Substituting selenocysteine for active site cysteine 149 of phosphorylating glyceraldehyde 3-phosphate dehydrogenase reveals a peroxidase activity

Sandrine Boschi-Muller[a], Sabine Muller[b], Alain Van Dorsselaer[c], August Böck[b], Guy Branlant[a,*]

[a] *Maturation des ARN et Enzymologie Moléculaire, UMR 7567 CNRS-UHP, Faculté des Sciences, P.O. Box 239, 54506 Vandoeuvre-Les-Nancy Cedex, France*
[b] *Lehrstuhl für Mikrobiologie der Universität München, Maria-Ward-Strasse 1a, D-80638 Munich, Germany*
[c] *Laboratoire de Spectrométrie de Masse Bio-Organique, UMR 7509 CNRS, Université Louis Pasteur, 1 Rue Blaise Pascal, 67008 Strasbourg, France*

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Abstract Replacing the essential Cys-149 by a selenocysteine into the active site of phosphorylating glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from *Bacillus stearothermophilus* leads to a selenoGAPDH that mimics a selenoperoxidase activity. Saturation kinetics were observed with cumenyl and *tert*-butyl hydroperoxides, with a better catalytic efficiency for the aromatic compound. The enzymatic mechanism fits a sequential model where the formation of a ternary complex between the holoselenoenzyme, the 3-carboxy 4-nitrobenzenethiol used as the reductant and the hydroperoxide precedes product release. The fact that the selenoGAPDH is NAD-saturated supports a binding of hydroperoxide and reductant in the substrate binding site. The catalytic efficiency is similar to selenosubtilisins but remains low compared to selenoglutathione peroxidase. This is discussed in relation to what is known from the X-ray crystal structures of selenoglutathione peroxidase and GAPDHs.

Key words: Selenocysteine; Artificial peroxidase; Cysteine active site; Glyceraldehyde 3-phosphate dehydrogenase

1. Introduction

Selenium is found in a number of proteins of organisms from all three kingdoms. When incorporated as a selenocysteine, selenium is generally involved in the oxidation-reduction process [1]. More information is now available about the role played by selenocysteine in several catalytic processes thanks to detailed kinetic studies and resolution of some X-ray crystal structures. In this regard, selenoglutathione peroxidase is one of the best characterised seleno-enzymes, so far, at the level of both enzymatic properties and 3D structure [2,3]. It protects cellular components against oxidative damage from hydrogen peroxide and various organic hydroperoxides. The catalytic mechanism involves the selenolate entity which forms an enzyme-selenenic acid intermediate (see Fig. 1). Regeneration of the reduced enzyme is accomplished via a two-step process involving reduced glutathione as reductant. The presence of a reactive and accessible selenol is crucial for the activity. This is the consequence of the low pK_a of the selenol (pK_a = 5.2) and of the great polarisability of the Se group. Thus, introducing a selenol entity into the active site of an enzyme can lead to it mimicking a peroxidase activity provided that the selenol is accessible and reactive and the active site can accommodate the substrates. This is the case for selenosubtilisin, a semi-synthetic enzyme obtained from the native protease by chemical modification of the catalytic serine residue [4,5]. More recently, the biochemical characterisation of selenium containing catalytic antibody as a glutathione peroxidase mimic has been described [6]. This antibody was prepared by converting the reactive serine residue of a monoclonal antibody raised against a glutathione derivative. But no artificial seleno-enzyme has been described so far where a catalytic cysteine residue is changed into a selenocysteine one. In this paper, phosphorylating glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has been chosen for such a modification. GAPDHs are homotetrameric enzymes which reversibly catalyse the oxidative phosphorylation of D-glyceraldehyde 3-phosphate (G3P) into 1,3-diphosphoglycerate in the presence of NAD and inorganic phosphate. Refined crystal structures have been determined for various sources [7–9]. In the active site, two catalytic amino acids have been characterised, namely Cys-149, the site of a covalent thioacyl enzyme intermediate, and His-176. The relative positioning of both residues is such that an efficient Cys^- His^+ ion pair is formed that lowers the pK_a of Cys-149 largely below pH 7 and thus increases the nucleophilicity of Cys-149, at physiological pH [10].

In the present work, selenoGAPDH of the Ser-153 mutant from *Bacillus stearothermophilus* has been produced by applying the method previously used to introduce selenocysteine into thioredoxin [11]. The kinetic properties of the selenoGAPDH are described and discussed in relation to what is known for selenoglutathione peroxidase and synthetic selenosubtilisins.

2. Materials and methods

2.1. Site-directed mutagenesis, production and purification of the Ser-153 mutant GAPDH of B. stearothermophilus

Site-directed mutagenesis were performed using the method of Kunkelet al. [12]. The *Escherichia coli* strain used for C153S mutant enzyme production was DH5α transformed with a pBluescript II SK containing the *gap* gene under the *lac* promoter (pSKBstII). Purification of C153S mutant enzyme was performed as previously described by Talfournier et al. [10]. Purity of the enzyme was checked by electrophoresis on a 10% SDS polyacrylamide gel and by mass spectrometry.

*Corresponding author. Fax: (33) 3 83 91 20 93.
E-mail: gbranlant@scbim.u-nancy.fr

Abbreviations: GAPDH, D-glyceraldehyde 3-phosphate dehydrogenase; G3P, D-glyceraldehyde 3-phosphate; ArS^-, 3-carboxy 4-nitrobenzenethiol; tBuOOH, *tert*-butyl hydroperoxide; CuOOH, cumenyl hydroperoxide

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2.2. Production and purification of selenocysteine mutant GAPDH of B. stearothermophilus

The strategies used for construction of the plasmid and production of selenocysteine mutant GAPDH have already been described for a selenocysteine variant of thioredoxin [11]. The gene encoding Ser-153 mutant GAPDH of *B. stearothermophilus* (*gap*) was set under the control of the T7 promoter of plasmid pT7-5. To this end, the 2.9 kb *Sac*I-*Hin*dIII fragment carrying *gap* was isolated from plasmid pSKBstII and ligated into the multicloning site of plasmid pT7-5 yielding plasmid pSM2. PSM2 was transformed into strain BL21*cysE51* and overexpression of *gap* in the presence of selenocysteine was performed as already described for $(Se)_2$-thioredoxin [11].

Purification of the selenocysteine mutant was performed as described for the wild-type GAPDH [10] except that after sonication a dialysis step was included before ammonium sulphate fractionation. Purification of the selenenyl sulphide form of selenocysteine containing enzyme was performed by addition of dithionitrobenzoate at a final concentration of 3 mM to all buffers used during the purification process. Purity of the enzymes was checked by electrophoresis on a 10% SDS-polyacrylamide gel and by electrospray mass spectrometry (ES/MS).

2.3. Enzyme assays and kinetics for Ser-153 mutant GAPDH

Initial rate measurements were carried out at 25°C on a Kontron Uvikon 933 spectrophotometer by following the absorbance of NADH at 340 nm. The temperature of the solutions was maintained at 25°C by thermostatted sample holders using a circulating bath water for all the measurements. The experimental conditions were 40 mM triethanolamine, 2 mM EDTA, 50 mM K_2HPO_4, pH 8.9. Turnover number (k_{cat}) was calculated using a molar extinction coefficient of 1.31×10^5 M^{-1} cm^{-1} at 280 nm. k_{cat} was expressed per site (N).

The initial rate data were fitted to the Michaelis-Menten relationship using least squares analysis to determine V_{max} and K_M. All K_M values were determined at saturating concentrations of the other substrates.

2.4. Enzyme assays and kinetics for selenoGAPDH

Initial rate measurements were carried out at 25°C on a Kontron Uvikon 933 spectrophotometer by following the absorbance of 3-carboxy 4-nitrobenzenethiol (ArS^-) at 410 nm in MES 100 mM, pH 5 buffer. The rates of reaction were corrected for the background reaction between hydroperoxide and ArS^-. ArS^- was prepared by reducing the corresponding disulphide using the procedure of Silver [13]. Selenoenzyme concentration was determined spectrophotometrically assuming that selenoGAPDH has the same extinction coefficient as the wild type and that isolated enzyme contains 70% of selenocysteine forms (see Section 3). Data were fitted to a sequential model with the program Sigmaplot (Jandel Scientific Software) as follows:

$$\frac{\nu}{E_o}=\frac{k_{max}\cdot[ArS^-]\cdot[ROOH]}{(K_{ROOH}\cdot K_{ArS^-}+K_{ArS^-}\cdot[ROOH]+[ArS^-]\cdot[ROOH])} \quad (1)$$

To investigate the pH dependence of k_{cat}/K_{tBuOOH}, the reaction rates were determined over a range of pHs (4.5–7) in a polybuffer containing 30 mM acetic acid, 30 mM imidazole, 120 mM Tris-HCl, and 0.2 mM EDTA at a constant ionic strength of 0.15 M. Substrate concentrations were 10 mM and 200 μM for *tert*-butyl hydroperoxide (tBuOOH) and ArS^-, respectively. k_{cat}/K_{tBuOOH} values were calculated directly at each pH by dividing the initial rate by the concentration of enzyme and of tBuOOH. The following equation was used to fit the experimental data:

$$(k_{cat}/K_{tBuOOH})_{app}=\frac{k_{max}/K_{tBuOOH}}{(1+10^{(pH-pK_a)})} \quad (2)$$

2.5. Electrospray mass spectrometry analyses

Molecular mass analyses were performed on a BioQ quadrupole mass spectrophotometer (Fisons, Manchester). Samples were solubilised in aqueous 50% CH_3CN containing 1% HCOOH at a final concentration of 20 pmol/μl. Data acquisition was performed in the multichannel acquisition mode.

3. Results

3.1. Production and isolation of the selenoGAPDH C153S mutant

The method used was derived from that which permitted an efficient synthesis of $(Se)_2$-thioredoxin [11]. It is based on the assumption that an efficient charging of $tRNA^{Cys}$ occurs with selenocysteine when cysteine is omitted. However, this strategy can present drawbacks for interpreting kinetic data when several selenocysteines are introduced. In the case of GAPDH from *B. stearothermophilus*, two cysteines are present, i.e. the catalytic Cys-149 and the Cys-153 which is located after one turn of the helix [7]. Therefore, before introducing selenocysteine at position 149, Cys-153 was changed into a serine. This substitution was expected to have no effect on the catalytic activity given a serine is already present in the GAPDH from *Thermus aquaticus* [14]. This was confirmed by the fact that

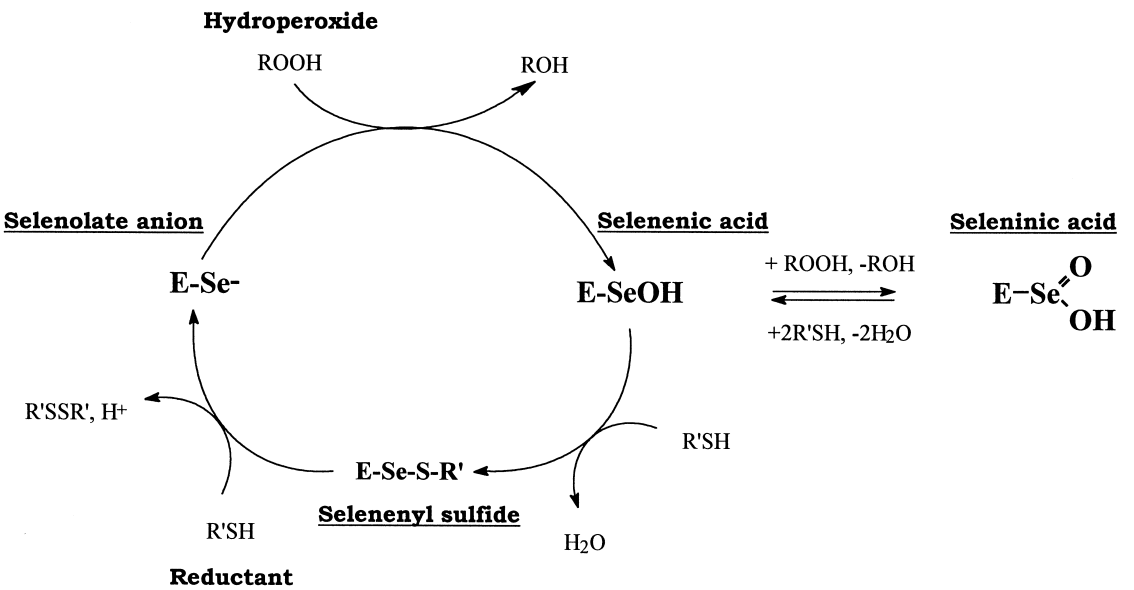

Fig. 1. Scheme of the mechanism of the peroxidase activity [3].

the K_M values of substrates and cofactor (K_M (G3P) = 1.7 ± 0.2 mM, K_M (Pi) = 21 ± 4 mM and K_M (NAD) = 98 ± 20 μM), and k_{cat} of the C153S mutant enzyme (93 ± 5 s^{-1}) are similar to those of the wild type [15].

The protocol applied to the purification of the seleninic ($ESeO_2H$) and the selenenyl sulphide (ESeSAr) forms of the C153S enzyme was similar to that used for the wild type [10]. To avoid loss of ArS^- during the purification process of the selenenyl sulphide form of the enzyme, ArS^- was added to all the buffers. A homogeneous preparation was obtained for both forms. A yield of 4 mg of purified protein was obtained per liter of culture for both forms which is 4-fold less than that obtained for selenothioredoxin. This relatively low yield remains to be explained. A ratio A_{280}/A_{260} of 1.1 was observed for all the selenoenzyme isolates. This ratio indicated that all GAPDH subunits were NAD saturated.

3.2. Biochemical characterisation of the purified protein isolates

Both selenoGAPDH C153S mutant enzymes were characterised by ES/MS and, in the case of the selenenyl sulphide form[1], also by titrating the enzyme active site since the appearance of ArS^- is easily monitored spectroscopically at 410 nm.

The ES/MS spectrum of both purified protein samples revealed the presence of two components. For the seleneninic isolate, the mass of the main component (36 018 Da) is that predicted for $EseO_2H$ form. The minor peak (35 934 Da) was then identified as the Ser-153 species (35 938 Da). A mass of 36 175 ± 8 was obtained by ES/MS for the selenenyl sulphide form which is in agreement to that expected (calculated mass = 36 183). From the relative peak intensities (curves not shown) it was estimated that both protein samples were composed of 70% Se GAPDH and 30% natural GAPDH. These yields are similar to that described for $(Se)_2$-thioredoxin [11]. The selenenyl sulphide isolate was titratable by reduction with excess of DTT. The shoulder at 326 mm which represents the contribution of Se-S and S-S forms, shifted to a maximum at 410 nm in the presence of DTT (curves not shown). The absorbance at 410 nm corresponded to 1 equivalent ArS^- per subunit, which reflects the contribution of the two populations namely 70% of the selenenyl sulphide form and 30% of the disulphur form.

3.3. Peroxidase activity of selenoGAPDH

The addition of tBuOOH and ArS^- to both forms of seleno isolates revealed the existence of a peroxidase activity. No activity was observed with wild-type GAPDH, thus proving that the peroxidase activity observed in both forms of seleno isolates is due to the active site selenocysteine introduced.

Table 1
Kinetic parameters for the peroxidase activity of selenoGAPDH

	k_{cat}	$K_{hydroperoxide}$	K_{TNB^-}	$k_{cat}/K_{hydroperoxide}$
TbuOOH	228	430	60	530
CuOOH	14	2	140	7 000
H_2O_2	N.D.	N.D.	N.D.	23 000 ± 1000

Reactions were carried out in MES 100 mM buffer, pH 5 at 25°C and followed spectrophotometrically as described in Section 2. For tBuOOH and CuOOH, the experimental data were fit to a sequential mechanism to obtain the parameters shown. For H_2O_2, $(k_{cat}/K_{H_2O_2})_{app}$ was obtained directly from the linear plot of v_0 versus $[H_2O_2]$ at 200 μM ArS^-. N.D.: not determined.

In order to probe the mechanism, detailed kinetic studies were undertaken on the selenenyl sulphide form. Three structurally distinct hydroperoxides, tBuOOH, cumenyl hydroperoxide (CuOOH) and H_2O_2, were tested in the presence of ArS^- as a convenient thiol to follow activity at 410 nm. Saturation kinetics were observed at all individual concentrations of ArS^-, tBuOOH and CuOOH investigated. Double-reciprocal plots of initial velocity versus ArS^-, tBuOOH and CuOOH concentration exhibited curves (Fig. 2) that fit well only a sequential model (Eq. 1) as observed for selenosubtilisin BPN′ and selenoglutathione peroxidase [16,17]. No saturation kinetics were observed with H_2O_2 concentrations up to 0.1 M. At higher concentrations, the rate for the spontaneous reduction of H_2O_2 by ArS^- becomes too high compared to the enzymatic peroxidase activity, thus preventing the determination of a K_M value. However, under conditions where $[H_2O_2] < 0.1$ M, a linear relationship of the enzymatic rate against $[H_2O_2]$ was observed. A $k_{cat}/K_{H_2O_2}$ value of 23 000 ± 1000 M^{-1} min^{-1} could be determined at 200 μM ArS^-. For the other substrates, apparent second order rate constants were calculated from the k_{cat} and K_M values deduced from fitting the experimental data to a sequential model (see data in Table 1).

The bimolecular reactions with the three hydroperoxides are about 3–5 orders of magnitude slower than for selenoglutathione peroxidase. However, compared to the chemical model done with tBuOOH as a substrate [18], the enzyme is at least 700-fold more efficient. No activity was observed with *tert*-butyl peroxide as already observed for selenosubtilisin and no inhibition of the enzymatic reaction was noticed when the radical trap 2,6-di-*tert*-butyl-4-methyl-phenol was added.

The pH dependence of the peroxidase k_{cat}/K_{tBuOOH} catalytic efficiency was studied from pH 4.5 to 7.2 at saturating concentration of ArS^- of 200 μM and low concentration of tBuOOH compared to the K_M value (10 mM vs 430 mM), thus ensuring that the observed rate is directly proportional to the tBuOOH concentration. As shown in Fig. 3, maximal efficiency is observed at acidic pH. The pH profile follows a single sigmoid curve with an apparent pK_a of 5.8. This suggests the involvement of an amino acid of pK_a 5.8 during the catalytic turnover. It is worth mentioning that selenoGAPDH and selenosubtilisin [18] behave similarly.

4. Discussion

SelenoGAPDH displays a catalytic efficiency similar to that

[1] It was expected from studies carried out on the model [23] that the seleninic acid form of GAPDH should react with 3 equivalents of ArS^- to give a selenenyl sulphide adduct and a dithionitrobenzoate molecule. However, under our experimental conditions, no significant decrease of absorbance at 412 nm was observed. Although no straightforward explanation can be advanced, it can be noted that seleninic acid in the model is likely more reactive than in the GAPDH active site, as already shown for selenosubtilisin [18]. The formation of an interaction between the anionic seleninate and the catalytic His-176 stronger than that observed in the subtilisin counterpart could also contribute to a strong decrease of the reactivity of seleninic GAPDH. This low reactivity does not necessarily contradict the fact that a turnover is observed in the presence of hydroperoxide. One can easily imagine that the simultaneous binding of the hydroperoxide and the reductant to the selenilate form of GAPDH can induce local conformational rearrangement that would render the ternary complex operative for reduction.

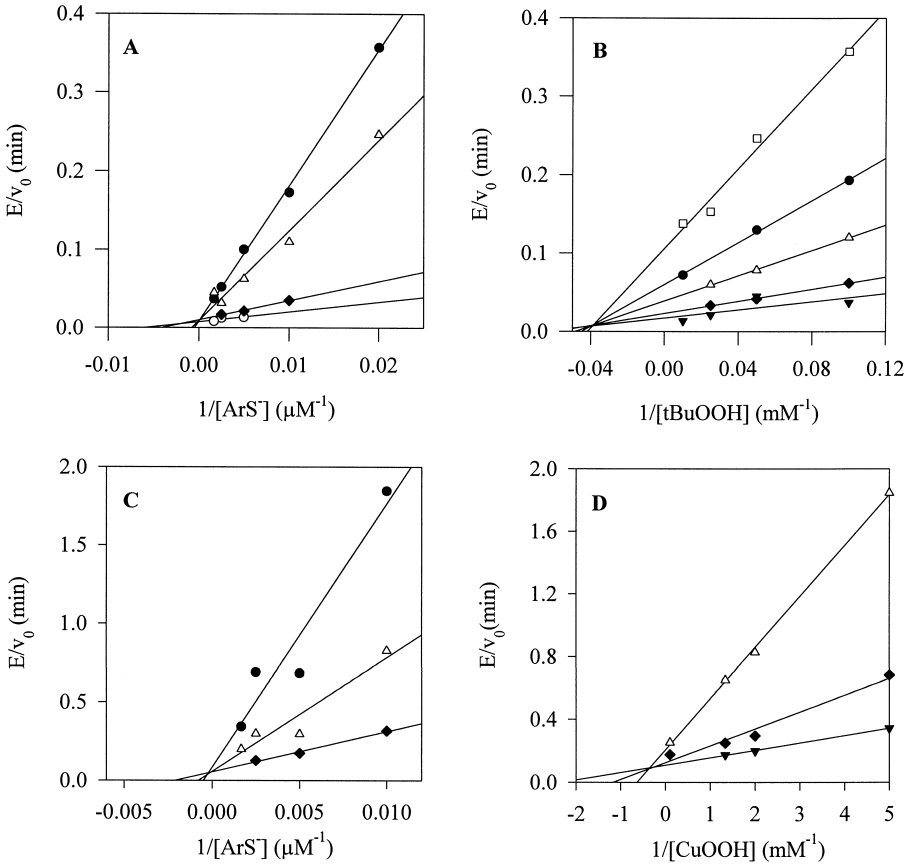

Fig. 2. Peroxidase activity of selenoGAPDH with different hydroperoxides as substrate. AI Plots of $[E]/v_0$ (min) versus $1/[ArS^-]$ (μM^{-1}) at [tBuOOH] = 10 mM (●), 20 mM (△), 40 mM (◆) and 100 mM (○). B: Plots of $[E]/v_0$ (min) versus 1/[tBuOOH] (μM^{-1}) at $[ArS^-]$ = 50 μM (□), 100 μM (●), 200 μM (△), 400 μM (◆) and 600 μM (▼). C: Plots of $[E]/v_0$ (min) versus $1/[ArS^-]$ (μM^{-1}) at [CuOOH] = 0.1 mM (●), 0.5 mM (△) and 0.75 mM (◆). D: Plots of $[E]/v_0$ (min) versus 1/[CuOOH] (μM^{-1}) at $[ArS^-]$ = 100 μM (△), 200 μM (◆) and 600 μM (▼).

of selenosubtilisins but low compared to natural selenoperoxidase [16,18,19]. The enzymatic mechanism satisfactorily fits a sequential model which is also likely the case for natural selenoperoxidase and selenosubtilisin [16,17]. SelenoGAPDH exhibits a significant difference in hydroperoxide recognition with a preference for CuOOH in contrast to selenosubtilisin which binds tBuOOH better than CuOOH. Together these

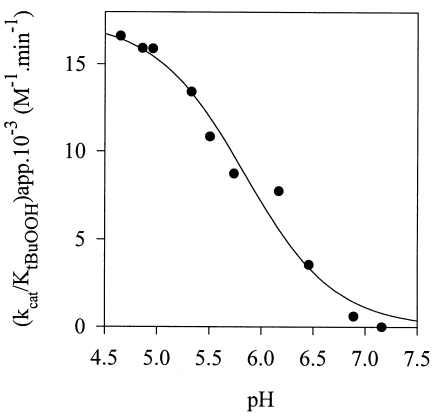

Fig. 3. pH dependence of $(k_{cat}/K_{tBuOOH})_{app}$. $(k_{cat}/K_{tBuOOH})_{app}$ (M^{-1} min^{-1}) versus pH was determined under experimental conditions as described in Section 2 where $[ArS^-]$ is 200 μM and [tBuOOH] 10 mM. The data were fit to Eq. 2, to give $pK_{app} = 5.8 \pm 0.1$ and $k_{max}/K_{tBuOOH} = 500 \pm 23$ M^{-1} min^{-1}.

results raise the questions of how the active site of selenoGAPDH accommodates the hydroperoxide and the reductant, and of the nature of the rate-limiting step and of the amino acids involved in the catalytic mechanism. Clearly, the fact that the enzymatic studies were carried out on the holoform supports binding of both substrates into the substrate binding sites themselves and not into the cofactor binding site. The formation of a kinetically significant ternary complex between selenoGAPDH, ArS^- and CuOOH or tBuOOH implies simultaneous binding of hydroperoxide and ArS^-. In fact, the substrate binding site of GAPDH is composed of two sites, one for G3P and one for inorganic phosphate. It can also accommodate phenyl derivatives as well, as revealed from experiments that showed esterolytic activity of GAPDH against *para*-nitrophenyl acetate with a K_M value of $\sim 10^{-3}$ M (unpublished result). Thus, it was expected that aromatic hydroperoxides like CuOOH can be bound stronger than tBuOOH.

The $k_{cat}/K_{R'OOH}$ values of H_2O_2, CuOOH and tBuOOH are low compared to those determined for glutathione peroxidase [19] but vary in the order $H_2O_2 > CuOOH > tBuOOH$ that superimposes the intrinsic reactivity of these hydroperoxides [20]. The fact that the affinity is better for the hydroperoxide while the k_{cat} value is decreased is not necessarily contradictory. In fact, one can easily imagine that the stronger the binding the less productive the positioning of the hydroperoxide relative to the selenolate for efficient attack. In that context, reactivity and accessibility of the selenolate anion

are factors that can also contribute to the catalytic efficiency. As already noted, the intrinsic pK_a of a selenol is around 5. It is thus ionised at physiological pH and should be reactive unless its nucleophilicity and/or accessibility are modified by its protein environment. In wild-type GAPDH from *B. stearothermophilus*, Cys-149 and His-176 form an efficient ion pair with pK_{app} values of 5.5 and 8.2, respectively. The formation of this ion pair permits the cysteine to exist as a thiolate species at acidic pH but its chemical reactivity is 20-fold lower relative to a free thiolate [10]. Assuming the formation of an ion pair between the selenolate anion and His-176, similar to that described for selenosubtilisin [21,22], a significant decrease of the chemical reactivity of selenolate ion within the active site of selenoGAPDH is expected to occur. Inspection of the active site of selenoglutathione peroxidase showed that the only interactions which stabilise the selenolate involve weak hydrogen bonding to Trp-158 and Gln-80 and possibly an α-helix macrodipole. No His residue is present in the vicinity of the selenolate, thus excluding the formation of an Se^- His^+ ion pair [3]. This suggests that the chemical reactivity of the selenolate ion in the natural enzyme is high compared to the selenoenzyme derived from GAPDH or subtilisin, both of which having a catalytic His. The fact that the active site of selenoglutathione peroxidase is located on the surface of the protein [3] while those of GAPDH and subtilisin are more buried can also contribute to the changes observed in the catalytic efficiency/pH profile of selenoGAPDH and selenosubtilisin. For selenoGAPDH and selenosubtilisin, optimal pH is observed at acidic pH, with an apparent pK_a of 5.8 and 7 [18], respectively. For glutathione peroxidase, an optimal pH at 8.8 has been reported [19], similar to that recently described for selenocatalytic antibody [6] and with almost no activity at acidic pH. Such differences remain to be explained at the molecular level. In particular, the nature of the amino acids responsible of the differences observed in the pH profile remains to be characterised. Knowledge of the pK_{app} and of the nucleophilicity of the selenol group within the GAPDH active site by an approach similar to that recently carried out on the thiol group of Cys-149 [10] would also be informative. This implies, in addition to solving various technical problems like working in the absence of oxygen, the possibility of easily isolating the selenol form. Finally, knowledge of the crystal structure of the selenoGAPDH and of the mutant in which His-176 is mutated will not only give new insights into these structural and mechanistic aspects but will also provide the opportunity to compare the active site structure of selenoGAPDH to those of selenosubtilisin and glutathione peroxidase.

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