Free Radical Biology and Medicine 156 (2020) 207–216

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original article

Reduction of sulfenic acids by ascorbate in proteins, connecting thiol-dependent to alternative redox pathways

Valesca Anschau[a], Gerardo Ferrer-Sueta[b,c], Rogerio Luis Aleixo-Silva[a], Renata Bannitz Fernandes[a], Carlos A. Tairum[a], Celisa Caldana Costa Tonoli[e], Mario Tyago Murakami[e], Marcos Antonio de Oliveira[d], Luis Eduardo Soares Netto[a,*]

[a] Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, 05508-090, São Paulo, Brazil
[b] Laboratorio de Fisicoquímica Biológica, Instituto de Química Biológica, Facultad de Ciencias, Universidad de La República, Iguá 4225, Montevideo, 11400, Uruguay
[c] Centro de Investigaciones Biomédicas (CEINBIO), Universidad de La República, Montevideo, Uruguay
[d] Instituto de Biociências, UNESP, Campus Do Litoral Paulista, São Vicente, 11330-900, São Paulo, Brazil
[e] Brazilian Biorenewables National Laboratory, National Center for Research in Energy and Materials, Campinas, Brazil

ARTICLE INFO

Keywords:
Ascorbate
Peroxiredoxin
Sulfenic acid
Peroxides

ABSTRACT

Sulfenic acids are the primary product of thiol oxidation by hydrogen peroxide and other oxidants. Several aspects of sulfenic acid formation through thiol oxidation were established recently. In contrast, the reduction of sulfenic acids is still scarcely investigated. Here, we characterized the kinetics of the reduction of sulfenic acids by ascorbate in several proteins. Initially, we described the crystal structure of our model protein (Tsa2-C170S). There are other Tsa2 structures in distinct redox states in public databases and all of them are decamers, with the peroxidatic cysteine very accessible to reductants, convenient features to investigate kinetics. We determined that the reaction between Tsa2-C170S-Cys-SOH and ascorbate proceeded with a rate constant of $1.40 \pm 0.08 \times 10^3\ M^{-1}\ s^{-1}$ through a competition assay developed here, employing 2,6–dichlorophenol-indophenol (DCPIP). A series of peroxiredoxin enzymes (Prx6 sub family) were also analyzed by this competition assay and we observed that the reduction of sulfenic acids by ascorbate was in the $0.4–2.2 \times 10^3\ M^{-1}\ s^{-1}$ range. We also evaluated the same reaction on glyceraldehyde 3-phosphate dehydrogenase and papain, as the reduction of their sulfenic acids by ascorbate were reported previously. Once again, the rate constants are in the $0.4–2.2 \times 10^3\ M^{-1}\ s^{-1}$ range. We also analyzed the reduction of Tsa2-C170S-SOH by ascorbate by a second, independent method, following hydrogen peroxide reduction through a specific electrode (ISO-HPO-2, World Precision Instruments) and employing a bi-substrate, steady state approach. The k_{cat}/K_M^{Asc} was $7.4 \pm 0.07 \times 10^3\ M^{-1}\ s^{-1}$, which was in the same order of magnitude as the value obtained by the DCPIP competition assay. In conclusion, our data indicates that reduction of sulfenic acid in various proteins proceed at moderate rate and probably this reaction is more relevant in biological systems where ascorbate concentrations are high.

1. Introduction

Hydrogen peroxide (H_2O_2) is an endogenous, diffusible oxidant that is generated as a by-product of the respiratory electron transport chain and enzymatically by some signaling enzymes, such as two NADPH oxidases, Nox4 and Duox [1,2]. There is current consensus on the role of H_2O_2 as a signaling molecule, alongside its potentially damaging character. H_2O_2 mediates several signaling pathways by oxidizing Cys residues in regulatory proteins, as reviewed by Ref. [3,4]. Sulfenic acids (-SOH) are the initial product of the two-electron oxidation of thiols by H_2O_2 and other oxidants such as organic hydroperoxides and hypohalites [5,6]. Therefore, for the full understanding of H_2O_2 signaling, the redox chemistry and biology of sulfenic acids needs to be investigated.

Sulfenic acids are unstable and readily react with thiols and other nucleophiles, therefore, they are not easily observed during the oxidation of low molecular weight thiols [7]. One of the major reactions that low molecular weight sulfenic acids can undergo is the very fast condensation reaction with thiols to form disulfides with rate constants >

Abbreviations: Cp, peroxidatic cysteine residue; DCPIP, 2,6–dichlorophenol-indophenol; FF, "fully folded" state of peroxiredoxins; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LU, "locally unfolded" state of peroxiredoxins; Prx(s), peroxiredoxin(s); Trx, thioredoxin

* Corresponding author.
E-mail addresses: nettoles@ib.usp.br, nettoluis@yahoo.com.br (L.E.S. Netto).

https://doi.org/10.1016/j.freeradbiomed.2020.06.015
Received 21 March 2020; Received in revised form 25 May 2020; Accepted 6 June 2020
Available online 29 June 2020
0891-5849/

10^5 $M^{-1}s^{-1}$ [8]. However, this reaction appears to be much slower for protein sulfenic acids, with the examples of glutathione reacting with the sulfenic acid of human peroxiredoxin 2 [9] or human serum albumin [10].

The persistence of sulfenic acids is also limited by their oxidation to sulfinic (-SO_2H) and sulfonic (-SO_3H) acids and by their self-condensation to yield thiosulfinates [11]. Furthermore, protein sulfenic acids can also undergo condensation reactions with backbone amides giving rise to sulfenamides [12]. In spite of that, sulfenic acids can gain stability through the presence of bulky substituents in organic compounds [6] or in proteinaceous microenvironments [13]. Indeed, sulfenic acids have been detected in several proteins [12] and in cellular systems such as cardiac rat tissue under basal conditions and in response to H_2O_2 [14] as well as in bacteria [15], yeast [16], HeLa cells [17] and plants [18].

Sulfenic acids are generated in the peroxidatic Cys residues (C_P) of peroxiredoxin (Prx) enzymes that react extremely fast with hydroperoxides, with second order rate constant in the 1×10^4 to 10^8 M^{-1} s^{-1} range [19–21].

The fate of the sulfenic acid in Prx enzymes depend on the number of Cys residues involved in catalysis. For 2-Cys Prx, sulfenic acids in C_P react with the resolving Cys residues (C_R), giving rise to a disulfide that in most cases is reduced by a thioredoxin (Trx) back to the thiolate form [22]. In contrast, the identities of the biological reductants of 1-Cys Prx enzymes remain poorly understood. The literature provides examples of sulfur-based reductants such as Trx, H_2S and GSH with or without the intervention of additional enzymes [23–25]. Sulfenic acids in mammalian 1-Cys Prx (Prx6) can be reduced by GSH, when these peroxidases are heterodimerized with π GST [26,27].

Ascorbate can also reduce sulfenic acids in 1-Cys Prx of the Prx6 family [28,29] and in other enzymes. For instance, direct reduction of sulfenic acids in glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by DTT and ascorbate was described a long time ago [30]. The reduction of papain sulfenic acid by ascorbate was also reported [31]. Notably, the reduction of sulfenic acids by ascorbate was revealed *in vivo* in a proteomic scale investigation, employing mice that are defective in protein disulfide isomerase (PDI) reoxidation [31].

To the best of our knowledge, all these reports are qualitative or indirect and do not provide the kinetic information needed to involve the reduction by ascorbate as a participant in the catalysis and the potential protection of critical cysteine residues in enzymes. We herein present the first quantitative assessment of the reduction of protein sulfenic acids by ascorbate. Our results indicated that in most cases, the rate constants lie in the 0.4–2.2 $\times$ 10^3 M^{-1} s^{-1} range, indicating that reduction of protein sulfenic acids is relevant in biological compartments where ascorbate concentration is high.

2. Results

2.1. Structural characterization of Tsa2-C170S as a model for protein sulfenic acid

We characterized a model protein-SOH for subsequent studies related to ascorbate reduction. We selected a 2-Cys Prx with the C_R residue mutated to a Ser residue (Tsa2-C170S), as the reactivity of sulfenic acids in similar proteins has been extensively characterized [32,33]. Additionally, Tsa2 displays the same quaternary structure (decamer) independently of its oxidative state, avoiding artifacts [34]. Therefore, possible variations in the peroxidatic activity due to different oligomeric states would be eliminated [34]. Furthermore, the recombinant protein for Tsa2-C170S is very stable and its expression in *Escherichia coli* yields a high amount of the enzyme. Previously, we showed that Tsa2 reduces H_2O_2 very rapidly (k = 1.3 $\times$ 10^7 M^{-1} s^{-1}) and the pKa value (6.3) of the thiol group of C_P was determined [35]. The kinetics of Tsa2-C170S were measured following the intrinsic fluorescence change upon oxidation with H_2O_2 and the rate constant was 3 ± 1 $\times$ 10^6 M^{-1} s^{-1} (data not shown).

It is worth to mention that 2-Cys Prx enzymes (Prx1/AhpC sub-family) [36] show a structural switch during their catalytic cycle. When these enzymes are reduced, the so-called “fully folded” (FF) state is stabilized, where C_P is in the first turn of an α-helix and C_P is located more than 10 Å away from the C_R. When oxidized to disulfide, 2-Cys Prx enzymes are stabilized in the so-called “locally unfolded” (LU) state, when the first turn of an α-helix containing C_P unfolds and C_P–SOH can get close to C_R to form the disulfide bond [37].

Two structures of Tsa2 are already available in Protein Data Bank

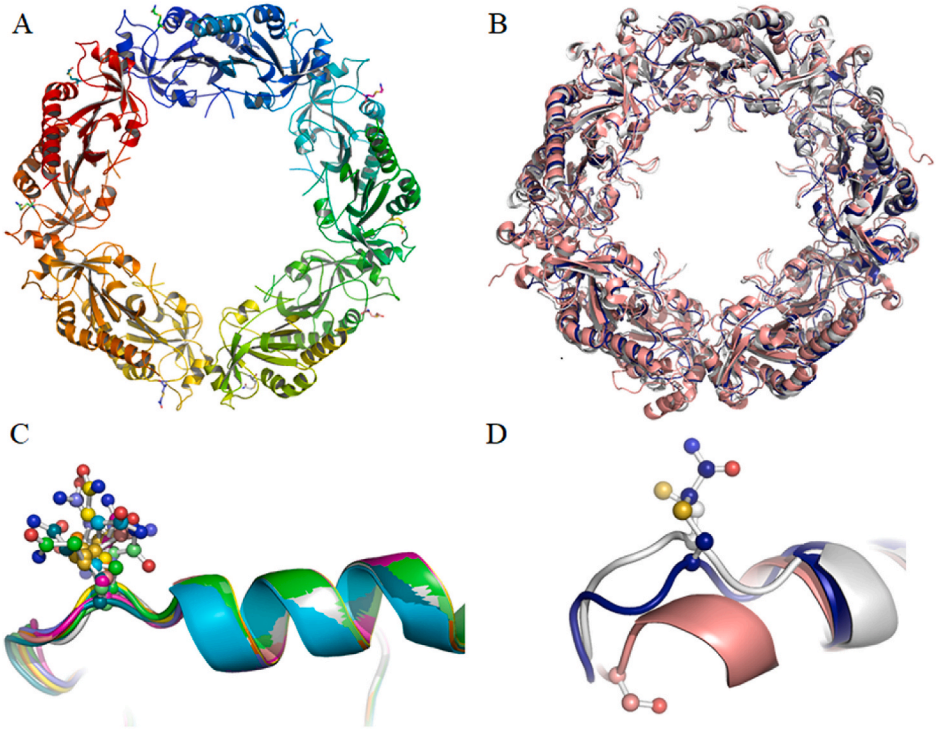


Fig. 1. Crystallographic structure of Tsa2-C170S. (A) Overall view of Tsa2-C170S structure, as a decameric assembly of five dimers. Each monomer is represented by a different color. (B) Superposition of all the three Tsa2 structures. Tsa2-C170S (blue), Tsa2-C48S (pink) and wild-type Tsa2 (white), showing very similar quaternary structures. (C) Superposition of monomeric chains of Tsa2-C170S in a region nearby C_P (From Ser45 to Ala58). As (A), each chain is represented by a different color. (D) Comparison of C_P positions among the three Tsa2 structures. The C_P alkylated with iodoacetamide in Tsa2-C170S (blue) is in similar position as C_P in the wild type Tsa2 (white), which is in the disulfide form and LU state. In contrast, Tsa2-C48S (pink) is in the FF state. In (C) and (D), the atoms of the S-(2-amino-2-oxoethyl)-L-cysteine (YCM) group are highlighted by balls and colored as follow: carbon = same color of the monomer, nitrogen = blue, oxygen = red, sulfur = orange. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(PDB): Tsa2-C48S (which presents a serine substituting C_P in the FF state in most of the ten chains) and wild-type Tsa2 (in disulfide form, with most of the chains in the LU state) [38]. The environment around

Table 1
Data collection and refinement parameters.

$Tsa2^{C170S}$	
Space group	P 1 21 1
Unit cell parameters	
a, *b*, *c* (Å)	53.4, 177.3, 115.3
α, β, ᵞ (°)	90.0, 103.3, 90.0
Resolution (outer shell)(Å)	47.4–2.6 (2.67–2.60)
I/σ(I)	6.6
Completeness (%)	97.73 (89,73)
CC1/2	0
Wilson B (Å2)	64.84
Refinement	
Resolution (Å)	47.400–2602
No. reflections	62338
Rwork/Rfree (%)	19.7/23.7
No. Atoms	
Protein	12793
Water	43
Average B-factors	
Protein	77.78
Water	78.02
r.m.s.d	
Bond lengths (Å)	0.007
Bond angles (°)	1.437
Ramachandran analysis (%)	
Favored regions	97.38%
Allowed regions	2.50%
Outliers	0.12%
PDB code	6UTL

C_P is under low steric hindrance effects in Tsa2, which is related to the fact that a Ser (Ser45), instead of a Thr residue, takes part of its catalytic triad [34].

Now, we also describe the crystal structure of Tsa2-C170S (Fig. 1A) at 2.6 Å resolution (Table 1), our model protein for studying C_P-SOH reduction by ascorbate. The overall structure of Tsa2-C170S is very similar to the other two structures (Tsa2-C48S, $Tsa2^{WT}$), all of them displaying a decameric form (Fig. 1B). To obtain homogeneous crystals of Tsa2-C170S, we treated the samples with iodoacetamide, a thiol alkylating agent. Remarkably, it was possible to assign the C_P modification by S-(2-amino-2-oxoethyl)-L-cysteine (YCM) in all of the ten subunits of the decamer (Fig. 1C).

Probably the modification of C_P with YCM, favored the active site of Tsa2-C170S to assume the LU state (Fig. 1D). Comparing the ten active sites of the three available structures for Tsa2 [34,38] (Fig. 1), we observed that the C_P loop possesses a high degree of conformational freedom, indicating that the C_P -SOH in the active site of Tsa2-C170S would be readily accessible to ascorbate.

2.2. Rate constant for the reaction between protein sulfenic acids and ascorbate by a competition assay

A competition assay using 2,6–dichlorophenol-indophenol (DCPIP) as competing oxidant was developed here to determine rate constants between ascorbate and protein sulfenic acids. The kinetics of DCPIP reduction by ascorbate have been studied previously and a moderately fast reaction was expected at neutral pH [39–41]. The mathematical model was built, considering Protein-SOH and DCPIP as two oxidants competing for the reduction of ascorbate under pseudo-first order conditions. In this system, DCPIP reduction was followed by the decay of its absorbance in the visible spectra, reflecting the consumption of the ascorbate in defect.

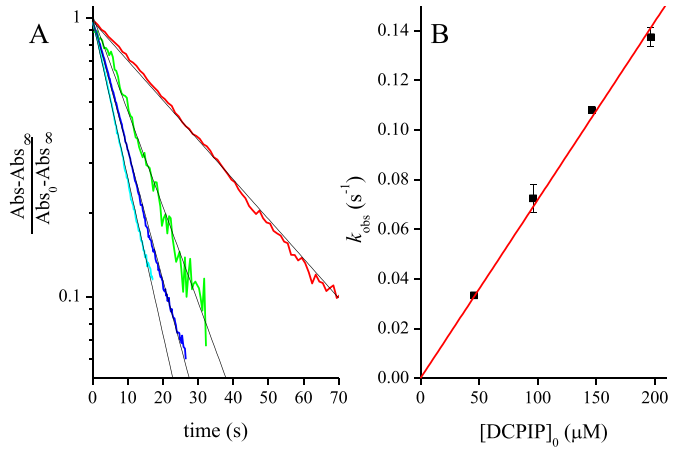


Fig. 2. Kinetics of DCPIP reduction by ascorbate.
(A) Time courses of DCPIP (46–197 μM) reacting with ascorbate (4 μM) in 50 mM potassium phosphate buffer, NaCl 50 mM, 100 μM DTPA pH 7.4 at 25 °C. (B) The k_{obs} of the reaction plotted *versus* DCPIP concentrations under the same experimental conditions.

Initially, we measured the rate constant of the reaction between DCPIP and ascorbate (in the absence of Protein-SOH). Under our experimental conditions, we obtained a value of $7.1 \times 10^2\ M^{-1}\ s^{-1}$ (k_1), which is consistent with previous reports (Fig. 2) [39].

Employing this DCPIP competition assay, we showed that ascorbate reduced sulfenic acid in Tsa2-C170S with a second order rate constant of $1410 \pm 79\ M^{-1}\ s^{-1}$ (Fig. 3). This is the first quantitative assessment of a Protein-SOH reduction by ascorbate.

2.3. Reduction of sulfenic acids in 1-Cys Prx (Prx6 enzymes)

Previously, we observed that the sulfenic acids of several 1-Cys Prx enzymes from different organisms are reduced by ascorbate [28], but no kinetic characterization was performed. Therefore, we assessed the reduction of 1-Cys Prx enzymes by ascorbate. Initially, we investigated LsfA, a 1-Cys Prx from *Pseudomonas aeruginosa* that is involved in bacterial virulence [42]. Through the DCPIP competition assay, a rate constant of $k = 2170 \pm 6\ M^{-1}\ s^{-1}$ for the reaction between LsfA and ascorbate was obtained (Fig. 4A). LsfA contains a single cysteine (Cys45 = C_P) in its sequence, which was mutated to an alanine residue (LsfA-C45A). No reaction was observed between ascorbate and LsfA-C45A, as expected (Fig. 4B).

TaPER1 is a 1-Cys Prx from wheat that is also reducible by ascorbate [29], but that had not been kinetically characterized before. Wild-type TaPER1-SOH was reduced by ascorbate with the second order rate constant equal to $2191 \pm 7\ M^{-1}\ s^{-1}$ (Fig. 4C), i.e., very similar to the value obtained for LsfA (Fig. 4A). TaPER1 contains three cysteines (Cys72, Cys147, Cys182), besides C_P (Cys46). Of the three mutant analyzed (TaPER1-C46S; TaPER1-C72S; TaPER1-C147S), only TaPER1-C46S failed to react with ascorbate, as expected (Fig. 4D, Fig. S1).

ScPrx1 is a mitochondrial 1-Cys Prx from *Saccharomyces cerevisiae* that is present in the matrix and intermembrane space [43,44]. Previously, we observed the reduction of ScPrx1 by ascorbate [28]. Now, we determined the rate constant of this reaction as $k = 449 \pm 2\ M^{-1}\ s^{-1}$ (Fig. 4E), therefore, lower than the corresponding values obtained for LsfA and TaPER1.

Mammalian enzymes are the most studied enzymes of the Prx6 subfamily. We determined before that the pKa value of the C_P of Prx6 is 5.2 and that the second-order rate constant of its reactions with H_2O_2 and peroxynitrous acid at pH 7.4 and 25 °C, were $3.4 \pm 0.2 \times 10^7\ M^{-1}\ s^{-1}$ and $3.7 \pm 0.4 \times 10^5\ M^{-1}\ s^{-1}$, respectively [45]. In both cases, the formation of sulfenic acids is expected. We also

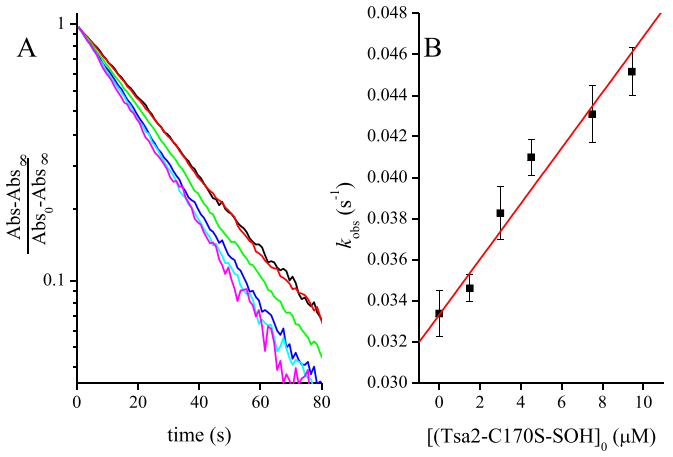


Fig. 3. Kinetics of Tsa2-C170S-SOH reduction by ascorbate studied by a competition kinetic approach.
(A) Time courses of DCPIP (45 μM) reduction by ascorbate (4 μM) in the presence of Tsa2-C170S-SOH (0–9 μM) in 50 mM potassium phosphate buffer, 50 mM NaCl, 100 μM DTPA pH 7.44 at 25 °C. The averages from at least four experimental traces are shown. (B) The k_{obs} of the reaction plotted *versus* the initial Tsa2-C170S-SOH concentration under the same experimental conditions.

described that Prx6 is reducible by ascorbate, again no kinetic characterization was performed [28]. By the DCPIP competition assay, we describe here that ascorbate reduced mammalian Prx6-SOH with a rate constant of 1009 ± 32 M^{-1} s^{-1} (Fig. 4F).

Therefore, the sulfenic acids of 1-Cys Prx of the Prx6-family were all reduced by ascorbate with rate constants in the 0.4 to 2.2 × 10^3 M^{-1} s^{-1} range and mutation of C_P abolished the reaction (Table 2).

The experiments of Tsa2-C170S-SOH and 1-Cys Prx-SOH reductions by ascorbate were all reproduced, utilizing another equipment in other institution and the results were essentially the same. The results described in Figs. 3 and 4 were performed by VA in UDELAR, Uruguay, whereas the results described in Fig. S2 were performed by RLA in USP, Brazil.

2.4. Reduction of sulfenic acids in non Prx enzymes by ascorbate

The reduction of sulfenic acids in proteins other than Prx were described before. Back in 1975, You et al. demonstrated that sulfenic acid at Cys149 of pig muscle GAPDH is reducible by ascorbate, which is involved in the reversible interconversion of dehydrogenase and acylphosphatase activities of this enzyme [30]. Now, we describe that the reduction of sulfenic acid in GAPDH by ascorbate proceeded with a second-order rate constant comparable with the values of Prx enzymes (Fig. 5A, Table 2). As a control, we observed that alkylation of GAPDH by NEM abolished the reaction, as expected (Fig. 5B).

One important study [31] described a non-canonical scurvy phenotype in mice with deficiency in endoplasmic reticulum thiol oxidase activities. The authors proposed that consumption of ascorbate by sulfenic acid is the mechanism underlying the non-canonical scurvy phenotype and described the reduction of papain-SOH by ascorbate as an example of this reaction [31]. However, once again no kinetic characterization was performed. Here, we described that papain-SOH is reduced by ascorbate with a rate constant of 783 ± 9 M^{-1} s^{-1} (Fig. 5C and Table 2) and no reaction was observed upon alkylation with NEM (Fig. 5D). Therefore, it appears that protein-SOH reduction by ascorbate is a general process that takes places in proteins other than Prx enzymes.

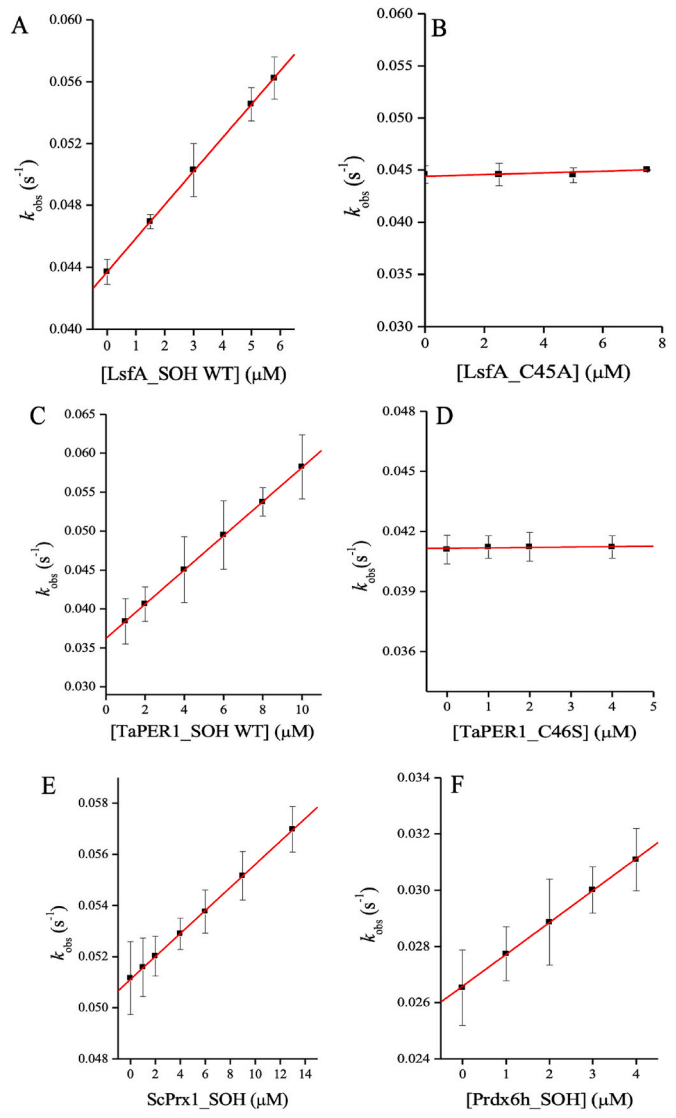


Fig. 4. Reduction of 1-Cys-SOH enzymes (Prx6 sub-family) by ascorbate, employing the DCPIP competition assay.
Pseudo-first order approach was employed and k_{obs} values were obtained by fitting single exponential to the time courses of 45 μM DCPIP reduction by 4 μM ascorbate in the presence of: (A) oxidized wild type LsfA-SOH, (B) LsfA-C45A, (C) wild type TaPER1_SOH, (D) TaPER1-C46S, (E) ScPrx1-SOH (F) human Prx6-SOH. Reactions were carried out in 50 mM potassium phosphate buffer, 50 mM NaCl, 100 μM DTPA at 7.44 and 25 °C. Each point represents the average of at least four experimental traces.

2.5. Tsa2-C170S reduction by ascorbate analyzed by steady state, bi-substrate approach

The reduction of sulfenic acids in proteins by ascorbate was also investigated through a second, independent approach. In this case, we followed H_2O_2 consumption, employing a specific electrode. Noteworthy, H_2O_2 determinations using the ISO-HPO-2 electrode (*World Precision Instruments*) is not interfered by ascorbate, as the responses of this electrode to H_2O_2 in the absence and presence of the reducing agent are essentially identical (SI Fig. 3).

We analyzed the reduction of our model sulfenic acid protein (Tsa2-C170S-SOH) by ascorbate, following H_2O_2 electrochemically by a steady state, bi-substrate approach. The scheme of reactions taking place in this setup is:

$$Tsa2\text{-}C170S\text{-}S^- + H_2O_2 \rightarrow Tsa2\text{-}C170S - SOH + H_2O \quad \text{(Reaction 1)}$$

Table 2
Rate constants of protein sulfenic acids with ascorbate measured through competition kinetics with DCPIP at pH 7.4 ± 0.1 and 25 °C.

Protein - SOH	Variant	k_2^{P-SOH} (M^{-1} s^{-1})
Prx6 family enzymes		
Prx1 (*Saccharomyces cerevisiae*)	WT	449 ± 2
TaPER1 (*Triticum aestivum*)	WT	2191 ± 7
	C46S	ND
	C72S	1273 ± 39
	C147S	1962 ± 6
LsfA (*Pseudomonas aeruginosa*)	WT	2170 ± 6
	C45A	ND
Prx6 (*Homo sapiens*)	WT	1009 ± 32
Prx1 family enzymes		
Tsa2 (*Saccharomyces cerevisiae*)	C170S	1410 ± 79
		1,4 ± 0,079
Non peroxidases		
GAPDH (*Oryctolagus cuniculus*)	WT	1845 ± 14
Papain (*Carica papaya*)	WT	783 ± 9

ND = below the detection limit of the technique.

Tsa2-C170S-SOH + ascorbate → Tsa2-C170S-S^- + dehydroascorbate (Reaction 2)

Therefore, in the overall reaction catalyzed by Tsa2-C170S, H_2O_2 and ascorbate are the two substrates, that probably operate through a ping-pong mechanism [28]. Initially, we determined the apparent enzymatic parameters, varying H_2O_2 concentration at distinct ascorbate levels. This Tsa2-C170S catalysis obeyed the Michaelis-Menten model (Fig. 6) and apparent K_M and k_{cat} values were determined (Table 3). Noteworthy, the double reciprocal plot indicated the mechanism of the catalysis is Bi-Bi Ping-Pong mechanism (Fig. S4), as expected.

We obtained the real K_M values for ascorbate and for H_2O_2 and the real k_{cat} [46]. The specificity constant of Tsa2-C170S reduction by ascorbate ($k_{cat}/K_{M\ asc}$) was 7.40 ± 0.15 × 10^3 M^{-1} s^{-1} (Fig. 7, Table 4) are in agreement with the second order rate constant determined by the DCPIP competition assay (Table 2). Therefore, by two independent assays, we observed that the reduction of TSA2-C170S sulfenic acid by ascorbate proceeds with a rate constant in the 10^3 M^{-1} s^{-1} range.

The catalytic efficiency of H_2O_2 reduction is relatively low (in the 10^4 M^{-1} s^{-1} range) in comparison with other determinations, employing other assays [19,35,47–49]. It is well known that steady-state assays may underestimate the second-order rate constants of the reaction between Prxs with hydroperoxides in cases where additional reaction or structural rearrangements are involved [35,48].

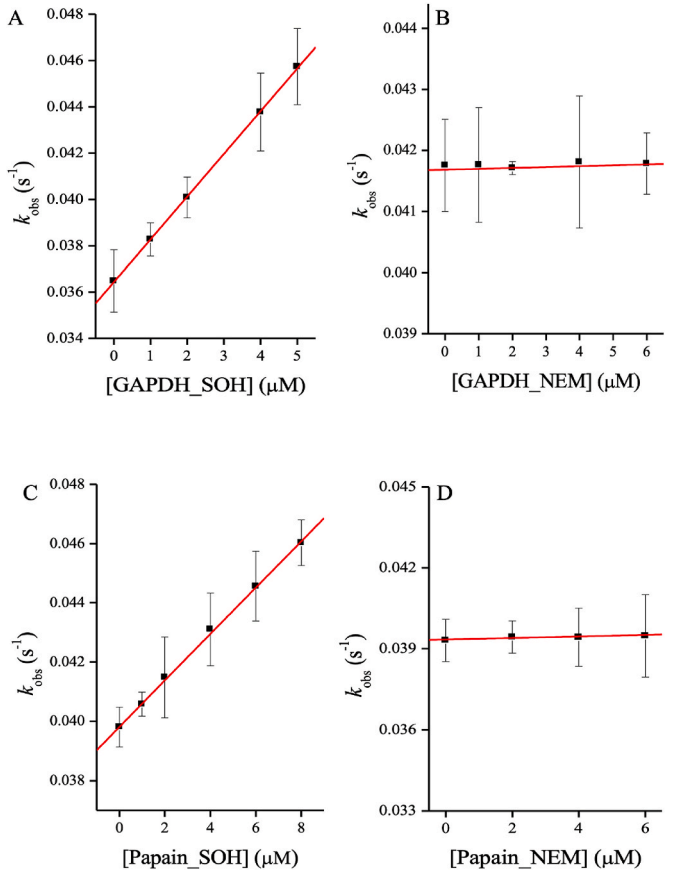


Fig. 5. Kinetics of GAPDH and papain reduction by ascorbate by the DCPIP competition assay.
Pseudo-first order approach was employed as described in the legend of Fig. 4. k_{obs} values were obtained by fitting single exponential to the time courses of 45 μM DCPIP reduction by 4 μM ascorbate in the presence of (A) GAPDH_SOH; (B) NEM-alkylated GAPDH; (C) Papain-SOH and (D) NEM-alkylated Papain in 50 mM potassium phosphate buffer, 50 mM NaCl and 100 μM DTPA, pH 7.4 at 25 °C. Each point represents the average from at least four experiments.

3. Discussion

The reduction of protein sulfenic acids by ascorbate has been frequently described [28–31,50], but so far there was no kinetic characterization of this reaction, precluding considerations about its biological relevance. Here, we described for the first time that the reduction of several proteins lie within the 0.4–2.2 × 10^3 M^{-1} s^{-1} range.

Therefore, ascorbate represents a broad-spectrum reductant for protein sulfenic acid, with human serum albumin as the only exception found so far [10]. Human serum albumin contains 35 Cys residues, 34 of them involved in disulfide bonds [51]. Cys34 is the single free thiol of human serum albumin (Cys34), whose gamma sulfur atom is buried to the interior of the protein, surrounded by side chains of Pro35, His39, Val77 and Tyr84 [10,51,52]. Possibly, if there is no restriction for ascorbate to access the sulfenic acid, the reduction will proceed at moderate rates. In the absence of steric hindrance, thiols may outcompete ascorbate, as the rate constant for the reduction of sulfenic acids by thiols is in the 10^5 M^{-1} s^{-1} range [8].

Given the rate constants determined herein, a question that emerges is if reduction of 1-Cys Prx enzymes by ascorbate is fast enough to sustain a competent peroxidase cycle. To address this question, we need to consider for each biological system: (i) the concentration of ascorbate, (ii) the concentration of the competing reductant and (iii) the rate

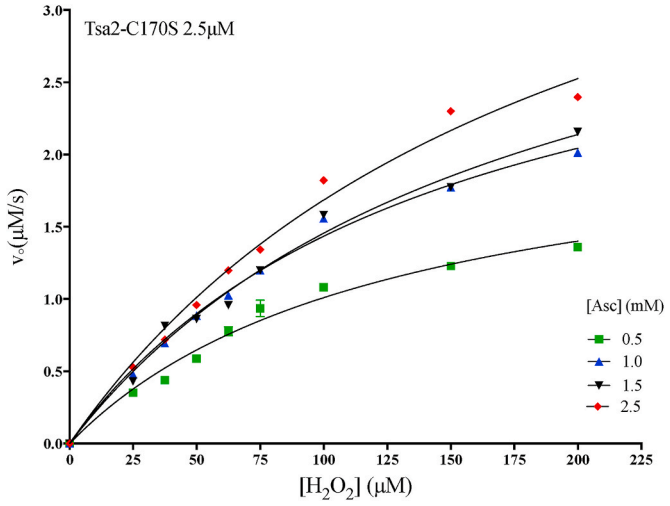


Fig. 6. Steady-state analysis for the ascorbate peroxidase activity of Tsa2-C170S.
The H_2O_2 reduction was measured at 30 °C in a reaction mixture containing 20 mM Tris/HCl, pH 7.4, 100 μM sodium azide and 100 μM DTPA. Henri-Michaelis-Menten plots for Tsa2-C170S (2.5 μM) catalysis at four different ascorbate concentrations. Reactions were started by the addition of H_2O_2. Non-linear regression used the Michaelis-Menten equation and GraphPad Prism 5 software. Each point represents triplicate data.

Table 3
Apparent kinetic constants for the reduction of the sulfenic acid formed in Tsa2-C170S by ascorbate, determined from Michaelis-Menten plots.

[Asc] (mM)	K_M^{app} (μM)	k_{cat} (s^{-1})
0.5	126.7 ± 16.5	0.91 ± 0.01
1	147.3 ± 11.6	1.41 ± 0.01
1.5	176.8 ± 21.8	1.61 ± 0.02
2.5	199.1 ± 25.6	2.01 ± 0.02

Experimental conditions described in the legend of Fig. 7.

constant of the competing reductant with protein sulfenic acid.

In the case of mammalian 1-Cys Prx (Prx6), the main pathway described for sulfenic acid reduction involves hetero-dimerization of this enzyme with πGST and GSH as the electron source [23,26,53]. The reported specific activity of 5 μmol min^{-1} (mg Prx6)$^{-1}$ can be translated to a k_{cat} of at least 2 s^{-1} [23]. In this case, the specificity constant (k_{cat}/K_M) for the reduction of hydroperoxides is in the range of 10^6 M^{-1} s^{-1} [53].

Since GSH concentrations are quite high in most mammalian cells, it is reasonable to assume that the GSH-πGST-Prx6 system would prevail over the ascorbate-Prx6 system. However, in cellular systems where πGST is expressed at low levels, Prx6 will remain as homo-dimers, which are not reducible by GSH [25] and ascorbate would emerge as possible reductant for this enzyme. Notably, in esophageal squamous carcinoma cells, Prx6 levels are elevated [54], whereas πGST is downregulated [55,56].

To contextualize and compare with other peroxide-consuming systems, we need to address the question of the catalytic cycle. Prx from the Prx6 family using ascorbate would have a two-step catalytic cycle involving the oxidation by H_2O_2 and the reduction by ascorbate. The rate of each cycle would depend on the available enzyme and the concentration of the substrates. Other Prx, for instance, human Prx1 and Prx2 from the Prx1 subfamily have a three-step catalytic cycle that, that besides oxidation by H_2O_2 and reduction by Trx, also involves the formation of the internal disulfide, the so called resolution reaction, that is frequently the rate limiting step. As a matter of fact, even though most Prx are extremely fast in the reaction with the peroxide, they are not too impressive in terms of turnover numbers (k_{cat}). The data deposited in the database of Brenda-enzymes.org shows that most values of k_{cat} are less than 100 s^{-1} and several below 1 s^{-1}. Human Prx2, for instance has a $k_{cat} \leq 0.25$ s^{-1} at neutral pH [57,58]. Human Prx6 could outperform Prx2 as a peroxidase in cell compartments with elevated ascorbate levels. Considering that the reduction step is rate-limiting in the Prx6 cycle, a 1 mM concentration of ascorbate yields a $k_{cat} = 1$ s^{-1}. Brain, liver, lung and adrenal tissue in humans all have reported to have ascorbate concentrations of 1 mM or more [59,60].

In the case of other organisms, less data is available thus difficulting the assessment of which system will prevail in the reduction of 1-Cys

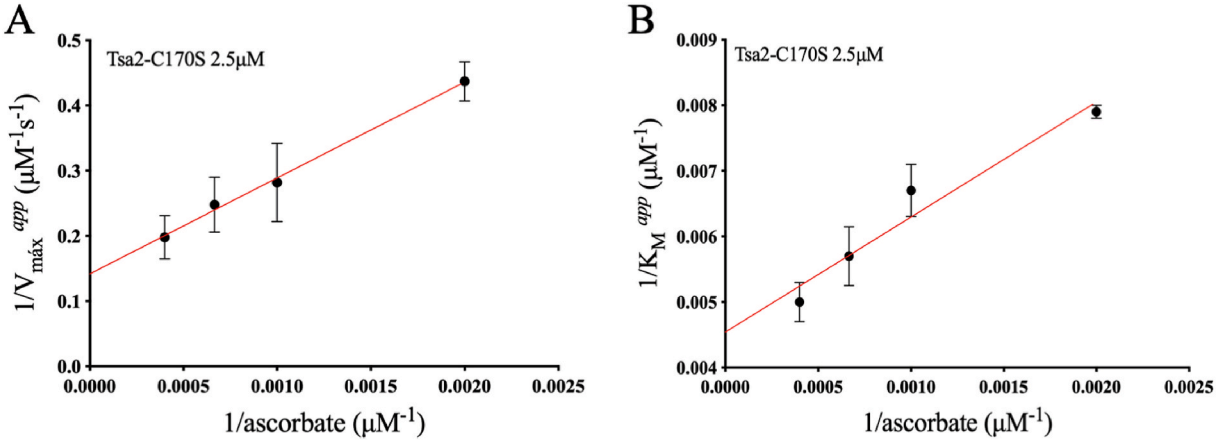


Fig. 7. Secondary plots (reciprocal values of (A) $1/V_{máx}^{app}$ *versus* the reciprocal of Asc concentration and (B) $1/K_M^{app}$ *versus* the reciprocal of Asc concentration) used for the determination of the kinetic constants of Tsa2-C170S.
Data set is the same described in Figs. 5 and 6. Parameters were calculated following a bi substrate, steady state kinetics for enzymes that display Bi-Bi Ping-Pong mechanism [46].

Table 4
Enzymatic parameters for the peroxidase activity of Tsa2-C170S supported by ascorbate.

				H_2O_2	Ascorbate
Enzyme (μM)	K_M^{Asc} (μM)	K_M^{H202} (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1} s^{-1}$)	k_{cat}/K_M ($M^{-1} s^{-1}$)
Tsa2-C170S – 2.5	386 ± 29.1	220.16 ± 16.6	2.82 ± 0.15	1.3 ± 0.15 × 10^4	7.4 ± 0.07 × 10^3

Data set is the same described in Figs. 6 and 7. Parameters were calculated following a bi-substrate, steady state kinetics for enzymes that display Bi-Bi Ping-Pong mechanism [46].

Prx enzymes. Interestingly, plant 1-Cys Prx enzymes are located in the nucleus of seed cells [29,61–63] and ascorbate has been related with seed development, ageing, and germination [reviewed in Ref. [64]]. Ascorbate levels in plants are quite high, attaining high millimolar levels in the nucleus [65,66], especially in photosynthetic tissues [67]. In addition, others have reported that ascorbate and also thioredoxin reductase can reduce the plant 1-Cys Prx [29].

1-Cys Prx (Prx6 sub-family) from bacteria are poorly studied. One of the few studies indicated that LsfA from *P. aeruginosa* is involved in bacterial virulence [42]. Attempts to measure sulfenic acid reduction of LsfA by the thioredoxin or glutaredoxin/GSH systems failed (our unpublished results). Therefore, our results identified the only possible biological reductant for LsfA. Not much information is available on the ascorbate levels in bacteria. In some cases, bacteria might synthesize this vitamin [68] and an ascorbate transporter was identified in *E. coli* [69].

In the case of yeast 1-Cys Prx (ScPrx1), the reduction of the sulfenic acid by ascorbate is probably not capable to compete with other reducing systems. ScPrx1 is located in the matrix and intermembrane space of mitochondria [43,44], where thioredoxin and glutaredoxin enzymes are also present and can reduce this 1-Cys Prx [24,43,70,71]. In yeast, a 5-carbon analog of ascorbate (named erythroascorbate) is present but not much information is available about its reducing properties. Furthermore, not much data is available on the levels of erythroascorbate in yeast mitochondria, especially in conditions where the biogenesis of this organelle is elevated [72].

Therefore, the variety of cellular aspects related to ascorbate levels as well as the biochemical properties and cellular location of 1-Cys Prx are enormous. Interestingly, Prx6 from *Arenicola marina* is rapidly oxidized by H_2O_2 and peroxynitrous acid, but no biological reductant was identified [73]. Since all the 1-Cys Prx that we analyzed here were reduced by ascorbate, it is plausible to assume that this vitamin can also reduce Prx6 from *A. marina*.

The reduction of sulfenic acids in proteins by ascorbate can have other functions than turning-over 1-Cys Prx enzymes. For instance, reduction of sulfenic acid by ascorbate in GAPDH inhibits the acyl phosphatase activity of this enzyme [30]. Remarkably, reduction of sulfenic acids by ascorbate were related to the non-canonical scurvy phenotype of mice lacking systems to reoxidize PDI [31]. More considerations on the physiological relevance of ascorbate – sulfenic acid pathway require kinetic analysis and determinations of ascorbate concentration. Anyway, it is tempting to speculate that ascorbate – sulfenic acid pair may represent a cross-talk between thiol and non-thiol redox systems.

4. Materials and methods

4.1. Procedures to obtain pure proteins

Plasmids utilized for the expression of recombinant proteins are described in SI Table 1. Prx enzymes were purified as described previously [29,34,42,44,74] and the purity of the preparations were assessed SDS-PAGE. GAPDH from rabbit muscle (G2267 Sigma-Aldrich) and Papain from *Carica papaya* (76218 Sigma-Aldrich) were purchased. Extinction coefficients for the proteins (SI Table 2) were determined by the use of the ProtParam tool [75].

4.2. Crystallization and structure refinement

For the crystallization process, Tsa2-C170S was submitted to an additional purification step by using size exclusion chromatography after purification by affinity chromatography. The sample was loaded in HiLoad 16/60 Superdex 200 column (GE Healthcare), containing 20 mM sodium phosphate buffer pH 7.4, 150 mM NaCl and 1 mM DTT. The purified sample (15 mg/ml) was treated with 10 μg/ml (final concentration) of trypsin at 4 °C. After 1 h, it was added 1 mM PMSF and the sample was centrifuged by 10 min/14000 rpm/4 °C. The supernatant was treated with 10 mM DTT for 30 min/RT and then 20 mM iodoacetamide was added. The crystals were obtained at 18 °C in 100 mM Hepes pH 7.5, 10% w/v PEG 6000 and 5% v/v 2-Methyl-2,4-pentanediol. Data were collected on the MX2 beamline at Brazilian Synchrotron Light Laboratory (LNLS). The decameric structure of Tsa2-C48S (PDB code = 5DVB) was used as molecular replacement model and the orientation of the molecule in asymmetric unit was obtained with MOLREP [76]. The space group selected was P 1 21 1 and the resolution was 2.6 Å. The manual model building and amino acid position correction was performed in Coot 0.8.2 [77] and refinement was executed by REFMAC 5 [78]. A TLS model was used in the refinement to identify each chain as a rigid body. Molecular model images were obtained by using Pymol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.). Table 1 presents the data collection and refinement statistics.

4.3. Reduction of proteins and quantification of their sulfhydryl groups

Proteins were treated with a 20-fold excess of DTT for at least 1 h at room temperature. Excess of DTT was removed using a PD-10 desalting column (GE Healthcare) or using two coupled HiTrap Desalting Columns (GE Healthcare). The amount of reduced thiols in proteins were determined spectrophotometrically by reaction with 4,4′-dithiodipyridine (DTDPy), which produces thiopyridone that can be quantified spectrophotometrically (ε_{324nm} = 21400 $M^{-1}cm^{-1}$) [79].

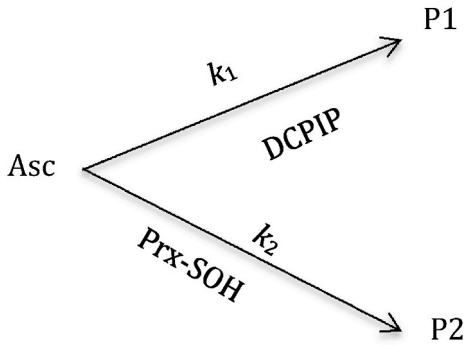
4.4. Preparation of proteins in the sulfenic acid form

Pre-reduced proteins were mixed with equimolar amounts of H_2O_2 to form the sulfenic form of the protein. This procedure was performed immediately before the competition assay.

4.5. Competition kinetics between protein sulfenic acids and 2,6-dichlorophenolindophenol for ascorbate

The assay is based on the competition between DCPIP and Protein-SOH for ascorbate with second order rate constants k_1 and k_2, respectively (Scheme 1).

According to Scheme 1, the rate of ascorbate (Asc) consumption would comply with the rate law:



Scheme 1. 2,6–dichlorophenol-indophenol (DCPIP) and Prx-SOH compete for ascorbate with second order rate constants k_1 and k_2, respectively.

$$-\frac{d[Asc]}{dt} = k_1[DCPIP]_0[Asc] + k_2[Protein - SOH]_0[Asc]$$

Under pseudo-first order conditions with DCPIP and Protein-SOH in excess over Asc, $[DCPIP]_0$ and $[Protein\text{-}SOH]_0$ can be assumed constant and grouped in the expression

$$-\frac{d[Asc]}{dt} = (k_1[DCPIP]_0 + k_2[Protein - SOH]_0)[Asc]$$

where

$$k_{obs} = k_1[DCPIP]_0 + k_2[Protein - SOH]_0$$

The rate law has the form of a pseudo-first order reaction, so the integrated form is an exponential function of the form:

$$y = Amp \times \exp(-k_{obs}t) + y_\infty$$

That can be used to fit the absorbance time courses in order to obtain the k_{obs}. In turn, plotting k_{obs} vs $[Protein\text{-}SOH]_0$ (with $[DCPIP]_0$ constant) one should obtain a linear trend with k_2 as the slope and k_1 as the y intercept.

In a daily prepared assay buffer (50 mM potassium phosphate pH 7.2, 50 mM NaCl), approximately 6 mg of DCPIP was incubated over 2 h with agitation. Ascorbate stock solution was prepared by adding 36 mg of this compound into 50 ml of MilliQ water. DCPIP and ascorbate concentrations were quantitated spectrophotometrically using $\varepsilon_{600nm} = 21500\ M^{-1}cm^{-1}$ and $\varepsilon_{265nm} = 14500\ M^{-1}cm^{-1}$, respectively. Reactions were followed at 522 nm using an *Applied Photophysics RX-2000* or SFA-20 rapid mixing accessory in a Varian Cary 50-Bio Spectrophotometer (Agilent Technologies).

To obtain the rate constant of DCPIP reacting with ascorbate, the ascorbate concentration was fixed at 4 μM with varying concentrations of DCPIP in excess. All the runs were performed at 24 ± 0.1 °C in 50 mM potassium phosphate buffer (pH 7.2), 50 mM NaCl and 0.1 mM DTPA. Rate constants (k_{obs}) were determined by nonlinear regression to the integrated pseudo first-order function:

$$Abs = (Abs_0 - Abs_\infty) \times \exp(-k_{obs}t) + Abs_\infty$$

To obtain the second order rate constant of the reaction between ascorbate and protein-SOH the experiment was repeated with constant $[DCPIP]_0 = 45$ μM and variable $[Protein\text{-}SOH]_0$. The k_{obs} values from distinct proteins were plotted against protein-SOH concentrations and the corresponding second order rate constant was determined from the slope of these linear fittings.

In a second set of experiments, 8 μM ascorbate was added using a Synergy H1(BioTek® Instruments, Inc.) plate reader injector, to a 4 μM final concentration on the well. Each well contained protein-SOH (at variable concentrations), DCPIP (45 μM) and the same potassium phosphate buffer described above. Each protein concentration was analyzed in at least nine replicates. After ascorbate injection, the plate was double orbitally stirred for 3 s. Reactions monitored at 600 nm for 2 min at 25 °C, reading each well individually. Noteworthy, as the k_{obs} values analyzed were very low, we used the highest possible concentrations of proteins. The values obtained by the spectrophotometer and the plate reader were essentially the same.

4.6. Bisubstrate steady-state approach

Peroxidase activity of Tsa2-C170S supported by ascorbate was monitored by H_2O_2 consumption in real-time detection using electrochemical probes for H_2O_2 (*Free Radical Analyser 4100 – World Precision Instruments)*. The instrument measures the amount of H_2O_2 oxidized on the surface of the sensor using a poise voltage of +400 mV. The calibration curve measured the current generated by addition of increasing amounts of H_2O_2. The reaction mixtures contained 20 mM Tris/HCl (pH 7.4), 0.1 mM DTPA, 0.1 mM azide, Tsa2-C170S (2.5 μM) and were carried out at 30 °C. Reactions were initiated by addition of H_2O_2 or ascorbate at various concentrations, producing essentially the same results. Initial rates were determined by considering the linear portion of the time-course curves. The experimental data were analyzed by nonlinear regression and fitted with the Michaelis-Menten equation, using GraphPad Prism 5 software (GraphPad Prism 7®, Software, Inc., San Diego). The same experimental data were also analyzed by the Lineweaver-Burk equation to identify the enzymatic mechanism of the bi-substrate process. Secondary plots of the apparent K_M and V_{max} values were created to obtain the K_M values for ascorbate and for H_2O_2 and V_{max}, as described previously [46].

Declaration of competing interest

The authors declare no conflict of interest

Acknowledgments

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) Grant 2013/07937-8 and CAPES-UDELAR – 54/2013.

The authors declare no conflict of interest. This article contains Supplementary information.

[1] Recipient of FAPESP Fellowship 2012-00629.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2020.06.015.

References

[1] L.E.S. Netto, F. Antunes, The roles of peroxiredoxin and thioredoxin in hydrogen peroxide sensing and in signal transduction, Mol. Cell. 39 (2016) 65–71, https://doi.org/10.14348/molcells.2016.2349.

[2] R.P. Brandes, N. Weissmann, K. Schröder, Nox family NADPH oxidases: molecular mechanisms of activation, Free Radic. Biol. Med. 76 (2014) 208–226, https://doi.org/10.1016/j.freeradbiomed.2014.07.046.

[3] M. Lo Conte, K.S. Carroll, The redox biochemistry of protein sulfenylation and sulfinylation, J. Biol. Chem. 288 (2013) 26480–26488, https://doi.org/10.1074/jbc.R113.467738.

[4] D.E. Heppner, Y.M.W. Janssen-Heininger, A. van der Vliet, The role of sulfenic acids in cellular redox signaling: reconciling chemical kinetics and molecular detection strategies, Arch. Biochem. Biophys. 616 (2017) 40–46, https://doi.org/10.1016/j.abb.2017.01.008.

[5] N.J. Kettenhofen, M.J. Wood, Formation, reactivity, and detection of protein sulfenic acids, Chem. Res. Toxicol. 23 (2010) 1633–1646, https://doi.org/10.1021/tx100237w.

[6] J.P.R. Chauvin, D.A. Pratt, On the reactions of thiols, sulfenic acids, and sulfinic acids with hydrogen peroxide, Angew. Chem. Int. Ed. 56 (2017) 6255–6259, https://doi.org/10.1002/anie.201610402.

[7] M.T. Ashby, P. Nagy, Revisiting a proposed kinetic model for the reaction of cysteine and hydrogen peroxide via cysteine sulfenic acid, Int. J. Chem. Kinet. (2007), https://doi.org/10.1002/kin.20211.

[8] P. Nagy, M.T. Ashby, Reactive sulfur species: kinetics and mechanisms of the oxidation of cysteine by hypohalous acid to give cysteine sulfenic acid, J. Am. Chem.

Soc. 129 (2007) 14082–14091, https://doi.org/10.1021/ja0737218.
[9] A.V. Peskin, P.E. Pace, J.B. Behring, L.N. Paton, M. Soethoudt, M.M. Bachschmid, C.C. Winterbourn, Glutathionylation of the active site cysteines of peroxiredoxin 2 and recycling by glutaredoxin, J. Biol. Chem. 291 (2016) 3053–3062, https://doi.org/10.1074/jbc.M115.692798.
[10] L. Turell, H. Botti, S. Carballal, G. Ferrer-Sueta, J.M. Souza, R. Durán, B.A. Freeman, R. Radi, B. Alvarez, Reactivity of sulfenic acid in human serum albumin, Biochemistry (2008), https://doi.org/10.1021/bi701520y.
[11] L.J. Alcock, M.V. Perkins, J.M. Chalker, Chemical methods for mapping cysteine oxidation, Chem. Soc. Rev. 47 (2018) 231–268, https://doi.org/10.1039/c7cs00607a.
[12] V. Gupta, K.S. Carroll, Sulfenic acid chemistry, detection and cellular lifetime, Biochim. Biophys. Acta Gen. Subj. 1840 (2014) 847–875, https://doi.org/10.1016/j.bbagen.2013.05.040.
[13] M. Zaffagnini, S. Fermani, M. Calvaresi, R. Orrù, L. Iommarini, F. Sparla, G. Falini, A. Bottoni, P. Trost, Tuning cysteine reactivity and sulfenic acid stability by protein microenvironment in glyceraldehyde-3-phosphate dehydrogenases of Arabidopsis thaliana, Antioxidants Redox Signal. 24 (2016) 502–517, https://doi.org/10.1089/ars.2015.6417.
[14] A.T. Saurin, H. Neubert, J.P. Brennan, P. Eaton, Widespread sulfenic acid formation in tissues in response to hydrogen peroxide, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 17982–17987, https://doi.org/10.1073/pnas.0404762101.
[15] C.L. Takanishi, L.H. Ma, M.J. Wood, A genetically encoded probe for cysteine sulfenic acid protein modification in vivo, Biochemistry (2007), https://doi.org/10.1021/bi701625s.
[16] C.L. Takanishi, M.J. Wood, A genetically encoded probe for the identification of proteins that form sulfenic acid in response to H2O2 in Saccharomyces cerevisiae, J. Proteome Res. (2011), https://doi.org/10.1021/pr1009542.
[17] S.E. Leonard, K.G. Reddie, K.S. Carroll, Mining the thiol proteome for sulfenic acid modifications reveals new targets for oxidation in cells, ACS Chem. Biol. (2009), https://doi.org/10.1021/cb900105q.
[18] N. Smirnoff, D. Arnaud, Hydrogen peroxide metabolism and functions in plants, New Phytol. (2018), https://doi.org/10.1111/nph.15488.
[19] M. Trujillo, A. Clippe, B. Manta, G. Ferrer-Sueta, A. Smeets, J.P. Declercq, B. Knoops, R. Radi, Pre-steady state kinetic characterization of human peroxiredoxin 5: taking advantage of Trp84 fluorescence increase upon oxidation, Arch. Biochem. Biophys. 467 (2007) 95–106, https://doi.org/10.1016/j.abb.2007.08.008.
[20] C.C. Winterbourn, A.V. Peskin, Kinetic approaches to measuring peroxiredoxin reactivity, Mol. Cell. 39 (2016) 26–30, https://doi.org/10.14348/molcells.2016.2325.
[21] A.M. Reyes, M. Hugo, A. Trostchansky, L. Capece, R. Radi, M. Trujillo, Oxidizing substrate specificity of Mycobacterium tuberculosis alkyl hydroperoxide reductase E: kinetics and mechanisms of oxidation and overoxidation, Free Radic. Biol. Med. 51 (2011) 464–473, https://doi.org/10.1016/j.freeradbiomed.2011.04.023.
[22] S.G. Rhee, Overview on peroxiredoxin, Mol. Cell. 39 (2016) 1–5, https://doi.org/10.14348/molcells.2016.2368.
[23] L.A. Ralat, Y. Manevich, A.B. Fisher, R.F. Colman, Direct evidence for the formation of a complex between 1-cysteine peroxiredoxin and glutathione S -transferase π with activity changes in both enzymes †, Biochemistry 45 (2006) 360–372, https://doi.org/10.1021/bi0520737.
[24] D. Greetham, C.M. Grant, Antioxidant activity of the yeast mitochondrial one-Cys peroxiredoxin is dependent on thioredoxin reductase and glutathione in vivo, Mol. Cell Biol. 29 (2009) 3229–3240, https://doi.org/10.1128/MCB.01918-08.
[25] I.V. Peshenko, H. Shichi, Oxidation of active center cysteine of bovine 1-Cys peroxiredoxin to the cysteine sulfenic acid form by peroxide and peroxynitrite, Free Radic. Biol. Med. 31 (2001) 292–303, https://doi.org/10.1016/S0891-5849(01)00579-2.
[26] Y. Manevich, A.B. Fisher, Peroxiredoxin 6, a 1-Cys peroxiredoxin, functions in antioxidant defense and lung phospholipid metabolism, Free Radic. Biol. Med. 38 (2005) 1422–1432, https://doi.org/10.1016/j.freeradbiomed.2005.02.011.
[27] S.Y. Krishnaiah, C. Dodia, E.M. Sorokina, H. Li, S.I. Feinstein, A.B. Fisher, Binding sites for interaction of peroxiredoxin 6 with surfactant protein A, Biochim. Biophys. Acta Protein Proteonomics 1864 (2016) 419–425, https://doi.org/10.1016/j.bbapap.2015.12.009.
[28] G. Monteiro, B.B. Horta, D.C. Pimenta, O. Augusto, L.E.S. Netto, Reduction of 1-Cys peroxiredoxins by ascorbate changes the thiol-specific antioxidant paradigm, revealing another function of vitamin C, Proc. Natl. Acad. Sci. Unit. States Am. 104 (2007) 4886–4891, https://doi.org/10.1073/pnas.0700481104.
[29] P. Pulido, R. Cazalis, F.J. Cejudo, An antioxidant redox system in the nucleus of wheat seed cells suffering oxidative stress, Plant J. 57 (2009) 132–145, https://doi.org/10.1111/j.1365-313X.2008.03675.x.
[30] K.S. You, L.V. Benitez, W.A. McConachie, W.S. Allison, The conversion of glyceraldehyde-3-phosphate dehyrogenase to an acylphosphatase by trinitroglycerin and inactivation of this activity by azide and ascorbate, BBA - Enzymol. 384 (1975) 317–330, https://doi.org/10.1016/0005-2744(75)90033-9.
[31] E. Zito, H.G. Hansen, G.S.H. Yeo, J. Fujii, D. Ron, Endoplasmic reticulum thiol oxidase deficiency leads to ascorbic acid depletion and noncanonical scurvy in mice, Mol. Cell. 48 (2012) 39–51, https://doi.org/10.1016/j.molcel.2012.08.010.
[32] H.R. Ellis, L.B. Poole, Roles for the two cysteine residues of AhpC in catalysis of peroxide reduction by alkyl hydroperoxide reductase from Salmonella typhimurium, Biochemistry 36 (1997) 13349–13356, https://doi.org/10.1021/bi9713658.
[33] L.B. Poole, The basics of thiols and cysteines in redox biology and chemistry, Free Radic. Biol. Med. 80 (2015) 148–157, https://doi.org/10.1016/j.freeradbiomed.2014.11.013.
[34] C.A. Tairum, M.C. Santos, C.A. Breyer, R.R. Geyer, C.J. Nieves, S. Portillo-Ledesma, G. Ferrer-Sueta, J.C. Toledo, M.H. Toyama, O. Augusto, L.E.S. Netto, M.A. De Oliveira, Catalytic Thr or ser residue modulates structural switches in 2-cys peroxiredoxin by distinct mechanisms, Sci. Rep. (2016), https://doi.org/10.1038/srep33133.
[35] R. Ogusucu, D. Rettori, D.C. Munhoz, L.E. Soares Netto, O. Augusto, Reactions of yeast thioredoxin peroxidases I and II with hydrogen peroxide and peroxynitrite: rate constants by competitive kinetics, Free Radic. Biol. Med. 42 (2007) 326–334, https://doi.org/10.1016/j.freeradbiomed.2006.10.042.
[36] K.J. Nelson, S.T. Knutson, L. Soito, C. Klomsiri, L.B. Poole, J.S. Fetrow, Analysis of the peroxiredoxin family: using active-site structure and sequence information for global classification and residue analysis, Proteins Struct. Funct. Bioinfor. 79 (2011) 947–964, https://doi.org/10.1002/prot.22936.
[37] A. Hall, K. Nelson, L.B. Poole, P.A. Karplus, Structure-based insights into the catalytic power and conformational dexterity of peroxiredoxins, Antioxidants Redox Signal. 15 (2011) 795–815, https://doi.org/10.1089/ars.2010.3624.
[38] M.H. Nielsen, R.T. Kidmose, L.B. Jenner, Structure of TSA2 reveals novel features of the active-site loop of peroxiredoxins, Acta Crystallogr. Sect. D Struct. Biol. 72 (2016) 158–167, https://doi.org/10.1107/S2059798315023815.
[39] M.I. Karayannis, Kinetic determination of ascorbic acid by the 2,6-dichlorophenolindophenol reaction with a stopped-flow technique, Anal. Chim. Acta 76 (1975) 121–130, https://doi.org/10.1016/S0003-2670(01)81993-0.
[40] P.J. Garry, G.M. Owen, D. Wayne Lashley, P.C. Ford, Automated analysis of plasma and whole blood ascorbic acid, Clin. Biochem. 7 (1974) 131–145, https://doi.org/10.1016/S0009-9120(74)91276-4.
[41] T.S. Rao, N.R. Kale, S.P. Dalvi, Kinetics and mechanism of the oxidation of L-ascorbic acid by 2,6-dichlorophenol-indophenol in aqueous solution, React. Kinet. Catal. Lett. 34 (1987) 179–184, https://doi.org/10.1007/BF02069221.
[42] G.H. Kaihami, J.R.F. de Almeida, S.S. dos Santos, L.E.S. Netto, S.R. de Almeida, R.L. Baldini, Involvement of a 1-cys peroxiredoxin in bacterial virulence, PLoS Pathog. 10 (2014) e1004442, , https://doi.org/10.1371/journal.ppat.1004442.
[43] J.R. Pedrajas, A. Miranda-Vizuete, N. Javanmardy, J.-Å. Gustafsson, G. Spyrou, Mitochondria of Saccharomyces cerevisiae contain one-conserved cysteine type peroxiredoxin with thioredoxin peroxidase activity, J. Biol. Chem. 275 (2000) 16296–16301, https://doi.org/10.1074/jbc.275.21.16296.
[44] F. Gomes, F.R. Palma, M.H. Barros, E.T. Tsuchida, H.G. Turano, T.G.P. Alegria, M. Demasi, L.E.S. Netto, Proteolytic cleavage by the inner membrane peptidase (IMP) complex or Oct1 peptidase controls the localization of the yeast peroxiredoxin Prx1 to distinct mitochondrial compartments, J. Biol. Chem. 292 (2017) 17011–17024, https://doi.org/10.1074/jbc.M117.788588.
[45] J.C. Toledo, R. Audi, R. Ogusucu, G. Monteiro, L.E.S. Netto, O. Augusto, Horseradish peroxidase compound i as a tool to investigate reactive protein-cysteine residues: from quantification to kinetics, Free Radic. Biol. Med. 50 (2011) 1032–1038, https://doi.org/10.1016/j.freeradbiomed.2011.02.020.
[46] I.H. Segel, Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, (1993).
[47] B.B. Horta, M.A. De Oliveira, K.F. Discola, J.R.R. Cussiol, L.E.S. Netto, Structural and biochemical characterization of peroxiredoxin Qβ from Xylella fastidiosa: catalytic mechanism and high reactivity, J. Biol. Chem. 285 (2010) 16051–16065, https://doi.org/10.1074/jbc.M109.094839.
[48] D. Parsonage, D.S. Youngblood, G.N. Sarma, Z.A. Wood, P.A. Karplus, L.B. Poole, Analysis of the link between enzymatic activity and oligomeric state in AhpC, a bacterial peroxiredoxin † , ‡, Biochemistry 44 (2005) 10583–10592, https://doi.org/10.1021/bi050448i.
[49] A.V. Peskin, F.M. Low, L.N. Paton, G.J. Maghzal, M.B. Hampton, C.C. Winterbourn, The high reactivity of peroxiredoxin 2 with H2O2 is not reflected in its reaction with other oxidants and thiol reagents, J. Biol. Chem. 282 (2007) 11885–11892, https://doi.org/10.1074/jbc.M700339200.
[50] J.A. Reisz, E. Bechtold, S.B. King, L.B. Poole, C.M. Furdui, Thiol-blocking electrophiles interfere with labeling and detection of protein sulfenic acids, FEBS J. 280 (2013) 6150–6161, https://doi.org/10.1111/febs.12535.
[51] S. Sugio, A. Kashima, S. Mochizuki, M. Noda, K. Kobayashi, Crystal structure of human serum albumin at 2.5 Å resolution, Protein Eng. 12 (1999) 439–446, https://doi.org/10.1093/protein/12.6.439.
[52] A.J. Stewart, C.A. Blindauer, S. Berezenko, D. Sleep, D. Tooth, P.J. Sadler, Role of Tyr84 in controlling the reactivity of Cys34 of human albumin, FEBS J. 272 (2005) 353–362, https://doi.org/10.1111/j.1742-4658.2004.04474.x.
[53] A. Fisher, C. Dodia, Y. Manevich, J. Chen, S. Feinstein, Phospholipid hydroperoxidase are substrates for non-selenium glutathione peroxidase, J. Biol. Chem. 274 (1999) 21326–21334, https://doi.org/10.1074/jbc.274.30.21326.
[54] Y. He, W. Xu, Y. Xiao, L. Pan, G. Chen, Y. Tang, J. Zhou, J. Wu, W. Zhu, S. Zhang, J. Cao, Overexpression of peroxiredoxin 6 (PRDX6) promotes the aggressive phenotypes of esophageal squamous cell carcinoma, J. Canc. 9 (2018) 3939–3949, https://doi.org/10.7150/jca.26041.
[55] S.Z. Wang Z, W. He, G. Yang, J. Wang, Z. Wang, J.M. Nesland, R. Holm, Decreased expression of GST pi is correlated with poor prognosis in patients with esophageal squamous cell carcinoma, BMC Canc. 10 (2010) 3408–3414, https://doi.org/10.1007/s10620-010-1212-7.
[56] R.K. Chandra, B.G. Bentz, G.K.H. Iii, A.M. Robinson, J.A. Radosevich, Expression of Glutathione S-Transferase Pi in Benign Mucosa, Barrett ' S Metaplasia, and Adenocarcinoma of the Esophagus, (2002), pp. 575–581, https://doi.org/10.1002/hed.10093.
[57] L.A.C. Carvalho, D.R. Truzzi, T.S. Fallani, S.V. Alves, J.C. Toledo, O. Augusto, L.E.S. Netto, F.C. Meotti, Urate hydroperoxide oxidizes human peroxiredoxin 1 and peroxiredoxin 2, J. Biol. Chem. 292 (2017) 8705–8715, https://doi.org/10.1074/jbc.M116.767657.

[58] S. Portillo-Ledesma, L.M. Randall, D. Parsonage, J.D. Rizza, P. Andrew Karplus, L.B. Poole, A. Denicola, G. Ferrer-Sueta, Differential kinetics of two-cysteine peroxiredoxin disulfide formation reveal a novel model for peroxide sensing, Biochemistry 57 (2018) 3416–3424, https://doi.org/10.1021/acs.biochem.8b00188.
[59] M. Lindblad, P. Tveden-Nyborg, J. Lykkesfeldt, Regulation of vitamin C homeostasis during deficiency, Nutrients 5 (2013) 2860–2879, https://doi.org/10.3390/nu5082860.
[60] J. Du, J.J. Cullen, G.R. Buettner, Ascorbic acid: chemistry, biology and the treatment of cancer, Biochim. Biophys. Acta Rev. Canc 1826 (2012) 443–457, https://doi.org/10.1016/j.bbcan.2012.06.003.
[61] C. Haslekås, R.A.P. Stacy, V. Nygaard, F.A. Culiáñez-Macià, R.B. Aalen, The expression of peroxiredoxin antioxidant gene, AtPer1, in Arabiodopsis thaliana is seed-specific and related to dormancy, Plant Mol. Biol. 36 (1998) 833–845, https://doi.org/10.1023/A:1005900832440.
[62] R.A.P. Stacy, T.W. Norcleng, F.A. Culiáñez-Macià, R.B. Aalen, The dormancy-related peroxiredoxin anti-oxidant, PER1, is localized to the nucleus of barley embryo and aleurone cells, Plant J. 19 (1999) 1–8, https://doi.org/10.1046/j.1365-313X.1999.00488.x.
[63] C. Haslekas, Seed 1-cysteine peroxiredoxin antioxidants are not involved in dormancy, but contribute to inhibition of germination during stress, Plant Physiol. 133 (2003) 1148–1157, https://doi.org/10.1104/pp.103.025916.
[64] M.C. De Tullio, O. Arrigoni, The ascorbic acid system in seeds: to protect and to serve, Seed Sci. Res. 13 (2003) 249–260, https://doi.org/10.1079/ssr2003143.
[65] N. Smirnoff, Ascorbic acid metabolism and functions: a comparison of plants and mammals, Free Radic. Biol. Med. 122 (2018) 116–129, https://doi.org/10.1016/j.freeradbiomed.2018.03.033.
[66] B. Zechmann, Subcellular distribution of ascorbate in plants, Plant Signal. Behav. 6 (2011) 360–363, https://doi.org/10.4161/psb.6.3.14342.
[67] E. Heyneke, N. Luschin-Ebengreuth, I. Krajcer, V. Wolkinger, M. Müller, B. Zechmann, Dynamic compartment specific changes in glutathione and ascorbate levels in Arabidopsis plants exposed to different light intensities, BMC Plant Biol. 13 (2013), https://doi.org/10.1186/1471-2229-13-104.
[68] B.A. Wolucka, D. Communi, Mycobacterium tuberculosis possesses a functional enzyme for the synthesis of vitamin C, L-gulono-1,4-lactone dehydrogenase, FEBS J. 273 (2006) 4435–4445, https://doi.org/10.1111/j.1742-4658.2006.05443.x.
[69] Z. Zhang, M. Aboulwafa, M.H. Smith, M.H. Saier, The ascorbate transporter of Escherichia coli, J. Bacteriol. 185 (2003) 2243–2250, https://doi.org/10.1128/JB.185.7.2243.
[70] J.R. Pedrajas, C.A. Padilla, B. McDonagh, J.A. Bárcena, Glutaredoxin participates in the reduction of peroxides by the mitochondrial 1-CYS peroxiredoxin in Saccharomyces cerevisiae, Antioxidants Redox Signal. 13 (2010) 249–258, https://doi.org/10.1089/ars.2009.2950.
[71] J.R. Pedrajas, B. McDonagh, F. Hernández-Torres, A. Miranda-Vizuete, R. González-Ojeda, E. Martínez-Galisteo, C.A. Padilla, J.A. Bárcena, Glutathione is the resolving thiol for thioredoxin peroxidase activity of 1-cys peroxiredoxin without being consumed during the catalytic cycle, Antioxidants Redox Signal. 24 (2016) 115–128, https://doi.org/10.1089/ars.2015.6366.
[72] W.K. Huh, B.H. Lee, S.T. Kim, Y.R. Kim, G.E. Rhie, Y.W. Baek, C.S. Hwang, J.S. Lee, S.O. Kang, D-erythroascorbic acid is an important antioxidant molecule in Saccharomyces cerevisiae, Mol. Microbiol. 30 (1998) 895–903, https://doi.org/10.1046/j.1365-2958.1998.01133.x.
[73] E. Loumaye, G. Ferrer-Sueta, B. Alvarez, J.F. Rees, A. Clippe, B. Knoops, R. Radi, M. Trujillo, Kinetic studies of peroxiredoxin 6 from Arenicola marina: rapid oxidation by hydrogen peroxide and peroxynitrite but lack of reduction by hydrogen sulfide, Arch. Biochem. Biophys. 514 (2011) 1–7, https://doi.org/10.1016/j.abb.2011.07.002.
[74] J.W. Chen, C. Dodia, S.I. Feinstein, M.K. Jain, A.B. Fisher, 1-Cys peroxiredoxin, a bifunctional enzyme with glutathione peroxidase and phospholipase A2 activities, J. Biol. Chem. 275 (2000) 28421–28427, https://doi.org/10.1074/jbc.M005073200.
[75] E. Gasteiger, C. Hoogland, A. Gattiker, S. Duvaud, M.R. Wilkins, R.D. Appel, A. Bairoch, Protein identification and analysis tools on the ExPASy server, Proteomics Protoc. Handb, 2009, https://doi.org/10.1385/1-59259-890-0:571.
[76] A. Vagin, A. Teplyakov, Molecular replacement with MOLREP, Acta Crystallogr. Sect. D Biol. Crystallogr. 66 (2010) 22–25, https://doi.org/10.1107/S0907444909042589.
[77] P. Emsley, B. Lohkamp, W.G. Scott, K. Cowtan, Features and development of Coot, Acta Crystallogr. Sect. D Biol. Crystallogr. 66 (2010) 486–501, https://doi.org/10.1107/S0907444910007493.
[78] G.N. Murshudov, P. Skubák, A.A. Lebedev, N.S. Pannu, R.A. Steiner, R.A. Nicholls, M.D. Winn, F. Long, A.A. Vagin, REFMAC5 for the refinement of macromolecular crystal structures, Acta Crystallogr. Sect. D Biol. Crystallogr. 67 (2011) 355–367, https://doi.org/10.1107/S0907444911001314.
[79] D.R. Grassetti, J.F. Murray, Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiodipyridine, Arch. Biochem. Biophys. 119 (1967) 41–49, https://doi.org/10.1016/0003-9861(67)90426-2.