Kinetic studies of peroxiredoxin 6 from Arenicola marina: Rapid oxidation by hydrogen peroxide and peroxynitrite but lack of reduction by hydrogen sulfide

Eléonore Loumaye a, Gerardo Ferrer-Sueta b, e, Beatriz Alvarez c, e, Jean-François Rees a, André Clippe a, Bernard Knoops a, Rafael Radi d, e, Madia Trujillo d, e, *

a Laboratory of Cell Biology, Institut des Sciences de la Vie, Université Catholique de Louvain, B-1348 Louvain-la-Neuve, Belgium
b Laboratorio de Fisicoquímica Biológica, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay
c Laboratorio de Enzimología, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay
d Departamento de Bioquímica, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay
e Center for Free Radical and Biomedical Research, Universidad de la República, Montevideo, Uruguay

Abstract

Arenicola marina lives in marine environments where hydrogen peroxide concentrations reach micromolar levels. The annelid also forms reactive species through metabolic pathways. Its antioxidant systems include a cytosolic peroxiredoxin, peroxiredoxin 6 (AmPrx6 or AmPRDX6) that shows high homology to the mammalian 1-Cys peroxiredoxin. Previous work confirmed the peroxidase activity of AmPrx6 in the presence of dihydrothreitol. Herein, we performed an in vitro kinetic characterization of the recombinant enzyme. AmPrx6 reduced hydrogen peroxide and peroxynitrite with rate constants of 1.1 ± 0.2 and 2 × 10^7 M^−1 s^−1, respectively, at pH 7.4 and 25°C. Reduction of tert-butyl hydroperoxide was slower. The pK₅₇ of the peroxidatic thiol of AmPrx6 was determined as 5.1 ± 0.2, indicating that it exists as thiolate, the reactive species, at physiological pH. The reductive part of the catalytic cycle was also explored. The enzyme sulfide, present in millimolar concentrations in marine sediments where the annelid lives and that is able to reduce the mammalian 1-Cys peroxiredoxin, did not support AmPrx6 peroxidase activity. The enzyme was not reduced by other potential physiological reductants tested. Our data indicate that in this annelid, Prx6 could contribute to peroxide detoxification in the presence of a so far unidentified reducing counterpart.

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subfamily [13]. Recently, a sequence-based classification of the
currently known 3516 Prx protein sequences into six distinct sub-

ties has become available through the PREX database (http://
www.csb.wfu.edu/prex/) [14]. In all Prxs, the peroxidatic cysteine
is located in an active site pocket and is activated for catalysis by
interacting with surrounding residues. The peroxidatic cysteines
have a low pK_a. Thus, they are deprotonated at physiological pH,
as required for the nucleophilic attack on the peroxide. For exam-
ple, the pK_a of peroxidatic thiols have been reported as <5, and
more recently as 5.9 for bacterial alkyl hydroperoxide reductase
C [15,16]; 5.2 for Mycobacterium tuberculosis alkyl hydroperoxide
reductase E and human Prx 5 [17,18] and 5.7 and 6.3 for yeast thi-
oredoxin peroxidase I and II, respectively [19]. Such low pK_a values,
however, are not enough to explain the high peroxidase efficiency
of Prxs [18,20], and other factors such as transition state stabiliza-
tion by active site microenvironment have started to be considered
as important contributors to their catalytic activity [21,22]. Taking
advantage of the ping-pong mechanism of the reactions catalyzed
by Prxs, the oxidizing and reducing parts of the catalytic cycle can
be studied separately. This has allowed the determination of the
kinetics of peroxide reactions by pre-steady state approaches.
Alternatively, catalytic efficiencies towards different peroxides
have been studied under steady-state conditions [23,24]. It is clear
that, depending on the Prx subfamily, the oxidizing substrates that
are used preferentially vary. Moreover, natural reducing substrates
for many Prxs, especially 1-Cys Prxs, are in many cases still
unknown.

A. marina peroxiredoxin 6 (AmPrx6, also abbreviated as
AmPRDX6) [2], which exhibits a high sequence homology with mamm-
alian 1-Cys Prxs (Prx6), was recently structurally characterized
[25]. The enzyme was able to catalyze the reduction of various hydro-
peroxides using the artificial substrate dithiotreitol (DTT) [8]. It is a
cysteolic enzyme highly expressed in tissues directly exposed to
the external environment, suggesting that it may be of particular
importance for protection against exogenous oxidative stress [8].
Herein, we report the first kinetic characterization of an annelid
Prx, AmPrx6 mutated at different cysteine residues, C45S, C86S, C183S were generated by PCR-mediated site-directed muta-
genesis as previously described [8].

Materials and methods

Materials

Horseradish peroxidase (HRP), cytosolic thioredoxin reductase
1 (TR1) from rat liver, H_2O_2, tert-butyl hydroperoxide (t-BuOOH),
glutathione (GSH), dihydroenitriamippentaacetic acid (dtpa),
dithiobiotetrol (DTT), 6-8-thioclic acid, reduced form (dihydro-
lic acid, DHLA), monobromobimane (mBBr) were purchased from
Sigma–Aldrich. Argon (~99.5% pure) was obtained from AGA Gas
Company, Montevideo, Uruguay. All other reagents were obtained
from standard commercial sources.

Expression and purification of AmPrx6

AmPrx6 (GenBank Accession No. DQ059567) was expressed in
Escherichia coli as a 6x His-tagged protein and purified as described
before [8]. AmPrx6 mutated at different cysteine residues, C45S,
C86S, C183S were generated by PCR-mediated site-directed muta-
genesis as previously described [8].

Peroxide and protein measurements

Peroxynitrite was synthesized as previously described [30,31].
Hydrogen peroxide remaining from the synthesis was eliminated by
treating stock peroxynitrite solutions with manganese dioxide.
Nitrile concentration in stock peroxynitrite solutions were less
than one third of peroxynitrite concentration, as measured by the
Griess assay [32]. The concentration of peroxynitrite was deter-
mained at 302 nm at alkaline pH (\(E_{240} = 1670 \text{ M}^{-1} \text{ cm}^{-1}\) [33]). The concentration of H_2O_2 stock solutions was measured at 240 nm
\(E_{230} = 43.6 \text{ M}^{-1} \text{ cm}^{-1}\) [34]), and HRP concentration at 403 nm
\(E_{403} = 1.02 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}\) [35]). Protein concentration was mea-
sured by the bicinchoninic assay using bovine serum albumin as
standard. AmPrx6 concentration was also measured by protein
absorption at 280 nm, considering a molar absorption coefficient
\(E_{280} = 28.210 \text{ M}^{-1} \text{ cm}^{-1}\) obtained with the ProtParam tool of the
ExPaSy software [36] with similar results.

Thiol reduction and quantification

AmPrx6 was reduced by incubation with DTT (5 mM) for 15 min
at room temperature. Excess DTT was removed immediately before
use by gel filtration either using HiTrap desalting columns or PD10
columns (GE-healthcare). Collected protein was kept on ice under
argon atmosphere to avoid reoxidation. Protein thiols were mea-
sured by the DTNB assay [37].

Determination of rate constants by competition kinetics

Unless otherwise indicated, all the experiments were carried out
in 100 mM sodium phosphate buffer plus 0.1 mM dtpa at pH 7.4 and 25 °C. The second-order rate constants for the reactions
between reduced AmPrx6 and oxidizing substrates (\(k_2\)) were
determined by a competitive assay using a stopped flow spectropho-
tometer (Applied Photophysics SX-17MV) as previously reported
[19]. HRP reacts rapidly with H_2O_2 or peroxynitrite to produce compound I [38,39]. If reduced AmPrx6 also reacts rapidly with the ox-
diants, and under conditions of oxidant in defect, the peroxiredoxin
should inhibit HRP-compound I formation, as indicated by reactions
Eqs. (1) and (2).

\[
\text{HRP + H}_2\text{O}_2/\text{peroxynitrite} \rightarrow \text{compound I} + \text{H}_2\text{O}/\text{NO}_2^-
\]

\(k_1\)  

1 \(k_2\) indicates the second-order rate constant of AmPrx6 with a peroxide substrate.
In particular, \(k_{2(\text{H}_2\text{O}_2)}\) and \(k_{2(\text{peroxynitrite})}\) are the second-order rate constants of
AmPrx6 reaction with peroxynitrite, hydrogen peroxide and t-BuOOH, respectively. \(k_2\) indicates the second-order rate constant of HRP oxidation to compound I. In particular, \(k_{2(\text{peroxynitrite})}\) and \(k_{2(\text{HRP})}\) are the second-order rate constants of HRP oxidation by peroxynitrite and hydrogen peroxide, respectively.

The second-order rate constants for the reactions of HRP with 
H₂O₂ and with peroxynitrite (k₁[H₂O₂] and k₁[ONOO⁻]) were 
confirmed under our experimental conditions by following 
the time courses of HRP oxidation to compound I at 398 nm 
(ε = 42,000 M⁻¹ cm⁻¹ [40]) at different HRP concentrations in 
excess. Two different batches of HRP (type VI Sigma–Alrich P8375 
250–330 U/mg solid and type I Sigma Aldrich P8125 50–150 U/ 
mg solid) were used that reacted with H₂O₂ with k₁[H₂O₂] of 
1 × 10⁷ and 5 × 10⁶ M⁻¹ s⁻¹, respectively, and with peroxynitrite 
with k₁[ONOO⁻] of 3 × 10⁶ and 1.2 × 10⁶ M⁻¹ s⁻¹, respectively, at 
pH 7.4 and 25 °C (data not shown), in agreement with previous 
reports [38,39].

If reactants are not used in large excess over the oxidant, 
the reactivity ratio is given by Eq. (3) [24]

\[
k₁ = \frac{\ln \left( \frac{[\text{HRP}_{\text{ox}}]}{[\text{HRP}_{\text{red}}]} \right)}{[\text{Compound I}]} \quad \text{(3)}
\]

where [HRP]₀ and [redAmPrx₆]₀ are initial HRP and reduced AmPrx₆ 
concentrations, respectively, [Compound I] is the final HRP-Com-
 pound I concentration and final oxidized AmPrx₆ concentration 
([redAmPrx₆]₀) was determined as [redAmPrx₆]₀ = [Peroxide]₀ − 
[Compound I]₀. Using the k₁ values indicated above, the k₂ values 
for each of the oxidizing substrates were determined.

Kinetics of the reaction between reduced AmPrx₆ and t-BuOOH 
was measured by competition with peroxynitrite as substrate, as 
described previously [18,24]. Peroxynitrite consumption by 
reduced AmPrx₆ in the absence or presence of different t-BuOOH 
concentrations was followed at 310 nm. The competing reactions 
were as follows:

\[
\text{redAmPrx₆ + peroxynitrite} \rightarrow \text{redAmPrx₆ + NO}_2 \quad \text{K} \quad \text{(4)}
\]

\[
\text{redAmPrx₆ + t-BuOOH} \rightarrow \text{redAmPrx₆ + t-BuOH} \quad \text{K} \quad \text{(5)}
\]

The concentration of t-BuOOH required to decrease AmPrx₆-
mediated peroxynitrite consumption by 50% was determined. At 
that concentration, the rate of reactions (4) and (5) are equal:

\[
k₂[\text{ONOO}^-] \times [\text{ONOO}^-] \sim k₂[\text{t-BuOOH}] \times [\text{t-BuOOH}] \quad \text{(6)}
\]

Then, using the k₂[ONOO⁻] value determined above, it was possible 
to approximate a rate constant for the reaction between the en-
zyme and t-BuOOH, k₂[t-BuOOH].

Kinetic simulations

The yields of HRP-compound I formation by peroxynitrite or 
hydrogen peroxide in the absence or presence of different con-
centrations of reduced AmPrx₆ were simulated according to a 
simple competition model using the Gepasi software (version 3.3) [41,42].

Determination of the acidity constant of the peroxidatic thiol in 
AmPrx₆

For the determination of the pKₐ of the peroxidatic thiol, we 
used two different methods. The first method consisted in meas-
uring the effect of pH on the rate of alkylation with mBBr that yields a 
fluorescent product ([ε₉₈ = 396 nm, ε₉₈ = 482 nm]) [43,44]. Using a 
384-well microplate, reduced AmPrx₆ (2 μM) was incubated with 
mBBr (1–5 μM) in a buffer containing 6 mM Tris, 3 mM MES, 
3 mM acetate and 144 mM NaCl. Different pHs in the range of 
3.8–8.5 were achieved by careful addition of 15 mM HCl or 
15 mM NaOH. This buffer was selected to maintain constant 
ionic strength (0.15 M) throughout the pH range [45]. The reaction 
was started by either the addition of Prx₆ (pH < 7) or NaOH (pH > 7) 
using the automatic dispenser of the microplate reader. Fluores-
cence was recorded simultaneously at all 16 pH values with a Var-
ioskan Flash microplate reader (Thermo Scientific). In order to 
increase the specificity of the rate determinations, we measured 
the emission of the Fluorescence Resonance Energy Transfer, from 
Trp to alkylated thiol ([ε₉₈ = 280 nm, ε₉₈ = 482 nm]) [46]. Fluores-
cence emission was followed for 40 min with points every 15 s, 
and the initial linear part of the time course was used to determine 
a slope that is proportional to the initial rate (ν₀). In order to 
compare the results from different experiments, the initial slope 
was divided by the initial concentration of reduced thiol and mBBr.

The slope was plotted as a function of pH and was fitted to either 
a one-pKₐ model or a two-pKₐ using Eqs. (7) and (8), respectively.

One-pKₐ model:

\[
v₀ = \frac{a₁[H]^+ + a₂K_{\text{a}}}{[H]^+ + K_{\text{a}}} \quad \text{(7)}
\]

where a₁ and a₂ are the initial reaction rates for the monoprotic 
and aprotic species, respectively, and Kₐ is the ionization constant 
for monoprotic species.

Two-pKₐ model:

\[
v₀ = \frac{a₁[H]^+ + a₂K_{\text{a}1} + a₃K_{\text{a}2}}{[H]^+ + K_{\text{a}1} + K_{\text{a}2}} \quad \text{(8)}
\]

where a₁, a₂, and a₃ are the initial reaction rates for the diprotic, 
monoprotic and aprotic species, respectively; Kₐ₁ and Kₐ₂ are the 
ionization constants for the diprotic and monoprotic species, 
respectively.

The second method relied on measurements of the rates of H₂O₂ 
reduction by AmPrx₆ at different pHs using the competition ap-
proach with HRP as previously reported [16]. Since among the dif-
ferent AmPrx₆ cysteine residues, only the peroxidatic thiol reac-
t fast enough to compete with HRP for H₂O₂, this method allows 
its specific pKₐ determination. Sodium phosphate buffer (100 mM 
plus 100 μM dtpa) was used for pHs 8.0 to 5.5, and sodium acetate 
buffer (100 mM plus 0.1 mM dtpa) for acidic pHs (5.5 to 4.7). Yields 
of HRP-compound I formation in the absence of competing AmPrx₆ 
were controlled at each pH. Rate constants of HRP-compound I 
formation, k₁[H₂O₂], decrease at pH < 5 and were corrected accord-
ingly as previously reported [16]. Rate constants of AmPrx₆ reaction 
with H₂O₂ at each pH were calculated using Eq. (3) and the pKₐ 
value of the peroxidatic thiol was calculated by taking into account 
that the thiolate and protonated peroxide are the reactive species 
and fitting the plot of rate constants versus pH to Eq. (9)

\[
K_{\text{pH independent}} = k_{\text{pH independent}} \times \frac{K_{\text{AmPrx₆}}}{K_{\text{AmPrx₆}} + [\text{H}^+]^a} \times \frac{[\text{H}^+]^a}{K_{\text{ROOH}} + [\text{H}^+]^a} \quad \text{(9)}
\]

where kₐpH dependent and kₐpH independent are the pH-dependent 
and pH-independent rate constants of peroxidatic thiol oxidation, 
respectively; Kₐ₆ is the peroxidatic thiol acidity constant and 
Kₐ₆ is the acidity constant of the peroxide (ROOH), in this case 
H₂O₂ (pKₐ = 11.7 for the first deprotonation of H₂O₂ at 25 °C).

Assessment of potential reducing substrates for AmPrx₆

Dihydrolipoic acid and dithiotreitol

The ability of DHLA to act as AmPrx₆ substrate was assessed. 
Briefly, DHLA (1.4 mM) was mixed with H₂O₂ (280 μM) in 
the absence or presence of AmPrx₆ (10 μM) in phosphate buffer 
100 mM pH 7.4 and 25 °C. The time courses of H₂O₂ decay were 
followed by removing aliquots at increasing times, stopping the 
reaction with HCl (0.2 M) and adding Fe(NH₄)₂(SO₄)₂ (5 mM) and 
KSCN (0.625 M). The absorbance at 480 nm was compared against
a calibration curve to calculate H₂O₂ concentrations. As a positive control we measured H₂O₂ consumption using DTT (1.4 mM) as reducing substrate under the same experimental conditions, as previously described [8].

**Glutathione and thioredoxin reductase**

AmPrx6 (1 mM) was mixed with glutathione (GSH) (1 mM), TR1 (0.2 μM) and NADPH (250 μM) in Hepes buffer 50 mM pH 7.0. The reaction was triggered by addition of H₂O₂ (500 μM) and followed by measuring NADPH oxidation at 340 nm (ε₃₄₀ = 6220 M⁻¹ cm⁻¹) [47].

**Hydrogen sulfide**

The possibility that H₂S could function as a reducing substrate of AmPrx6 was explored by two methods.

Firstly, we determined the rate constant for the oxidation of H₂S by H₂O₂. This reaction has an uncatalyzed rate constant of 0.73 ± 0.03 M⁻¹ s⁻¹ (pH 7.4, 37 °C) [48]. The amount of H₂S remaining after exposure to H₂O₂ in the presence or absence of AmPrx6 was measured through the methylene blue method as previously [48]. Briefly, in closed vials, H₂S (220 μM) was mixed with H₂O₂ (100 μM) in Tris buffer (0.1 M) plus dtpa (0.1 mM), pH 7.4, with or without AmPrx6 (4 μM). At increasing times, aliquots were mixed with Zn²⁺ (6 mM) and catalase (k = 0.1 s⁻¹) for stopping the reaction. The concentration (k) of catalase solutions was determined according to [49]. Then, N,N-dimethyl-p-phenylenediamine (7.5 mM from 20 mM stock in 7.2 M HCl) and FeCl₃ (11.25 mM from 30 mM stock in 1.2 M HCl) were added. The absorbance was measured at 750 nm and compared against a calibration curve containing known concentrations of NaHS. Alternatively, the amount of H₂S remaining was measured [50]. Briefly, H₂O₂ (280 μM) was mixed with H₂S (0.5 or 1 mM) in the absence or presence of AmPrx6 (5 or 10 μM) in Tris buffer (0.1 M) plus dtpa (0.1 mM), pH 7.4. The time courses of H₂O₂ decay were followed colorimetrically as indicated above for DHLA.

Secondly, we measured whether H₂S was able to reduce thiol groups in oxidized AmPrx6. Thiol content of reduced AmPrx6 (200 μM) was determined without any treatment, exposed to H₂O₂ (150 μM) or exposed to H₂O₂ and then treated with H₂S (1.5 mM) for 5 min was measured by Ellman’s assay [37].

**Results**

**Kinetics of AmPrx6 oxidation by oxidizing substrates**

**Hydrogen peroxide**

Wild type AmPrx6 dose-dependently inhibited the formation of HRP-compound I from H₂O₂ (1 mM) and HRP (3 mM) (Fig. 1). According to Eq. (3), a rate constant of H₂O₂ reduction by AmPrx6 of (1.1 ± 0.3) × 10⁶ M⁻¹ s⁻¹ at pH 7.4 and 25 °C was calculated (Fig. 1A, inset). We obtained a similar rate constant value of (1.8 ± 0.5) × 10⁶ M⁻¹ s⁻¹ for C86S AmPrx6, in agreement with previous reported data indicating that Cys86 was not required for the enzymatic activity (Fig. 1B) [8]. In contrast, the peroxidatic mutant C45S was inactive and therefore did not inhibit HRP-compound I formation by H₂O₂ (Fig. 1A).

**Peroxynitrite**

The formation of HRP – compound I from the reaction between peroxynitrite (1 μM) and HRP (5 μM) was also inhibited by AmPrx6 (Fig. 2). From Eq. (3), a rate constant of (2 ± 0.7) × 10⁶ M⁻¹ s⁻¹ and 3.4 ± 0.5 × 10⁶ M⁻¹ s⁻¹ were determined for wild type and C86S AmPrx6, respectively (Fig. 2A and B). As expected, the C45S mutant did not inhibit peroxynitrite-mediated compound I formation (data not shown).

**Tert-butyl hydroperoxide**

The rate constant of AmPrx6 reaction with t-BuOOH was also investigated. A concentration of t-BuOOH of 120 μM inhibited by 50% the consumption of 15 μM peroxynitrite by AmPrx6, indicating that the rate constant between the enzyme and the organic peroxide was slower than with peroxynitrite (≈2.5 × 10⁹ M⁻¹ s⁻¹) (Fig. 3). Low t-BuOOH concentrations had no effect on peroxynitrite decomposition rate, as previously reported and did not affect AmPrx6 absorbance at 310 nm (data not shown). However, increasing t-BuOOH concentration to mM levels, led to a maximum of only 70% inhibition of absorbance decay at 310 nm, probably because of interferences of these high concentrations of organic peroxide.

**Determination of the acidity constant of AmPrx6 peroxidatic thiol**

**Thiol alkylation with mBBR**

A first approximation to the enzyme pKₐ was obtained by the rate of alkylation with mBBR, a reaction that labels all the reduced cysteines of AmPrx6 and thus allows the pKₐ determination of all the protein thiols in the range from 3.9 to 8.6. The initial rate versus pH plot (Fig. 4) shows that the reaction is faster at alkaline pHs, as expected since the thiolate was the reactive species. Two inflection points were observed that fitted to a two-pKₐ model (Eq. (8)) and that would correspond to the peroxidatic thiol (pKₐ1 = 6.2 ± 1.2) and to a combined value for the rest of cysteine residues (pKₐ2 = 8.1 ± 0.2). The high degree of uncertainty of the more acidic pKₐ arises from the fact that the experimental data is fitted to a five-parameter function. Interestingly, when the C45S mutant was studied in a similar experiment, the more acidic ionization corresponding to the peroxidatic cysteine was not observed and essentially identical results were obtained above pH 7 (Fig. 4).

**Peroxidatic thiol oxidation by H₂O₂**

To specifically measure the pKₐ value of the peroxidatic cysteine, the effect of pH on the rate constant of AmPrx6 oxidation by H₂O₂ was determined. By fitting the plot of rate constant of oxidation versus pH to Eq. (9), the pKₐ of the peroxidatic thiol in wild type AmPrx6 was determined as 5.1 ± 0.2 (Fig. 5). As expected from this pKₐ value and considering that more than 99% of H₂O₂ would be protonated at pHs lower than 8, the pH-independent rate constant of AmPrx6 oxidation by H₂O₂ was almost identical to that determined at pH 7.4. Moreover, the pH-independent rate constant of AmPrx6 oxidation by peroxynitrite was calculated as 1 × 10⁶ M⁻¹ s⁻¹, according to Eq. (9) and using the experimentally determined rate constant at pH 7.4 and pKₐ values of 5.1 (determined herein) and 6.8 [30] for peroxidatic thiol and peroxynitrous acid, respectively.

**Investigation of AmPrx6 reduction by potential reducing substrates of physiological relevance**

**DHLA**

DHLA (1.4 mM) caused a slow H₂O₂ (280 μM) reduction that did not accelerate in the presence of AmPrx6 (10 μM) (not shown). Under the same experimental conditions, AmPrx6 catalyzed the reduction of H₂O₂ by DTT as previously reported [8].

**Glutathione and cytosolic thioredoxin reductase**

It was recently reported that the yeast mitochondrial 1-Cys peroxiredoxin 1 can be reduced by GSH in the presence of mitochondrial thioredoxin reductase (TR2) [47]. Since AmPrx6 is a cytosolic enzyme, we tested the ability of GSH plus cytosolic thioredoxin reductase (TR1) to reduce the enzyme. AmPrx6 did not catalyze NADPH oxidation by H₂O₂ in the presence of GSH and TR1 (not shown).
The possibility that H$_2$S could act as a reducing substrate of AmPrx6 was investigated by two different approaches, namely (a) investigation of the ability of AmPrx6 to catalyze H$_2$O$_2$ reduction by H$_2$S and (b) investigation of the ability of H$_2$S to reduce oxidized AmPrx6.

As shown in Fig. 6, no increases in the rate of H$_2$S oxidation by H$_2$O$_2$ were detected in the presence of AmPrx6. No increases were detected when H$_2$O$_2$ was measured instead of H$_2$S (data not shown). Accordingly, the amount of reduced thiols present in the enzyme did not increase upon incubation with millimolar concentrations of H$_2$S (data not shown). Thus, no evidence was obtained that H$_2$S could act as a reducing substrate of AmPrx6.

**Discussion**

In this work, we present the results of different assays aimed at getting insights into the catalytic activity of AmPrx6. We report for the first time the second order rate constants for the reaction of the enzyme with different oxidizing substrates and the pK$_a$ of the peroxidatic thiol. The fast reactions with H$_2$O$_2$ and peroxynitrite, $(1.1 \pm 0.3) \times 10^7$ and $(2 \pm 0.7) \times 10^6$ M$^{-1}$s$^{-1}$ at pH 7.4 and 25°C for wild type enzyme, respectively (Figs. 1A and 2A) suggest that AmPrx6 works as an efficient antioxidant enzyme. The reaction was 5-fold faster with H$_2$O$_2$ than with peroxynitrite at pH 7.4, and pH-independent rate constants were similar for both oxidants. This is against the trend reported previously for peroxide-mediated GSH and serum albumin thiol oxidation, where reactivity correlated with the acidity of the leaving group conjugate acid (HNO$_2$, pK$_a = 3.15$ [51] versus H$_2$O, pK$_a = 15.7$ [52]) [53], and indicates the existence of protein factors that direct preferential specificity for the oxidizing substrates in Prxs. In this sense, human Prx2 was about ten times more efficient in the reduction of H$_2$O$_2$, $(k_2 \sim 1.0 \pm 0.1 \times 10^7$ M$^{-1}$s$^{-1}$), than in the reduction of peroxynitrite $(k_2 \sim 1.4 \pm 0.8 \times 10^6$ M$^{-1}$s$^{-1}$) at pH 7.4 [54]. On the contrary, human Prx5 reacted faster with peroxynitrite than with H$_2$O$_2$ [18]. Furthermore, the organic peroxide t-BuOOH reacted with AmPrx6 at a slower rate than the other peroxides tested, suggesting that the enzyme is probably not so efficient in the reduction of lipid
hydroperoxides in cell membranes (Fig. 3). The peroxidatic thiol of the enzyme exhibits a $pK_a$ of 5.1 ± 0.2 (Fig. 5), similar to those of other Prxs tested so far [16–19]. Thus, the peroxidatic thiol would be deprotonated at physiological pH. Since thiolate is the reactive species towards peroxide substrates, a $pK_a$ of 5.1 indicates that >99% of the enzyme would be under the form required for catalysis at pH around 7.4. However, pH independent reactivities of thiols towards peroxynitrite are known to decrease for those thiols with the lower $pK_a$ [18], and therefore, low $pK_a$ values would in fact not increase but rather could decrease thiolate reactivity, if only $C_p$ $pK_a$ values were to be considered. It is clear that active site factors are contributing to the catalytic power of peroxiredoxins [18]. These include transition state stabilization [21] and specific activation of the thiolate upon substrate binding, as recently proposed [22]. In this work, we also investigated the potential of different reductants to act as $AmPrx6$ substrate. Previous data indicated that neither the conventional mechanism of mammalian 1-Cys Prxs, GSH loaded on glutathione-S-transferase pi, or thioredoxin, which is usually the reductant of 2-Cys Prxs, were able to reduce $AmPrx6$ [8]. We then hypothesized that another reductant, well conserved in all living organisms, like DHLA, could act as reductant for $AmPrx6$, as demonstrated for other thiol-containing peroxidase [28,55]. However, DHLA was not able to act as $AmPrx6$ reducing substrate. Cytosolic thioredoxin reductase plus GSH were also unable to support the catalytic activity of $AmPrx6$. This annelid lives in the muddy and intertidal sediments in which organic matter is under decomposition, producing high amounts of sulfides.
Hydrogen sulfide could diffuse across cell membranes to penetrate inside the cells [56]. Moreover, enzymatic routes producing H$_2$S at significant rates have been demonstrated in this annelid, the actual concentration of H$_2$S in animal tissues being probably in the μM range [27]. Thus, we investigated the putative role of H$_2$S as a reductant for AmPrx6, as it was reported previously that it can reduce bovine Prx6 [57]. In our hands, AmPrx6 was not reduced by H$_2$S (Fig. 6).

Altogether, our data confirm that AmPrx6 is a highly efficient Prx that is very likely involved in the protection of the annelid against peroxides formed endogenously, and also exogenously during tide cycles, considering the abundance of the enzyme in tissues directly exposed to the environment [8]. Finally, a physiological reductant for AmPrx6 is still to be identified.

Acknowledgments

This work was supported by Grants from CSIC (Uruguay) to B.A. and R.R., from Howard Hughes Medical Institute to R.R. and from “Fonds de la Recherche Scientifique” (Belgium), “Fonds de la Recherche Fondamentale Collective” (Belgium), “Communauté française de Belgique-Action de Recherches Concertées” (Belgium), and the French “Ministère des Affaires Etrangères” under the Integrated Program Action called Tournesol (No. 05365 GT) to JFR.

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