. Nevertheless, Tsa1^{T44A} remained quite reactive towards hydroperoxides ($10^4 \text{ M}^{-1} \text{ s}^{-1}$) (Figure 7B; Table 2; see Supplementary Fig. 8A) as compared with ordinary thiols, such as free Cys or GSH.

2-Cys Prx enzymes display complex structural and biochemical properties, involving distinct quaternary structures and oxidation states, including overoxidized states. Our contribution here is to show that the active site Thr is involved in the stabilization of 2-Cys Prx decamers through the CH- π hydrogen bonds with Tyr/Phe residues of adjacent dimers in addition to be a member of the catalytic triad.

Acknowledgments

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Author Contributions

C.A.T.Jr and M.C.S. performed point mutations expressed and purified the proteins. C.A.T.Jr, M.C.S., C.A.B., and M.H.T. performed steady state kinetic analysis and SEC and CD.; Spectrofluorometric approaches were performed C.A.T.Jr, R.R.G., J.C.T.Jr, C.J.N., S.P.L., and G.F.S.; C.A.T.Jr and M.A.O. executed the structural analysis. L.E.S.N., O.A. and M.A.O provided overall project supervision and wrote the paper along with C.A.T.Jr and M.C.S.

Competing Financial Interests Statement

The authors declare no competing financial interests.

Figure 1

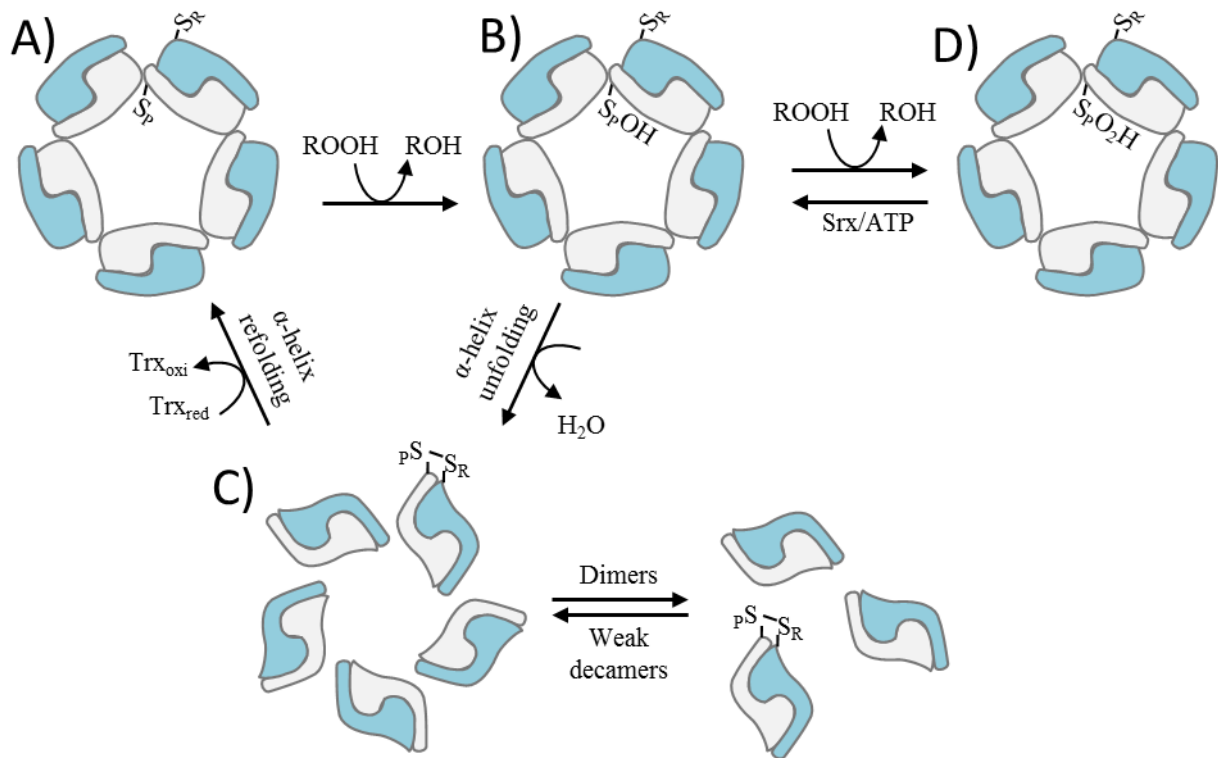


Figure 1. Proposed mechanism for 2-Cys Prx (AhpC-Prx1) reduction of hydroperoxides.

(a) Cys_p in the fully folded (FF) form (S_p⁻) displays high reactivity towards hydroperoxides and favor the maintenance of the decamer in typical 2-Cys Prx. (b) The oxidation product (sulfenic acid - S_pOH) can have two outcomes: condensation or overoxidation. (c) Since the two Cys residues are far away in the FF form, a local unfolding should take place for the intermolecular disulfide bond formation, which induces the impairment of the decamer, being possible the appearance of dimers. The reduced form of typical 2-Cys Prx is regenerated by Trx, restarting the catalytical cycle. Additionally, sulfenic state of 2-Cys Prx (b) can be overoxidized (d) by reaction with a second molecule of hydroperoxide, inactivating the enzyme. This process can be reversed by Srx, with consumption of ATP.

Figure 2

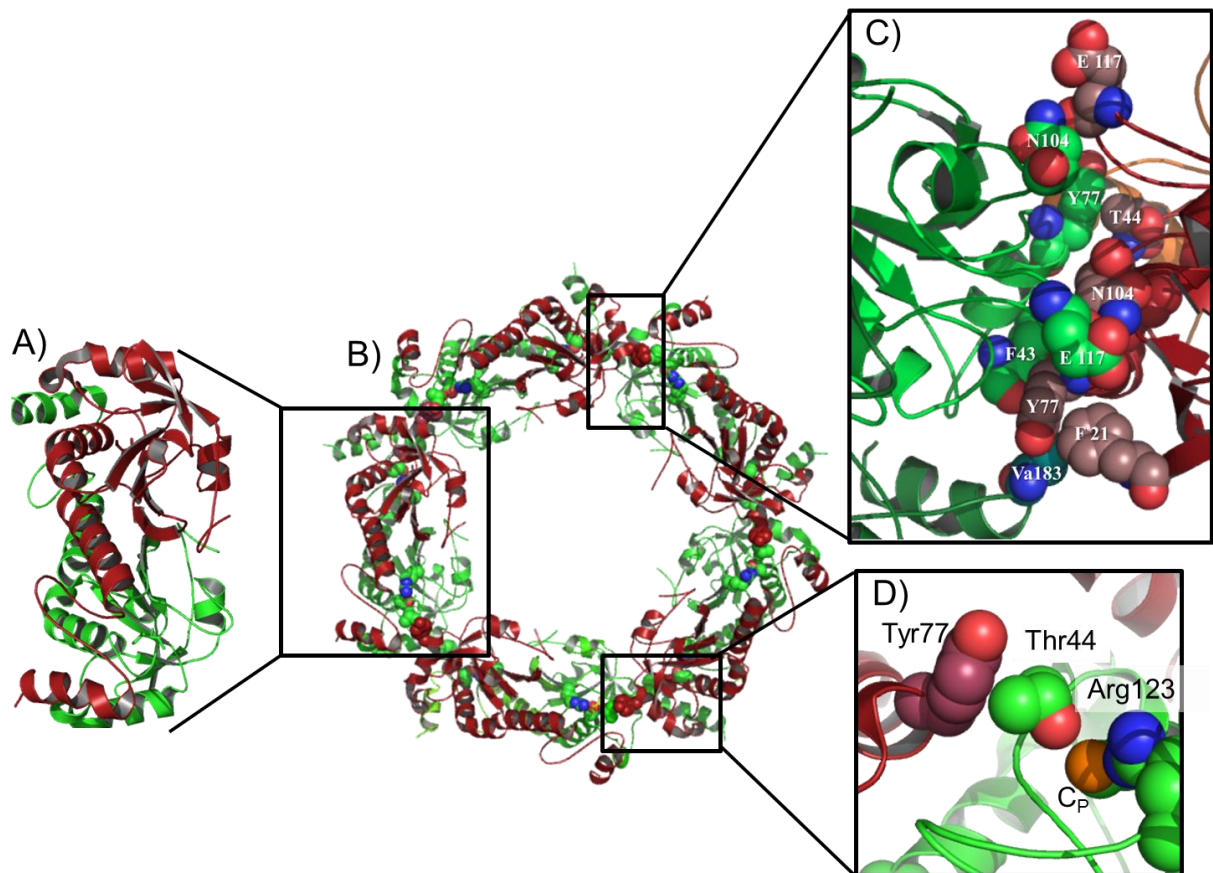


Figure 2. Decameric *Tsal*^{C47S} structure and decamer stabilization. (a) *Tsal* homodimer represented in cartoon with a monomer colored in green and the other in red. (b) *Tsal* decamer as a pentamer of homodimers. (c) Molecular interactions at the dimer-dimer interface in the *Tsal*^{C47S} crystallographic structure. Amino acid residues involved in the interactions between dimers are represented with spheres. (d) Thr44 CH- π bond with the Tyr77 of the adjacent dimer. The opposite extremity of Thr44 is involved with polar interactions with Cys_P S _{γ} , which in turn is stabilized by guanidine group of the Arg123. For (c) and (d), the carbon atoms are represented by colors similar to the polypeptide chain to which they belong, whereas N = blue, O = red and S = orange.

Figure 3

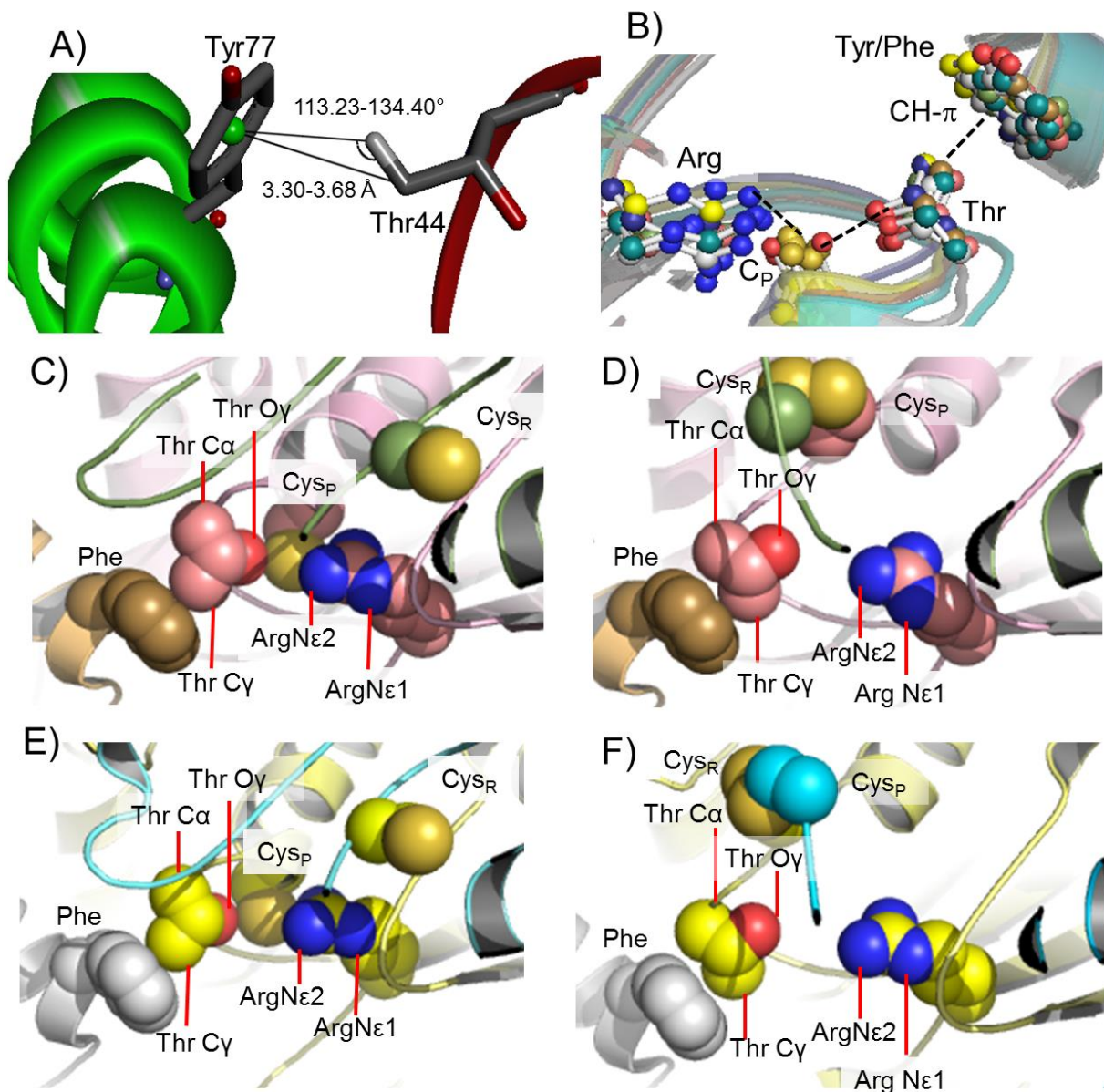


Figure 3. Interactions between Thr and Tyr (Phe) in reduced (FF state) or disulfide (LU state) forms of typical 2-Cys Prxs. (a) Diagram of Thr44 and Tyr77 from Tsa1 at the dimer-dimer interface, depicting the corresponding CH- π hydrogen bond. The graphic representation and the distances/angles were accessed using the program Discovery Studio 4.0 (Accelrys Software Inc., Discovery Studio Modeling Environment). (b) Overlap of the catalytic triad residues (Cys_P = C_P, Arg and Thr) and CH- π hydrogen bonds between Thr and Phe/Tyr residues of several 2-Cys Prx crystallographic structures. The enzymes are on FF state and depicted by different colors as follow: *Salmonella typhimurium* AhpC (yellow; 4MA9), *Leishmania major* PrxII (brown; 4K1F), *Schistosoma mansoni* Prx (white; 3ZTL),

Pseudosciaena crocea Prx4 (cyan; 3QPM), *Homo sapiens* sulfinilate form of Prx2 (red; 1QMV), *Rattus norvegicus* Prx1^{C51S} (dark green; 2Z9S) and *S. cerevisiae* Tsa1^{C47S} (dark blue; 2Z9S). The crystallographic structures of 2-Cys Prx in FF state of the wild type enzymes from *S. typhimurium* (**c**; 4MA9) and human Prx4 (**e**; 3TKP) shows an intimate hydrogen bond network between Phe-Thr-Cys_p-Arg which is lost with the disulfide formation of the LU state in *S. typhimurium* (**d**; 1YEP) and human Prx4 (**f**; 3TJB). The structures are represented in cartoon (pink and gold = AhpC; yellow and gray = Prx4) and the residues of catalytic triad and the conserved Tyr/Phe are represented by spheres. Carbon atoms are in same color of the cartoon representations. The other atoms follow the pattern: N = blue, O = red, S = orange.

Figure 4

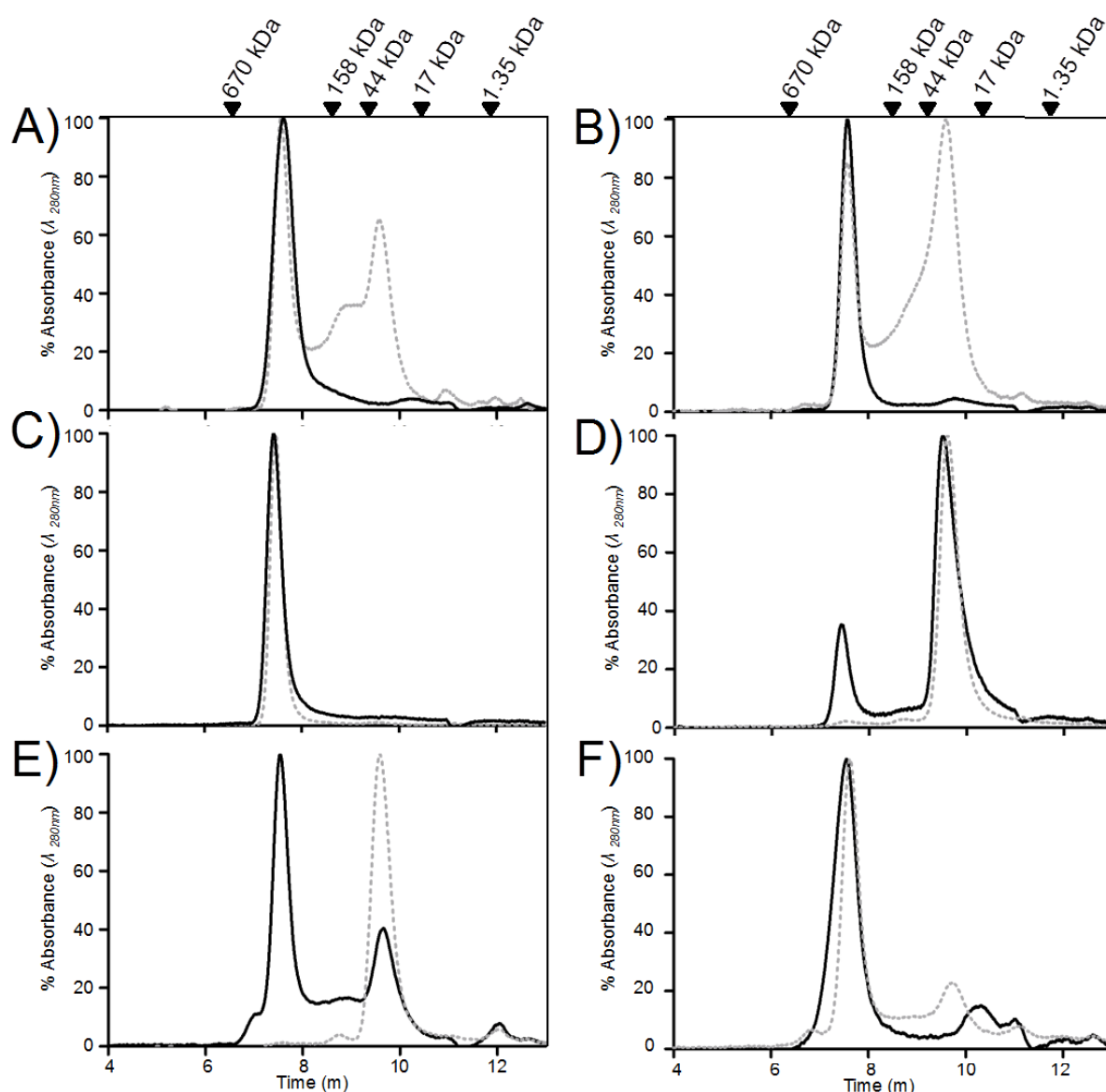


Figure 4. Size exclusion chromatography (SEC) profiles of Tsa1^{WT} and mutants in reduced and oxidized states. Samples were reduced with TCEP (5 mM; solid lines) or previously reduced with TCEP (5 mM) and then oxidized with hydrogen peroxide (1.2 molar equivalents; dashed lines) by 30 min at 25°C. Tsa1^{WT} (a), Tsa1^{T44A} (b), Tsa1^{T44S} (c), Tsa1^{T44V} (d), Tsa1^{R123G} (e) and Tsa1^{R123K} (f) at 50 μM were injected into HPLC system equipped with Phenomenex BioSep-SEC-S3000 column with a flow rate of 1 mL/min. The molecular standards used were: bovine thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), vitamin B₁₂ (1.35 kDa) and are assigned on the top of the figure.

Figure 5

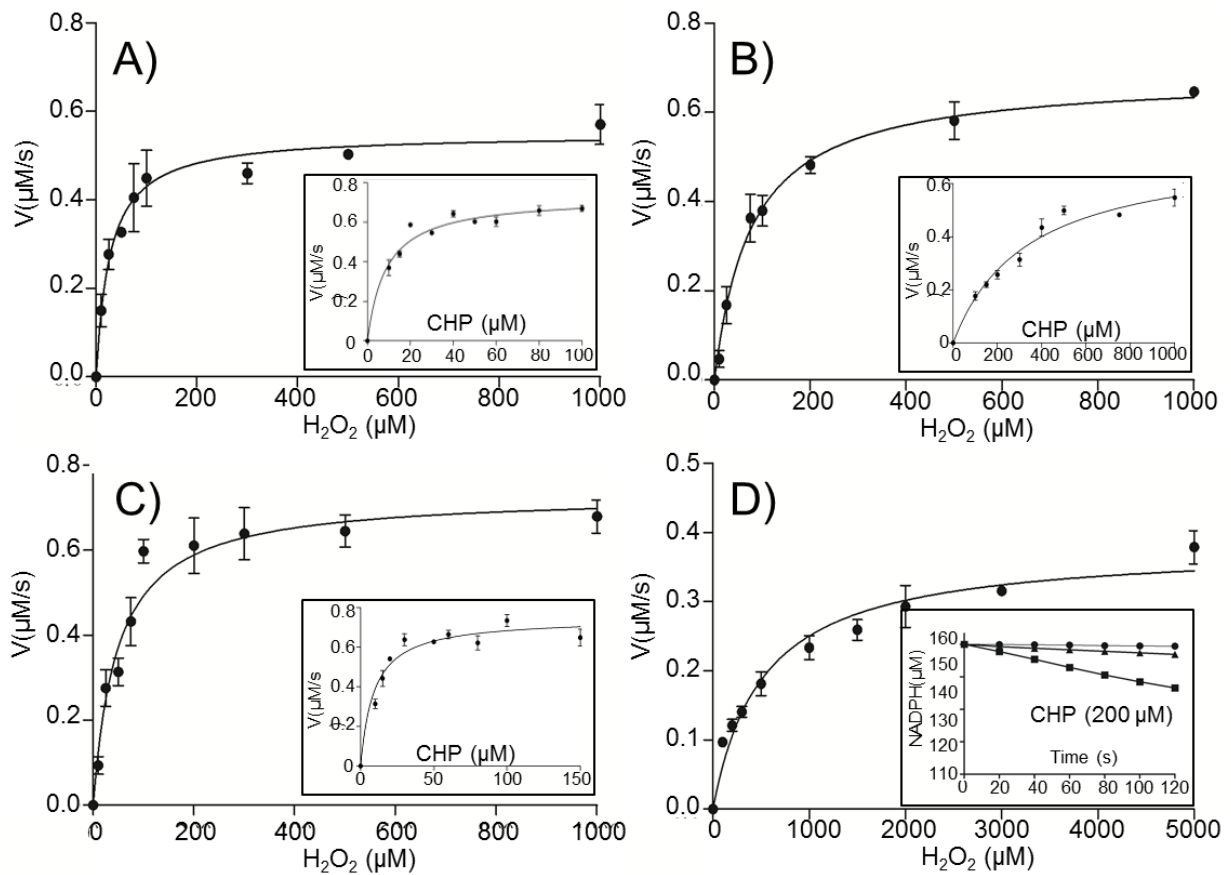


Figure 5. Steady state kinetics analysis of reduction by the Trx system of Tsa1 and mutants with increasing concentrations of hydrogen peroxide or cumene hydroperoxide (CHP). Tsa1^{WT} (a), Tsa1^{T44A} (b), Tsa1^{T44S} (c) and Tsa1^{T44V} (d) were incubated with Trx system (Prx = 1 μM ; Trx1 = 2 μM ; TrxR1 = 0.3 μM ; NADPH = 150 μM) in 50 mM HEPES (pH 7.4), 100 μM DTPA and 1 mM sodium azide at temperature of 30°C and variable concentrations of hydroperoxides as indicated in the x axis. The reactions were monitored by the consumption of NADPH spectrophotometrically at 340 nm. In the inset graphics, reactions using CHP instead of H_2O_2 are represented under the same experimental conditions, except for (d), since no NADPH consumption was observed. In this case, as an example, it is presented the reaction of 200 μM CHP with Tsa1^{T44V} (●), Tsa1^{WT} (■) as a positive control and in the absence of enzyme as a negative control (▲). The initial rate values to each reaction were adjusted in a Michaelis-Menten curve to obtain the kinetic parameters.

Figure 6

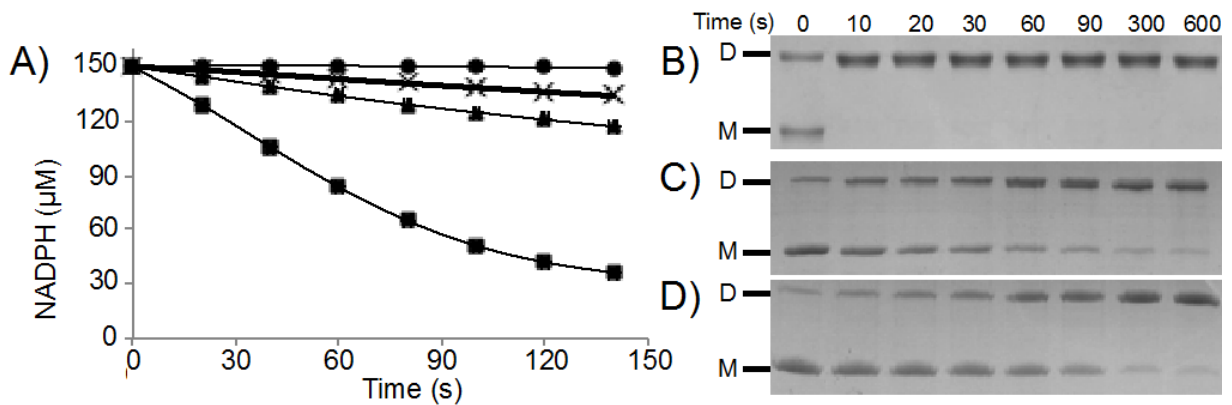


Figure 6. Kinetic characterization of Tsa1 mutants for Arg123. (a) Trx coupled peroxidase assay. Reactions were followed by the oxidation of NADPH ($Abs_{340\text{ nm}}$) at 30°C in a $100\ \mu\text{l}$ volume containing $150\ \mu\text{M}$ NADPH; $200\ \mu\text{M}$ hydrogen peroxide; $2\ \mu\text{M}$ of *S. cerevisiae* Trx1; $0.3\ \mu\text{M}$ of *S. cerevisiae* TrxR1 and $1\ \mu\text{M}$ $Tsa1^{WT}$ (■), $Tsa1^{R123G}$ (×) or $Tsa1^{R123K}$ (▲). Reactions without Tsa1 addition were also monitored as control (●). (b) to (d) non reducing SDS-PAGE assay for disulfide formation. Enzymes (b = $Tsa1^{WT}$; c = $Tsa1^{R123G}$; d = $Tsa1^{R123K}$) were pre-reduced with $20\ \text{mM}$ DTT the excess was removed by gel filtration. Tsa1 and mutants ($10\ \mu\text{M}$) were treated with three equivalents of H_2O_2 . The experiments were performed in $10\ \text{mM}$ HEPES (pH 7.4), $100\ \mu\text{M}$ DTPA, $1\ \text{mM}$ sodium azide at 25°C . To stop the reaction, buffer containing NEM ($50\ \text{mM}$), 4% SDS, 10% glycerol, $62.5\ \text{mM}$ Tris-HCl (pH 6.8) was added at the times indicated at the top of the figure (10-600 s). D = dimer; M = monomer.

Figure 7

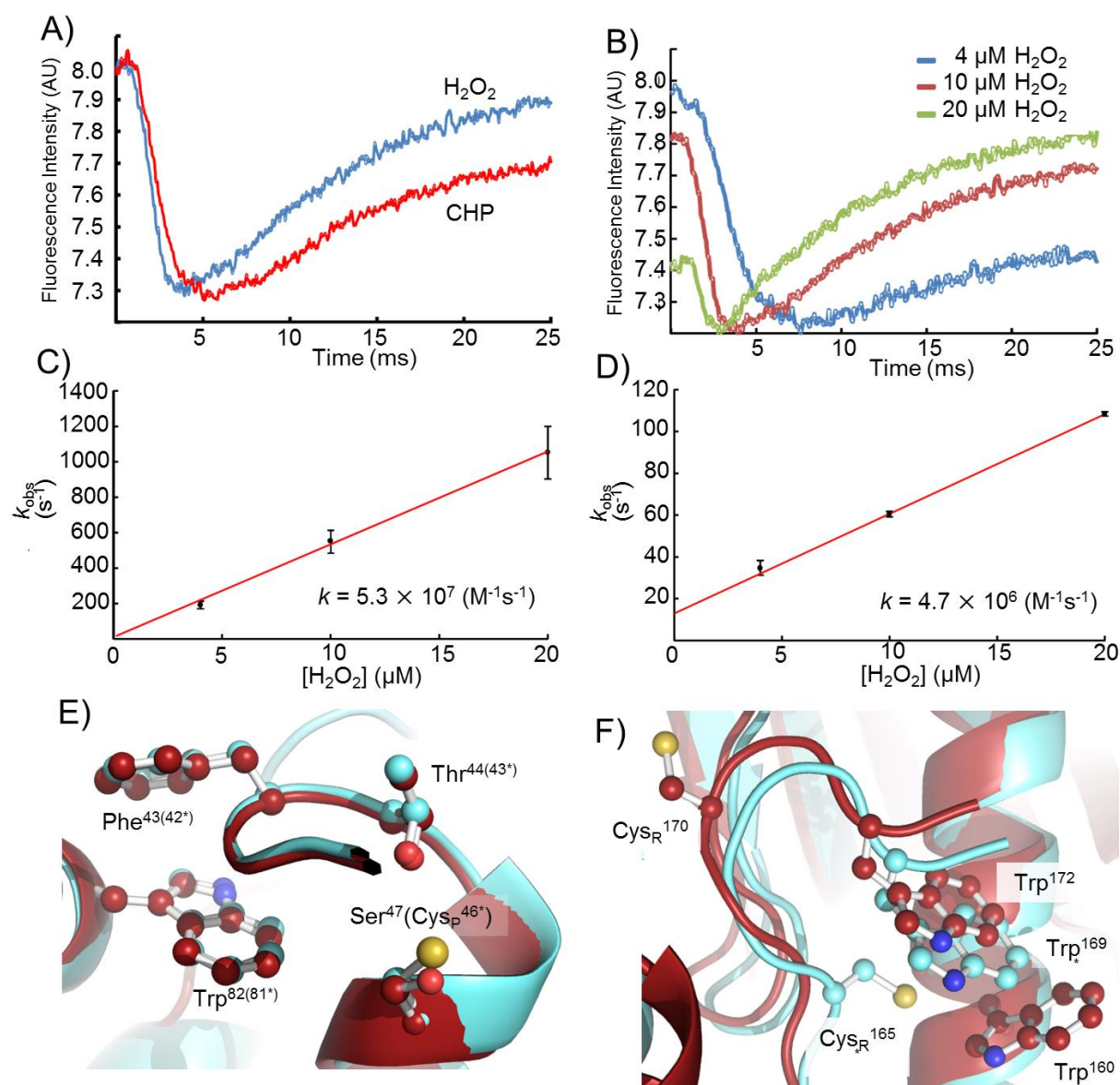


Figure 7. Hydroperoxide reduction by Tsa1 followed by changes in its intrinsic fluorescence. (a) Representative time course of Tsa1^{WT} (1 μM) oxidation by H₂O₂ or CHP (20 μM). (b) Oxidation of Tsa1^{WT} (1 μM) by increasing levels of H₂O₂ (4, 10 or 20 μM). Enzymes were previously reduced with 20 mM DTT for 30 minutes at room temperature, and the excess was removed by gel filtration. Total intrinsic fluorescence decay upon oxidation by hydroperoxides were followed ($\lambda_{ex} = 280 \text{ nm}$) at 25°C. The assays were performed in 40 mM phosphate buffer (pH 7.4). The plot of the k_{obs} obtained to the first (c) and second (d) phases demonstrates the dependence on hydroperoxide concentration. The k_{obs} values were obtained by fitting the stopped-flow data to single exponential; second order rate constant was calculated from the slope of k_{obs} plot versus oxidant concentration. Structural comparison of

Trp residues between Tsa1 (3SBC, red) and StAhpC (4MA9, light blue) in the vicinity of Ser47 (substituting Cys_P) (e) and Cys_R (f). Striking differences are found in the position of Tsa1 Trp¹⁷² and Cys_R¹⁷⁰, and Trp¹⁶⁹ and Cys_R¹⁶⁵ of StAhpC. Furthermore, an additional Trp residue in Tsa1 is found close to Cys_R. The residues numbering refer to Tsa1 residues and those between parentheses and containing an asterisk are relative to StAhpC residues. The structures are represented in cartoon, while Trp and Cys residues are represented by balls and sticks. C = same color of the cartoon representations, N = blue, O = red, S = orange.

Figure 8

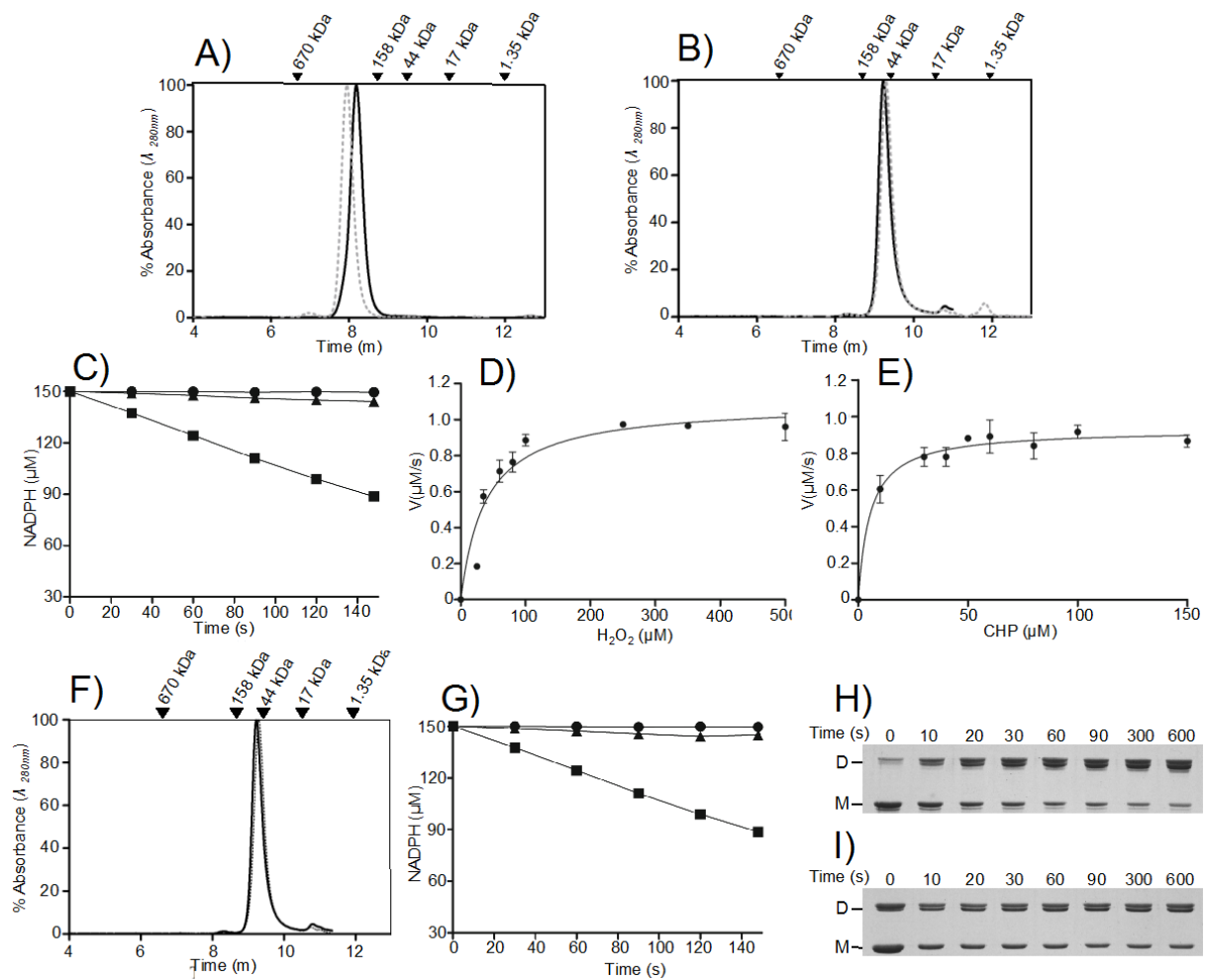


Figure 8. Structural and kinetic characterization of Tsa2, Tsa1^{Y77A} and Tsa1^{S78D}. Elution profiles of reduced (solid) and oxidized (dashed) Tsa2 (a) and Tsa1^{Y77A} (b) at 50 μ M concentration in SEC, as described in Figure 4. (c) Trx coupled assay for H₂O₂ reduction; Tsa1^{WT} (■) or Tsa1^{Y77A} (▲) (1.0 μ M). As control, reactions without Tsa1 were also monitored (●). Reactions were initiated by the addition of 200 μ M H₂O₂. Trx coupled assay for Tsa2 using H₂O₂ (d) or CHP (e). In (c), (d) and (e), the experimental conditions and Michaelis-Menten plot were performed the same as presented in Figure 5. (f) SEC profiles of Tsa1^{S78D} in reduced (solid) and oxidized (dashed) states, under the same conditions as (a). (g) Trx coupled assay using Tsa1^{S78D}. Reactions containing Tsa1^{WT} (■), Tsa1^{S78D} (▲) or no Tsa1 (●) were performed as previously described for Tsa1^{Y77A}. Non reducing SDS-PAGE assay for disulfide formation after H₂O₂ treatment of the Tsa1^{Y77A} (h) and Tsa1^{S78D} (i). The pre-reduced and desalted enzymes (10 μ M) were treated with three equivalents of H₂O₂. The experiments were performed at 25 °C in 10 mM HEPES (pH 7.4), 100 μ M DTPA, 1 mM sodium azide. Reactions were stopped by addition of buffer containing 50 mM NEM, 4% SDS, 10%

glycerol, 62.5 mM Tris-HCl (pH 6.8) at the times indicated at the top of the figure (10-600 s).

D = dimer; M = monomer.

Figure 9.

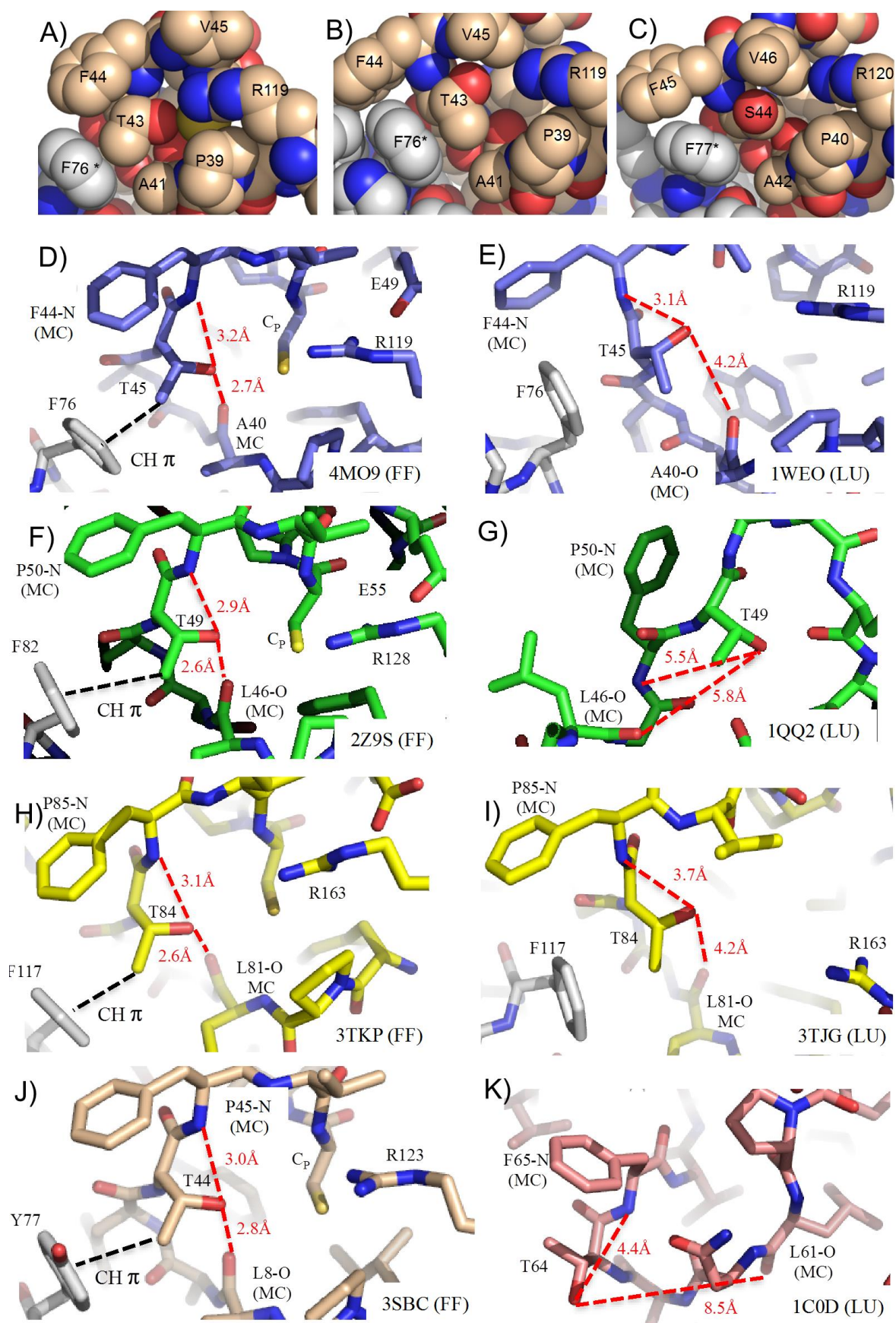


Figure 9. Environment around active site Thr/Ser residue in oxidized and reduced states of the 2-Cys Prxs. StAhpC active site pocket environment represented in spheres in reduced (**a**; 4MA9) and oxidized states (**b**; 1YEP). Structure of *A. xylophilus* 2-Cys Prx (presents natural replacement of active site Thr by Ser) in oxidized state (**c**; 1WE0). Atoms are colored as follow: C = beige, N = blue, O = red, S = orange. Polar interactions of the Thr O γ with the main chain amino (Phe) or carbonyl group (Ala/Leu) of *S. typhimurum* 2-Cys Prx (**d**, **e**), *R. norvegicus* Prx1 (**f**, **g**), human Prx4 (**h**, **i**) and *S. cerevisiae* Tsa1 (**j**) in FF (reduced; **d**, **f**, **h**, **j**) or LU (oxidized; **e**, **g**, **i**) state. As a consequence of FF-LU transition, the distances of polar interactions increase significantly. The higher distances among the enzymes analyzed in LU are from *R. norvegicus* Prx1 (**g**) and mitochondrial *Plasmodium falciparum* 2-Cys Prx (mPfPrx) (**k**). The carbon atoms to each monomer are represented by different colors: AhpC = purple and white; Prx1 = green and white; Prx4 = yellow and white; Tsa1 = beige and white; mPfPrx = rose and white. The remaining atoms are colored as follow: N = blue, O = red, S = orange. Red dashed line denotes the distance among the atoms in angstroms to perform polar interactions. The black dashed line denotes CH- π bond. MC denotes main chain. PDB code and oxidation state is discriminate at the right bottom of the figure.

Tables

Table 1. Apparent Michaelis-Menten parameters for Tsa1^{WT} and mutants. Mono-substrate approach was employed varying hydroperoxide concentrations. Rates were followed by NADPH oxidation. Tsa1^{WT} or mutants (1.0 μM), Trx1 (2 μM), TrxR1 (0.3 μM), NADPH (150 μM) and H₂O₂ or CHP. The experiments were performed at 30 °C. The results represent three independent experiments for each enzyme.

	H ₂ O ₂			CHP		
	K _m (μM)	k _{cat} (s ⁻¹)	k _{cat} /K _m (M ⁻¹ s ⁻¹)	K _m (μM)	k _{cat} (s ⁻¹)	k _{cat} /K _m (M ⁻¹ s ⁻¹)
Tsa1 ^{WT}	27.58 ± 5.18	0.55 ± 0.02	2.0 ± 0.3 × 10 ⁴	8.79 ± 0.05	0.72 ± 0.1	8.19 ± 1.1 × 10 ⁴
Tsa1 ^{T44A}	77.28 ± 12.21	0.68 ± 0.03	8.8 ± 0.2 × 10 ³	334.3 ± 102.70	0.73 ± 0.1	2.18 ± 0.28 × 10 ³
Tsa1 ^{T44S}	48.64 ± 8.77	0.73 ± 0.03	1.5 ± 0.2 × 10 ⁴	9.09 ± 3.40	0.74 ± 0.1	8.14 ± 1.4 × 10 ⁴
Tsa1 ^{T44V}	519.9 ± 82.26	0.38 ± 0.01	7.3 ± 1.4 × 10 ²	-	-	-

Table 2. Correlation of the oligomeric state and peroxidatic activity.

	Oligomerization State		Peroxidase Activity		Apparent second order rate constant	
	Reduced	Oxidized	Trx System		(M ⁻¹ s ⁻¹)	
			H ₂ O ₂	CHP	H ₂ O ₂	CHP
Tsa1 ^{WT}	Decamer	Dimer/Decamer	2.0 × 10 ⁴	8.2 × 10 ⁴	5.0 × 10 ⁷	3.3 × 10 ⁷
Tsa1 ^{T44A}	Decamer	Dimer*/Decamer	8.8 × 10 ³	2.2 × 10 ³	4.0 × 10 ⁴	8.6 × 10 ⁴
Tsa1 ^{T44S}	Decamer	Decamer	1.5 × 10 ⁴	8.1 × 10 ⁴	2.3 × 10 ⁷	1.4 × 10 ⁷
Tsa1 ^{T44V}	Dimer*/Decamer	Dimer	7.3 × 10 ²	0.0	ND	ND
Tsa1 ^{R123G}	Dimer/Decamer*	Dimer	0.0	0.0	ND	ND
Tsa1 ^{R123K}	Dimer/Decamer*	Dimer/Decamer*	ND	ND	ND	ND
Tsa1 ^{Y77A}	Dimer	Dimer	0.0	0.0	1.4 × 10 ⁴	1.3 × 10 ³
Tsa1 ^{S78D}	Dimer	Dimer	0.0	0.0	4.0 × 10 ¹	4.0 × 10 ¹
Tsa2 ^{WT}	Decamer	Decamer	2.8 × 10 ⁴	1.7 × 10 ⁵	1.3 × 10 ⁷ (‡)	ND

*Denote the predominant quaternary species; ND= Not Determined e; ‡ Determined by HRP competition assay.

Online Methods

Site directed mutagenesis - The pET15b/*Tsa1* plasmid was used as template to generate the individual *Tsa1* mutants carrying Thr44 substitutions to Ala (*Tsa1*^{T44A}), Ser (*Tsa1*^{T44S}) and Val (*Tsa1*^{T44V}); Arg123 to Gly (*Tsa1*^{R123G}) and Lys (*Tsa1*^{R123K}); Tyr77 to Ala (*Tsa1*^{Y77A}) and Ser78 to Asp (*Tsa1*^{S78D}). The mutagenesis protocols were performed according manufacturer instructions, using Quick Change II Kit (Stratagene) and the following primers: *Tsa1*^{T44A}_F (5' TGGCCTTCGCTTTCGTCTGT 3'), *Tsa1*^{T44A}_R (5' ACAGACGAAAGCGAAGGCCA 3'); *Tsa1*^{T44S}_F (5' TTGGCCTTCAGTTCGTCTGT 3'), *Tsa1*^{T44S}_R (5' ACAGACGAAACTGAAGGCCAA 3'); *Tsa1*^{T44V}_F (5' TTGGCCTTCGTTTTTCGTCTG 3'), *Tsa1*^{T44V}_R (5' CAGACGAAAACGAAGGCCAA 3'); *Tsa1*^{R123K}_F (5' GTCGCCTTGAAAGGTTTGTTTC 3'), *Tsa1*^{R123K}_R (5' GAACAAACCTTTC AAGGCGAC 3'); *Tsa1*^{R123G}_F (5' GTCGCCTTGGGAGGTTTGTTTC 3'), *Tsa1*^{R123G}_R (5' GAACAAACCTCCCAAGGCGAC 3'); *Tsa1*^{S78D}_F (5' TCCGAATACGACCTTTTGGCA 3'), *Tsa1*^{S78D}_R (5' TGCCAAAAGGTCGTATTCGGA 3'), *Tsa1*^{Y77A}_F (5' GACTCCGAAGCCTCCCTTTTG 3'), *Tsa1*^{Y77A}_R (5' CAAAAGGGAGGCTTCGGAGTC 3'). The reaction products were treated with *DpnI* to remove methylated original plasmids and *E. coli* XL1-Blue strain was used as host in the transformations. Single colonies were selected and their plasmids extracted and sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit using the automatic sequencer ABI 3730 DNA Analyser (Thermo Scientific) to confirm codons substitutions. The plasmids carrying the point mutations were transformed in *E. coli* BL21 (*DE3*) strain by electroporation.

Protein expression and purification - Single colonies of *E. coli* BL21 (*DE3*) strain containing the pET15b/*tsa1*, pET15b/*tsa1*^{T44A}, pET15b/*tsa1*^{T44S}, pET15b/*tsa1*^{T44V}, pET15b/*tsa1*^{R123G}, pET15b/*tsa1*^{R123K}, pET15b/*tsa1*^{Y77A}, pET15b/*tsa1*^{S78D} or pPROEx/*tsa2* plasmids were

inoculated in LB medium (20 ml) containing 0.1 mg ampicillin/mL overnight at 37 °C/250 rpm, transferred to 1 L of fresh LB medium, and cultured further until OD₆₀₀ reached 0.6–0.8. Then the expression of all proteins was induced by the addition of 0.3 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 37°C/3hs/250 rpm in an orbital shaker. Cells were harvested by centrifugation and cell pellets were resuspended in start buffer (50 mM sodium phosphate buffer, pH 7.4, 500 mM NaCl, 20 mM imidazole and 2 mM phenylmethyl sulfonyl fluoride - PMSF) and disrupted by sonication. The cell extracts were kept in ice during streptomycin sulfate (1%) treatment for 20 min. The supernatants were clarified by centrifugation, homogenized by filtration and purified by immobilized metal affinity chromatography (IMAC) using HisTrap column (GE Healthcare). Imidazole was removed by gel filtration using PD10 desalting column (GE Healthcare) and the purity of recombinant proteins was verified by SDS-PAGE.

Protein quantification - The purified enzymes were quantified by the molar extinction coefficient for reduced *S. cerevisiae* Tsa1 and mutants ($\epsilon_{280} = 23,950 \text{ M}^{-1} \text{ cm}^{-1}$), except Tsa1^{Y77A} ($\epsilon_{280} = 22,460 \text{ M}^{-1} \text{ cm}^{-1}$) obtained using the ProtParam tool (<http://www.expasy.ch/tools/protparam.html>).

Protein reduction – Wild type and mutant proteins were initially reduced with 50 mM TCEP in 50 mM sodium phosphate buffer (pH 7.4) 50 mM NaCl, 100 μM diethylenetriaminepentaacetic acid (DTPA) and 1 mM sodium azide, for 30 minutes, at room temperature. In some cases TCEP was replaced by DTT (20 mM). After reduction, excess of TCEP or DTT was eliminated using a PD10 column (GE Healthcare).

Circular dichroism spectroscopy of Tsa1^{WT} and mutants carrying Thr44 or Arg123

substitutions - The CD spectra of Tsa1^{WT} and mutant proteins were obtained using a 0.1 cm path length cuvette containing 10 μ M of protein sample in 10 mM Tris buffer (pH 7.4) and 100 mM NaF. The assays were carried out at 25 °C in a Jasco J-810 spectropolarimeter (Jasco Inc.). Spectra were presented as an average of eight scans recorded from 190 to 260 nm. The content of secondary structures in each protein was estimated using the CDNN 2.1 software³².

Size-exclusion chromatography - Size-exclusion chromatography was performed by analytical HPLC (Jasco LC-2000Plus) equipped with a PU 2880 Plus injector and a PDA MD 2018 detector (Jasco). The samples (50 μ M in 100 mM Tris-HCl at pH 7.4) were separated by a system containing a Phenomenex BioSep-SEC-S3000 column (7.8 \times 300 mm, 5 μ m, resolution range of 15 to 2000 kDa, Phenomenex, Inc., Torrance, California, USA) using a flow of 1.0 mL/min in 100 mM Tris-HCl buffer (pH 7.4) and 50 mM NaCl. The elution profile was monitored by absorbance (λ = 280 nm). Bovine thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B₁₂ (1.35 kDa) were used as molecular standards (Bio-Rad). The chromatograms were analyzed using Jasco BORWIN, version 1.50, software (Jasco). The enzymes were reduced or oxidized by treatment with 5 mM TCP or 1.2 molar excess of hydrogen peroxide, respectively, for 30 min/25°C.

Determination of Trx-linked peroxidase activity of Tsa1 mutants – Thioredoxin peroxidase activities were monitored by NADPH oxidation in a coupled assay²⁴. *S. cerevisiae* Tsa1^{WT} and mutants (1.0 μ M) were incubated with Trx1 (2 μ M), TrxR1 (0.3 μ M) and NADPH (150 μ M) in 50 mM HEPES buffer (pH 7.4) containing sodium azide (1mM) and DTPA (100 μ M), at 30°C. Reactions were initiated by addition of hydrogen peroxide or cumene hydroperoxide

(CHP). All kinetic data were analyzed by non-linear regression using Michaelis-Menten equation (GraphPad Prism 5 software, GraphPad Software, Inc., San Diego).

Kinetics of Tsa1 oxidation using the intrinsic fluorescence of the enzyme - Reduced Tsa1^{WT}, Tsa1 mutants and Tsa2 enzymes (1 μ M) in 40 mM sodium phosphate buffer (pH 7.4) were rapidly mixed with either H₂O₂ or CHP in excess in an Applied Photophysics SX-17MV stopped-flow spectrometer with a mixing time of approximately 2 ms. The reaction was followed by total fluorescence intensity decrease (λ_{ex} = 280 nm) of the enzymes upon oxidation. Observed rate constants (k_{obs}) were determined by fitting the stopped-flow data to single exponential functions. The apparent second order rate constants of the oxidation of the enzymes by hydroperoxides (k_{ox}) were determined from the slope of k_{obs} values plotted against hydroperoxide concentrations.

Non-reducing SDS PAGE assays – To study reduction of peroxiredoxins by DTT and Trx1, wild-type Tsa1 and mutant proteins were previously reduced as described before and treated with hydrogen peroxide at 1.2 molar ratio in 50 mM sodium phosphate buffer (pH 7.4) containing 50 mM NaCl, 100 μ M DTPA and 1 mM sodium azide. Next, the enzymes were reduced by increasing concentrations of DTT (10 μ M to 10 mM) or Trx (2.5 to 25 μ M) during 1 minute. The reaction was stopped with denaturing buffer (62.5 mM Tris-HCl, pH 6.8, containing 4% SDS and 10% glycerol) together with alkylation by 50 mM N-ethylmaleimide (NEM). Oxidized Tsa1^{WT} (intermolecular disulfide) runs as a dimer in non-reducing 12% SDS-PAGE, whereas reduced Tsa1^{WT} (dithiol) runs as a monomer in the same conditions. In order to evaluate the disulfide formation in the Tsa1^{WT} and mutants, the proteins were initially reduced as described above. The oxidation assays were performed using 10 μ M of protein and 30 μ M of H₂O₂, in 100 μ L of final volume at room temperature, in 50 mM sodium phosphate

buffer (pH 7.4) containing NaCl 50 mM, 100 μ M DTPA and 1 mM sodium azide. The reactions were stopped with 10 μ L of denaturing buffer and 50 mM NEM to alkylate the thiols of the enzymes, precluding formation of unspecific disulfide bonds (25) at the intervals of 0, 10, 30, 60, 90, 120, 300 and 600 seconds. Results were evaluated by non-reducing SDS-PAGE stained with Coomassie blue.

Analysis of Prx1/AhpC crystallographic structures - Analysis of available crystallographic structures and figures were performed using Pymol (<http://www.pymol.org>) and Discovery Studio 4.0. Distances and angles of non-conventional hydrogen bonds (CH- π interactions) in Prx structures were determined using Discovery Studio 4.0 software (<http://accelrys.com/products/discovery-studio>) according to (26).

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SUPPLEMENTARY INFORMATION

THE THREONINE RESIDUE OF PEROXIREDOXIN CATALYTIC TRIAD STABILIZES ENZYME DECAMERIC STRUCTURE

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Supplementary Figures

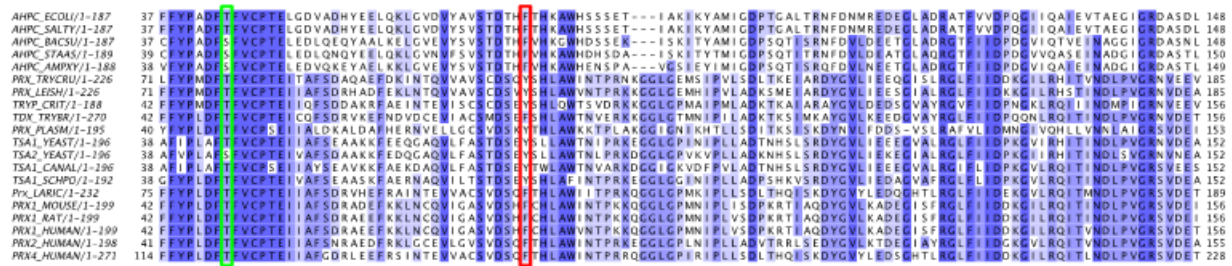


Figure 1. Sequence alignment of several 2-Cys Prx reveals conservation of catalytic triad Thr/Ser and Phe/Tyr. Amino acid sequence alignment, using ClustalX (figure generated by Jalview). Identical residues are shaded in blue and sequence similarity is indicated by a blue gradient based on the conservation of physicochemical characteristics. The species abbreviation initials are given in italics and are as follows: *Escherichia coli* = AHPC_ECOLI (NCBI accession number: P0AE08.2); *Salmonella typhimurium* = AHPC_SALTY (P0A251.2); *Bacillus subtilis* = AHPC_BACSU (P80239.2); *Staphylococcus aureus* = AHPC_STAAS (Q6GC91.1); *Leishmania major* = PRX_LEISH (XP_001683326.1); *Trypanosoma cruzi* = PRX_TRYCRU (CAA06923.1); *Crithidia fasciculata* = TRYPCRIT (AAC72300.1); *Trypanosoma brucei* = TDX_TRYBR (Q26695.1); *Plasmodium vivax* = PRX_PLASM (XP_001616002.1); *Saccharomyces cerevisiae* = TSA1_YEAST (P34760.3) and TSA2_YEAST (Q04120.3); *Candida albicans* = TSA1_CANAL (Q9Y7F0.1); *Schizosaccharomyces pombe* = TSA1_SCHPO (O74887.1); *Larimichthys crocea* = PRX_LARIC (377656258); *Mus musculus* = PRX1_MOUSE (P35700.1); *Rattus norvegicus* = PRX1_RAT (Q63716.1); *Homo sapiens* = PRX1_HUMAN (Q06830.1), PRX2_HUMAN (P32119.5) and PRX4_HUMAN (Q13162.1). The green box denotes the catalytic triad Thr/Ser and the red box the Tyr/Phe.

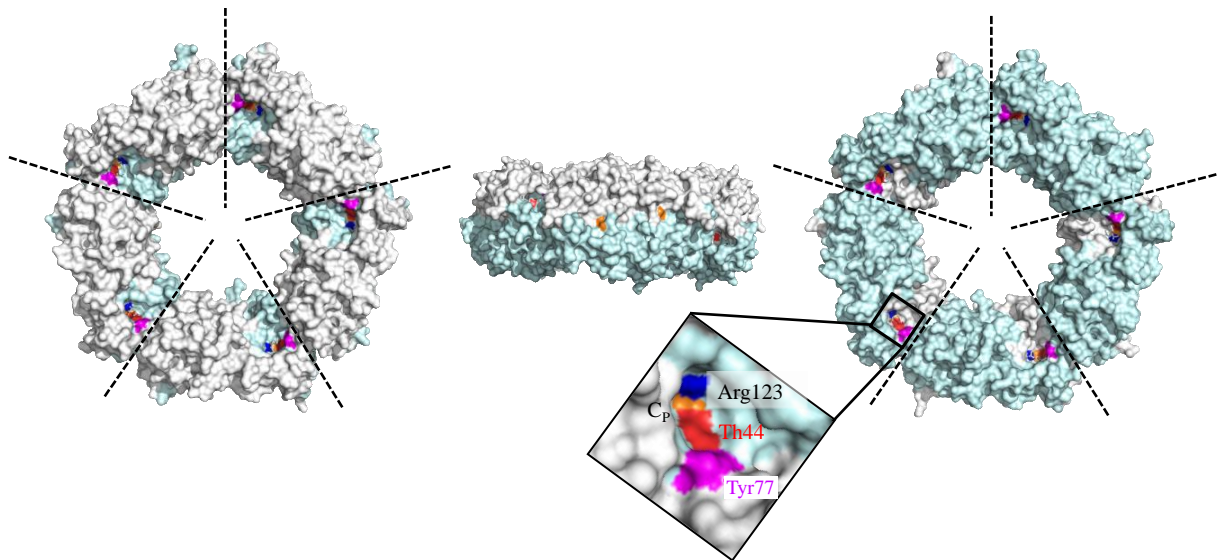


Figure 2. Position of the catalytic triad and Tyr⁷⁷ at *S. cerevisiae* Tsal molecular surface. A monomer of obligate homodimer is colorized in white and the other in light blue. The dashed lines delimit the homodimers evidencing the D interface. The surface of the Arg¹²³ is represented in blue, the C_p in orange and Thr⁴⁴ in red. The Tyr⁷⁷ of the adjacent dimer is colorized in magenta.

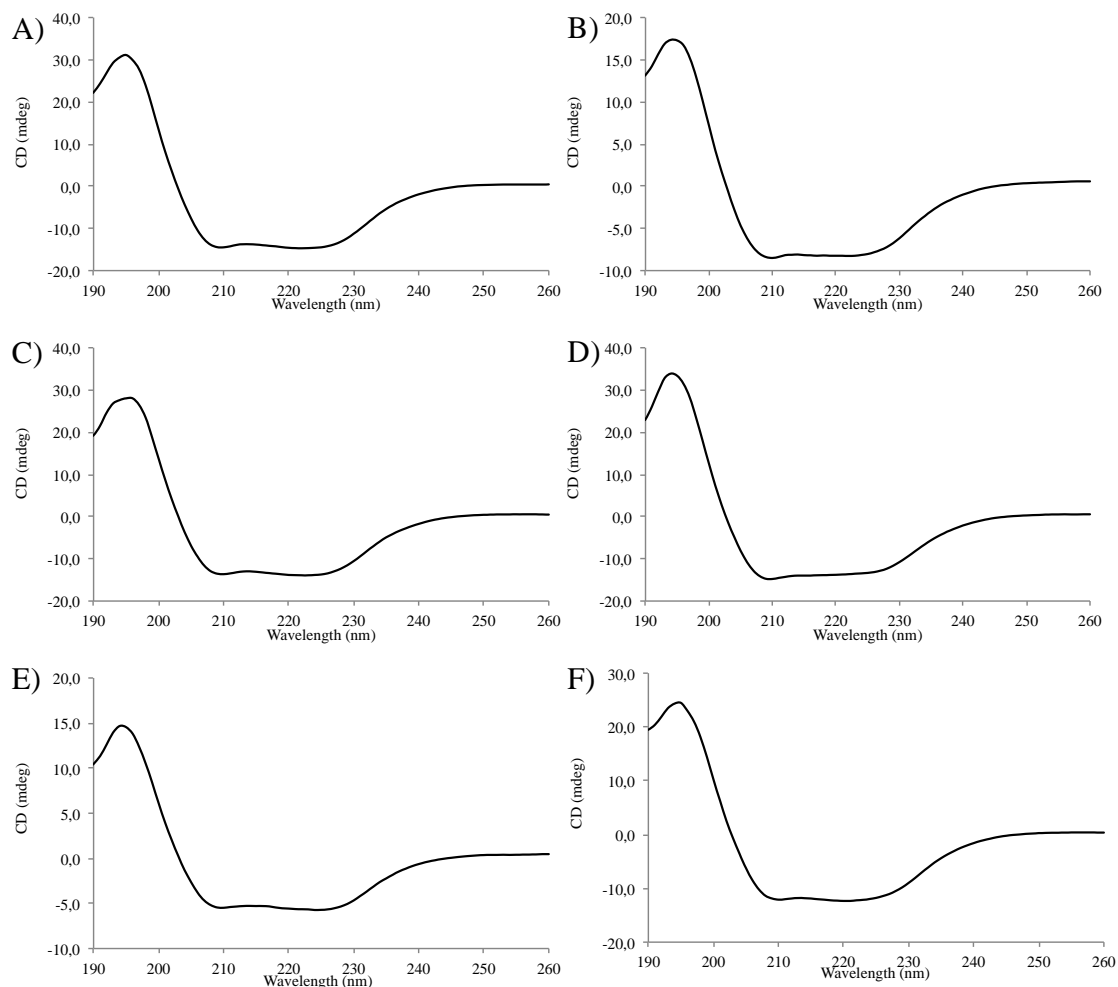


Figure 3. CD spectra of native samples of Tsal^{WT} and mutants containing Thr⁴⁴ and Arg¹²³ substitutions. Spectra of the enzymes Tsal^{WT} (A), Tsal^{T44A} (B), Tsal^{T44S} (C), Tsal^{T44V} (D), Tsal^{R123G} (E) and Tsal^{R123K} (F). The proteins concentration used in experiments were 10 μ M in 10 mM Tris buffer (pH 7.4) and 100 mM sodium fluoride. All spectra were recorded at 25°C and corrected against the buffer. The graphical representations are averages from eight consecutive scans.

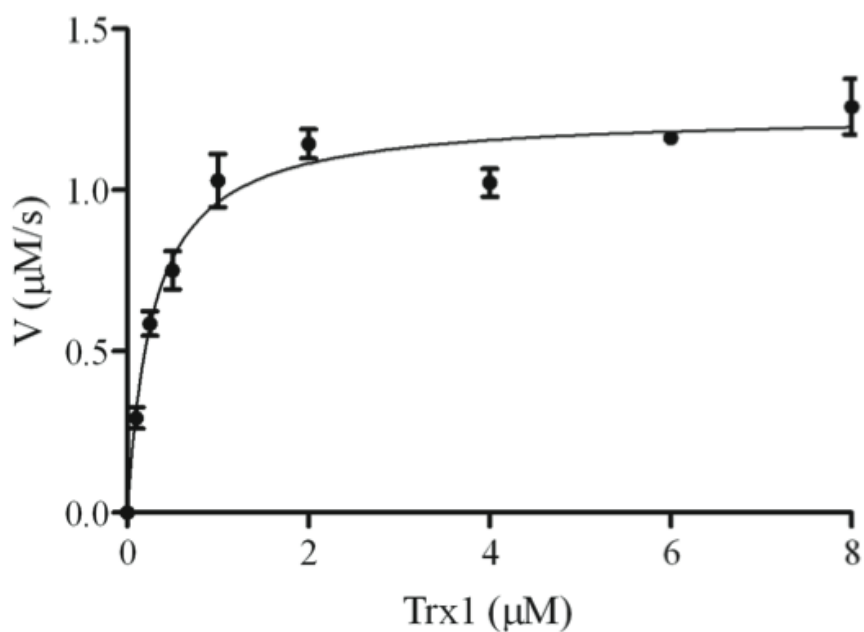


Figure 4. Dependence of Tsa1 peroxidase activity on thioresdoxin concentration. Hydrogen peroxide reduction rates were obtained in reaction mixtures containing NADPH (150μM), TrxR (0.3μM), Tsa1 (1μM), in HEPES 50mM (pH=7.4) and sodium azide (1mM) and DTPA (100μM), using variable concentrations Trx1 (0.1, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0μM). The reactions were performed at 30°C.

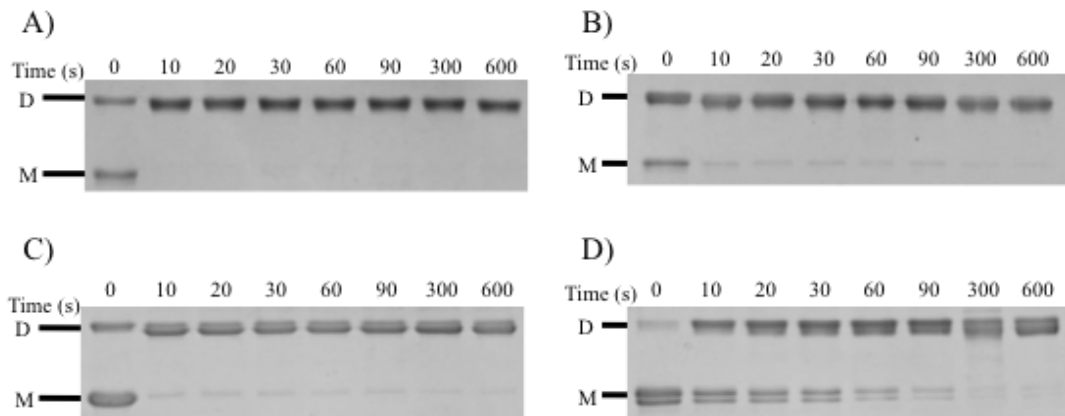


Figure 5. Monomer-dimer conversion of Tsa1 and mutants as a function of the time after oxidation by H_2O_2 . Tsa1^{WT} (A), Tsa1^{T44A} (B), Tsa1^{T44S} (C) and Tsa1^{T44V} (D) (10 μ M) were treated with three equivalents of H_2O_2 . Proteins were previously reduced with 20 mM DTT for 30 minutes at room temperature and the excess was removed by gel filtration. Assays were performed at RT in 10 mM HEPES (pH 7.4), 100 μ M DTPA, 1mM sodium azide. Reactions were stopped by the addition of buffer containing NEM (50 mM) 4% SDS, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8) at the times indicated at the top of the figure. D = dimer and M = monomer.

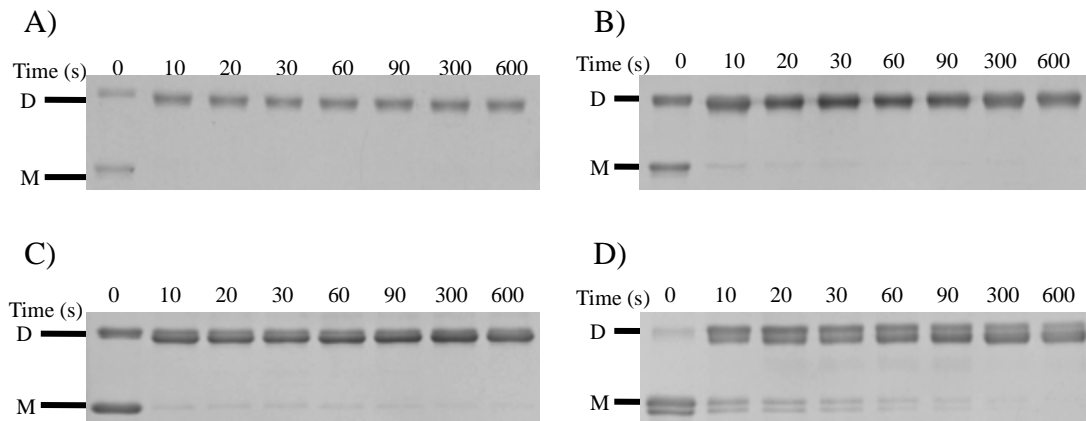


Figure 6. Monomer-dimer conversion of Tsa1 and mutants as a function of the time after oxidation by CHP. Reactions of Tsa1^{WT} (A) and mutants Tsa1^{T44A} (B), Tsa1^{T44S} (C) and Tsa1^{T44V} (D) (10 μ M) were treated with three equivalents of CHP. Proteins were previously reduced with 20 mM DTT for 30 minutes at room temperature and the excess was removed by gel filtration. Assays were performed at RT in 10 mM HEPES (pH 7.4), 100 μ M DTPA, 1mM sodium azide. Reactions were stopped by the addition of buffer containing NEM (50 mM) 4% SDS, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8) at the times indicated at the top of the figure. D = dimer and M = monomer.

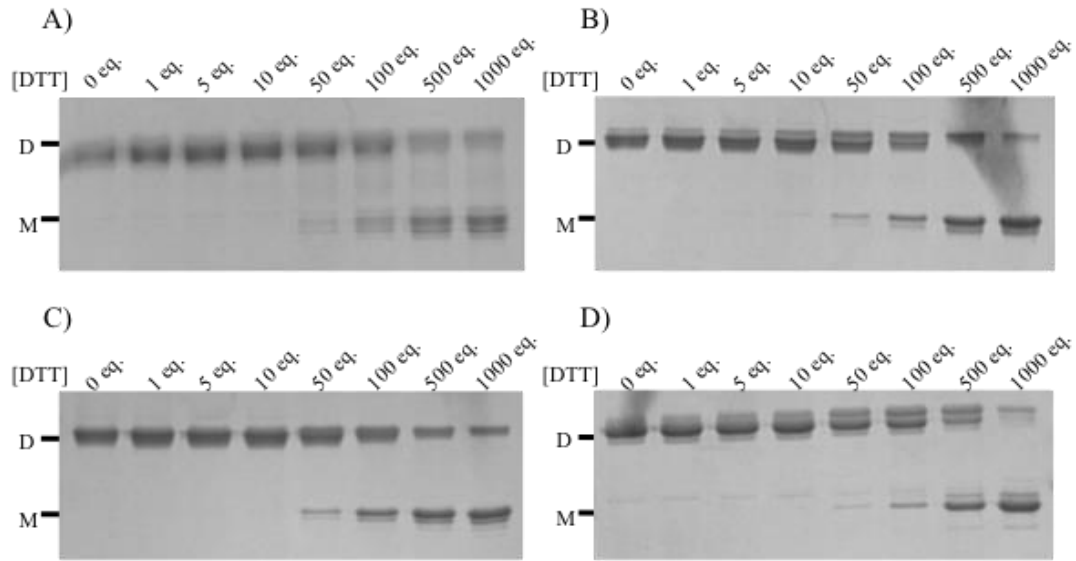


Figure 7. SDS-PAGE analysis of the reduction of Tsa1 and mutants by DTT. DTT-reduced Tsa1^{WT} (A), Tsa1^{T44A} (B), Tsa1^{T44S} (C) and Tsa1^{T44V} (D) (10 μ M) were previously oxidized with 1.2 eq. of hydrogen peroxide for 30 minutes at RT and reduced again for 1 minute at different concentrations of DTT as described in the upper part of the gels. Lane 1 contains the protein oxidized with 1.2 eq. of hydrogen peroxide and lanes 2-8 the product of reduction reaction with variable amounts of DTT. External left subtitles are: D = dimer and M = monomer.

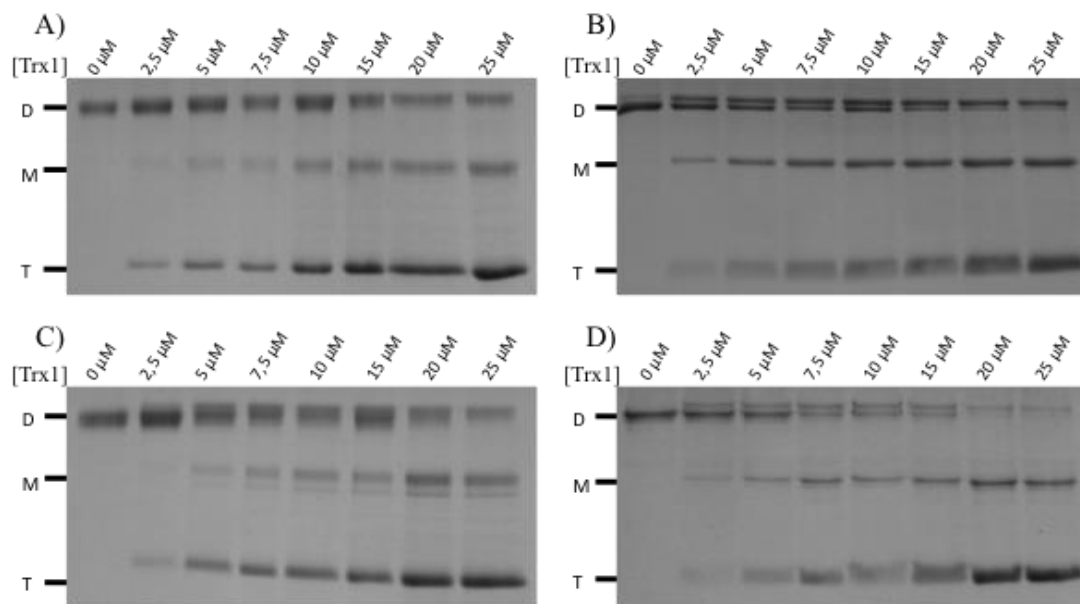


Figure 8. SDS-PAGE analysis of the reduction of Tsa1 and mutants by Trx1. Trx-reduced Tsa1^{WT} (A), Tsa1^{T44A} (B), Tsa1^{T44S} (C) and Tsa1^{T44V} (D) (10 μM) were previously oxidized with 1.2 eq. of hydrogen peroxide for 30 minutes at RT and reduced again for 1 minute at different concentrations of Trx1 as described in the upper part of the gels. Lane 1 contains the protein oxidized with 1.2 eq hydrogen peroxide. D = dimer, M = monomer and T=thioredoxin.

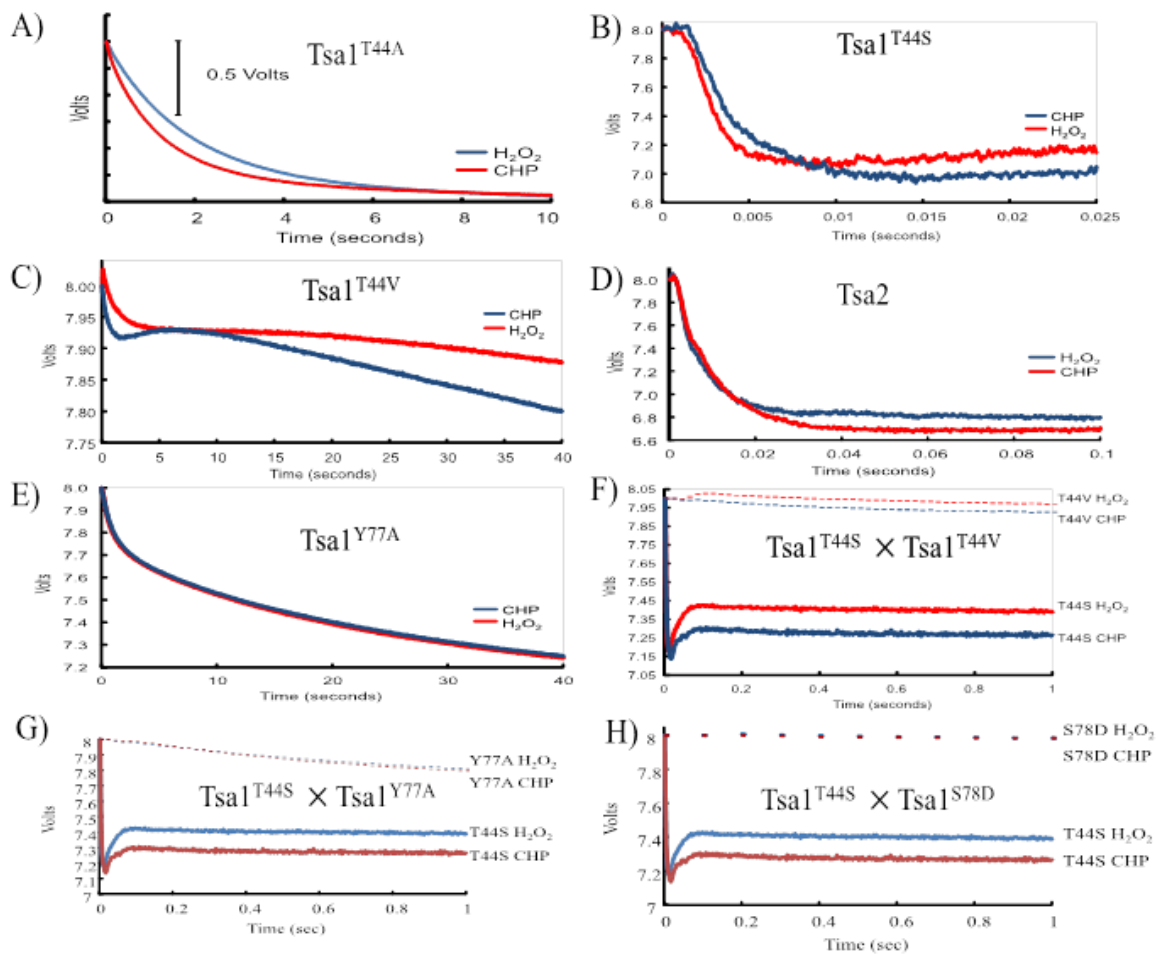


Figure 9. Representative examples of the rate of the reaction of Tsa1 mutants with hydrogen peroxide and cumene hydroperoxide. Samples were previously reduced with 20 mM DTT by 30 minutes at RT and the excess of reducing agent was removed by gel filtration. In the reaction, 20 μM of H_2O_2 (red trace) or CHP (blue trace) was added to 1 μM of Tsa1^{T44A} (A), Tsa1^{T44S} (B), Tsa1^{T44V} (C), Tsa2 (D), and Tsa1^{Y77A} (E). Comparative traces of Tsa1^{T44S} with Tsa1^{T44V} (F), Tsa1^{Y77A} (G) and Tsa1^{S78D} (H). The assays were performed in phosphate buffer (40 mM) pH = 7.4 at 25 °C. The rates were followed by the total fluorescence decay ($\lambda_{\text{ex}} = 280 \text{ nm}$). Similar experiments were performed with different concentrations of hydrogen peroxide or CHP to determine k_{obs} and k_{ox} for each enzyme as described in the experimental procedures.

APÊNDICE B

Lopes, A.M.; Nascimento, L. O.; Ribeiro, A.; Tairum, C.A.; **Breyer, C.A.**; Oliveira, M. A. Monteiro, G.; Souza Motta, C. M.; Magalhães, P. O. Avendaño, J. G. F.; Cavaco-Paulo, A.M.; Mazzola, P. G.; Rangel-Yagui, C. O.; Sette, L.D.; Converti, A.; Pessoa-Júnior, A. **THERAPEUTIC L-ASPARAGINASE: UPSTREAM, DOWNSTREAM AND BEYOND**. Artigo aceito para publicação no periódico *Critical Reviews in Biotechnology*.

6 REVIEW ARTICLE

9 **Therapeutic L-asparaginase: upstream, downstream and beyond**

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25 **Abstract**

27 L-asparaginase (L-asparagine amino hydrolase, E.C.3.5.1.1) is an enzyme clinically accepted as an
28 antitumor agent to treat acute lymphoblastic leukemia and lymphosarcoma. It catalyzes
29 L-asparagine (Asn) hydrolysis to L-aspartate and ammonia, and Asn effective depletion results in
30 cytotoxicity to leukemic cells. Microbial L-asparaginase (ASNase) production has attracted
31 considerable attention owing to its cost effectiveness and eco-friendliness. The focus of this
32 review is to provide a thorough review on microbial ASNase production, with special emphasis
33 to microbial producers, conditions of enzyme production, protein engineering, downstream
34 processes, biochemical characteristics, enzyme stability, bioavailability, toxicity and allergy
35 potential. Some issues are also highlighted that will have to be addressed to achieve better
36 therapeutic results and less side effects of ASNase use in cancer treatment: (a) search for new
37 sources of this enzyme to increase its availability as a drug; (b) production of new ASNases with
38 improved pharmacodynamics, pharmacokinetics and toxicological profiles, and (c) improve-
39 ment of ASNase production by recombinant microorganisms. In this regard, rational protein
40 engineering, directed mutagenesis, metabolic flux analysis and optimization of purification
41 protocols are expected to play a paramount role in the near future.

42 **Introduction**

44 L-asparaginase (ASNase) is an enzymatic drug and an
45 essential component of the combination chemotherapy
46 against diseases such as acute lymphoblastic leukemia
47 (ALL), lymphosarcoma, Hodgkin's disease, acute myelogen-
48 ous leukemia, acute myelomonocytic leukemia, chronic
49 lymphocytic leukemia, reticulosarcoma and melanosarcoma
50 (Avramis & Tiwari, 2006; Kumar et al., 2014; Pui & Evans,
51 2006; Verna et al., 2007). This drug depletes L-asparagine
52 (Asn) in blood, blocking protein synthesis and inhibiting
53 DNA and RNA synthesis in cancer cells. As a result, cell

86 **Keywords**

87 Acute lymphoblastic leukemia, antineoplastic
88 activity, biopharmaceutical, L-asparaginase,
89 microbial L-asparaginase production

90 **History**

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103 functions are impaired resulting in apoptosis (Bussolati et al.,
104 1995).

105 Normal cells, however, are able to synthesize Asn and are
106 less affected by its depletion by treatment with ASNase.
107 Nonetheless, when used for long-term treatment, it may cause
108 hypersensitivity leading to allergic reactions such as skin
109 rashes, respiratory disorders, low blood pressure, sweating
110 and loss of consciousness (Sarquis et al., 2004) as well as
111 anaphylaxis (Verna et al., 2007). Different ASNase prepar-
112 ations from *Escherichia coli* [native (EcA) and PEGylated
113 form] or *Erwinia chrysanthemi* [native form (ErA)] are
114 available on the market (Tong et al., 2014). Additionally, a
115 PEGylated recombinant *E. chrysanthemi*-derived ASNase is
116 currently in Phase I clinical trials (<http://www.clinicaltrials.gov/show/NCT01551524>).

117 Mashburn & Wriston (1963) proved that purified *E. coli*
118 ASNase exerted the same therapeutic effect as guinea pig
119 serum (Jain et al., 2012), with the advantage of easier
120

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121 production at lower cost (Schalk & Lavie, 2014). Asn has also
122 shown to be a fundamental nutritional requirement for the
123 *in vitro* growth of both Walker carcinosarcoma 256 and
124 leukemic mice cells.

125 In recent years, there has been increased interest in
126 ASNase use to treat ALL in adults, particularly young adults
127 (Rytting, 2012). Out of the 4000 ALL cases diagnosed yearly
128 in the USA, approximately two-thirds are children and
129 adolescents, making ALL the most common cancer among
130 this age group (Apostolidou et al., 2007; Pui & Evans, 2006).
131 Long-term improvement in children was reported to be
132 around 80%, and the overall survival rate 90%, while in adults
133 these figures were 38% and 50%, respectively (De Bont et al.,
134 2004; Pui & Howard, 2008). In recent decades, there has been
135 considerable progress in leukemia treatment. However,
136 because of issues related to the access to treatment, signifi-
137 cant differences in survival are reported among populations.
138 The five-year survival rate is 43% in the USA and Western
139 Europe, 25% in Japan, 24% in South America, 19% in India,
140 15% in Thailand, and 14% in sub-Saharan Africa. In areas
141 with access to treatment, the five-year survival rate in
142 children can reach 80% (Datusus, 2013; INCA, 2014).

143 ASNase is widely distributed in nature, it being found not
144 only in microorganisms, but also in plants and in tissues
145 (liver, pancreas, brain, ovary or testes, kidneys, spleen and
146 lungs) of several animals like fishes, mammals and birds.
147 However, microbes are a better source than animals or plants,
148 considering their ability to grow easily on rather simple and
149 inexpensive substrates. Furthermore, they offer easy opti-
150 mization of culture conditions for enzyme bulk production,
151 easy genetic modification to increase the yield, economically-
152 viable extraction and purification, good stability and
153 consistency (Thakur et al., 2014), coupled with the *ex situ*
154 preservation.

155 Taking into account this scenario, the aim of this review is
156 to provide a thorough review of microbial ASNase produc-
157 tion. More specifically, it focuses on microorganisms,

181 conditions for enzyme production by either submerged or
182 solid-state fermentation, applications, biochemical character-
183 istics and manipulation, and downstream processes.

184 Upstream processing – ASNase production

185 Bacterial fermentation

186 Although several species of bacteria are reported to produce
187 ASNase, *E. coli* and *E. chrysanthemi* are at present the main
188 microbial agents for industrial-scale production. ASNases
189 from these microorganisms have similar mode of action,
190 antineoplastic activity and toxicity, but are serologically and
191 biochemically distinct and have different pharmacokinetic
192 profiles (Kumar & Sobha, 2012).
193

194 ASNase has mainly been produced by bacterial or fungal
195 submerged fermentation (SF). Experimental evidence has
196 shown that this production is greatly influenced by various
197 factors such as type and concentration of carbon and nitrogen
198 sources, pH, temperature, fermentation time, aeration and
199 mainly the microbial agent (Basha et al., 2009; Gurunathan &
200 Sahadevan, 2012; Zia et al., 2013).
201

202 A comprehensive review on the main aspects of ASNase
203 production by bacterial SF was published by Kumar & Sobha
204 (2012), to which the reader is invited to refer. Therefore, a
205 simple list of the most significant bacteria able to extra-
206 cellularly express ASNase is provided (Table 1) (Abdel-Fatah,
207 1997; Abdel-Fatah et al., 1998; Alegre & Minim, 1993;
208 Amena et al., 2010; Basha et al., 2009; Fisher & Wray, 2002;
209 Geckil & Gencer, 2004; Geckil et al., 2006; Gladilina et al.,
210 2009; Gunasekaran et al., 1995; Kenari et al., 2011; Koshi
211 et al., 1997; Kotzia & Labrou, 2005; Kotzia & Labrou, 2007;
212 Kumar et al., 2010; Lebedeva & Berezov, 1997; Lubkowski
213 et al., 1996; Mahajan et al., 2012; Manna et al., 1995;
214 Narayana et al., 2008; Nawaz et al., 1998; Pinheiro et al.,
215 2001; Prakasham et al., 2007; Pritsa & Kyriakidis, 2001;
216 Ramaiah & Chandramohan, 1992; Sidoruk et al., 2011; Sinha
217 et al., 1991; Thenmozhi et al., 2011)
218

158
159 Table 1. Main bacteria employed in SF for L-asparaginase production.

160 Taxon	161 Reference	162 Taxon	163 Reference
164 Actinomycetes (Marine)	(Basha et al. 2009)	165 <i>Pseudomonas</i> spp.	
166 <i>Bacillus</i> spp.		167 <i>P. aeruginosa</i>	(Geckil et al. 2006)
168 <i>B. cereus</i>	(Thenmozhi et al. 2011)	169 <i>P. aurantiaca</i>	(Lebedeva & Berezov 1997)
170 <i>B. licheniformis</i>	(Mahajan et al. 2012)	171 <i>P. stutzeri</i>	(Manna et al. 1995)
172 <i>B. subtilis</i>	(Fisher & Wray 2002)	173 <i>Staphylococcus</i> spp.	(Prakasham et al. 2007)
174 <i>Enterobacter</i> spp.		175 <i>Streptomyces</i> spp.	
176 <i>E. aerogenes</i>	(Geckil & Gencer 2004)	177 <i>S. albidoflavus</i>	(Narayana et al. 2008)
178 <i>E. cloacae</i>	(Nawaz et al. 1998)	179 <i>S. gulbargensis</i>	(Amena et al. 2010)
180 <i>Erwinia</i> spp.		181 <i>S. longsporoflavus</i>	(Abdel-Fatah 1997)
182 <i>E. aroideae</i>	(Alegre & Minim 1993)	183 <i>S. phaeochromogenes</i>	(Abdel-Fatah et al. 1998)
184 <i>E. carotovora</i>	(Kotzia & Labrou 2005)	185 <i>S. plicatus</i>	(Koshi et al. 1997)
186 <i>E. chrysanthemi</i>	(Kotzia & Labrou 2007)	187 <i>Thermus</i> spp.	
188 <i>Escherichia coli</i>	(Kenari et al. 2011)	189 <i>T. thermophilus</i>	(Pritsa & Kyriakidis 2001)
190 <i>Helicobacter</i> spp.		191 <i>Vibrio</i> spp.	
192 <i>H. pylori</i>	(Gladilina et al. 2009)	193 <i>V. fisheri</i>	(Ramaiah & Chandramohan 1992)
194 <i>Nocardia</i> spp.		195 <i>V. hawveyi</i>	
196 <i>N. asteroides</i>	(Gunasekaran et al. 1995)	197 <i>V. proteus</i>	(Sinha et al. 1991)
198 <i>Pectobacterium</i> spp.		199 <i>Wolinella</i> spp.	
200 <i>P. carotovorum</i>	(Kumar et al. 2010)	201 <i>W. succinogenes</i>	(Lubkowski et al. 1996)
202 <i>Photobacterium</i> spp.		203 <i>Yersinia</i> spp.	
204 <i>P. leiognathi</i>	(Ramaiah & Chandramohan 1992)	205 <i>Y. pseudotuberculosis</i>	(Sidoruk et al. 2011)
206 <i>P. phoshoreum</i>		207 <i>Zymomonas</i> spp.	
		208 <i>Z. mobilis</i>	(Pinheiro et al. 2001)

Members of the Enterobacteriaceae family are undoubtedly the best ASNase producers among the eubacteria. The addition of 6% *n*-dodecane under dissolved oxygen levels above 80% increased the *E. coli* cell concentration by 12.7% and ASNase activity (up to 60.80 IU/mL) by 21% (Wei & Liu, 1998). Among the other Enterobacteriaceae, when 10 g/L lactose or cheese whey was used as the carbon source for bench-scale batch cultivation of *Erwinia aroidae*, the addition of Asn and yeast extract or tryptone remarkably stimulated ASNase production (Alegre & Minim, 1993; Minim & Alegre, 1992). Also, the Gram-negative, rod-shaped bacterium *Enterobacter cloacae* was able to successfully utilize Asn either as the sole carbon and nitrogen source or in combination with L-fructose, D-galactose, sucrose or maltose and expressed ASNase intracellularly (Nawaz et al., 1998). ASNase production was also reported in *Enterobacter aerogenes* cultured on different carbon sources such as glucose, lactose, mannitol and glycerol (Geckil & Gencer, 2004; Geckil et al., 2005), but its activity was low (0.60 U/mL).

Another eubacterium able to express high levels of ASNase is *Pectobacterium carotovorum* MTIC 1428 (Kumar et al., 2009); when grown under optimized conditions on a medium containing glucose, yeast extract, peptone and Asn, it exhibited a maximum activity of 15.39 U/mL (specific activity of 27.88 U/mg) after only 12 h along with the highest volumetric productivity (1.282 U/mLh) reported in the literature, to the best of our knowledge.

Although reports on ASNase production by filamentous bacteria are quite scarce, some of them, especially marine actinomycetes, were shown to be effective producers of this enzyme, among which is the thermophilic soil isolate S3 that displayed a ASNase activity as high as 49.20 U/mL at pH 7.5 and 50 °C (Basha et al., 2009). These results open a new scenario in the search for more heat-resistant ASNases from thermophilic bacteria.

In recent years, solid-state fermentation (SSF) has emerged as a valid alternative to SF for the production of extracellular

enzymes (Venil & Lakshmanaperumalsamy, 2009), because it allows employing agroindustrial wastes as nutrient sources and holds potential for the production of secondary metabolites, especially in developing countries (Sangeetha et al., 2004). In comparison with SF, only a few reports are available on SSF for ASNase production, mainly using agroindustrial wastes, among which are soy bean meal (Abdel-Fattah & Olama, 2002; Basha et al., 2009; El-Bessoumy et al., 2004), rice bran (Venil & Lakshmanaperumalsamy, 2009), gram husk and coconut (Hymavathi et al., 2009). The most significant results found in the literature on SSF for ASNase production by bacteria are summarized in Table 2 (Abdel-Fattah & Olama, 2002; El-Bessoumy et al., 2004; Ghosh et al., 2013; Hymavathi et al., 2009; Venil & Lakshmanaperumalsamy, 2009; Vuddaraju et al., 2010).

To provide only the most significant examples, optimization of SSF for ASNase production by *Pseudomonas aeruginosa* and *Serratia marcescens* has been reported by several authors. El-Bessoumy et al. (El-Bessoumy et al., 2004) obtained, under optimal conditions, ASNase activity of 165.1 IU/mL (specific activity of 17.90 IU/mg) after 96 h of fermentation by *P. aeruginosa* 50071 in a medium with 40% moisture content containing 22 g/L soy bean meal, at pH 7.4 and 37 °C. When 3.11% (w/v) casein hydrolyzate and 3.68% (w/v) corn steep liquor were added as supplementary nutrients to the same medium (20 g/L soy bean meal), but with higher moisture content (50%), this strain exhibited, under comparable conditions (pH 7.0; 37 °C; 96 h), 38% lower specific activity (Abdel-Fattah & Olama, 2002), which confirms the influence of both environmental and nutritional conditions on SSF performance.

Fungal fermentation

Because all available ASNase therapeutic preparations are from prokaryotic sources, anaphylactic and other immunological side effects as well as enzyme inactivation are frequent events. In this sense, the search for ASNase sources

Table 2. Summary of fermentation conditions and results of L-asparaginase production by bacteria in SSF.

Microorganism	Substrates/conditions	L-asparaginase production	Reference
<i>P. aeruginosa</i> 50071	Soy bean meal 20 g/L; moisture content 50%; casein hydrolysate 3.11% (w/v); corn steep liquor 3.68% (w/v); pH 7.0; 37 °C; 96 h	11.15 IU/mg of proteins	(Abdel-Fattah & Olama 2002)
	Soy bean meal 22 g/L; moisture content 40%; pH 7.4; 37 °C; 96 h.	17.90 IU/mg of proteins; 165.1 IU/mL of crude extract	(El-Bessoumy et al. 2004)
<i>Bacillus circulans</i> MTCC 8574	Red gram husk; moisture content 99.5%; glucose 0.234% (w/w); Asn 0.248% (w/w); pH 7.0; 36.3 °C; 24 h.	2.322 U/gds	(Hymavathi et al. 2009)
<i>S. marcescens</i> SB08	Rice bran 10 g; moisture content 40%; Asn 0.01%; yeast extract 0.5%; pH 7.0; 30 °C; 36 h.	79.84 U/gds	(Venil & Lakshmanaperumalsamy 2009)
<i>S. marcescens</i> NCIM 2919	Sesame oil cake 40 g/L; moisture content 68.64%; glucose 3.1% (w/w); NaNO ₃ 1.01% (w/w); pH 7.0–7.5; 30 °C; 48 h.	110.8 U/gds	(Vuddaraju et al. 2010)
	Coconut oil cake 6 g; moisture content 40%; pH 6; 37 °C.	3.87 U/gds	(Ghosh et al. 2013)

Units are different because studies report values expressed in different units. IU, international unit; gds, grams of dried substrate.

Table 3. Main yeasts and filamentous fungi employed for L-asparaginase production in SF.

Taxon	References	Taxon	Reference
<i>Aspergillus</i> spp.		<i>Fusarium</i> spp.	
<i>A. niger</i>	(Zia et al. 2013, Alhussaini 2013)	<i>F. moniliforme</i>	(Tippani & Sivadevuni 2012)
<i>A. tamari</i>	(Sarquis et al. 2004)	<i>F. semitectum</i>	(Alhussaini 2013)
<i>A. terreus</i>	(Gurunathan & Sahadevan 2012)	<i>Paecilomyces</i> spp.	(Gupta et al. 2009)
<i>A. flavus</i>	(Alhussaini 2013)	<i>Penicillium</i> spp.	
<i>Bipolaris</i> spp.	(Lapmak et al. 2010)	<i>P. chrysogenum</i>	(Alhussaini 2013)
<i>Candida</i> spp.		<i>P. crustosum</i>	
<i>C. utilis</i>	(Kil et al. 1995)	<i>P. digitatum</i>	(Shrivastava et al. 2012)
<i>Cladosporium</i> spp.	(Kumar & Manonmani 2013)	<i>P. olsonii</i>	(Alhussaini 2013)
<i>Cylindrocarpon</i> spp.		<i>Rhodospiridium</i> spp.	
<i>C. obtusisporum</i>	(Raha et al. 1990)	<i>R. toruloides</i>	(Ramakrishnan & Joseph 1996)

from eukaryotic microorganisms can lead to enzymes with lower side effects to humans (Shrivastava et al., 2012). To achieve this purpose, the use of screening techniques is an important step of any prospecting process, which can be made based on function-driven analysis (e.g. color changes, fluorescence, presence of inhibition zones) or on sequence-driven analysis (e.g. gene detection by PCR, hybridization with specific probes). For both approaches, high- or medium-throughput screening has to be considered when a high number of biological materials or targets are the prospection focus (Sette et al., 2013).

Endophytic fungi have been reported as interesting ASNase producers. Theantana et al. (Theantana et al., 2007; Theantana et al., 2009), who investigated the major fungi present in Thailand medicinal plants, found the main producers of this enzyme in the genera *Colletotrichum*, *Eupenicillium*, *Fusarium*, *Penicillium* and *Talaromyces*. Marine environment, as well, appears to be a prolific source of ASNase-producing fungi. Thirunavukkarasu et al. (Thirunavukkarasu et al., 2011) found that this enzyme is secreted by seaweeds endophytes of the genera *Alternaria*, *Chaetomium*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Nigrospora*, *Paecilomyces*, *Phaeotrichoconis*, *Phoma* and *Pithomyces*. Sudha (Sudha, 2009) reported that 17 fungal strains recovered from mangrove soil were able to produce ASNase, and Sundaramoorthi et al. (Sundaramoorthi et al., 2012) selected five fungal strains isolated from different soils from Arabian Sea that exhibited the same activity.

A list of yeasts and filamentous fungi able to produce ASNase in SF is provided in Table 3 (Alhussaini, 2013; Gupta et al., 2009; Gurunathan & Sahadevan, 2012; Kil et al., 1995; Kumar & Manonmani, 2013; Lapmak et al., 2010; Raha et al., 1990; Ramakrishnan & Joseph, 1996; Sarquis et al., 2004; Shrivastava et al., 2012; Tippani & Sivadevuni, 2012; Zia et al., 2013).

Tippani & Sivadevuni (2012) detected ASNase activities of 404.0 and 376.0 IU/mL for *Fusarium semitectum* and *Fusarium moniliforme* using proline and glucose as nitrogen and carbon sources, respectively, while maximum production by *Fusarium oxysporum* (360.0 IU/mL) occurred with sodium nitrate. These results appear to be a clear proof of the leading role of nutritional factors in SF by fungi.

As far as the members of the *Aspergillus* genus are concerned, *Aspergillus niger* (Mishra, 2006), *Aspergillus terreus* and *Aspergillus tamari* (Sarquis et al., 2004) were shown to have great potential to produce ASNase. Limiting

the comparison only to the best results collected in the literature, *A. terreus* MTCC 1782 exhibited L-ASNase activity in the range 16.05–43.99 IU/mL when cultivated for 58–96 h in SF on Czapek–Dox medium (pH 6.0–6.3) supplemented with Asn, L-proline, glucose or sucrose at 30–35 °C and 140–160 rpm (Gurunathan & Sahadevan, 2012; Gurunathan & Renganathan, 2011). Lower ASNase activity was reported for *A. niger* (5.45 IU/mL) in the same medium (pH 6.5) supplemented with glucose and Asn at 35 °C and 120 rpm after 96 h (Zia et al., 2013) as well as for *A. flavus* (1.76 IU/mL) in a glucose-Asn medium (pH 4.5) supplemented with starch under static conditions at 30 °C (Patro et al., 2014).

As regards to other fungal genera, *Bipolaris* BR438 isolated from brown rice was reported by Lapmak et al. (Lapmak et al., 2010) to be the best ASNase producer among other genera, exhibiting ASNase activity of 6.30 IU/mL when cultivated in the Czapek–Dox medium containing 1% Asn and 0.4% glucose at 30 °C for 72 h.

Regarding SSF by fungi, several studies attempted to maximize ASNase production using different substrates and conditions. These results are summarized in Table 4 (Hosamani & Kaliwal, 2011; Mishra, 2006; Nair et al., 2013; Rani et al., 2012; Uppuluri & Reddy, 2009; Uppuluri et al., 2013). The best enzyme producers in SSF belong to the *Aspergillus* genus. Rani et al. (Rani et al., 2012) obtained maximum specific activity of 70.67 U/g with *Aspergillus* sp. KUFS20 using orange peel as a substrate, while *A. terreus* MTCC 1782 yielded no less than 253.0, 110.0 and 85.00 U/g on moistened pomegranate, wheat bran and coconut oil cake, respectively, after 72–120 h (Nair et al., 2013). Mishra (2006) reported, under aerobic conditions, a maximum ASNase specific activity of 40.90 U/g using *glycine max* bran with 70% moisture content and a mean particle size of 1205–1405 µm, after 96 h of SSF by *A. niger* at pH 6.5 and 30 °C.

The highest ASNase specific activity was reported for *A. niger* (344.0 U/g) cultivated in SSF on agro-wastes in a column bioreactor using sesame (black) oil cake as a substrate at 32 °C, 0.4 vvm aeration and 22 cm bed thickness (Uppuluri & Reddy, 2009; Uppuluri et al., 2013).

These results, taken together with those of SSF by bacteria, are very promising from the ASNase activity viewpoint; however, since SSF requires complex media, it appears to be still very far from satisfying the high purity requirements of ASNase for pharmaceutical and medical applications. Furthermore, in our opinion, the large availability of

Table 4. Summary of fermentation conditions and results of L-asparaginase production by filamentous fungi in SSF.

Microorganism	Substrates/conditions	L-asparaginase production	References
<i>A. niger</i>	Bran of <i>Glycine max</i> ; moisture content 70%; pH 6.5; 30 °C; 96 h.	40.90 U/gds	(Mishra 2006)
	Column bioreactor; sesame (black) oil cake; aeration of 0.4 vvm; bed thickness of 22 cm; 32 °C.	344.0 U/gds	(Uppuluri & Reddy 2009, Uppuluri et al. 2013)
<i>Fusarium equiseti</i>	Soya bean meal; particle size 3 mm; 0.5% glucose; 0.5% (NH ₄) ₂ SO ₄ ; 0.5% yeast extract; moisture 70%; 20% (v/v) inoculum; 45 °C; 48 h	8.51 IU/mL of crude extract	(Hosamani & Kaliwal 2011)
<i>Aspergillus</i> sp. KUFS20	Orange peel; 0.01 M phosphate buffer; moisture 50%; inoculum 10 ⁶ spores/mL; pH 6.2; 30 °C; 96 h	70.67 U/gds	(Rani et al. 2012)
<i>A. terreus</i> MTCC 1782	Moistened pomegranate; inoculum 10 ⁷ spores/mL; 30 °C; 120 h	253.0 U/gds	(Nair et al. 2013)
	Moistened wheat bran; inoculum 10 ⁷ spores/mL; 30 °C; 72 h	110.0 U/gds	
	Moistened coconut oil cake; inoculum 10 ⁷ spores/m; 30 °C; 120 h	85.00 U/gds	

Units are different because studies report values expressed in different units. IU, international unit; gds, grams of dried substrate.

molecular biology tools makes the screening of fungal isoforms, rather than the cultivation of filamentous fungi, an interesting issue for future construction of a recombinant organism for ASNase production.

Downstream processing

Processes for producing therapeutic proteins have achieved substantial advances in the last decades. Nonetheless, purification processes deserve the same attention (Dutra-Molino et al., 2014). Downstream steps might reach 50–80% of the total production costs of proteins, and the best step combination can result in significant enhancement in terms of purification and process economy.

Protein precipitation

Separation by precipitation from an aqueous extract is the most traditional method to recover and purify biomolecules (Golunski et al., 2011). This method results in a temporary disruption of protein secondary and tertiary structures, leading to its precipitation. It is a technique easy to scale-up, with simple equipment requirements, low costs and possibility to use a large number of precipitants. Moreover, the precipitant agent can be recycled in the final process by distillation, evaporation or heat drying, thus reducing the environmental impact associated to its disposal.

ASNase recovery from different sources often employs precipitation. Several authors have purified ASNase up to apparent homogeneity by (NH₄)₂SO₄ precipitation (Amena et al., 2010; Basha et al., 2009; Gervais et al., 2013; Manna et al., 1995; Mishra, 2006; Moorthy et al., 2010). In general, partial purification starts with the addition of finely powdered (NH₄)₂SO₄ to the crude extract containing the enzyme up to saturation, causing ASNase precipitation. Salt concentration ranges from 35% to 100%, resulting in different yields depending on the ASNase source. Moorthy et al. (Moorthy et al., 2010) recovered up to 96.2% ASNase from *Bacillus* sp. grown on glucose, with purification fold of 10.9 after precipitation. Salt precipitation is usually followed by

centrifugation and column filtration to increase the purification yield. Production and purification of *Streptomyces gulbargensis* ASNase yielded 50.6%, with 1.8 purification fold. Purification was increased to 26.9-fold after precipitate was collected by centrifugation and dialyzed against buffer followed by Sepharcyl S-200 column filtration (Amena et al., 2010). Similar strategy was employed by El-Bessoumy et al. (El-Bessoumy et al., 2004) in the attempt to produce, isolate and purify ASNase from *P. aeruginosa* after SSF. Purification after (NH₄)₂SO₄ precipitation resulted in 5.2-fold purification and increased over five times after gel filtration through Sephadex G-100.

Other precipitant agents such as ethanol can also be successfully employed to pre-purify and concentrate the target biomolecule. Ethanol is widely produced in Brazil and worldwide (Golunski et al., 2011) and can be recycled after precipitation reducing the environmental impact.

Precipitation is one of the first steps in the downstream process and it is usually combined with traditional techniques to enhance biomolecules purification fold and process yield. Precipitation can also concentrate the target molecule reducing the volume for later stages (Glatz, 1990). Nonetheless, innovative techniques should be investigated aiming at faster and less expensive purification processes.

Liquid-liquid extraction

One interesting alternative to be exploited in separation science for biomolecules extraction/purification is liquid-liquid extraction (LLE) by aqueous two-phase systems (ATPS). LLE is defined as the removal of a solute from a liquid (or liquid mixture) phase when in contact with another immiscible or partially soluble liquid (or liquid mixture) where the component (solute) is preferentially soluble. Its partition can be improved using simple tools such as, for instance, the addition of affinity ligands (Albertsson, 1986; Lam et al., 2004). ATPS can be obtained when mixtures of water-soluble polymers are combined with another polymer or with certain inorganic salts above critical concentrations.

601 They can also be formed using other materials such as
602 surfactants, block copolymers and ionic liquids. Several
603 physicochemical properties influence protein partitioning in
604 two-phase systems, namely isoelectric point, surface hydro-
605 phobicity and molar mass of system components. Also,
606 partitioning depends on polymer or surfactant concentration,
607 pH and salt addition (Albertsson, 1986).

608 Few attempts were made to employ ATPS to purify
609 ASNase. Qin and Zhao (2003) described a combined strategy
610 to release and separate ASNase from *E. coli* ATCC 11303
611 cells by aqueous two-phase micellar systems (ATPMS). Cells
612 treated with 9.4% (w/v) K_2HPO_4 and 15% (w/v) Triton X-100
613 at 25 °C for 15–20 h released nearly 80% of the enzyme, while
614 keeping whole. Considering the structure of *E. coli* cells and
615 that ASNase is located in the periplasmic space, those authors
616 proposed that Triton X-110 micelles might disrupt the outer
617 membrane and cause the release of the enzyme from the
618 periplasmic space. For this system (surfactant/phosphate/
619 water), phase separation into a micelle-rich top phase and a
620 salt-rich bottom phase was observed above critical micelle
621 concentration. Most of the released enzyme was recovered in
622 the bottom, phosphate-rich phase. In spite of the potential
623 offered by ATPMS to release/purify ASNase, no data on
624 purification factor or contaminants/whole cell partitioning
625 behavior was reported.

626 Jian-Hang et al. (2007) proposed another strategy combin-
627 ing cell disruption by high-pressure homogenization and
628 product capture by ATPMS for the extraction of intracellular
629 ASNase from *E. coli*. They employed triblock copolymers of
630 poly(propylene oxide) (PPO) and poly(ethylene oxide) (PEO),
631 PEO–PPO–PEO, to form primary ATPS for enzyme purifi-
632 cation. ASNase purification via this novel *in situ* process
633 resulted in increased enzyme yield (from 52% to 73%) and
634 specific activity (from 78.60 to 94.80 U/mg) compared with
635 the conventional process, including cell disruption, centrifu-
636 gal clarification and subsequent ATPMS.

637 ATPS still needs further investigation to be employed for
638 commercial ASNase purification, but the results obtained
639 with this technique already point to its potential. Furthermore,
640 we believe that there is a need to further investigate ATPS
641 composed of ionic liquids for ASNase purification, which
642 seems to be a particularly promising alternative (Souza et al.,
643 2015).

644 Chromatography

647 Intravenous enzymes preparations such as ASNase require
648 high levels of purity, thus a sequence of purification processes
649 is necessary. A high degree of enzyme purity is also important
650 from the viewpoint of process control, but steps required for
651 purification, in general, result in enzyme activity loss and
652 increased final cost (Gräslund et al., 2008).

653 ASNase from *P. aeruginosa* 50071 obtained by SSF was
654 purified by $(NH_4)_2SO_4$ fractionation, Sephadex G-100 gel
655 filtration and CM-Sephadex C50 ionic exchange, and the
656 specific activity increased from 17.90 (crude extract) to
657 1900 IU/mg (final preparation) (El-Bessoumy et al., 2004). A
658 similar method was used by Singh et al. (2013) to purify
659 extracellular ASNase from the protease-deficient *Bacillus*
660 *aryabhatai* ITBHU02 strain. After $(NH_4)_2SO_4$ fractionation

and DEAE-Sepharose fast flow and Seralose CL-6B column 661
chromatographic steps, the enzyme was 68.9-fold purified 662
with specific activity of 680.47 U/mg. Warangkar and 663
Khobragade (2010), who extracted and purified ASNase 664
from *E. carotovora* by $(NH_4)_2SO_4$ fractionation (60–70%), 665
followed by chromatographic steps using Sephadex G-100, 666
CM cellulose and DEAE Sephadex, observed an 667
increase in the enzyme specific activity from 1.36 to 668
1034 IU/mg, with a minimum mass loss and a final recovery 669
of 36.5%. 670

Penicillium digitatum was also used to produce extracel- 671
lular ASNase. After protein precipitation and desalting, gel 672
filtration chromatography with Sephadex G-25, followed by 673
Sephadex G-100, resulted in a 60.9-fold purification. Loureiro 674
et al. (2012) purified ASNase from *Aspergillus* sp. by ion 675
exchange chromatography (DEAE Sepharose) followed by gel 676
filtration (Sephacryl S-200HR) at different flows, resulting in 677
a 12% final yield and a 7.72 purification factor. An additional 678
purification step by reapplying the pooled fraction to the same 679
Sephacryl S-200HR column resulted in even lower yield 680
(7.28%) with a purification factor of 10.7. 681

In recent work, ASNase has been obtained from the genus 682
Cladosporium. Precipitation followed by DEAE cellulose ion 683
exchange and subsequent size exclusion chromatography 684
ensuring a final specific activity of 83.3 U/mg (Kumar & 685
Manonmani, 2013). 686

This overview reveals that protocols for ASNase purifica- 687
tion from different sources lead to different purification 688
performances; therefore, any comparison is not straightfor- 689
ward. Nonetheless, one can see that gel filtration and ion- 690
exchange chromatography are the most employed purification 691
steps. They are often preceded by precipitation with 692
 $(NH_4)_2SO_4$ as a first pre-chromatographic step, which, in 693
our opinion, may be replaced by some ATPS. 694

695 ASNase formulation

696 ASNase PEGylation

697 Conjugation of proteins with polyethylene glycol (PEG), or 698
PEGylation, has become a well-established technology in the 699
field of biopharmaceutical formulations to increase half-life. 700
It reduces the urinary excretion of a biomolecule (Yang et al., 701
2004) as well as its enzymatic degradation due to the 702
increased steric bulk (Veronese & Pasut, 2005). In addition, 703
PEGylated bioproducts often exhibit reduced affinity for the 704
target receptor compared with the native precursor, which can 705
lead to a lower clearance by target-mediated clearance 706
mechanisms. Finally, the addition of PEG moieties might 707
enhance the immunological profile of a biomolecule by 708
reducing its ability to raise antibodies (Mehvar, 2000). 709

710 Since PEG polymers are highly hydrated, with 2–3 water 711
molecules per ethylene glycol unit, their hydrodynamic radii 712
is approximately 5- to 10-fold greater than would be 713
predicted based on their nominal molecular weight (Harris 714
& Chess, 2003), underlying a dramatic increase in the 715
effective molecular size of PEG–protein conjugates. PEG 716
provides protein protection from proteases and peptidases, by 717
impairing access of proteolytic enzymes. Conjugation with 718
PEG creates a hydration shell surrounding proteins, whose 719
size determines the degree of hydration and depends on PEG 720

721 structure, in that branched configuration has higher hydration
722 level compared with the random coil one (Tirosch et al., 1998).
723 Such a dynamic hydrated PEG shell is also responsible for
724 protection from proteolysis. Despite this, the high flexibility
725 of the PEG backbone chain usually enables high affinity
726 interactions between enzyme and substrate. Consequently,
727 PEGylated therapeutic proteins may retain efficacy while
728 acquiring greater stability in plasma (Fishburn, 2008).

729 PEG-ASNase was approved by the US Food and Drug
730 Administration in 1994 for the treatment of patients exhibit-
731 ing hypersensitivity to the native *E. coli* enzyme and in 2006
732 as a first-line treatment for patients with ALL. *E. coli*
733 ASNase, one of the first PEGylated proteins, was produced by
734 a random process coupling several 5 kDa PEG chains with the
735 enzyme surface. As a result, polydispersity is considerable in
736 PEG-ASNase formulations (Pasut et al., 2008).

737 Monomethoxylated PEG is generally used in protein
738 conjugation because its monofunctionality yields cleaner
739 chemistry. However, PEG diol impurities, with molecular
740 weight approximately twice that of the monofunctional
741 methoxy-PEG, are always formed in a percentage (1–10%)
742 that rises with PEG molecular weight, hence increasing the
743 polydispersity of conjugates (Veronese, 2001).

744 Enzyme PEGylation may be accompanied by loss of
745 biological activity of the conjugate compared with the native
746 enzyme, owing to sterical hindrance of the active site, which
747 may be somehow prevented by site-specific modifications
748 (Veronese & Pasut, 2005). In spite of these drawbacks, PEG-
749 ASNase formulations have longer half-life compared with
750 native enzymes (5 and 10 times longer than free *E. coli* and
751 *Erwinia* enzymes, respectively) (Dinndorf et al. 2007) and
752 increased thermostability (Soares et al., 2002). PEG-ASNase
753 activity in plasma is detectable 1 h after injection and
754 elimination half-life is 5.5–7.0 days, while native *E. coli*
755 and *Erwinia* ASNases have elimination half-lives of 26–30
756 and 16 h, respectively (Avramis & Panosyan, 2005).

757 After intramuscular injection, PEG-ASNase level grad-
758 ually increases, whereas that of Asn gradually falls over
759 several days. Intravenous injection of PEG-ASNase is better
760 tolerated and may lessen discomfort to patients and provide
761 immediate Asn depletion. Although ASNase dosage strongly
762 depends on its half-life, the effect of route of administration is
763 not so evident (Avramis & Panosyan, 2005).

764 In spite of increased half-life, decreased dosage and
765 frequency of PEG-ASNase, the degree to which PEGylation
766 reduces the enzyme immune response is unclear, and there are
767 reports where patients developed hypersensitivity to the
768 PEGylated form. Antibodies against PEG were found in
769 some patients with undetectable ASNase activity after
770 receiving PEG-ASNase and were proposed as the cause of
771 rapid clearance of conjugates and absence of enzyme activity
772 in serum (Pasut & Veronese, 2009).

773 Associated with the longer half-life of PEG-ASNase in
774 serum and, as a result of enzyme action, there is the risk of an
775 increase of ammonia blood concentrations to clinically
776 significant levels. Hyperammonemia, after ASNase therapy,
777 was first reported by Leonard and Kay in 1986 (Heitink-Pollé
778 et al., 2013), but the ASNases preparations they used (EcA
779 and ErA) had short half-life, and ammonia was rapidly
780 removed from circulation. Due to the longer PEG-ASNase

781 half-life, the ammonia concentration could not return to 781
782 normal levels between doses, and ammonia toxicity accumu- 782
783 lated, with the appearance of hyperammonemia symptoms 783
784 that disappeared after cessation of enzyme administration 784
785 (Heitink-Pollé et al., 2013). Based on this background, despite 785
786 the benefits of conjugating therapeutic proteins with PEG, in 786
787 our opinion, drug pharmacodynamics and pharmacokinetics 787
788 have to be better characterized to ensure therapeutics 788
789 efficiency of PEG-ASNases with minimal negative side 789
790 effects. 790

791 Freeze-dried formulations 792

793 Parenteral enzyme formulations must comply with their 793
794 specifications during long-term storage. Water-dispersed 794
795 enzymes, however, might be degraded through peptide bond 795
796 hydrolysis and deamination, among other reactions, which are 796
797 favored by a temperature increase (Singh et al., 2009). 797
798 ASNase is susceptible to these reactions, which reduce its 798
799 long-term or even short-term stability. For example, it was 799
800 shown that partial deamination of ErA and EcA changes their 800
801 isoelectric point and plasma half-life, although with no 801
802 changes in enzymatic activity (Gervais et al., 2013). 802

803 Freeze-drying can prevent most water-related reactions by 803
804 sublimating water from the frozen product under vacuum, 804
805 also allowing sterile drying without heating or chemical 805
806 sterilization. This process, however, suffers from other 806
807 undesired effects such as cold denaturation, freeze denatur- 807
808 ation and osmotic pressure increase due to dehydration and 808
809 cryoconcentration (Singh et al., 2009), whose relative 809
810 importance can be evaluated by splitting the process into 810
811 freezing and drying. 811

812 As regards the freezing process, ErA activity was lost up to 812
813 40% when the enzyme was subject to freeze–thaw cycling, 813
814 restored 24 h after freeze–thaw at -20°C and partially 814
815 restored when the freezing temperature reached -40°C . 815
816 Transient activity loss depends on the tetramer dissociation 816
817 rate (Gervais et al., 2013), whereas cleavage of dissociated 817
818 monomers leads to aggregation and permanent activity loss 818
819 (Jameel et al., 1997). Reconstituted EcA (Elspar[®]) was shown 819
820 to retain *in vitro* activity after cold storage for at least 14 days 820
821 (4°C) and freezing (-20°C) for at least 6 months, besides 821
822 resisting repeated freeze–thaw cycles (Wypij & Pondenis, 822
823 2013). Moreover, the presence of mannitol in Elspar[®] 823
824 formulation may contribute to prevent cold denaturation. 824

825 Freeze-drying without protectants is rarely an option for 825
826 proteins. Hellman et al. (1983) tested several protectants at 826
827 different concentrations, which had been submitted to the 827
828 same freeze-drying procedure as ErA. Glucose, mannose and 828
829 sorbitol showed 100% tetramer and activity retention after 829
830 reconstitution at pH 10, while 20% and 50% retentions were 830
831 observed in the absence of any protectant and in the presence 831
832 of mannose contained in commercial formulations, respect- 832
833 ively. Protein retention reached a maximum value when the 833
834 enzyme was freeze dried without protectants and reconsti- 834
835 tuted at pH 7.5. Conversely, Izutsu et al. (Izutsu et al., 1994) 835
836 observed 50% activity retention with the naked enzyme and 836
837 proposed mannitol as a good protectant under natural 837
838 conditions, as long as the amorphous form is maintained. 838
839 Such discordance might be related to different 839
840 840

841 freeze-drying protocols employed by these research-groups,
842 in that the former froze the product at -35°C for 24 h, while
843 the latter quickly froze with liquid nitrogen.

844 The collapse temperature is an essential parameter for
845 freeze-drying; formulations must in fact be dried below their
846 collapse temperature to maintain stability, easy reconstitution
847 and dry cake form. Adams and Ramsay (Adams & Ramsay,
848 1996) characterized ErA formulations with a variety of
849 protectants in terms of collapse temperature, cake structure
850 and activity/tetramer retention. Lactose was shown to be the
851 most suitable protectant among those tested, together with
852 lactose/mannitol combination. These authors performed a
853 cycle optimization to reduce the drying time, based on the
854 collapse temperature. Lactose effectiveness as a protectant
855 during ErA freeze-drying confirmed the results of a previous
856 study that demonstrated the potential of other disaccharides as
857 enzyme stabilizers such as trehalose and maltose (Adams &
858 Irons, 1993).

859 Lyoprotection mechanisms are not fully elucidated. Based
860 on the current hypothesis that protectants substitute water in
861 protein hydration shell, Ward et al. (1999) proposed that each
862 highly polar residue of ASNase requires one molecule of
863 protectant, and this way they were able to estimate sugar
864 concentration, but failed to predict PEG amount.

865 Instead of adding protectants, it is possible to conjugate
866 them to improve thermostability, among other properties.
867 For instance, PEGylated EcA activity was entirely
868 recovered when reconstituted after freeze-drying (Soares
869 et al., 2002). EcA conjugation with inulin, on the other
870 hand, led to improvement in freeze-thawing resistance with
871 no significant change in freeze-drying (Tabandeh & Aminlari,
872 2009).

873 Literature on EcA freeze-drying is scarce and outdated,
874 while ErA is more explored. Papers are generally broken
875 down into formulation screening or process parameters, with
876 large disparities in freeze-drying methodology. Patents were
877 deposited but, for obvious reasons, information is not
878 straightforward. So far, no model is adequate enough to
879 predict freeze-drying behavior of protein solutions; therefore,
880 empirical testing is still needed to obtain an adequate
881 formulation.

884 **Structural features, undesirable characteristics and** 885 **protein engineering of bacterial ASNases**

886 Despite being a widely used drug, ASNase possesses a
887 secondary L-glutaminase activity (GLNase), and several side
888 effects are associated with ASNase-based treatments, includ-
889 ing immunological reactions, hepatotoxicity, neurotoxicity,
890 coagulation abnormalities, among others. Additionally, its
891 administration to patients often results in a rapid decay of
892 circulating ASNase levels, leading to high administration
893 frequency. Determination of crystallographic structure of
894 several ASNases has assisted decisively in a better elucidation
895 of the enzyme features and the catalytic process. Moreover,
896 these data have currently enabled approaches involving
897 rational enzyme engineering based on structural data and
898 *in silico* methods, aiming to obtain more efficient and specific
899 bacterial ASNases.

Enzyme structure and catalytic mechanism

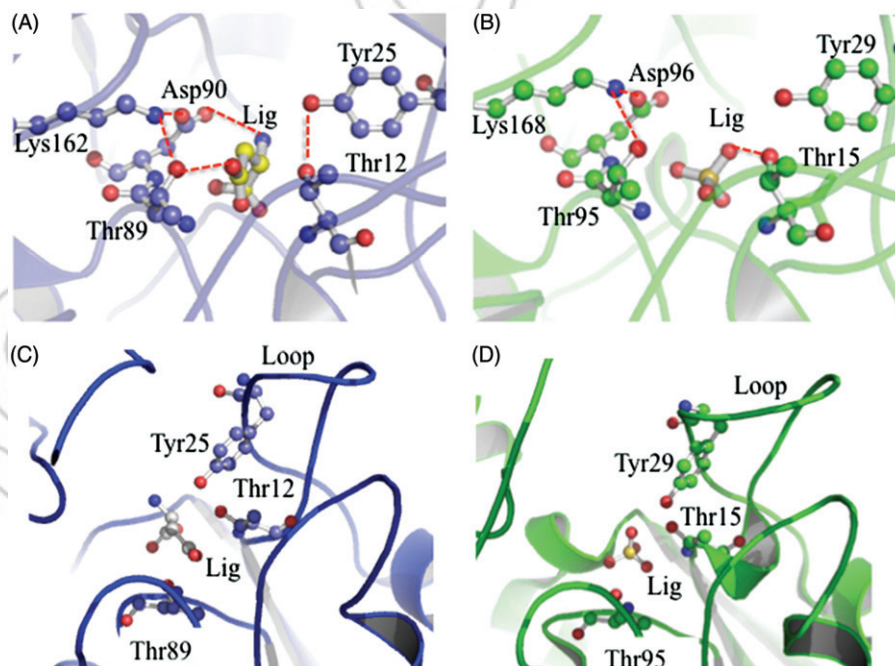
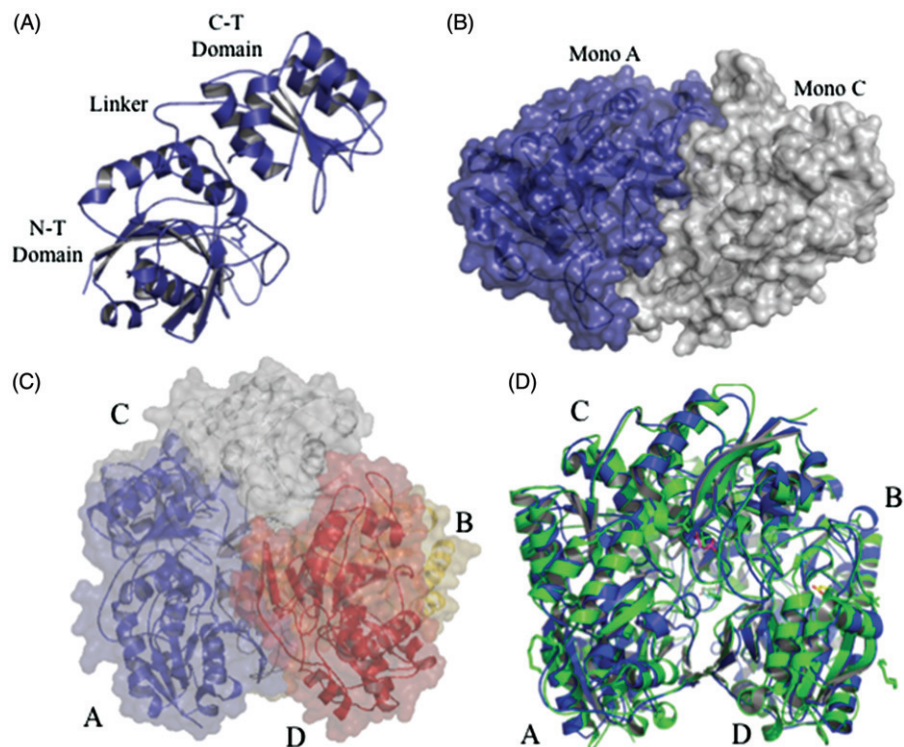
901 EcA was the first bacterial ASNase whose high-resolution
902 crystallographic structure was determined (Swain et al.,
903 1993). Structural studies revealed a well-organized homo-
904 tetrameric enzyme, with each monomer containing ~ 330
905 amino acids arranged in two domains (N- and C-terminal),
906 both belonging to the α/β class. The N-terminal domain
907 comprises the residues 1–190 and is connected to the smaller
908 C-terminal domain (213–326) by a large linker (191–212)
909 (Figure 1A). Monomers are able to associate tightly with each
910 other forming intimate dimers characterized by an extensive
911 interface between the subunits that are held together by
912 several interactions, mainly van der Waals and electrostatic
913 interactions (Figure 1B). Finally, the association of two
914 dimers results in the tetrameric biological unit, which is kept
915 together by molecular interactions similar to those found in
916 homodimers (Figure 1C) (Jaskólski et al., 2001; Swain et al.,
917 1993). Several macromolecular ASNase structures are avail-
918 able at Protein Data Bank (PDB) (<http://www.rcsb.org>),
919 including ErA (Lubkowski et al., 2003; Sanches et al.,
920 2003), which shares high structural homology with the *E. coli*
921 counterpart (r.m.s.d. = 0.89) (Figure 1D).

922 ASNases active sites are located at the interface of intimate
923 dimers, with each intimate dimer containing two active site
924 pockets formed by amino acids from both subunits (Palm
925 et al., 1996; Swain et al., 1993). Structural and functional
926 studies revealed that the so-called catalytic triad composed of
927 three polar amino acids, namely Thr⁸⁹-Lys¹⁶²-Asp⁹⁰ in EcA,
928 is essential for enzyme activity (Jaskólski et al., 2001).

929 Examination of ASNase structure with ligand molecules in
930 the active site revealed the formation of an intricate hydrogen
931 network with ligands (Figure 2A and B) and disclosed two
932 additional residues of importance for the catalytic mechanism
933 (Thr¹² and Tyr²⁵ in EcA) (Michalska & Jaskólski, 2006).
934 These residues are located in a large loop (amino acids 10–32
935 in EcA) that operates as a lid for the active site, probably
936 assisting the correct substrate binding and thus favoring
937 catalysis (Figure 2C and D).

Immunological side effects and enzyme instability

938 ASNase administration can promote a number of harmful side
939 effects including immunological responses, ranging from
940 allergic reactions to fatal anaphylactic shock, coagulation
941 disorders, pancreatitis, hyperglycemia, hepatotoxicity and
942 protein synthesis inhibition (Rizzari et al., 2013). As far as
943 the immunological side effects are concerned, the decay of
944 ASNase antitumoral activity is directly associated with the
945 production of ASNase antibodies by the patient, which leads
946 to the drug clearance from the bloodstream and reduces the
947 treatment efficacy. In this respect, early studies have shown
948 high circulating levels of ASNase by ELISA with low enzyme
949 activity, which was initially attributed to ASNase denaturation
950 (Asselin et al., 1993). However, more recent studies suggest
951 that ASNase clearance may be a result of protease cleavage
952 (Offman et al., 2011). Protein stability and immunogenic
953 effects are apparently closely related. The proteolytic cleavage
954 of ASNase may be responsible for additional epitopes
955 exposure, which are involved in the patients' immune
956 response.



961 Figure 1. Structural features of bacterial
 962 ASNase. (A) Cartoon representation of
 963 ASNase monomer from *E. coli*, composed by
 964 the N-terminal and C-terminal domains con-
 965 nected by a large loop. (B) Molecular surface
 966 of intimate dimer of EcA with one monomer
 967 colored in blue and the other in light gray. (C)
 968 Homotetrameric quaternary structure of EcA.
 969 Each monomer is represented by a different
 970 color (A, dark blue; B, yellow; C, light gray;
 971 D, red). The enzyme cartoon representation is
 972 depicted inside the molecular surface. (D)
 973 Superposition of crystallographic structures
 974 of ASNases from *E. coli* (blue) and *E.*
 975 *chrysanthemi* (green). Graphic models were
 976 generated by PyMOL software ([http://](http://www.pymol.org)
 977 www.pymol.org) using the coordinates 3ECA
 978 (*E. coli*) and 107J (*E. chrysanthemi*) from the
 979 PDB (<http://www.rcsb.org>).

987 Figure 2. ASNases active site pocket struc-
 988 tural features and ligand binding. The active
 989 site regions of EcA (blue) (A) and ErA
 990 (green) (B) reveal extensive structural hom-
 991 ology of amino acids involved in catalysis
 992 (Thr¹², Tyr²⁵, Thr⁸⁹, Asp⁹⁰ and Lys¹⁶² in
 993 *E. coli*, and Thr¹⁵, Tyr²⁹, Thr⁹⁵, Asp⁹⁶ and
 994 Lys¹⁶⁸ in *E. chrysanthemi*). Detail of the
 995 active site region of EcA (C) and ErA (D)
 996 showing the position of the loop at the
 997 entrance of active site pocket.
 998 Representations are in cartoon and catalytic
 999 amino acids are represented by ball and stick
 1000 and colored by CPK (O, red; N, blue; C, same
 1001 color of the enzyme). For the ligands aspar-
 1002 tate (C) and SO₄ (D) carbons are in yellow,
 1003 sulfur in orange, oxygens in red and nitrogen
 1004 in blue. Graphic models were generated by
 1005 PyMOL software (<http://www.pymol.org>)
 1006 using the coordinates 3ECA (*E. coli*) and
 1007 107J (*E. chrysanthemi*) from the PDB ([http://](http://www.rcsb.org)
 1008 www.rcsb.org).

1011 response (Offman et al., 2011; Patel et al., 2009). In this
 1012 respect, cysteine proteases such as asparagine endopeptidase
 1013 (AEP) and cathepsin B are able to hydrolyze bacterial
 1014 ASNase.

1015 ASNase antibody production has been observed in 60% of
 1016 patients treated with EcA. ASNase hypersensitivity clinical
 1017 symptoms include anaphylaxis, pains, angioedema, hives,
 1018 rash and pruritus. Hypersensitivity occurs more frequently
 1019 when the treatment is interrupted or discontinued, with
 1020 children presenting less hypersensitivity and antibody

1021 production when compared to adolescents and adults
 1022 (Pieters et al., 2011; Shrivastava et al., 2015).

1023 Replacement or modification of ASNases to mitigate 1024 the immune side effects

1025 The immunological side effects of ASNases can be partially
 1026 minimized using enzymes from different bacteria (e.g.
 1027 replacing EcA for ErA) (Avramis & Tiwari, 2006).
 1028 In addition, new experimental protocols of enzyme
 1029

1081 encapsulation into erythrocytes or entrapment into liposomes
1082 showed lower side effects (Agrawal et al., 2013; Kwon et al.,
1083 2009; Patel et al., 2009).

1084 As mentioned before, three ASNase formulations are used
1085 to treat ALL and other lymphoid malignancies, namely EcA,
1086 ErA and PEGylated EcA. Native EcA is the most commonly
1087 used worldwide and considered first-line therapy in Europe,
1088 with usual dosage of 6000 IU/m² thrice a week and elimination
1089 half-life of 26–30 h. PEGylated EcA, which is the first-
1090 line therapy in USA, has a half-life (5.5–7.0 days) five times
1091 longer than native EcA and, consequently, its dosage is lower
1092 (2000–2500 IU/m² every 2 or 4 weeks). ErA has been used at
1093 a dosage of 6000 IU/m² thrice a week as a therapeutic option
1094 in patients with side effects from EcA, because it elicits lower
1095 immunological response and induces minor coagulation
1096 disorders. The main disadvantage of ErA is its half-life of
1097 only 16 h (Shrivastava et al., 2015).

1098 Side effects and GLNase activity

1100 An important side effect of ASNase treatment is related to the
1101 nonspecific ASNase amidohydrolase activity. Patients treated
1102 with bacterial enzymes also exhibit low levels of L-glutamine
1103 (Gln) in the bloodstream, resulting in decreased levels of
1104 protein synthesis (Offman et al., 2011). Despite the signifi-
1105 cantly lower k_m of ASNase amidohydrolase activity for Asn
1106 (30-fold lower in EcA) (Avramis & Tiwari, 2006), Gln
1107 represents 50% of all free amino acids in the body being an
1108 important nitrogen source (Ramya et al., 2012). The general
1109 effect of lowering protein synthesis is directly associated with
1110 several side effects, among which are as immunosuppression,
1111 thromboembolism and neurological disorders (Kafkewitz &
1112 Bendich, 1983; Villa et al., 1986).

1113 In spite of toxicity, Offman et al. (2011) demonstrated,
1114 using molecular dynamics and site direct mutagenesis, that
1115 GLNase activity of EcA is necessary for antitumor effect, and
1116 similar results were observed for *Helicobacter pylori* ASNase
1117 (HpASNase) (Parmentier et al., 2015). However, GLNase
1118 activity of EcA was shown to be irrelevant for antitumor effect
1119 against cell lines that do not express asparagine synthetase
1120 (ASNS), but required in cell lines with high ASNS levels
1121 (Chan et al., 2014).

1123 Resistance to ASNase treatment

1125 Many patients are refractory to treatment with ASNases,
1126 which is ultimately related to differences in genetic back-
1127 ground and gene expression profile, including especially
1128 NF- κ B related genes. This indicates very complex genetic,
1129 biochemical and cellular relationships involved in ASNase
1130 resistance mechanism (Chien et al., 2015; Holleman et al.,
1131 2004). Several studies demonstrated that ASNase resistance is
1132 associated to ASNS up-regulation in ALL cells that are
1133 refractory to bacterial ASNase treatment (Chan et al., 2014;
1134 Chien et al., 2015). Conversely, the decrease of *asns* mRNA
1135 expression levels enhances the sensitivity of HL-60 cells to
1136 EcA treatment (Hashimoto et al., 2009).

1137 Studies with ASNS inhibitors also indicate the role of this
1138 enzyme in the resistance to ASNase treatment. For example,
1139 analogues of adenylated sulfoximine, an intermediate of
1140 ASNS catalytic mechanism, are able to enhance the *in vitro*

1141 cytotoxicity of bacterial ASNases to resistant cell lines
1142 (MOLT-4) (Gutierrez et al., 2006; Ikeuchi et al., 2009;
1143 Ikeuchi et al., 2012).

1144 As discussed above, recently some authors have argued
1145 that glutaminase activity of ASNases is fundamental for
1146 cytotoxicity against several types of ALL cancer cells (Chan
1147 et al., 2014; Chien et al., 2015), so the Gln intracellular levels
1148 may be also important for treatment efficacy. In this context,
1149 among several drugs used in combination with ASNases are
1150 the glucocorticosteroids, stimulators of glutamine synthetase
1151 (GLNS) expression (Gaynon & Carrel, 1999). Despite the
1152 lack of studies on the effects of GLNS in ALL cells, inhibition
1153 of Gln uptake was recently suggested to play a role in the
1154 treatment of acute myeloid leukemia, since bacterial ASNase
1155 up-regulates GLNS expression. Additionally, GLNS inhibi-
1156 tion by methionine-L-sulfoximine in six different human
1157 sarcoma cell lines with distinct sensitivity to ASNase
1158 increased cytotoxicity (Tardito et al., 2007). Nonetheless,
1159 more studies on GLNase activity of ASNases are necessary
1160 to verify the molecular mechanisms involved in enzyme
1161 regulation and cancer cell proliferation.

1163 Mutational studies and rational enzyme engineering

1164 Several mutational approaches were performed in ASNase
1165 aiming to investigate aspects such as catalysis, substrate
1166 binding, turnover and affinity, antigenicity and oligomeriza-
1167 tion, among others. Table 5 summarizes the structural effects
1168 of amino acids mutations in EcA (Aung et al., 2000; Borek
1169 et al., 2014; Chan et al., 2014; Derst et al., 1992; Derst et al.,
1170 1994; Derst et al., 2000; Harms et al., 1991; Jaskólski et al.,
1171 2001; Jianhua et al., 2006; Mehta et al., 2014; Offman
1172 et al., 2011; Palm et al., 1996; Verma et al., 2014; Wehner
1173 et al., 1992).

1175 Excluding the pioneering studies involving covalent
1176 modifications by inhibitors, peptide sequencing and other
1177 techniques to identify amino acids involved in catalysis
1178 (Harms et al., 1991; Wehner et al., 1992), the vast majority of
1179 the site mutagenesis approaches were in fact performed after
1180 the determination of EcA crystallographic structures. These
1181 include structures showing ligands at the active site pocket
1182 (Jaskólski et al., 2001), demonstrating the importance of
1183 protein structure knowledge to rationally evaluate the amino
1184 acids involved in substrate binding and catalysis as well to
1185 plan replacements strategies.

1186 The amino acids substitutions that affect the kinetic
1187 parameters of bacterial ASNases are concentrated, close to
1188 the active site, in the intimate dimer interface (Figure 3A and
1189 B). However, there is a lack of mutational studies on the dimer
1190 complementary region, which is also part of the active site
1191 pocket (Figure 3C and D). This observation is important,
1192 since the active site volume is closely related to substrate(s)
1193 binding, aspects that will be addressed later.

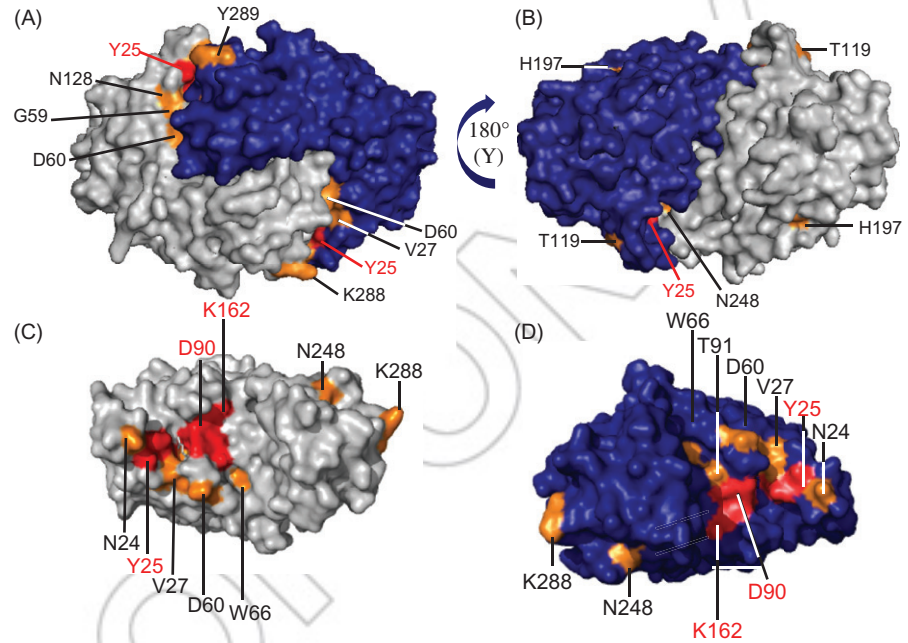
1194 The molecular surface mapping of amino acids substitu-
1195 tions that cause alterations of enzyme stability reveals that
1196 several amino acids are located at the enzyme surface, some
1197 of them between the dimers (Asn²⁴, Asp¹³⁸ and Tyr²⁵⁰) or
1198 tetramer contacts (Asn³⁷, Asn¹²⁴, Lys¹³⁹, Tyr¹⁸¹ and Lys²⁰⁷)
1199 (Figure 4). Rational efforts were also performed aiming to
1200 enhance enzyme stability and half-life. Replacement of Asn²⁴

Table 5. Continued

Amino acid	Substitution(s)	Functional and or structural effects	Reference
		Reduction of antigenicity and immunogenicity/Rapid depletion of Asn and down-regulation of ASNS transcription/decrease of GLNase activity	
K288/Y176	R288/F176	Decrease of GLNase activity	
Y289	F289	No effect	(Derst et al. 1994)
Y326	Stop	Instability of tetramer	

ASNase, L-asparaginase; GLNase, L-glutaminase; Asn, L-asparagine.

Figure 3. *E. coli* ASNase surface structural representations of mutational approaches influencing enzyme activity. (A and B) EcA dimer with one monomer colored in gray and the other in dark blue, showing the positions of amino acids whose substitutions resulted in altered enzyme activity over the substrates (orange) (e.g. Asn, Gln and AHA). Amino acids involved in catalytic activity are colored in red (Thr¹², Tyr²⁵, Thr⁸⁹, Asp⁹⁰ or Lys¹⁶²). (C and D) EcA monomer representations showing the amino acids in monomers interfaces. Molecular graphics were generated by PDB coordinates 3ECA (*E. coli*) (<http://www.rcsb.org>).



by Ala or Thr, located in the lid loop containing Tyr²⁵, increased enzyme stability, rendering higher resistance to AEP and increasing enzyme activity (Offman et al., 2011).

Regarding antigenicity, hydrophilic protein regions associated with long side chain-amino acids are closely related to antigenic sites. Studies involving EcA antigenicity showed that several amino acids fit into the characteristics described above (Tyr¹⁷⁶, Arg¹⁹⁵, Lys¹⁹⁶, His¹⁹⁷ and Lys²⁸⁸) (Figure 4A–D) (Jianhua et al., 2006; Mehta et al., 2014). Additionally, approaches using two distinct techniques to identify immunogenic peptides report an EcA fragment containing several bulky amino acids as the most immunogenic one (Werner et al., 2005). Interestingly, analysis of EcA tetramer structure reveals that some amino acids involved in the antigenicity are buried in the enzyme structure (Figure 4E and F). It is likely that, at very low concentrations such as those used in therapy, in addition to the tetrameric form, there are significant amounts of dimers with additional accessible residues, as previously demonstrated *in vitro* (Werner et al., 2005).

Recently, Mehta et al. (2014) demonstrated that EcA double substitution of Lys²⁸⁸Ser/Tyr¹⁷⁶Phe decreases significantly the enzyme immunogenicity, since the patient antibodies binding was reduced to less than 40%. As expected, Lys²⁸⁸ is located at the tetramer surface

(Figure 4E and F), but curiously Tyr⁷⁶ (Figure 4A and B) is buried between the dimers of the tetramer. Again, the tetramer dissociation may be related with this phenomenon.

As described previously, several ASNase undesirable characteristics have been ascribed to its GLNase activity (Aghaiypour et al., 2001; Chan et al., 2014; Derst et al., 2000; Mehta et al., 2014). In ErA, substitution of Glu⁶³ and Ser²⁵⁴ led to a decrease in GLNase activity (Derst et al., 2000). In EcA, substitution of Asp²⁴⁸ by Ala revealed an effective decrease in Gln hydrolysis (Derst et al., 2000). However, the mutant also showed a significant decrease in Asn hydrolysis (about ~12% of the wild type).

Another study using molecular dynamics simulations combined with structural analysis and site directed mutagenesis, demonstrated that the double substitution Asn²⁴Ala and Tyr²⁵⁰Leu resulted in negligible GLNase activity and ~30% decrease of ASNase activity (Offman et al., 2011). As mentioned before, Asn²⁴ is located close to Tyr²⁵ from the lid loop (Figure 5A) and its substitution by Ala or Thr is related with proteolytic resistance to AEP. The GLNase activity decrease was associated with the active site cavity volume (Asn²⁴Ala, substitution) and tetramer compactness (Tyr²⁵⁰Leu, substitution) (Offman et al., 2011). It is noteworthy that the cytotoxicity of mutant EcA was significantly

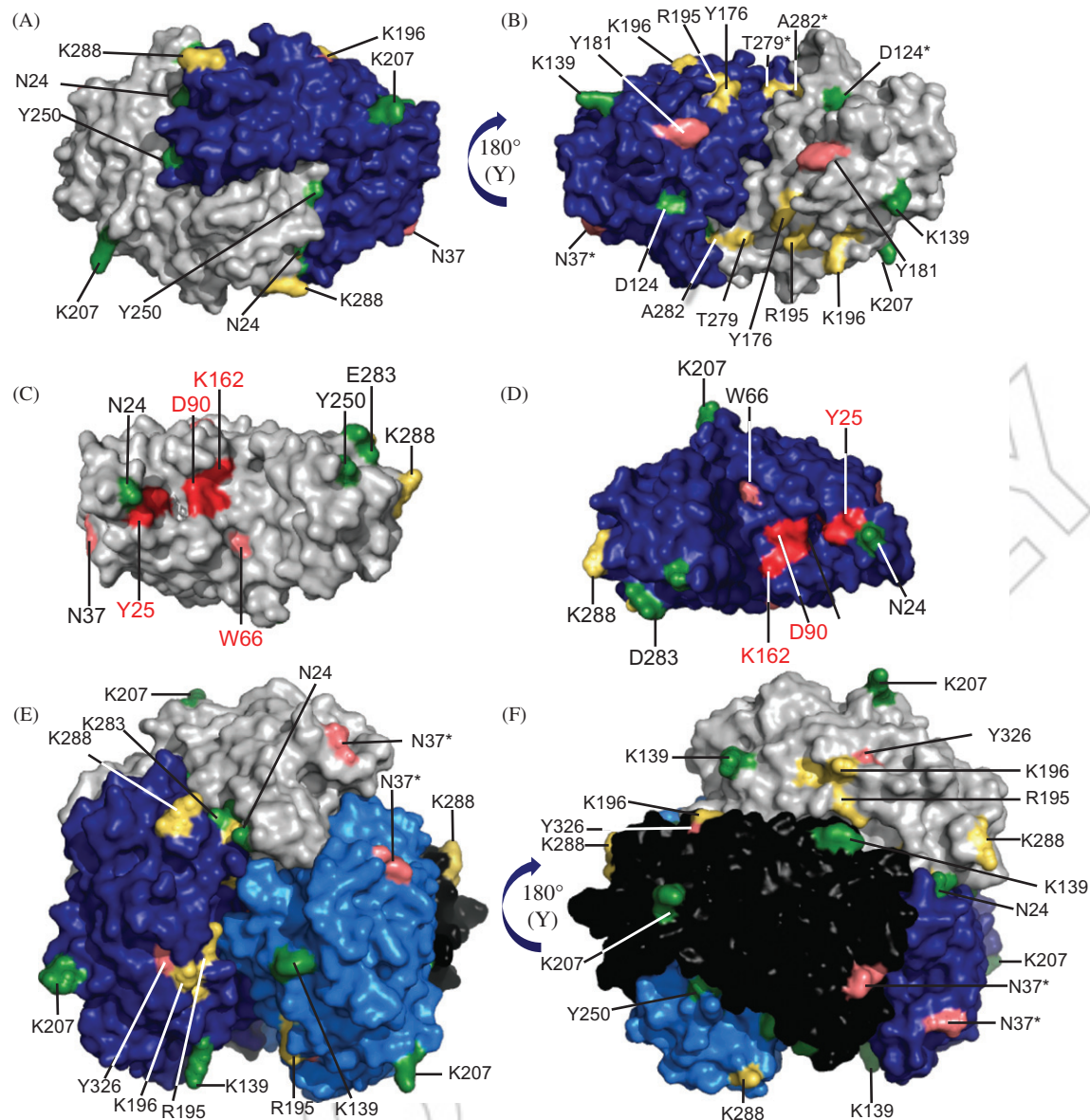


Figure 4. EcA structural surfaces representations of mutational approaches influencing enzyme stability and antigenicity. (A and B) EcA intimate dimer showing the surface mapping of amino acids whose replacement increases or decreases thermal (green) or proteolytic (salmon) enzyme stability or antigenicity reduction (yellow). (*) Amino acids substitutions performed in other bacterial species. (C and D) EcA monomer representations showing amino acids in monomers interfaces. (E and F) Tetramer interface with one dimer colored in gray and dark blue and the other in black and light blue. Molecular graphics were generated by PDB coordinates 3ECA (*E. coli*).

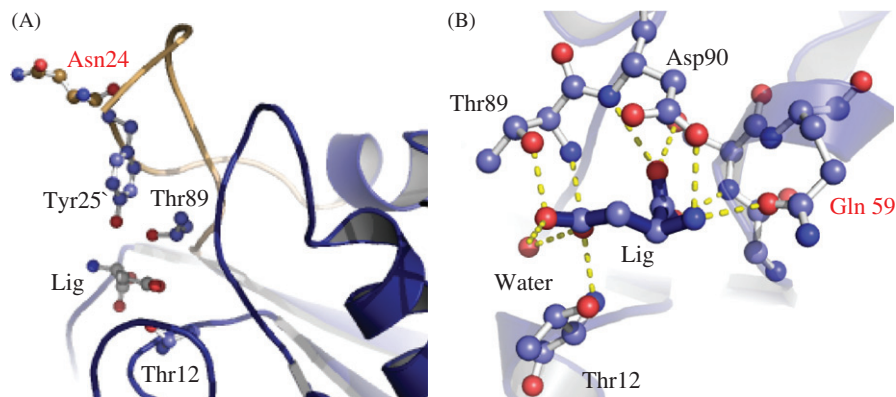
lower than that of the wild type enzyme. The authors argued that EcA dual activity is also associated with therapeutic toxicity, since mutants with lower GLNase activity also exhibited lower cytotoxicity (Offman et al., 2011).

Chan et al. (2014) also investigated additional factors of cytotoxicity of EcA mutants deficient in GLNase activity. Molecular dynamics simulation approaches combined with saturation site directed mutagenesis allowed the authors to identify an EcA glutamine residue (Gln⁵⁹) in the active site pocket able to perform saline interactions with the ligand (Figure 5B). It was demonstrated that the mutant enzyme carrying Gln⁵⁹ substitution by a Leu (EcA^{Q59L}) retains ~60% of ASNase activity, but no detectable GLNase activity.

The mutant EcA^{Q59L} was able to kill efficiently cell lines with low ASNS levels, but did not display cytotoxicity against cell lines with high expression of this enzyme (Chan et al.,

2014). ASNS is able to catalyze the conversion of aspartate and glutamine to asparagine and glutamate through an ATP-dependent reaction, thus supplying ALL cells with Asn. Some studies report that ASNase-resistant forms of the disease may be associated with ASNS up-regulation in some patients (Richards & Kilberg, 2006). Nevertheless, the demonstration that EcA variants without GLNase activity are able to kill cancer cells with low ASNS levels is very important and may represent a promising therapy with less side effects for patients affected by tumors with this characteristic (Chan et al., 2014).

Recently, Verma et al. (2014) demonstrated that substitution of EcA Tyr¹⁷⁶Phe and Trp⁶⁶Tyr residues promotes a substantial decrease of GLNase activity, but ASNase activity is almost the same as that of the wild type enzyme. Tyr¹⁷⁶ and Trp⁶⁶ are very far from the enzyme active sites, at both dimers, and the effects of their replacements were related to



1561 Figure 5. Amino acids involved in EcA
 1562 enzyme stability and activity. (A)
 1563 Representation of active site region of *E. coli*
 1564 ASNase showing the position of residues
 1565 (Thr¹², Asn²⁴, Tyr²⁵, Thr⁸⁹) and the ligand
 1566 (aspartate). The loop containing Asn²⁴ and
 1567 Tyr²⁵ are colored in light brown. Active site
 1568 amino acids and aspartate are represented by
 1569 CPK (O = red; N, blue; C, blue). Asn carbons
 1570 are colored in light brown and the other
 1571 amino acids in light blue. (B) Molecular
 1572 binding aspartate in EcA. Red characters
 1573 indicate Gln⁵⁹, whose replacement results in
 1574 residual GLNase activity. The *E. coli* enzyme
 1575 (PDB code 1NNS) is colored in blue and
 1576 represented in cartoon. Amino acids are
 1577 represented in ball and stick and colored by
 1578 CPK (O = red and C = blue). Graphic models
 1579 were generated by PyMOL ([http://](http://www.pymol.org)
 1580 www.pymol.org).

1579 an intricate network of indirect molecular interactions, which
 1580 affects the enzyme quaternary folding with consequences in
 1581 its activity (Figure 4). In subsequent studies, it was
 1582 demonstrated that, contrary to the wild type enzyme, the
 1583 mutant enzymes EcA^{W66Y}, EcA^{Y176F} and EcA^{K288S/Y176F} are
 1584 able to down-regulate ASNS expression, however the reasons
 1585 for this phenomenon are not yet understood (Mehta et al.,
 1586 2014).

1587 The studies presented in this section provide amazing
 1588 examples of how EcA rational protein engineering can assist
 1589 in a better understanding of catalytic mechanisms, substrate
 1590 binding, enzyme folding and their relationship with cytotox-
 1591 icity and immunogenicity. Engineered enzymes or new
 1592 variants derived from studies presented here may have high
 1593 potential for future alternative therapeutic treatments using
 1594 recombinant ASNases. Additional studies involving structure
 1595 determination and analysis of mutant enzymes with improved
 1596 properties should provide a better understanding of EcA
 1597 functional and structural features and open perspectives of
 1598 creating new enzymes with personalized characteristics
 1599 for different groups of patients. In addition to the rational
 1600 modification based on structure analysis, emerging
 1601 approaches based on computational analyses involving
 1602 molecular dynamics, docking of substrates, among others *in*
 1603 *silico* techniques, have proven to be very promising to guide
 1604 rational enzyme modifications.

1605 Concluding remarks and future perspectives

1608 In general, ASNase is mainly produced by bacterial fermenta-
 1609 tion, and the native and pegylated forms of ASNase
 1610 continue to be the most used in ALL therapy. Despite the
 1611 benefits of conjugating ASNase with PEG, parallel negative
 1612 side effects could be solved, in our opinion, through a better
 1613 characterization of pharmacodynamics and pharmacokinetics,
 1614 which would increase drug efficiency.

1615 Nonetheless, due to ASNase importance in the treatment of
 1616 several types of cancers, in particular leukemia, it is essential
 1617 to search for new sources of this enzyme in order to increase
 1618 its availability as a drug and reduce side effects. Considering
 1619 the advantages of the use of microorganisms in bioprocesses
 1620 and that very little is known regarding the magnitude of

1630 microbial diversity, they may be considered a target source of
 1631 genomic innovation in the search for new ASNases with
 1632 improved properties compared with those currently employed
 1633 in therapy. In this sense, more effort should be devoted, in our
 1634 opinion, to the screening of new ASNase-producing
 1635 microorganisms.

1645 Rational protein engineering based on protein structure is
 1646 another promising strategy to produce ASNases with
 1647 improved pharmacodynamics, pharmacokinetics and toxico-
 1648 logical profiles. Indeed, approaches involving site directed
 1649 mutagenesis of residues in the active site were able to produce
 1650 recombinant enzymes with good ASNase activity, but negli-
 1651 gible GLNase activity. Additional procedures involving the
 1652 introduction of structural disulfides and deletion of proteases
 1653 cleavage sites may allow the production of more robust
 1654 enzymes. There is little information on *Saccharomyces*
 1655 *cerevisiae* ASNase and, giving the ease of cultivation and
 1656 possibility of genetic manipulation of this yeast, we believe
 1657 that such an enzyme deserves to be better investigated as an
 1658 alternative to the existing bacterial ASNases. In particular,
 1659 special attention should be paid to its better structural and
 1660 kinetic characterization as well as to the rational engineering
 1661 of the yeast enzymes by means of site-directed and random
 1662 mutations.

1663 One interesting technological approach that may contribute
 1664 to improve ASNase production by recombinant microorgan-
 1665 isms is the metabolic flux analysis (MFA), a powerful tool to
 1666 estimate the metabolic state constrained by exchange of
 1667 nutrient fluxes between cells and environment (Antoniewicz,
 1668 2013). This analysis has been successfully used to identify
 1669 key nodes in the primary metabolism, which are characterized
 1670 by significant changes to the partitioning of the flux under
 1671 different conditions, and thus it can be considered as a
 1672 potential control point manipulation (Boghigian et al., 2010;
 1673 Goudar et al., 2014). Additionally, *Pichia pastoris*, which has
 1674 been developed as an excellent host for heterologous genes
 1675 using alcohol oxidase as a promoter, has potential for high
 1676 cell density cultivations with high levels of protein expression
 1677 and efficient secretion (Cregg et al., 2000). Therefore, MFA
 1678 applied to ASNase production by recombinant *P. pastoris*
 1679 seems to us an interesting alternative to be investigated, with
 1680 determination of optimal culture conditions in terms of

1681 temperature, methanol concentration and pH. This may be
 1682 achieved through modeling of metabolic fluxes related to
 1683 methanol-metabolizing pathway, which is important in the
 1684 induction of the heterologous protein as well as yeast growth
 1685 (Anasontzis et al., 2014).

1686 The information gathered here also demonstrates a gap on
 1687 novel alternatives and optimized protocols for ASNase
 1688 bioproduction and purification. Therefore, research efforts
 1689 should be addressed to these topics, with special focus on
 1690 cultivation parameters and novel and cheaper purification
 1691 strategies such as liquid–liquid extraction with ionic liquids.
 1692 We hope that, in a near future, novel alternatives to bacterial
 1693 ASNases can be available for ALL treatment, with better
 1694 therapeutic results and less side effects.

1695 Declaration of interest

1696 The authors report no conflict of interest. This work was
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APÊNDICE C

De Paula, C. P.; Santos, M. C.; Tairum, C. A; **Breyer, C. A.**; Gaeta, H.; Toyama, M. H.; Oliveira, M. A. **Functional characterization and heterologous expression of a new rhodanase like protein from *Xylella fastidiosa***. Manuscrito em fase final de elaboração que será submetido ao periódico *PLOS ONE*.

FUNCTIONAL CHARACTERIZATION AND HETEROLOGOUS EXPRESSION OF A NEW RHODANESE LIKE PROTEIN FROM *XYLELLA FASTIDIOSA*.

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Abbreviations used: Grx, glutaredoxin; Rho, rhodanese; ORFs, open reading frames; ROS, reactive oxygen species; Grx, glutaredoxin; Trx, thioredoxin; TrxR, thioredoxin reductase; GR, glutathione reductase; GSH, glutathione; *t*-BOOH, *t*-butyl hydroperoxide; DTNB, 5,5'-dithio-bis (2-nitrobenzoic acid); NEM, N-ethylmaleimide; IPTG, isopropyl-1-thio-β-D-galactopyranoside; DTT, 1,4-dithiothreitol; DTPA, diethylenetriaminepentaacetic acid; HPLC, high performance liquid chromatography.

Keywords: Glutaredoxin (Grx), Reactive Oxygen Species (ROS), Glurho, Rhodanese (Rho), *Xylella fastidiosa*.

Abstract: The *Xylella fastidiosa* is a phytopathogen and the causal agent of diseases in several commercial plants, accounting for high financial losses. The genome analysis of *X. fastidiosa* ORFs revealed a new putative gene, present in all strains sequenced to date, whose product may possess three domains: the N-terminal domain has a high similarity with the monothiol glutaredoxins (Grx), which are related to several redox processes. The C-terminal portion presents similarity to Rhodanases (Rho), involved in the sulfur (S) metabolism and the central region presents similarity to Fe-S cluster superfamily. Due to these characteristics this ORF was nominated *glurho*. The ORF translation revealed N-terminal and the C-terminal domains also contain two cysteines residues (Cys³³ and Cys²⁶⁶), which are essential to Grx and Rho activity in enzymes described to date, indicating that both domains may be functional. Additionally, proteomic studies revealed that the Glurho protein is highly expressed in *X. fastidiosa* inside the cell and in its biofilm. Since that reactive oxygen species and cyanide production by the host are very common processes in defense against pathogen infection and predation, this protein may play a key role in bacterial protection and resistance against the host defenses. In this work, we performed databases searches, which revealed that Glurho homologues are only present in prokaryotes, most of which are pathogenic to plants and animals, due to these characteristics, Glurho protein may represent promising targets for therapeutical drugs. Corroborating with this view, individual sequence alignment with well characterized glutaredoxins, Fe-S cluster containing proteins and rhodanases, revealed that the domains share low identity with their counterparts, but the Grx and rhodanese domain possess very conserved domains related to the biochemical activity of the proteins. In order to characterize the biochemical properties of Glurho, the recombinant enzyme was expressed as His-tagged protein and purified by immobilized metal affinity chromatography (IMAC) to homogeneity. To evaluate the activity, supposedly conferred by Grx domain, the disulfide reduction and peroxidase activity glutathione dependent assays were performed but none activity was detected. However, enzymatic evaluation of the rhodanese function revealed that Glurho protein is able to detoxify efficiently cyanide to thiocyanate *in vitro* with higher efficiency (5 ×) than bovine rhodanese. We also demonstrated that the cysteines residues are essential to rhodanese activity, since the NEM alkylation annihilated enzyme activity. Finally, the substitution of the cysteine residue from rhodanese domain (Cys²⁶⁶) by a serine abolished the rhodanese activity, indicating that the residue is essential to the enzyme functionality. Meanwhile the Cys³³ replacement by serine increased the rates of the rhodanese activity in 3-fold. We also investigate the *in vivo* effects of KCN tolerance by the heterologous enzyme expression in *E. coli* BL21 (DE3) cells, which revealed that Glurho is able to protect the bacterial cells against high concentrations of cyanide. However, contrastingly with the *in vitro* rhodanase mutants biochemical data, the *E. coli* BL21 (DE3) cells expressing Glurho^{C33S} and Glurho^{C266S}, indicating that *in vivo* cyanide resistance phenotype is dependent of Cys³³ integrity.

Introduction

Xylella fastidiosa is a gram-negative bacterium which infects the plant xylem of many taxonomic groups of economic importance such as citrus, alfalfa, coffee, grape, almond, peach and pear [1–4]. The bacterium is transmitted by contaminated plants through cicada, a specific vector which the mechanism of interaction with the host is until now poorly unknown [2,5,6].

The *X. fastidiosa* genomes contains several genes related to virulence, adhesion and response to plant defenses, among them, several genes related to the ROS metabolism represented by antioxidant enzymes [7–10]. Smolka *et al.* [11], using two-dimensional gel electrophoresis allied to mass spectrometry, demonstrated that a substantial amount of antioxidant proteins are expressed in high concentrations in the strain of the *X. fastidiosa* 9a5c. Among them, the ORF XF2394, a hypothetical protein, was detected in large amounts in both, intracellular and extracellular contents. The sequence analysis of the ORF XF2394 using CDD program (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>), revealed that it possesses three domains: the N-terminal domain has a high similarity to the monothiol glutaredoxins, which are related to several redox processes; the C-terminal portion presents similarity to Rhodanases, involved in the sulfur (S) and cyanide (CN⁻) metabolism; and the central region is similar to Fe-S cluster biosynthesis superfamily, related to sulfur assimilation and oxidative stress defense. Due to these features, in this study we have named *Glurho* (for Glutarhodanase) the product of the ORF.

Glutaredoxins (Grx) are low molecular weight oxidoreductases (~12KDa) that have at least a cysteine residue in its active site used in oxireduction processes [12,13]. Grx proteins can be classified according to the number of cysteine residues as dithiolic (Cys-X-X-Cys motif) or monothiolic (Cys-X-X-Ser) [12,14–17]. Using the reactive cysteines, dithiol Grxs perform thiol-disulfide exchanges reducing their disulfide containing substrates. The reduction mechanism requires glutathione (GSH), glutathione reductase (GR) and NADPH reducing equivalents [18,19]. Additionally, to the disulfide reduction, it has been demonstrated that some yeast Grx are able to act as glutathione peroxidases, revealing an additional protective role of these enzymes to the organisms [20,21].

The *X. fastidiosa* *Glurho* protein presents resemble monothiol glutaredoxins which is characteristic of [19] which is highly conserved from bacteria to humans [16,22]. Therefore it

is possible that the monothiol glutaredoxin domain of Glurho could exhibit the same mechanism of action of dithiol glutaredoxins since the reduction mechanism of Grxs may be monothiolic even to some dithiolic Grx [19,23].

Relative to the central domain in Glurho the iron-sulfur assembly proteins, a variety of proteins widely distributed in nature, from bacteria to mammals, are able to synthesize the Fe-S cluster [74,75]. They serve a variety of biological roles, including electron transport, catalytic, structural, and sensory roles [76]. The free iron can generate oxygen radicals via Fenton-like reactions that may result in damage macromolecules [74,75,76]. The connection between Fe metabolism and oxidative stress during infection of pathogens has been demonstrated, since some microorganisms require the enzymes necessary for the synthesis of Fe-S to respond to oxidative stress or in the case of phytopathogen *Erwinia chrysanthemi* maintain a strong virulence. As the Grx proteins the Fe-S activity is also centered in conserved cysteine residues [74,75,76].

It is well known that the synthesis of CN^- , together with ROS production represent defense strategies widely used by plants against pathogens and predators [24–26]. Among the enzymes involved in detoxification of CN^- , the rhodanese (Rho) are one of the most extensively studied since they are widely distributed in nature [27–29]. The enzyme is able to catalyze the reaction between thiosulfate and cyanide leading to the formation of sulfite and the less toxic thiocyanate [28]. In bacteria, such as *Escherichia coli*, the most studied representative rhodanese protein is GlpE, which has been proposed to possess great importance on organism homeostasis since plays essential biochemical roles acting as thiosulfate-cyanide sulfurtransferase [30].

The Glurho rhodanese-like domain contains a cysteine residue at C-terminal portion on the position 266, which might be involved in cyanide (CN^-) detoxification and/or sulfur metabolism [27,28,31,32]. Although almost all rhodanese researches focus on cyanide resistance, the occurrence of so many rhodanese-like proteins suggests alternative functions, including sulfur and selenium metabolism and biosynthesis of prosthetic groups in iron-sulfur proteins [28,31–33].

Cipollone, *et al.* 2007 [28] divided the rhodanese superfamily into 4 groups according to some common characteristics. Although the amino acids can differ significantly at the sequence level, the three-dimensional structure has common characteristics even among different organisms [28]. The Group 3 is represented by enzymes containing the catalytic

rhodanese domain combined with other globular protein domain. In this case the involvement of a rhodanese domain in a given process seems to be related to the functional properties of the accompanying domain [36–38].

Protein domains represent the basic evolutionary units that form proteins. The duplication and exchange by recombination and fusion, followed by divergence are the most frequently mechanisms for protein evolution [39]. Thus, the recombination of existing domains may be a major mechanism that modifies protein function and increases proteome complexity. The fusion of the proteins related to consecutive metabolic steps seems to be of great importance for some pathogens. An important example is the Trx System, which is constituted by two enzymes: the thioredoxin (Trx) and thioredoxin reductase (TrxR), a ubiquitously system spread among all organisms [40–43] responsible for the reduction of the peroxiredoxins, which are very important enzymes to the cell redox homeostasis [44–46].

Curiously, in some pathogens the two enzymes of Trx system are fused. In *Mycobacterium leprae* thioredoxin reductase is a natural hybrid that contains a Trx molecule attached to the C-terminal portion [47]. The alkyl hydroperoxide reductase F (AhpF), found in several bacteria such as *Salmonella typhimurium*, is also a modified TrxR, which has an additional N-terminal domain (NTD), produced by the fusion of two thioredoxin molecules [48]. AhpF belong to the AhpR complex that is formed by the AhpF and the peroxiredoxin AhpC. In fact AhpF is an enzyme specialized in AhpC reduction [49]. In both the cases, it has been shown that these fused enzymes are of great importance in resistance to pathogen infection and establishment in the host [50,51].

Thus, the *X. fastidiosa* Glurho appears to be a unique protein found only among prokaryotes which can be involved in the resistance of pathogens and vectors of plant defenses. Additionally, it can be related to the sulfur metabolism and training of clusters Fe-S [28,32,52].

Since reactive oxygen species and cyanide production by the host are very common processes in defense against pathogen infection and predation, this protein may play a key role in bacterial protection. In this work, we show that Glurho homologues are only present in prokaryotes, most of which are pathogenic to animals and plants, due to these characteristics, Glurho protein may represent promising targets for therapeutical drugs. Corroborating with this view, individual sequence alignment of Glurho domains with proteins Grx, Fe-S cluster assembling proteins and rhodanases of several organisms, revealed that the domains share low

identity with their counterparts. In order to characterize the biochemical properties of Glurho, the recombinant enzyme was expressed as His-tagged protein and purified to homogeneity. To evaluate the activity, supposedly conferred by Grx domain, the disulfide glutathione dependent reduction and peroxidase activity assays were performed but none activity was detected. However, enzymatic evaluation of the rhodanese function revealed that Glurho protein is able to transfer efficiently sulfur to cyanide forming thiocyanate *in vitro* with higher efficiency (5 ×) than the well known bovine rhodanese. We also demonstrated that the cysteines residues are essential to rhodanese activity, since the NEM alkylation annihilated enzyme activity. The substitution of the cysteine residue from rhodanese domain (Cys²⁶⁶) by a serine abolished the rhodanese activity, revealing that the Cys²⁶⁶ residue is essential to the enzyme functionality. Curiously, the Cys³³ replacement by serine increased the rhodanese activity in 3-fold, suggesting a regulatory role of the residue. We also investigate the *in vivo* effects of KCN tolerance by the heterologous enzyme expression in *E. coli* cells, which revealed that Glurho is able to protect the bacterial cells against cyanide compounds even at high concentrations (1.5 mM). However, contrastingly with the *in vitro* data cells expressing Glurho^{C33S} and Glurho^{C266S} were very susceptible to KCN toxicity, indicating that *in vivo* cyanide resistance phenotype is dependent of Cys³³ integrity.

Materials and methods

Glurho amplification, cloning and sequence - The open reading frame (ORF) XF2394 (NCBI accession number: KIA57599.1) was amplified by PCR using with template genomic DNA from *X. fastidiosa* 9a5c strain. The oligonucleotides XF2394_F-5'-CGCGATCCCATATGATGCCGCAGTGCGGTTTTTCTGC-3' and XF2394_R-5'-CGCAAGCTTGGATCCTCAATACTTCGGCACACTCGC-3' containing *Nde* I and *Bam* HI cloning adapters (underlined sequence) were designed based on gene sequence presents at *X. fastidiosa* database (<http://www.xylella.lncc.br/>). Amplified DNA fragments was digested with *Nde* I and *Bam* HI and cloned into *Nde*I-*Bam* HI-digested pET15b vector. An *E. coli* DH5 α strain was transformed with the construct and cells and cultured to increase plasmid production. The resulting pET15b/*Glurho* vector was sequenced in an ABI 3730 DNA Analyzer using the BigDyeTerminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The plasmids containing the correct constructs were used to transform *E. coli* BL21 (DE3) strains.

Site Directed Mutagenesis. The pET15b/*Glurho* plasmids were used to generate the two individually *Glurho* mutant proteins carrying cysteines (Cys³³ and Cys²⁶⁶) substitutions to serines. The substitutions were performed using the Quick Change II kit (Stratagene) and the mutagenic primers *Glurho*^{C33S}_F (5' ATGCCGCAGTCCGGTTTTTCT 3'), *Glurho*^{C33S}_R (5' AGAAAAACCGGACTGCGGCAT 3'); *Glurho*^{C266S}_F (5' GCATTCTTGTCTTCATCAC GGC 3') and *Glurho*^{C266S}_R (5' GCCGTGATGAGACAAGAATGC 3'). The reaction products were used to transform *E. coli* XL1-Blue strain and single colonies were grown on liquid media during 16hs and then the plasmids were extracted, purified and sequenced to confirm the codons substitutions, and used to transform *E. coli* BL21 (DE3) strains.

Protein Expression and purification .Single colonies of *E. coli* BL21 (DE3) strains containing the pET15b/*glurho*, pET15b/*glurho*^{C33S} and pET15b/*glurho*^{C266S} plasmid were inoculated in LB medium (20 ml) containing 0.1 mg ampicillin/ml and grown overnight at 37 °C. Afterwards the cell cultures were transferred to 1 liter of fresh LB medium, and geowed until OD₆₀₀ reached 0.6–0.8. The expression of the recombinant proteins were induced by the addition of 0.3 mM IPTG, and the cells were maintained at 18 °C/16hs/250 rpm in a orbital shaker. After this period, cells were harvested by centrifugation, and the pellets were

resuspended in start buffer (50 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl, 20 mM imidazole and 2 mM phenylmethyl sulfonyl fluoride (PMSF) and the cells were disrupted by sonication. The cell extracts were kept in ice during streptomycin sulfate 1% treatment for 20 min. The supernatants were clarified by centrifugation, homogenized by filtration and purified by nickel affinity chromatography using the HisTrap column (GE Healthcare) by imidazole gradient (50-500 mM). The Glurho^{WT}, Glurho^{C33S} and Glurho^{C266S} recombinant proteins were predominantly eluted at 500 mM imidazole fractions. Imidazole was removed using a PD10 desalting column (GE Healthcare) and purified proteins were kept in 50 mM sodium phosphate buffer (pH 7.4) containing 50 mM NaCl. The enzymes purity were confirmed by SDS-PAGE and the concentrations were determined by the molar extinction coefficient for reduced *X. fastidiosa* Glurho ($\epsilon_{280} = 25.565 \text{ M}^{-1} \text{ cm}^{-1}$) using the ProtParam tool (<http://www.expasy.ch/tools/protparam.html>)

Evaluation of the Glutaredoxin Disulfide Reduction Activity. The Grx activity conferred by Glurho was evaluated by the standard assay for Grxs which measures the ability of the protein to reduce the mixed disulfide formed between a GSH molecule and β -hydroxyethyl disulfide (HED) spectrophotometrically ($\lambda=340 \text{ nm}$) at 30 °C [53]. The reaction mixture containing 0.1 M Tris-HCl pH 7.4; 1 mM GSH; 6 $\mu\text{g/ml}$ GR and 0.7 mM HED was incubated at 30 °C for three minutes for the formation of the mixed HED disulfide and then 0.2 mM NADPH was added, the reaction was started by the addition of Glurho 1 μM and followed by the decrease in the absorbance at 340 nm due to the oxidation of NADPH during 250 seconds.

An additional disulfide reduction assay was performed using as substrate mixed disulfide already formed between Cys and GSH (Cys-S-SG). The reaction mixture containing GSH 1 mM, GR 6 $\mu\text{g/ml}$, Tris-HCl pH7.4 at 100 mM, NADPH 0.2 mM, Cys-SSG 100 μM and Glurho protein at 1 μM was measured spectrophotometrically ($\lambda=340 \text{ nm}$). The mix solution containing all the reagents except the NADPH and the Cys-SSG disulfide was incubated at 37 °C for 1 minute and then the NADPH was added, after the stabilization the Cys-SSG disulfide was added and the absorbance was recorded by 250 seconds to evaluate the NADPH oxidation.

Evaluation of Glurho GSH dependent Peroxidase Activity. The GSH dependent peroxidase activity of Glurho with *t*-BOOH was evaluated *in vitro* by NADPH oxidation assay. The

components of the reactions were 0.4 mM NADPH, 1 mM GSH, 6 µg/ml Glutathione reductase, 1 µM Glurho and 1 mM *t*-BOOH. The reactions were performed in buffer Tris HCl, 1mM (pH 7.4) in a final volume of 1 ml. The reaction was started by the addition of Glurho and the absorbance at 340 nm was recorded for 200 seconds.

Evaluation of DTT dependent peroxidase activity. The rate of DTT oxidation catalyzed by Glurho in the presence of the H₂O₂ as substrate was evaluated by monitoring the change in absorbance at 310 nm for 250 seconds due to formation of the DTT disulfide [54]. The assay contained: 100 mM Tris-HCl pH7,4, H₂O₂ 1 mM, Glurho 1 µM, and 10 mM DTT in a 1 ml quartz cuvette at 37 °C.

Cyanide sulfurtransferase activity. To evaluate the rhodanese activity of Glurho, Glurho^{C266S} and Glurho^{C33S} proteins to convert CN⁻ to SCN⁻ was accomplished spectrophotometrically as described previously [55,56]. The reaction consisted of potassium phosphate (KH₂PO₄) 40 mM, KCN 50 mM, sodium thiosulfate (Na₂S₂O₃) 50 mM and Milli Q water in 1 ml of reaction. The reactions were initiated with the addition of 0-1.2 µM of Glurho, Glurho^{C33S} or Glurho^{C266S} and after 5 minutes the reactions were stopped by adding formaldehyde 2,5%, and then by adding ferric nitrate (100g of Fe(NO₃)₃.9H₂O and 200 ml of 65% HNO₃ per 1500 ml) in a final volume of 3ml. In the reaction the cyanide is converted to thiocyanate that in acidic conditions and in the presence of Fe⁺³ results in a red intermediated, which is quantified spectrophotometrically (λ=460 nm). As a positive control were used the same concentrations of bovine rhodanese and to the negative control no enzyme was added to reaction mixtures.

Glurho heterologous expression in KCN⁻ containing media. Cultures of *E. coli* BL21 (DE3) harboring the plasmid pET15b (empty vector) and pET15b/*Glurho*, pET15b /*Glurho*^{C266S} and pET15b /*Glurho*^{C33S} (recombinant vectors) were grown in LB medium containing 100µg ampicillin/ml overnight at 37 °C/ 250 rpm. After this period the cultures were transferred to 15 ml of fresh LB broth and the cell culture were grown until OD₆₀₀ reached 0.6–0.8. The cultures in exponential phase were diluted to OD₆₀₀ = 0.02 with or without IPTG 0.1 mM and were exposed to 0.3 mM, 1.3 mM, 1.5 mM and 2.5 mM of KCN⁻ during 24h, and the OD₆₀₀ of the cultures were recorded.

Evaluation of sulfur acceptors. To evaluate the Glurho sulfur transfer activity, NADPH oxidation assay was performed. The assays were performed in a final volume of 1 ml containing potassium phosphate buffer, 50 mM (pH=7.4); *E. coli* TrxR, 0.2 μ M; NADPH, 150 μ M; *X. fastidiosa* Tsnc or *E. coli* Trx, 10 μ M. The reactions were initiated by the addition of sodium thiosulfate, 80 mM and Glurho, 10 μ M, and the absorbance was monitored spectrophotometrically ($\lambda=340$ nm) by 150 seconds.

Assessment of secondary structure by circular dichroism spectroscopy. Secondary structural elements of Glurho and mutants were determined experimentally by circular dichroism (CD) using a Jasco J-720 spectropolarimeter (Jasco, Easton, MD). Purified recombinant wild type and mutant enzymes (10 μ M) in 10 mM NaH₂PO₄ buffer (pH 7.5) were used for the Far-UV CD analysis from 195 to 260 nm at 20°C in a 0.1-cm-gap cuvette. The molar ellipticity values (θ) were calculated using a molar concentration of and the number of 328 amino acids. The Glurho and mutants secondary structures content were determinate using the K2D software (42).

Results and discussion

X. fastidiosa Glurho homologues are restricted to prokaryotes

The homology analysis of Glurho protein with translated genebank sequences using the program BlastX (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) revealed that this protein is present only in a small group of organisms (21), excluding the *X. fastidiosa* strains, all of them are prokaryotes (**Fig. 1**). Our results also shows that the vast majority of organisms that possess the *glurho* gene belongs to the genus *Xanthomonas* and are causative agents of several diseases in plants. Additionally, some human pathogens of the *Stenotrophomonas* genus, the causative agent of respiratory diseases, also possesses homologues to Glurho. The remaining species are found from environmental genomic projects and biological data are unavailable.

Sequence alignment and conserved domain analysis

As mentioned before, the analysis of Glurho using the conserved domain database, (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) [57] revealed that Glurho protein is formed by three distinct domains comprising a Grx like (N-terminal), Fe-S cluster assembly (central) and a rhodanese like domains (C-terminal). To investigate the conservation of these putative domains we performed alignments of the Glurho protein with well-characterized counterparts of other organisms. The alignment of the Grx like domain revealed that the Grx domain is very conserved among eukaryotes and prokaryotes, including the catalytic cysteine which is embedded in the CGFS motif (**Fig. 2A**, red asterisk), characteristic of monothiolic glutaredoxins [19,58]. The Fe-S cluster assembly like domain, is not very conserved among the organisms and the alignment was performed using only bacterial counterparts. An important observation is the fact that Fe-S cluster assembly like domain of Glurho do not posses multiple cysteine residues as the other proteins analyzed (**Figure 2B**, green asterisk). As an example, the *Escherichia coli* ISCa (Iron-Sulfur Cluster Assembly protein) presents three cysteine residues that are essential to the assembly of Iron-Sulfur cluster, since their substitutions to serines abolishes the protein activity [59–61]. Therefore, despite the similarity presented with Fe-S cluster assembly detected by the CCD database, this domain does not appear to be functional. Finally, the analysis of the primary sequence of the rhodanese-like domain, shows that the similarities with other rhodaneses are very low. However, the

conserved cysteine residue essential to enzyme activity is present at Glurho domain (Cys²⁶⁶) (**Fig. 2C**, blue asterisk). Since that the *in silico* analysis suggests that at least two domains may be functional, we decided to express and purify the recombinant Glurho protein to assess its biochemical properties.

Glurho is not effective in disulfide reduction or hydroperoxide decomposition.

Initially, we tested the oxidoreductase activity of the Glurho Grx domain, using the standard assay of the mixed disulfide disulfide reduction formed between β -hydroxyethyl disulfide (HED) and glutathione (β -ME-SG) [23,62]. Our results demonstrate that no significant differences were observed between the negative control (without protein) and the reaction containing Glurho, indicating that Glurho is not able to reduce the β -ME-SG disulfide (**Figure S1A**). Since that β -ME-SG disulfide uses a synthetic compound (HED), we also tested the Glurho oxidoreductase activity using as substrate a mixed disulfide between cysteine and glutathione (Cys-S-SG), which may resemble more substantially the endogenous substrate of the Grx, however, no activity was detected (**Figure S1B**). Despite that Gluro Grx domain not having shown activity as a disulfide reductase, is well known that some monothiolic Grx presents low or null affinity in reducing mixed glutathione disulfides, which seems to be related to slight structural differences at their active sites environment [19]. Additionally, recent studies performed by Li and colleagues 2010, [63] using structural comparisons and sequence alignments of monothiol and dithiol Grx, showed that all monothiol Grx (such as those with the catalytic motif CGFS), possess an insertion of 5 amino acids, not conserved in sequence, in close vicinity in the sequence to the catalytic cysteine. In fact all monothiolic Grx that have these five additional amino acids, coincidentally do not possess activity in the HED assay [63]. On the other hand, dithiol Grx as well some monothiol ones, as the yeast ScGrx6, ScGrx7 and poplar GrxS12, whose lacks the 5 additional amino acids exhibit activity in the HED assay. The analysis of the protein sequence of the Glurho Grx like domain makes clear the existence of the additional amino acids close to the CGFS motif (**Figure 2A**), indicating that the absence of disulfide reductase activity may rely in this structural feature.

It was previously demonstrated that the yeast glutaredoxins Grx1 and Grx2 can directly reduce hydroperoxides acting as glutathione peroxidases [21]. Furthermore *grx1* and *grx2* yeast mutants are sensitive to oxidative stress induced by hydroperoxides [21]. Therefore

peroxidase activity assays were carried out to verify the possibility of the Grx domain of Glurho to act as GSH dependent peroxidase. To test the peroxidase activity over organic peroxide (tert-butyl hydroperoxide) we employed the NADPH oxidation assay using glutathione reductase (GR) and GSH. The results demonstrated that Glurho was not able to decompose the *t*-BOOH (**Figure S1C**). We also tested the peroxidase activity using the dithiolic reductant DTT, by the oxidation of DTT assay using hydrogen peroxide as substrate. However no enzymatic activity over hydroperoxide decomposition was detected (**Figure S1D**). Together our results demonstrated that Glurho is not effective as a peroxidase.

Glurho is able to convert cyanide to promote cyanide detoxification

Rhodanases are a group of enzymes that uses a cysteine residue to catalyze the reaction between thiosulfate and cyanide leading to the formation of sulfite and thiocyanate [27,28,30]. To evaluate the rhodanase activity of Glurho we employed the method described by Westley (1981) in which the thiocyanate formation in acidic conditions and in the presence of Fe^{+3} results in a red colored intermediate that can be quantified spectrophotometrically. The reactions were carried out using growing Glurho concentrations and our results demonstrated that the thiocyanate formation increases proportionally with Glurho concentration (**Fig. 3A**).

Since that the Glurho presented rhodanase activity, we also decided to compare the Glurho rhodanase activity with one of the most well studied enzymes of this class: the bovine liver rhodanase (Rhobov) [56,64]. For this purpose, equivalent concentrations of the enzymes (1 μM) were used in the thiocyanate formation assay and reactions were stopped after five minutes. Our results demonstrated that the *X. fastidiosa* Glurho exhibited a specific activity five folds higher than the Rhobov (**Fig. 3B**). These results are very interesting since that cyanide production in response to grazing and predation by a variety of plants are well known [70,71], and Glurho may act as an defense front to enable the insect resistance to plant defenses. In fact some species that are infected by *X. fastidiosa* like almond, alfalfa and peach trees are able to produce cyanogenic compounds in their stem (e.g. prussic acid) [72]

Evaluation of additional sulfur acceptors

It has been demonstrated that the additionally to the cyanide, the rhodanases are able to exerts the sulfurtransferase activity over another substrates [32,36,37,38]. Recent studies

demonstrated that the thioredoxin (Trx) is an efficient rhodanese sulfur acceptor [65]. In this case, the sulfur atom form a mixed disulfide with Trx cysteine which is reduced by the thioredoxin reductase enzyme (TrxR) using reducing equivalents from NADPH, producing hydrogen sulfide (H₂S) (**Figure S2A**). To evaluate if Glurho is able to act as sulfurtransferase to the Trx, we performed a NADPH oxidation assay, using *E. coli* Trx and *E. coli* TrxR or Tsnc, a Trx from *X. fastidiosa*. The results demonstrated that EcTrx and Tsnc were not able to accept sulfur atoms from Glurho (**Figure S2B**).

Although the results have shown that Glurho is not capable to transferring sulfur atoms to *E. coli* Trx, the results are inconclusive to Tsnc. Since that the *E. coli* TrxR was used in the NADPH oxidation assay, the sulfur transfer by Glurho to the Tsnc could have occurred but was not detected due to an inefficiency of the *E. coli* TrxR to reduce *X. fastidiosa* Tsnc, because the proteins are from different species, a phenomena related before [73].

In reason of observations presented above, additional cyanide sulfurtransferase assays were performed using higher Tsnc concentrations (1 or 2 molar equivalents). In addition to Tsnc, the low molecular weight thiolic compounds DTT and GSH were also tested as sulfur acceptors. The sulfur transfer to the potential acceptors catalyzed from Glurho would lead to a decrease of thiocyanate formation due to the competition with KCN⁻ for sulfur atoms. The results revealed the reduction of thiocyanate formation when DTT (**Figure S3C**) and GSH (**Figure S3B**) are added to the reactions, while no differences were observed when Tsnc is added (**Figure S3A**), indicating that Tsnc is not able to accept sulfur atoms from Glurho. On the other hand, the Glurho was able to exert the sulfurtransferase activity over thiolic compounds, which may indicate that the CN⁻ may not be the sole substrate for the enzyme.

Cysteines integrity is essential for Glurho sulfurtransferase activity

As mentioned before, all the rhodaneses described to date uses a cysteine residue to capture a sulfur atom and transfer to their substrates [27,28,30,56,64]. To confirm that the Glurho activity is centered on a cysteine residue, previously to ferrithiocyanate assay, the enzyme cysteines were alkylated using N-ethylmaleimide (NEM). Our results revealed that the cysteine alkylation abolished entirely the rhodanese activity (**Fig. 4A**), confirming that the cysteines integrity is essential to the enzymatic properties of Glurho.

Since that the Cys²⁶⁶ residue is inserted in rhodanese domain is reasonable to think that it is responsible for the sulfurtransferase activity of Glurho (**Fig. 2C**). Moreover, since there is

no activity was detected for the oxidoreductase domain Grx, it is possible that the residue Cys³³ may play a structural role in the enzyme. Thus their replacement could affect the enzyme structure reducing or even eliminating their activity. In order to unequivocally identify the Cys²⁶⁶ as the residue as responsible for rhodanese activity and assess whether Cys³³ exerts a structural role on Glurho, we have performed site directed mutagenesis replacing individually the cysteines residues by serines.

Therefore the sulfurtransferase activity assay was performed to evaluate the enzyme activity of the mutants Glurho^{C33S} and Glurho^{C266S} compared to the wild type protein. As expected, the rhodanese activity of Glurho^{C266S} was virtually abolished (**Fig. 4B**). On the other hand the Glurho^{C33S} presented an unexpected increase of the rhodanese specific activity 3-fold higher than the wild type enzyme (**Fig. 4B**).

The higher rhodanese activity of the Glurho^{C33S} mutant, indicates that despite the Cys³³ of the Grx domain do not play roles in oxidoreductase processes or over the enzyme structure, it may play a regulatory role in enzyme rhodanese activity. An example is ThiI, a bifunctional enzyme required for the biosynthetic pathway of 4-thiouridine, a modified base present in certain bacterial tRNAs and Thiazole moiety of Thiamine [24]. Most of the protein related to ThiI family presents an N-terminal THUMP domain involved in a variety of RNA modifications and a C-terminal rhodanese domain. In the reaction mechanism proposed for ThiI, desulfurase (IscS) first catalyses the transfer of sulfur from a free Cys to the Cys catalytic residue of the rhodanese of ThiI (Cys⁴⁵⁶), and then residue Cys³⁴⁴ acts as a nucleophile forming a Cys⁴⁵⁶-Cys³⁴⁴ disulfide releasing 4-thiouridine [36,37,38].

Since the enzyme ThiI also represents a rhodanese fusion protein with two domains containing cysteines, which have as intermediate a disulfide between the cysteines of both domains. Therefore, the Glurho Cys³³ could form a disulfide as an intermediate in the cycle of sulfur transfer to the CN⁻, and thus modulate the activity of the enzyme. In this context, the disulfide would need to be reduced to the enzyme could perform another round of sulfur transfer. To test this hypothesis assays were conducted, fixing Glurho concentration varying the time of the experiment without the addition of a reductant. As can be seen in Figure 5, even without the presence of a reducing agent the amount of formed thiocyanide increased with time, indicating that no disulfides are formed between Cys³³ and Cys²⁶⁶.

Heterologous expression of Glurho confers high cyanide resistance to E. coli cells

It has been reported 1 mM of cyanide causes significant inhibition of aerobically growing of *E. coli*. Therefore, to verify any protective effect of Glurho against cyanide toxicity, exponential phase cultures of *E. coli* BL21 (DE3) containing the empty vector (pET15b) or carrying the plasmid with *glurho* gene (pET15b /Glurho) were adjusted to $OD_{600} = 0.01$ and challenged with 1.5 mM of KCN in the presence of IPTG (0.1mM). Since that the basal expression levels of heterologous proteins are significantly high in *E. coli* BL21 (DE3) cells, we also performed the same experiment without the IPTG addition [77]. After 24 hours of growing of the cells cultures the optical density of the cells cultures were measured. The results demonstrated that optical density of the cultures cells carrying the recombinant vector but uninduced with IPTG, presented growing rates 83% higher than the cells carrying the empty vector. To IPTG induced cells the rates were 91% higher than the cells carrying the empty vector, indicating that cyanide resistance are dependent of recombinant protein inside the cell (**Fig. 11A**).

The ability to cyanide detoxification *in vivo* of Glurho^{C33S} and Glurho^{C266S} were also tested using the same conditions described before to non-IPTG induced cells. Curiously, the *E. coli* cells strains carrying the recombinant vectors with cysteine substitutions (pET15b/*Glurho*^{C33S} and pET15b/*Glurho*^{C266S}) were not able to confer any resistance to the cells under inhibitory cyanogenic conditions (**Fig.11B**). Indicating that the Cys³³ may play an important role in cyanide resistance *in vivo*.

Concluding Remarks

In this work, we performed the first characterization of the Glurho a multi domain enzyme, presenting homologues only in prokaryotes, most of which are pathogenic to animals and plants. Despite that no classical oxidoreductase or structural role was detected to Grx domain, the evaluation of the rhodanese function revealed that Glurho is able to detoxify efficiently cyanide to thiocyanate *in vitro* with higher efficiency (5 ×) than bovine rhodanese. Since *X. fastidiosa* is a 2-host pathogen the high resistance to cyanide may be very important to the insect vector, since some *X. fastidiosa* target plants produces cyanogenic compounds as a way to protect themselves from insect predation. Additional importance to the Glurho sulfurtransferase activity may be related to the large amounts of sulfur on *X. fastidiosa*'s biofilm [35] and the presence of Glurho on extracellular environment [11], which may

indicate an important role on bacterial adhesion and maintenance on xylem vessel. Curiously, the *glurho* gene position on *X. fastidiosa* genome are very close to several genes related to biofilm extracellular polysaccharides (EPS) biosynthesis such as Xanthan gum [66, 67].

Additional importance, also be related to the fact that the integrity of both cysteines residues is essential to rhodanese activity *in vivo*. The necessity of the Cys³³ to cyanide resistance *in vivo* still needs research to the understanding its role to the cell defenses. However, it is interesting to mention that some members of the monothiol Grxs family, including yeast Grx3 and Grx4, were recently shown to form [2Fe-2S]-bridged homodimers using as Fe ligands the active site cysteines residue assisted by two GSH molecules [58]. These unusual GSH-linked Fe-S clusters in monothiol Grxs provide a direct link between iron homeostasis with Fe-S cluster assembly and thiol redox regulation. Finally, by all the arguments presented above, the *glurho* protein may be an important pathogen enzyme involved several strategies for the attack and neutralization of host defenses.

Acknowledgments

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Figure 1 - Sequence alignment of Glurho homologues. The deduced amino acid sequences were obtained at the ncbi genebank (<http://www.ncbi.nlm.nih.gov>), the sequence alignment was performed using the Clustal W2 (www.ebi.ac.uk/Tools/msa/clustalw2) and the alignment scoring and graphical representation was performed using the Jalview (www.jalview.org). Identical residues are shaded in purple and sequence similarities are indicated by a purple gradient based of the conservation of physicochemical characteristics of the amino acids. The abbreviations used to organisms and proteins are as follow: GLP1 – Glurho protein from *X. fastidiosa*; GLP2 – Glutaredoxin-like protein from *X. campestris pv. Campestris*; GLP3 – Glutaredoxin-like protein from *X. campestris musacearum*; GLP4 – Glutaredoxin-like protein from *X. gardneri*; GLP5 – Glutaredoxin-like protein from *X. vesicatoria*; GLP6 – Glutaredoxin-like protein from *X. Oryzae oryzicola*; GLP7 – Glutaredoxin-like protein from *X. Oryzae oryzae*; GLP8 – Glutaredoxin-like protein from *X. fuscans aurantifolii*; GLP9 – Glutaredoxin-like protein from *X. campestris pv. musacearum*; GLP10 – Glutaredoxin-like protein from *X. axonopodis pv. citri*; GLP11– Glutaredoxin-like protein from *X. axonopodis pv. punicae*; GLP12 – Glutaredoxin-like protein from *X. citri pv. mangiferaeindicae*; GLP13 – Glutaredoxin-like protein from *X. sacchari*; GLP14 – Glutaredoxin-like protein from *X. albilineans*; GLP15 – Glutaredoxin-like protein from *X. translucens pv. graminis*; GLP16 – Glutaredoxin-like protein from *Stenotrophomonas maltophilia*; GLP17– Glutaredoxin-like protein from *Stenotrophomonas* sp.; GLP18 – Glutaredoxin-like protein from *Pseudomonas geniculata*; GLP19– Glutaredoxin-like protein from *Pseudomonas spadix*; GLP20 – Glutaredoxin-like protein from *Pseudoxanthomonas suwonensis*; GLP21 – Glutaredoxin-like protein from *Rhodanobacter* sp.; GLP22 – Glutaredoxin-like protein from *Frateuria aurantia*; GLP23 – Glutaredoxin-like protein from *Haliangium ochraceum*; GLP24 – Glutaredoxin-like protein from *Plesiocystis pacifica*; GLP25 – Glutaredoxin-like protein from *Sorangium cellulosum*.

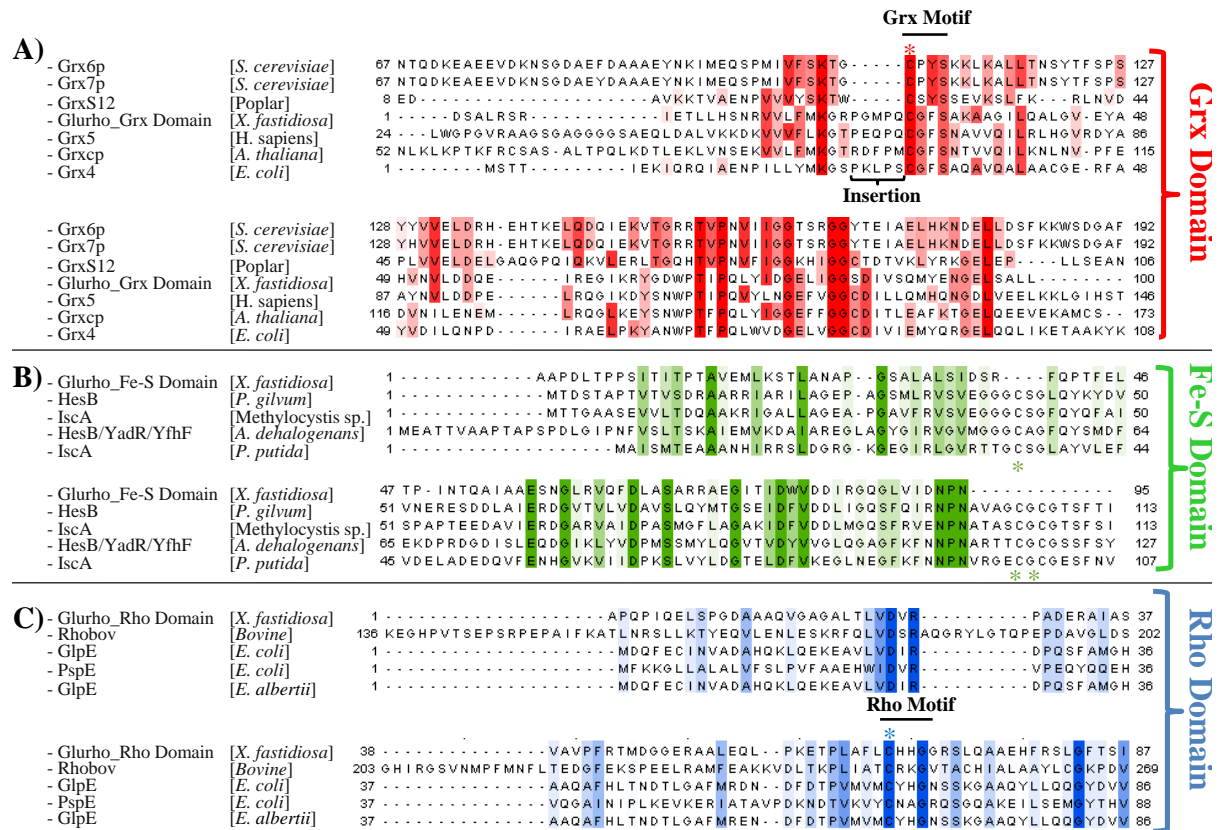


Figure 2 - Similarity analysis of Glurho domains. To perform the analysis the Glurho *X. fastidiosa* deduced amino acid sequence of the protein was divided in three independent segments as defined by the CCD database. Protein sequences of well known Grx, Fe-S cluster assembly and rhodanese representatives were obtained at the ncbi database (<http://www.ncbi.nlm.nih.gov>), the sequence alignment was performed using the Clustal W2 (www.ebi.ac.uk/Tools/msa/clustalw2) and the alignment scoring and graphical representation was performed using the Jalview (www.jalview.org). Identical residues are shaded in red, green and blue, to Grx, Fe-S cluster assembly and rhodanese, respectively. Sequence similarities are indicated by a gradient based of the conservation of physicochemical characteristics of the amino acids. **A)** Alignment of amino acid sequences of monothiol from *S. cerevisiae* (Grx6p and Grx7p), Poplar (GrxS12) *E. coli* (Grx4) *H. sapiens* (Grx5), *A. thaliana* (Grxcp) and Grx domain of Glurho from *X. fastidiosa*. Identical amino acids are highlighted in red and catalytic motif is pointed out (CGFS). **B)** Amino acid sequences alignment of proteins related to Fe-S clusters biosynthesis, *Polymorphum gilyum* (HesB), *Methylocystis* sp. (ISCA), *Anaeromyxobacter dehalogenans* (HesB / YADR / YfhF) and Fe-S_biosynthesis domain of Glurho from *X. fastidiosa*. Identical amino acids are highlighted in green. **C)** Amino acid alignment sequences of *Bos Taurus* Rodanases (Rhobov), *E. coli* (PspE), *E. coli* (GlpE), *E. albertii* (GlpE) and Rho domain of Glurho from *X. fastidiosa*. Identical amino acids are highlighted in blue and catalytic motif is pointed out (CHHG in the case of Glurho). The red, green and blue asterisks highlight characteristic cysteines residues of glutaredoxins, Fe-S assembly proteins and rhodanases, respectively. The black bar near to the Grx motif is relative to the insertion of monothiolic Grx.

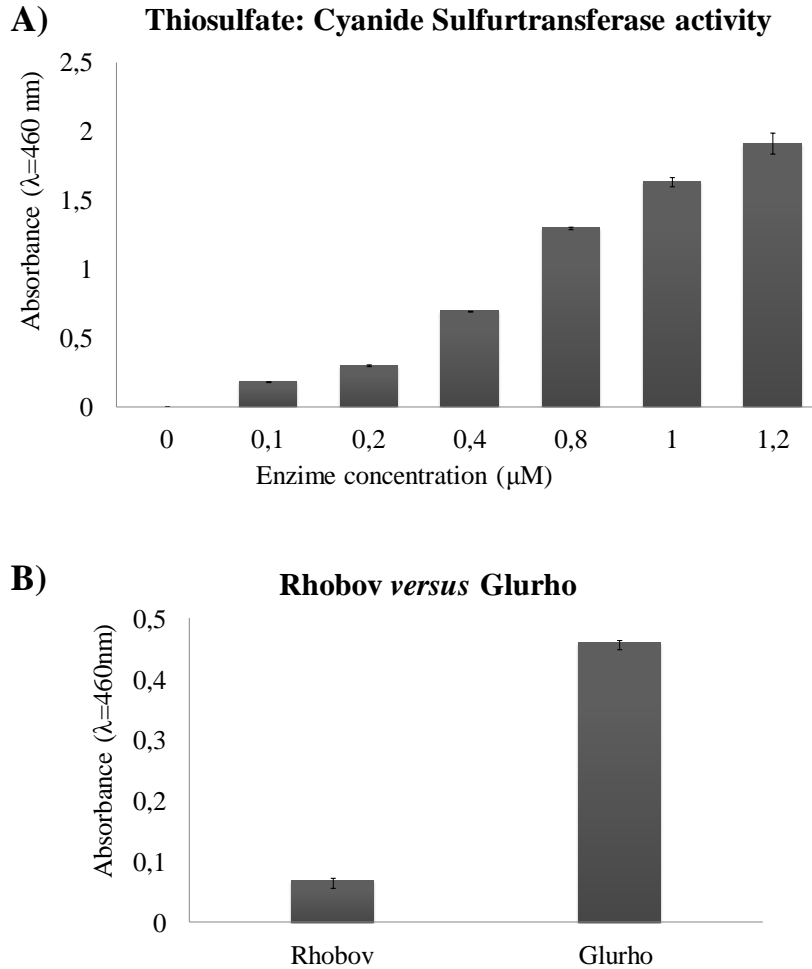


Figure 3 – Evaluation of Glurho rhodanese activity. **A)** Determination of Thiosulfate – cyanide sulfurtransferase activity by Glurho. The reactions were performed in 1ml containing 40 mM potassium phosphate, 50 mM cyanide, 50 mM sodium thiosulfate and Milli Q water (q.s.p). The reactions were initiated by adding 0-1.2 μM of the enzyme. After 5 minutes the reaction were stopped by adding formaldehyde 2.5% (300μl), and then by adding ferric nitrate (100g of $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ and 200 ml of 65% HNO_3) (200μl). The ferrithiocyanide formation was determinate spectrophotometrically ($\lambda=460\text{nm}$). **B)** Comparative rhodanese activity of Glurho and bovine rhodanese (Rhobov). The reaction was carried out in the same conditions described above for 1min using 1 μM of Glurho or 1 μM of Rhobov. In both assays no enzyme was added to control reactions. All assays were performed in triplicate for three times.

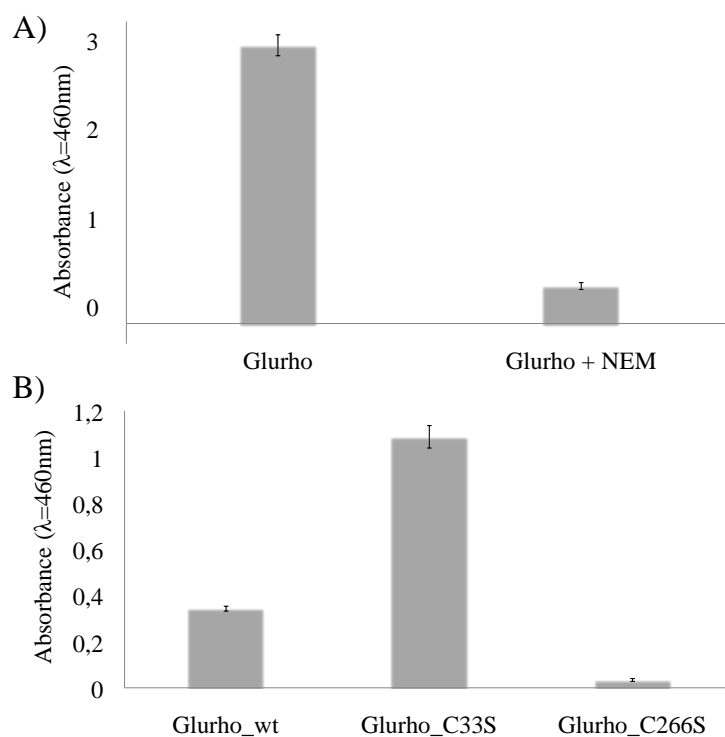


Figure 4 – Involvement of Cys-33 and Cys-266 in Glurho sulfurtransferase activity. A) Determination of Glurho^{C33S} and Glurho^{C266S} sulfurtransferase activity. The assays were performed in 1ml containing 40 mM potassium phosphate, 50 mM potassium cyanide, 50 mM sodium thiosulfate and Milli Q water (q.s.p). The reactions were initiated by adding 1 μM of the wild type Glurho enzyme or Glurho^{C33S} and Glurho^{C266S}. After five minutes (the reactions were stopped by adding formaldehyde 2.5% (300μl), followed by ferric nitrate (100g of Fe(NO₃)₃·9H₂O and 200 ml of 65% HNO₃) (200μl). The ferrithiocyanide formation was determinate spectrophotometrically (λ=460nm). The assays were performed in triplicate for three times.

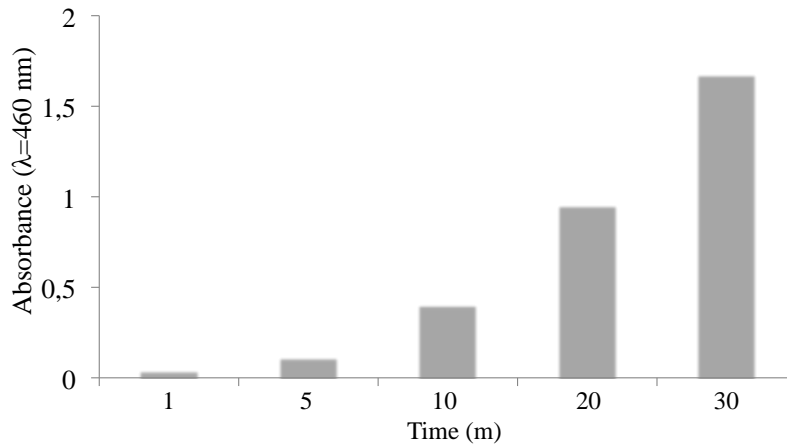


Figure 5 – Evaluation of thiocyanate formation catalyzed by Glurho at different time intervals. The reactions were performed in 1ml containing 40 mM potassium phosphate, 50 mM potassium cyanide, 50 mM sodium thiosulfate and Milli Q water (q.s.p). The reactions were initiated by adding 1 μ M of the Glurho enzyme. After different period of times (1, 5, 10, 20 and 30 minutes) the reactions were stopped by adding formaldehyde 2.5% (300 μ l), followed by ferric nitrate (100g of $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ and 200 ml of 65% HNO_3) (200 μ l). The ferrithiocyanide formation was determinate spectrophotometrically ($\lambda=460\text{nm}$).

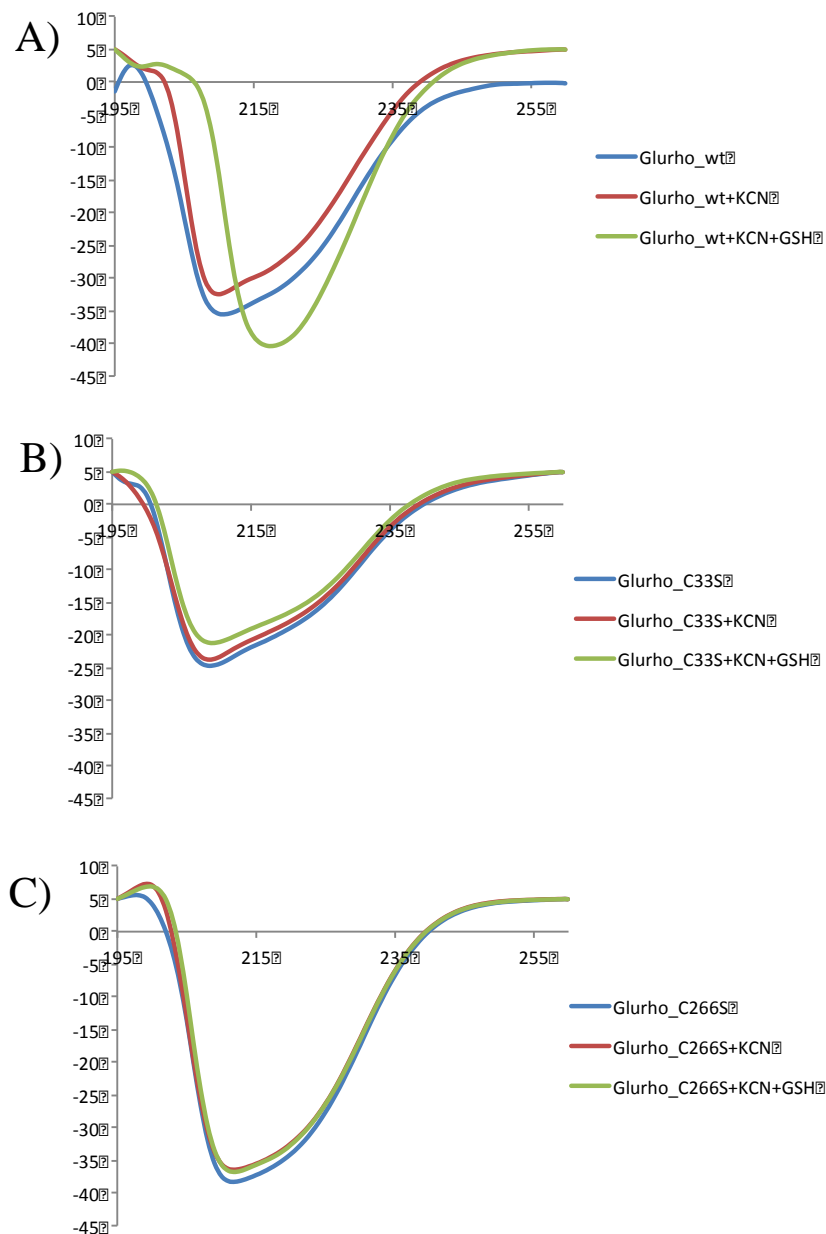


Figure 6. CD spectra of reduced and oxidized samples of Tsa1^{WT} and mutants containing Thr⁴⁴ and Arg¹²³ substitutions. Spectra in the reduced form with 5 mM of TCEP (solid line) and treated with 1.2 eq of hydrogen peroxide (dashed line) of the enzymes Tsa1^{WT} (A), Tsa1^{T44A} (B), Tsa1^{T44S} (C), Tsa1^{T44V} (D), Tsa1^{R123G} (E) and Tsa1^{R123K} (F). The proteins concentration used in experiments were 10 μ M in 20 mM sodium fluoride buffer (pH 7.4). All spectra were recorded at 25°C and corrected against the buffer. The graphical representations are averages from eight consecutive scans.

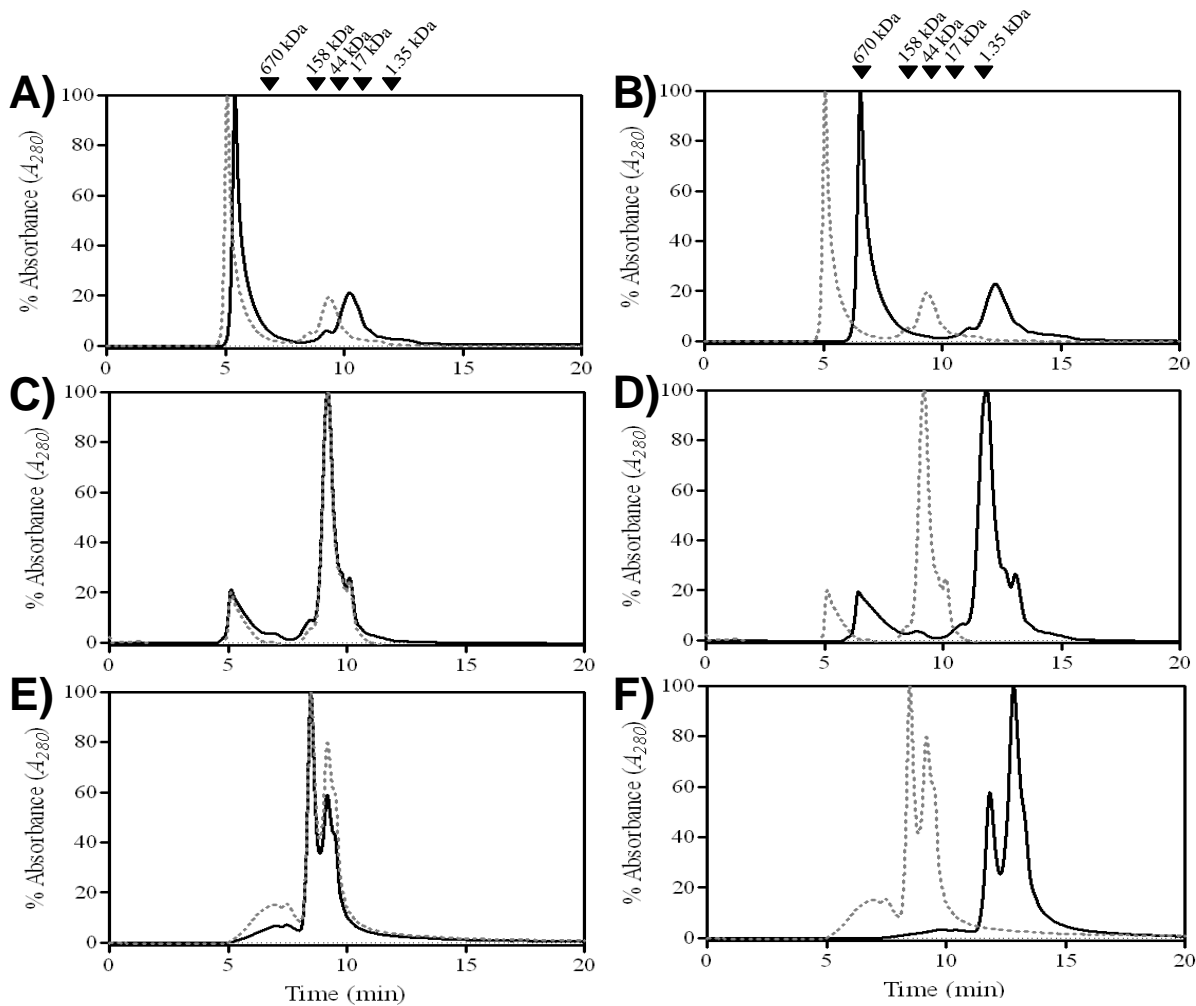


Figure 7. Size-exclusion chromatography - size-exclusion chromatography was performed by analytical hplc using a jasco series equipped with a pu 2880 plus injector and a pda md 2018 detector (jasco). the samples (15 μ mol in 100 mm tris-hcl at ph 7.4) were separated by a system containing a phenomenex biosep-sec-s3000 column (7.8×300 mm, 5 μ m, resolution range of 15 to 2000 kda, phenomenex, inc., torrance, california, usa) using a flow of 0.5 ml/min in tris-hcl buffer (ph 7.4) and 50 mm nacl. the elution profile was monitored at a wavelength of 280 nm. bovine thyroglobulin (670 kda), bovine gamma globulin (158 kda), ovalbumin (44 kda), myoglobin (17 kda) and vitamin b₁₂ (1.35 kda) were used as molecular standards (bio-rad). the chromatograms were analyzed using the class-vp software (shimadzu co.). the redox treatments for tsal and mutant proteins were 5 mm tcep or 1.2 molar equivalents of hydrogen peroxide for 2hs/30°C.

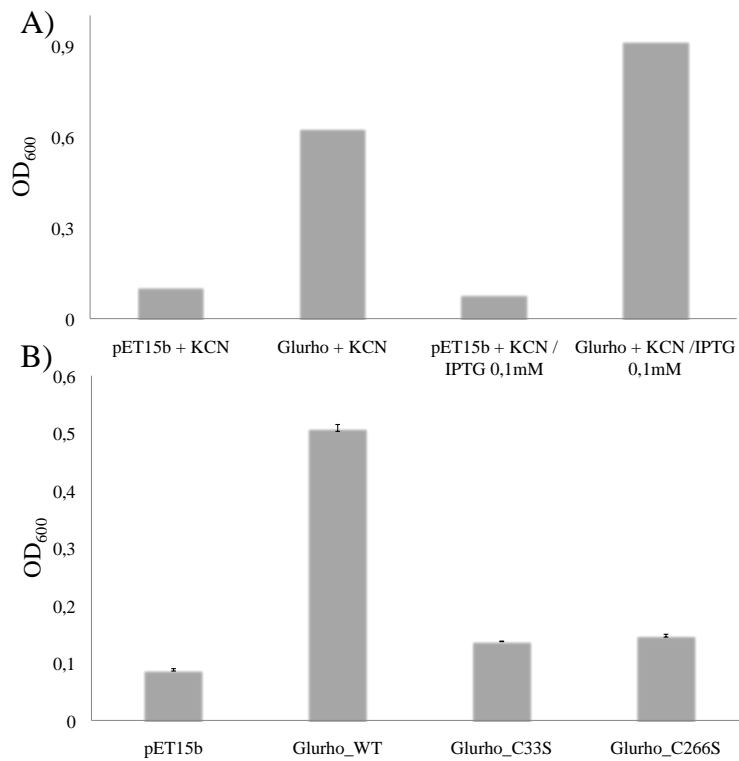


Figure 11 - Only the heterologous expression of intact Glurho protein confers to *E. coli* cells a high resistance to cyanide . A) Cell cultures of *E. coli* BL21 (DE3) containing the empty plasmid (pET15B) or harboring pET15b/Glurho in exponential phase were diluted to OD₆₀₀=0.1, exposed to KCN⁻ (1.3 mM) and grown at 37°C, with the addition or not of IPTG 0.1mM to evaluate the protective effect of basal expression on cell. The growth rate was determined after 24 hs. **B)** Effects of cysteines substitutions over the cyanide resistance of *E. coli* cells harboring the pET15b/Glurho^{C33S} or pET15b/Glurho^{C266S} plasmids. Comparative resistance of *E. coli* cells containing pET15B, pET15b/Glurho, pET15b/Glurho^{C33S} or pET15b/Glurho^{C266S}. The experimental conditions were the same as presented in (A). All assays were performed in triplicate for three times.

SUPPLEMENTARY MATERIAL

FUNCTIONAL CHARACTERIZATION AND HETEROLOGOUS EXPRESSION OF A NEW RHODANESE LIKE PROTEIN FROM *XYLELLA FASTIDIOSA*.

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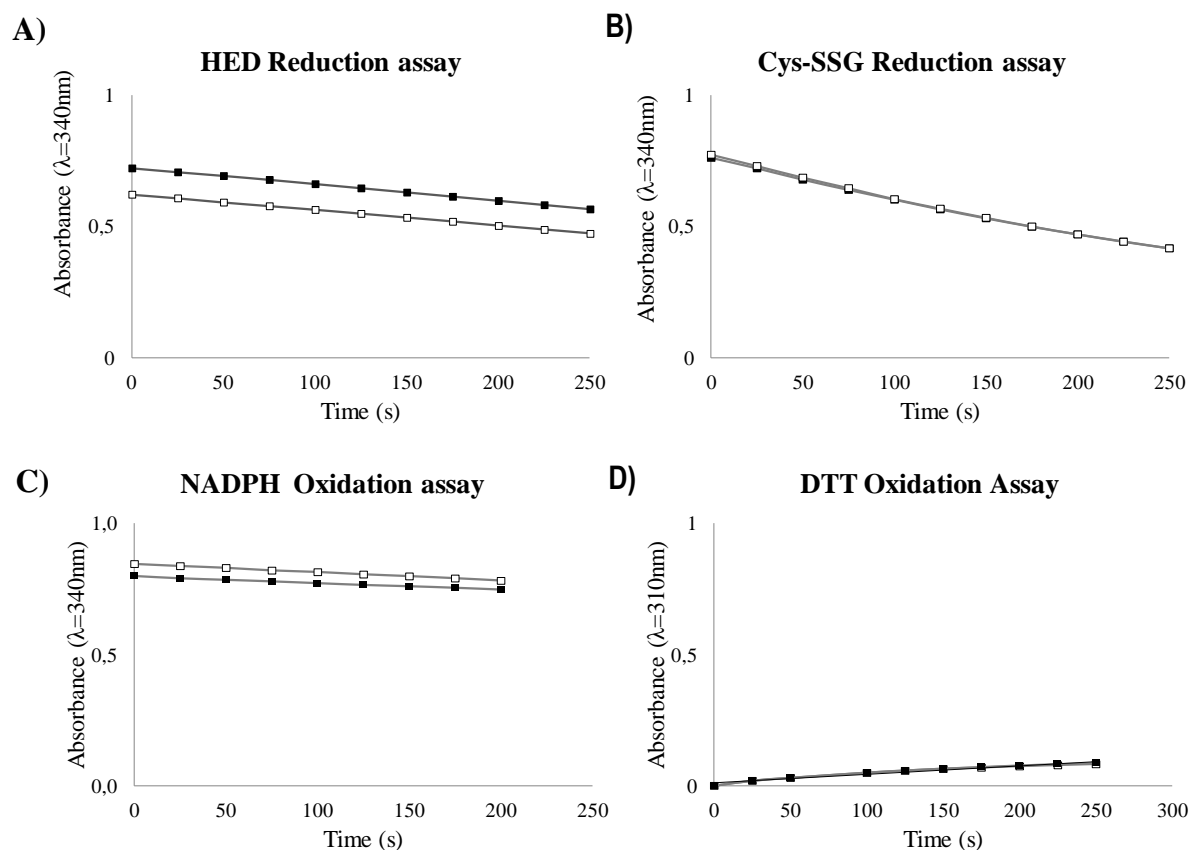
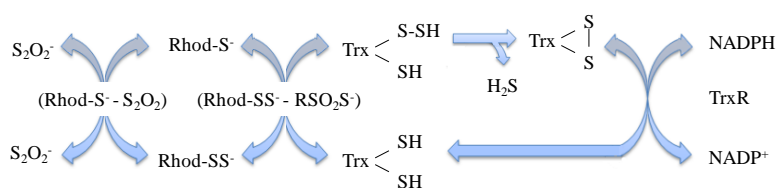


Figure S1 - Evaluation of glutaredoxin activity by different assays. **A) HED assay.** The Glurho ability to reduce the mixed disulfide formed between GSH and β -hydroxyethyl disulfide (HED) was measured spectrophotometrically ($\lambda=340\text{nm}$). The reactions mixtures containing 0.1 M Tris-HCl (pH 7.4); 1mM GSH; 6 $\mu\text{g/ml}$ GR and 0.7 mM HED were incubated at 30°C for three minutes for the formation of the HED mixed disulfide and then 0.2 mM NADPH was added, the reaction was started by the addition of Glurho and followed by the decrease in the absorbance at 340 nm due to the NADPH oxidation. **B) Cys-SSG reduction assay.** The Glurho ability to reduce the mixed disulfide formed between GSH and Cys was monitored spectrophotometrically ($\lambda = 340\text{nm}$) in 1ml reaction containing: 1mM GSH, Gr 6 $\mu\text{g/ml}$, 100mM Tris-HCl (pH 7.4), 0.2mM of NADPH, 100 μM of CysS-SG and 1 μM of Glurho. **C) NADPH oxidation assay.** The *t*-BOOH decomposition was measured spectrophotometrically ($\lambda=340\text{nm}$) due to the oxidation of NADPH, the reaction contained: 0.1 M Tris-HCl (pH 7,4); 1mM GSH; 6 $\mu\text{g/ml}$ GR, 0.2 mM NADPH, 1 μM of Glurho and 1mM of *t*-BOOH. **D) Evaluation of thiol peroxidase activity by DTT oxidation assay.** The H_2O_2 decomposition assay was measured spectrophotometrically ($\lambda=310\text{nm}$), graphic of absorbance \times time was obtained from the DTT oxidation. The reaction contained: 10mM of DTT, 100mM Tris-HCl (pH7.4), 1mM of H_2O_2 and 1 μM of Glurho. The blank squares (\square) represent the complete reaction whereas the black squares (\blacksquare) are relative to the negative controls (with no addition of Glurho). All the experiments were performed using triplicates.

A)



B)

NADPH oxidation Assay - Trx as Sulfur Acceptor

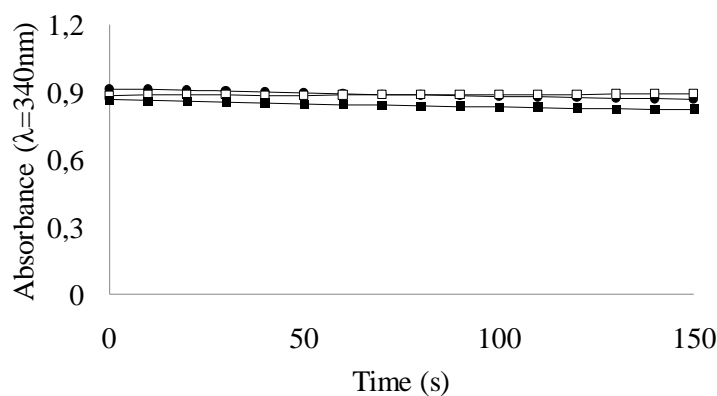


Figure S2 – Evaluation of *E. coli* Trx and *X. fastidiosa* as sulfur acceptor to Glurho. **A)** Mechanism of sulfur transfer by rhodanese to Trx. Schematic representation of rhodanese sulfur transfer from thiosulfate (S₂O₂⁻) using Trx as acceptor. Initially, the rhodanese captures the sulfur and transfer to the Trx that becomes oxidized and releases a hydrogen sulfide. The oxidized Trx is reduced by the TrxR using NADPH reducing equivalents. **B)** Glurho NADPH oxidation assay. The evaluation of Glurho uses the protein Trx was monitored spectrophotometrically (λ= 340 nm) by the NADPH oxidation assay for 120 seconds. The reaction was performed in 1ml containing: potassium phosphate buffer 50 mM, TrxR (*E. coli*) 0.2 μM, NADPH 150 μM, *X. fastidiosa* Trx (Tsnc) 10 μM or *E. coli* Trx 10 μM, 80 mM of sodium thiosulfate and 10 μM of Glurho. The black circles (●) are relative to the negative control reaction (without Glurho). The black squares (■) are relative to the reaction using *X. fastidiosa* Trx (Tsnc) and white squares (□) the reactions performed with *E. coli* Trx. The assays were performed in triplicate.

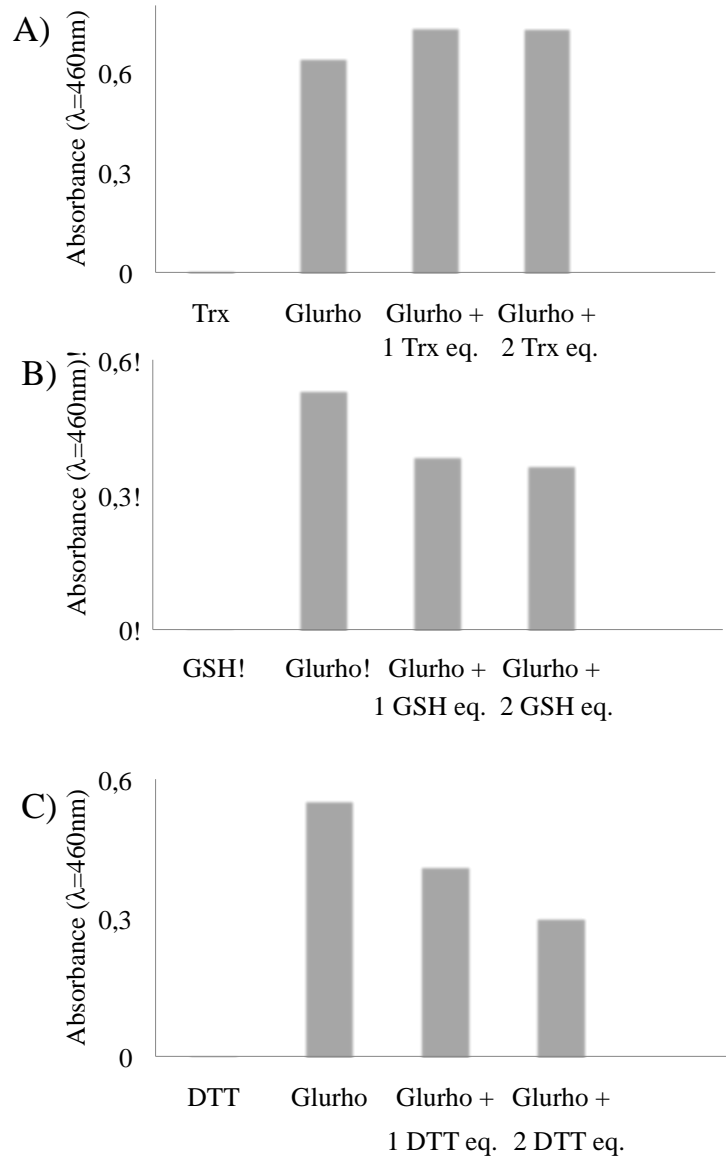


Figure S3 – Evaluation of Glurho sulfur transferase activity to thiolic acceptors. Evaluation of sulfurtransferase activity of Glurho over *X. fastidiosa* Trx (TsnC) (A) or the low molecular weight thiolic compounds GSH (B) or DTT (C). The reactions were performed essentially as described in Material and Methods to ferrithiocyanide assay. As the positive control just Glurho (1 μM) was added to the reactions and for negative control only the thiolic compounds. The ferrithiocyanide formation was determined spectrophotometrically ($\lambda=460\text{nm}$). Were tested 1 or 2 molar equivalents of each thiolic compound. The results are representative of three independent experiments.