Hydroperoxide and peroxynitrite reductase activity of poplar thioredoxin-dependent glutathione peroxidase 5: kinetics, catalytic mechanism and oxidative inactivation

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Gpxs (glutathione peroxidases) constitute a family of peroxidases, including selenocysteine- or cysteine-containing isoforms (SeCys-Gpx or Cys-Gpx), which are regenerated by glutathione or Trxs (thioredoxins) respectively. In the present paper we show new data concerning the substrates of poplar Gpx5 and the residues involved in its catalytic mechanism. The present study establishes the capacity of this Cys-Gpx to reduce peroxynitrite with a catalytic efficiency of $10^9$ M$^{-1}$·s$^{-1}$. In PtGpx5 (poplar Gpx5; Pt is Populus trichocarpa), Glu79, which replaces the glutamine residue usually found in the Gpx catalytic tetrad, is likely to be involved in substrate selectivity. Although the redox midpoint potential of the Cys$^{44}$–Cys$^{92}$ disulfide bond and the $pK_a$ of Cys$^{44}$ are not modified in the E79Q variant, it exhibited significantly improved kinetic parameters ($K_m$ and $k_{cat}$) with tert-butyl hydroperoxide. The characterization of the monomeric Y151R variant demonstrated that PtGpx5 is not an obligate homodimer. Also, we show that the conserved Phe$^{90}$ is important for Trx recognition and that Trx-mediated recycling of PtGpx5 occurs via the formation of a transient disulfide bond between the Trx catalytic cysteine residue and the Gpx5 resolving cysteine residue. Finally, we demonstrate that the conformational changes observed during the transition from the reduced to the oxidized form of PtGpx5 are primarily determined by the oxidation of the peroxidatic cysteine into sulfenic acid. Also, MS analysis of in-vitro-oxidized PtGpx5 demonstrated that the peroxidatic cysteine residue can be over-oxidized into sulfinic or sulfonic acids. This suggests that some isoforms could have dual functions potentially acting as hydrogen-peroxide- and peroxynitrite-scavenging systems and/or as mediators of peroxide signalling as proposed for 2-Cys peroxiredoxins.

Key words: glutathione peroxidase, over-oxidation, peroxynitrite, redox property, site-directed mutagenesis, thiol peroxidase.

INTRODUCTION

In plants, various metabolic pathways or physiological processes, such as photosynthesis and respiration, produce ROS (reactive oxygen species) in different subcellular compartments such as mitochondria, chloroplasts or peroxisomes [1]. ROS can be different species, such as singlet oxygen, hydroxy radicals, superoxide radical anions (O$_2^-$) or hydrogen peroxide (H$_2$O$_2$). On the other hand, plants produce nitric oxide (NO) via several metabolic pathways or physiological processes, such as those occurring during environmental constraints, their concentrations can increase dramatically and lead to conditions of oxidative or nitro-oxidative stress. All organisms, and particularly plants, have developed a wide range of detoxification processes to tightly control cellular ROS and peroxynitrite concentrations [4,5]. Among these scavenging enzymes are Tpxs (thiol-dependent peroxidases), which use a reactive cysteine residue to reduce various types of peroxides (from H$_2$O$_2$ to phospholipid hydroperoxides and peroxynitrite). Five classes of thiol peroxidases have been defined in plants, grouping both the so-called Gpxs (glutathione peroxidases) and Prxs (peroxiredoxins), the latter separated into 1-Cys Prx, 2-Cys Prx, type II Prx and Prx Q subgroups [6]. Whatever the substrate reduced, the regeneration of the sulfenic acid formed during catalysis is dependent on thiol compounds, usually glutathione time they are toxic compounds leading to random or targeted oxidation of DNA and proteins, or to lipid peroxidation with associated potential drastic modifications of the physico-chemical properties of these macromolecules [1,2]. Reactive species are continuously produced by cells, but in particular conditions, such as those occurring during environmental constraints, their concentrations can increase dramatically and lead to conditions of oxidative or nitro-oxidative stress. All organisms, and particularly plants, have developed a wide range of detoxification processes to tightly control cellular ROS and peroxynitrite concentrations [4,5]. Among these scavenging enzymes are Tpxs (thiol-dependent peroxidases), which use a reactive cysteine residue to reduce various types of peroxides (from H$_2$O$_2$ to phospholipid hydroperoxides and peroxynitrite). Five classes of thiol peroxidases have been defined in plants, grouping both the so-called Gpxs (glutathione peroxidases) and Prxs (peroxiredoxins), the latter separated into 1-Cys Prx, 2-Cys Prx, type II Prx and Prx Q subgroups [6]. Whatever the substrate reduced, the regeneration of the sulfenic acid formed during catalysis is dependent on thiol compounds, usually glutathione

Abbreviations used: ABA, abscisic acid; ABI2, ABA INSENSITIVE 2; AP1, activator protein 1; COOH, cumene hydroperoxide; Cr, Chlamydomonas reinhardtii; Dm, Drosophila melanogaster; DTPA, diethylenetriaminepenta-acetic acid; DTT, dithiothreitol; ESI, electrospray ionization; Gpx, glutathione peroxidase; Glx, glutamate; HRP, horseradish peroxidase; IAM, iodoacetamide; mBBr, monobromobimane; NBD-Cl, 4-chloro-7-nitro-2,1,3-benzooxadiazole; PDT-bimane, (2-pyridyl)dithiobimane; Prx, peroxiredoxin; Pt, Populus trichocarpa; RNS, reactive nitrogen species; ROS, reactive oxygen species; Tb, Tryptosarcoma brucei; t-BOOH, t-butyl hydroperoxide; Tc, Trypanosarcoma cruzi; TCA, trichloroacetic acid; Tpx, thiol-dependent peroxidase; Trx, thioredoxin; WT, wild-type; Xf, Xylella fastidiosa.

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or thiol-containing proteins such as Trxs (thioredoxins), Grxs (glutaredoxins) or tryptaredoxins. Whereas most, if not all, Tpxs are able to reduce H$_2$O$_2$, the capacity to reduce peroxynitrite to nitrite has only been reported for some mammalian, protozoa, plant and bacterial members [5,7,8].

With regard to the Gpx class, they are usually monomeric or tetrameric enzymes distributed into two major types, depending on the presence of a selenocysteine (SeCys-Gpx) or cysteine (Cys-Gpx) as the catalytic residue [9]. SeCys-Gpxs are present in mammals or algae such as *Chlamydomonas reinhardtii*, whereas Cys-Gpxs are found in non-vertebrate organisms, such as bacteria, fungi, insects and plants. Despite being classified as Gpxs, most, if not all, Cys-Gpxs are in fact regenerated by Trxs, but not, or very poorly, by glutathione [6,10–12]. Cys-Gpxs usually contain three conserved cysteine residues, but only two of these cysteine residues are essential for activity [11]. The catalytic mechanism employed by Cys-Gpxs is a two-step process. The first step consists of a nucleophile attack of the substrate by the most N-terminal cysteine defined as the peroxidatic cysteine residue (Cy$_{SP}$), which is concomitant with the formation of a sulfenic acid on this residue and with the release of a water or of an alcohol molecule, depending on the nature of the substrate. Then, the most C-terminal cysteine residue, named the resolving or recycling cysteine (Cy$_{SR}$) attacks the sulfenic acid moiety forming an intramolecular disulfide bond [11,13–15]. The recycling of the reduced active form usually proceeds via a dithiol/disulfide exchange with Trxs. As observed for some other Tpxs, such as 2-Cys Prxs and for some related enzymes belonging to the methionine sulfoxide reductase protein family, the transition from the reduced to the oxidized form in the dimeric poplar Gpx5 (termed PtGpx5; *Pt* is *Populus trichocarpa*) is accompanied by an important conformational change [16–19]. Previous studies on Gpxs have shown that three residues glutamate/glutamine, tryptophan and asparagine, govern the reactivity of Cy$_{SP}$, forming a catalytic tetrad [13–15]. Although the residues are different, the principle is very similar to Prxs, where a threonine and an arginine a catalytic tetrad [13–15]. Although the residues are different, the principle is very similar to Prxs, where a threonine and an arginine stabilize the active site pocket [9,17,19].

In the present study, an in-depth biochemical and enzymatic analysis has been performed on PtGpx5 to dissect different steps of the catalytic and Trx-dependent recycling mechanisms. This analysis has shed light on five important properties of this Cys-Gpx: (i) it efficiently reduces peroxynitrites, (ii) it is reduced by Trx both in monomeric and dimeric forms, (iii) the conformational changes observed during the transition from the reduced to the oxidized form are dependent on the oxidation state of Cy$_{SP}$, (iv) Cy$_{SR}$ is susceptible to over-oxidation into sulfinic or sulfonic forms, and (v) an engineered enzyme, where one of the residues of the catalytic tetrad, Glu$^{79}$, is replaced by a glutamine residue, is a better catalyst for the reduction of organic hydroperoxides.

**Peroxide and protein quantification**

The concentration of H$_2$O$_2$ stock solutions was measured at 240 nm ($\varepsilon_{240} = 43.6$ M$^{-1}$ cm$^{-1}$). The peroxynitrite concentration was determined at alkaline pH at 302 nm ($\varepsilon_{302} = 1670$ M$^{-1}$ cm$^{-1}$) [3]. Protein concentrations were measured spectrophotometrically using a molar absorption coefficient at 280 nm of 20065 M$^{-1}$ cm$^{-1}$ for PtGpx5 WT, the single cysteine mutants, and the E79Q, D89K and F90E variants; 19940 M$^{-1}$ cm$^{-1}$ for the double cysteine mutant; and 18575 M$^{-1}$ cm$^{-1}$ for PtGpx5 Y151R, as determined from their primary sequences (http://www.expasy.ch/tools/protparam.html). The concentration of HRP (horseradish peroxidase) was determined from its Soret band absorption ($\varepsilon_{403} = 1.02 \times 10^4$ M$^{-1}$ cm$^{-1}$).

**Steady-state activity measurement**

The Trx-dependent peroxidase activity of WT and mutated PtGpx5 was measured using an NADPH-coupled spectrophotometric method at 25 °C as described previously [11]. The assays were carried out in a total volume of 500 μl containing TE buffer [30 mM Tris/HCl (pH 8.0) and 1 mM EDTA], 200 μM NADPH, 1 μM Arabidopsis thaliana NTRB (NADPH thioredoxin reductase B), 25 μM poplar Trxh1, 200 nM PtGpx5 and various peroxide concentrations. The catalytic parameters for one substrate have been obtained by varying its concentration at saturating concentrations of the other substrate [typically between 1 and 100 μM for Trxh1, and between 40 μM and 12 mM for the peroxides, either H$_2$O$_2$, t-BOOH (t-butyl hydroperoxide) and COOH (cumene hydroperoxide)]. The decrease in absorbance was followed at 340 nm. The peroxidase activity was determined after subtracting the spontaneous reduction rate observed in the absence of PtGpx5, and the number of micromoles of NADPH oxidized per second per micromole of enzyme (i.e. turnover number, s$^{-1}$) was calculated using the molar absorption coefficient of NADPH ($\varepsilon_{340} = 6230$ M$^{-1}$ cm$^{-1}$). Two or three independent experiments were performed at each substrate concentration. The $k_{cat}$ and $K_m$ values of PtGpx5 for Trxh1 or peroxides have been calculated by non-linear regression using GraFit (Erithacus Software). Inactivation of PtGpx5 and Arabidopsis 2-Cys Prx (50 nM and 1.5 μM respectively) was evaluated under the same conditions using H$_2$O$_2$ concentrations ranging from 50 μM to 2.5 mM by monitoring the mean rate of NADPH oxidation between 30 s and 1 min after addition of thiol peroxidase.

**Reduction of PtGpx5**

PtGpx5 was reduced immediately before use by incubation with 1 mM DTT (dithiothreitol) for 30 min at 4°C. Excess reductant was removed by gel filtration using a HiTrap column (Amersham Bioscience) and UV–visible detection at 280 nm. Samples were extensively purged with argon once collected.

**Pre-steady-state investigation of the oxidative part of the PtGpx5 catalytic cycle**

All pre-steady-state kinetic measurements were made using a stopped-flow apparatus with a mixing time of 1.1 ms and coupled with spectrophotometric and fluorometric detection (Applied Photophysics SV20). Experiments were performed in 100 mM sodium phosphate buffer plus 0.1 mM DTPA (diethylenetriaminepenta-acetic acid), at pH 7.4 and 25 °C, unless otherwise indicated.

The kinetics of H$_2$O$_2$-mediated PtGpx5 oxidation was studied by two pre-steady-state approaches. The first approach was a direct determination that made use of the increase in
PtGpx5 intrinsic tryptophan-dependent fluorescence intensity that occurred upon enzyme oxidation. Reduced enzyme (2 μM) was rapidly mixed with different concentrations of H2O2 in excess. The time courses of total fluorescence increase (λem = 295 nm) were followed and experimental curves were fitted to increasing exponentials from which observed rate constants (kobs) were determined. From the slope of the plot of kobs values against H2O2 concentrations, the rate constant of H2O2-mediated PtGpx5 oxidation at pH 7.4 and 25°C was determined. The second approach was an indirect competition method which has been described previously [20]. Briefly, we investigated the ability of reduced PtGpx5 to inhibit HRP oxidation to compound I by H2O2, a reaction with a reported rate constant (kH2O2/HRP) of 1.7 × 107 M-1·s-1 [21]. The time courses of compound 1 formation from the reaction of 2 μM HRP and 0.65 μM H2O2 in the absence or presence of 28.5 μM reduced PtGpx5 were followed at 398 nm. Experimental traces were fitted to exponential curves and observed rate constants (kobs) of compound 1 formation were obtained. Since kobs relates to the rate constant of H2O2-mediated oxidation of HRP (kH2O2/HRP) and to the rate constant of H2O2-mediated oxidation of PtGpx5 (kH2O2/PtGpx5) using kobs = kH2O2/HRP × [HRP] + kH2O2/PtGpx5 × [PtGpx5], both rate constants were determined.

The kinetics of peroxynitrite-mediated PtGpx5 oxidation was also investigated by two pre-steady-state approaches. First, we followed the initial rate of peroxynitrite decomposition in the absence or presence of reduced PtGpx5 at 310 nm as reported previously [22]. However, the reaction rate was too fast to be accurately followed by this methodology. Therefore we used a competition approach, by determining the inhibitor effect of increasing reduced PtGpx5 concentrations on peroxynitrite-mediated HRP oxidation to compound I in a similar way as performed with H2O2. From the plot of kobs against PtGpx5 concentrations, kH2O2/HRP and kH2O2/PtGpx5 were determined.

Pre-steady-state investigation of the reductive part of the PtGpx5 catalytic cycle

The kinetics of the reactions of oxidized PtGpx5 with GSH, poplar thioredoxin h1 (Trxh1) and h5 (Trxh5) were investigated by a direct fluorescence approach, where reduced PtGpx5 was firstly oxidized by treatment with an almost equimolar H2O2 concentration (~0.8:1) followed by mixing with different concentrations of reductant in excess. The time courses of total fluorescence intensity decrease (λem = 295 nm) were fitted to exponential curves and kobs values of fluorescence change were plotted against the reductant concentration. From the slopes of the latter plots, the rate constants of the PtGpx5 reaction by GSH, Trxh1 or Trxh5 at pH 7.4 and 25°C were determined.

Detection of sulfenic acid with NBD-Cl (4-chloro-7-nitro-2,1, 3-benzoxadiazole)

NBD-Cl was used to detect the formation of sulfenic acids on purified proteins after treatment with H2O2 [23]. Stock solutions of NBD-Cl (100 mM) were prepared in DMSO and stored in the dark. The recombiant proteins were reduced by incubation with 5 mM DTT for 1 h at 25°C. After reduction, proteins were subjected to successive dilution/concentration steps in TE buffer by ultrafiltration in order to remove excess DTT. Then, 50 μM reduced proteins were oxidized by incubation in 500 μM reaction mixtures with various concentrations of H2O2, from 250 μM to 5 mM. NDB-Cl (250 μM) was added after 1 min and the reaction mixture was incubated in the dark for 1 h at 25°C. The proteins were separated from excess NBD-Cl by precipitation for 15 min on ice with 10% (v/v) TCA (trichloroacetic acid).

After centrifugation (14,000 g for 15 min at 20°C), the pellets were washed with 1 ml of 2% TCA. After another centrifugation step (14,000 g for 15 min at 20°C), proteins were resuspended in 500 μl of 1 M Tris/HCl and 2% SDS for 30 min and their UV-visible spectra were recorded on a Cary 50 spectrophotometer (Varian).

ESI (electrospray ionization)–MS analysis of oxidized and reduced PtGpx5

Solutions of PtGpx5 WT or C7392S at 100 μM were either reduced by 150 μM DTT or oxidized in the presence of a mixture of 150 μM, 1.5 mM or 7.5 mM of both DTT and H2O2 for 5 min at room temperature (20°C). High-resolution ESI–MS spectra of treated and untreated proteins were obtained on a Bruker microTOF-Q spectrometer (Bruker Daltonik), equipped with an Apollo II ESI source with ion funnel, operated in the negative-ion mode. The concentrated sample (approximately 100 μl of the 100 μM solution) in formic acid was injected at a flow rate of 10–20 μl·min-1. Data were analysed with the DataAnalysis software (Bruker).

Determination of the oligomerization state

The oligomerization state of WT and mutated Gpxxs was analysed by size-exclusion chromatography on a Supersize 12 HR 10/30 column connected to an FPLC system (Pharmacia Fine Chemicals). Proteins (200 μl) at a concentration of 10 mg·ml-1 were loaded on to the column equilibrated in 50 mM Tris/HCl (pH 8.0) containing 150 mM NaCl and 1 mM EDTA. The flow rate was fixed at 0.25 ml·min-1 and detection was recorded at 280 nm. The column was calibrated by loading 100 μl of bovine milk lactoacerum [24].

Determination of the midpoint redox potential

Oxidation/reduction titrations, using the fluorescence of adducts formed between protein thiol groups and mBBr (monobromobimane), were carried out at room temperature in 500 μl reaction mixtures containing 100 μg of protein in 100 mM Hepes/NaOH buffer (pH 7.0) and defined mixtures of oxidized and reduced DTT to set the ambient potential (E0) with an overall concentration in DTT of 2 mM [11].

pKa of PtGpx5 sulfhydryl groups

Reactions were started by the addition of PDT-bimane [(2-pyridyl)dithiobimane] to a final concentration of 25 μM into a cuvette containing 10 μM pre-reduced proteins in 500 μl of sodium citrate or phosphate buffer ranging from pH 3.0 to 8.0, following a procedure described in [25]. The reaction of PDT-bimane with cysteine residues forms pyridine-2-thione, which has a maximum absorption wavelength at 343 nm.

Circular dichroism

CD spectra of the different enzymes [25 μM in 10 mM phosphate buffer (pH 7.1)], either reduced by DTT or oxidized by an almost equimolar H2O2 concentration, were recorded at 25°C in a quartz cuvette (1-mm pathlength) between 200 and 250 nm, with a bandwidth of 10 nm using a Jobin–Yvon CD6 spectropolarimeter. The mean residue molar ellipticity [θ] was expressed in deg·cm2·dmol−1.

In-gel digestion and MS analysis of the PtGpx5–Trxh1 heterodimer

The formation of heterodimers between PtGpx5 and poplar Trxh1 C42S was induced by incubating a pre-reduced Trxh1 C42S and native (oxidized) PtGpx5 at 1 μg/μl in 50 μl of 50 mM
Tris/HCl (pH 8.0) for 1 h at room temperature. Pre-reduced Trxh1 C42S was obtained after reduction of 500 μM protein using 10 mM DTT for 30 min and desalting on a Sephadex G-25 column. Approximately 7.5 μg of each protein was loaded on to non-reducing SDS/PAGE. The gel band corresponding to the PtGpx5–Trxh1 heterodimer was excised, washed and alkylated by IAM (iodoacetamide) in the presence or absence of DTT. Finally, it was digested in-gel with sequencing grade modified trypsin (Promega/SDS Biosciences) or chymotrypsin (Roche). The resulting peptides were extracted, dried and dissolved in 0.1% formic acid for mass spectrometric analysis.

Peptide analysis was performed by reverse-phase liquid chromatography ESI–MS/MS (tandem MS) using a nanoACQUITY UPLC (ultra-performance liquid chromatography) system coupled to a QTOF (quadrupole time-of-flight) mass spectrometer (Q-TOF Ultima, Waters Corporation). Raw data were processed by ProteinLynx Global Server (version 2.2.5) software for database searches. Proteins were identified by an in-house Mascot server and the Mascot Daemon application (version 2.1.6; Matrix Science, http://www.matrixscience.com) using the Populus protein database. The following settings were used for the database search: trypsin- or chymotrypsin-specific digestion with one missed cleavage allowed, carbamidomethylated cysteine, oxidized methionine, dioxygenated and trioxidized cysteine in variable mode, peptide tolerance of 80 p.p.m., and fragment tolerance of 0.1 Da. Peptides with Mascot ion scores exceeding in variable mode, peptide tolerance of 80 p.p.m., and fragment tolerance of 0.1 Da were extracted, dried and dissolved in 0.1% formic acid for mass spectrometric analysis.

### RESULTS

We previously determined the involvement of two cysteine residues (Cys44 and Cys82, formerly classified as Cys147 and Cys155 when including the plastidial targeting sequence) out of the three conserved cysteine residues in the catalytic mechanism of plant Cys-Gpxs using PtGpx3 as a model [11]. Moreover, we have been able to solve the three-dimensional structure of PtGpx5, a paralogue isoform, in both reduced and oxidized forms, which confirmed first that Cys44 and Cys82 of PtGpx5 are the CysP and CysS, respectively, and which indicated that a large conformational change, i.e. unwinding of the beginning of the α1-helix and of the whole α2-helix, occurs in order to bring the two distant cysteine residues together to form an intramolecular disulfide bond [18]. This change in oxidation state is also associated with a modification of the intrinsic fluorescence of the protein and with a shift in migration in non-reducing SDS/PAGE [11]. In the present study, we have generated different mutated PtGpx5 proteins, substituting first individually all cysteine residues by serine residues (Gpx5 C44S, C73S and C92S variants), and then combining two mutations to generate protein variants in which only CysP (C73/92S) or CysS (C44/73S) remained. In addition, by the aim of dissecting some steps of the catalytic cycle, we have generated other mutated proteins for residues presumably involved in dimerization (Y151R), in Trx recognition (F90E), in the reactivity of CysP (E79Q) and in the instability of α2-helix (D89K).

### Substrate specificities of PtGpx5

Using activity measurements achieved under steady-state conditions, we have first confirmed that, as previously reported for some plant Gpxs, the recombinant form of PtGpx5 cannot appreciably reduce peroxides using glutathione as a reductant (results not shown) [10,11,26]. The kinetic parameters of PtGpx5 have thus been determined using a NADPH/NTR/Trxh1 reduction system and various peroxides. The catalytic efficiency ($k_{cat}/K_m$) of PtGpx5 towards these peroxides is quite variable with values of 133, 78 and 3 × 10$^{-3}$ M$^{-1}$ s$^{-1}$ for H$_2$O$_2$, COOH and t-BOOH respectively (Table 1). The marked difference observed with t-BOOH (a difference of a factor 25 to 45 compared with the other peroxides tested) essentially correlates with a difference in the apparent substrate affinity, with $K_m$ values of 32 and 81 μM for H$_2$O$_2$ and COOH, and 1.9 mM for t-BOOH. The comparison of these values with those determined previously for two different Cys-Gpxs from photosynthetic organisms, PtGpx3 and CrGpxH (Cr is Chlamydomonas reinhardtii), indicates that all three enzymes have very similar properties, t-BOOH being the poorest substrate in all cases (Table 1) [11,27].

We took advantage of the fact that, similarly to PtGpx3, PtGpx5 exhibits fluorescence changes between the reduced and the oxidized state, to study the kinetic of H$_2$O$_2$ reduction independently of the presence of the recycling system. Addition of excess H$_2$O$_2$ to reduced PtGpx5 caused a rapid increase in its intrinsic fluorescence intensity that did not occur when PtGpx5 C44S, a variant mutated on CysP, was used (Figure 1A, inset). Addition of increasing concentrations of H$_2$O$_2$ led to a linear dose-dependent increase of the observed rate constants of PtGpx5 intrinsic fluorescence change (Figure 1A), indicating that the bimolecular reaction between the enzyme and the oxidant was the rate-limiting step of the process leading to the change observed in tryptophan fluorescence. From the slope of the plot, a second-order rate constant of (5 ± 1) × 10$^4$ M$^{-1}$ s$^{-1}$ at pH 7.4 and 25°C was determined. These results were confirmed by measuring the inhibition of HRP compound I formation by PtGpx5. Indeed, reduced PtGpx5 (28.5 μM) caused only a marginal decrease in the yield of HRP (2 μM) oxidation to compound I by H$_2$O (0.65 μM) and a slight increase in the $k_{obs}$...
value of the process \( \text{from } 23 \text{ s}^{-1} \text{ in the absence of PtGpx5} \) (consistent with a rate constant value of \( 1.2 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1} \text{ in agreement with previously reported data [21]} \)) to \( 27.7 \text{ s}^{-1} \), i.e., \( 4.7 \text{ s}^{-1} \) increase in the observed rate constant of HRP compound I formation, that divided into PtGpx5 concentration \( (28.5 \mu \text{M}) \) indicated a rate constant value of \( 1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} \text{ at pH 7.4 and 25°C} \) that is consistent with a rate constant of \( \sim 1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} \) (Supplementary Figure S1 at http://www.BiochemJ.org/bj/442/bj4420369add.htm).

With regard to the apparent affinity of PtGpx5 towards poplar Trxh1, a \( K_m \) value of 5.2 \( \mu \text{M} \) was obtained in steady-state conditions, slightly better than the value determined with PtGpx3 (12 \( \mu \text{M} \)). This led to a \( k_{\text{cat}}/K_{\text{m}} \) of \( 1.33 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1} \). Using intrinsic fluorescence measurements, the kinetics of GSH or Trx oxidation by PtGpx5 was measured by mixing oxidized PtGpx5 or reduced PtGpx5 (as a control) with various amounts of GSH or pre-reduced Trxh1 or Trxh5. These substrates led to changes in PtGpx5 intrinsic fluorescence intensity that fitted to exponential curves, and observed rate constants of the process were linearly dependent on the oxidant concentration, indicating that the bimolecular reaction was rate-limiting the process leading to the observed change in protein fluorescence. With this method, the second-order rate constants of the PtGpx5 reaction with oxidants were \( 56 \pm 5 \text{ M}^{-1} \cdot \text{s}^{-1} (1.00 \pm 0.02) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} \) and \( (4.3 \pm 0.04) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} \) for GSH, Trxh1 and Trxh5 respectively (Figures 1B and 1C).

Next, the capacity of PtGpx5 to reduce peroxynitrite was assessed. When reduced PtGpx5 (10 \( \mu \text{M} \)) was mixed with peroxynitrite (15 \( \mu \text{M} \)), a rapid decrease in peroxynitrite concentration was observed, indicating its very fast reduction by the enzyme (Supplementary Figure S2A at http://www.BiochemJ.org/bj/442/bj4420369add.htm). This decrease was too fast for estimating the initial rate of peroxynitrite decay by stopped-flow techniques (more than 10\% substrate consumption in the first 10 ms). In this context, the rate constant of the reaction of PtGpx5 with peroxynitrite was determined by a competition approach as described in [20]. As expected, reduced PtGpx5 inhibited the yield of peroxynitrite-dependent formation of HRP compound I (Figure 2, inset) and caused a dose-dependent increase in the observed rate constant of HRP compound I formation (Figure 2). The rate constant of peroxynitrite-mediated HRP oxidation was \( 3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} \) at pH 7.4 and 25°C, in agreement with previously reported data [28]. The rate constant of peroxynitrite-mediated PtGpx5 oxidation was \( 1.4 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} \) at pH 7.4 and 25°C.

**Sulfenic acid formation and over-oxidation of the peroxidatic cysteine residue of PtGpx5**

Activity measurements achieved under steady-state conditions with \( \text{H}_2\text{O}_2 \) indicated that the rate of the reaction catalysed by PtGpx5 decreased with time, although substrate amounts were not limiting, suggesting that some enzyme inactivation occurred (results not shown). Hence, we have established a dose-dependent response by increasing the concentrations of \( \text{H}_2\text{O}_2 \) from 50 \( \mu \text{M} \) to 2.5 mM and using, for comparison, a 2-Cys Prx from *A. thaliana*, as this class of Tpxs is known to be sensitive to over-oxidation [29]. For both proteins, a significant loss of activity was observed (Figure 3). Although some inactivation of 2-Cys Prxs was detected at lower oxidant concentrations (visible at 500 \( \mu \text{M} \)), both proteins lost approximately 60\% of activity at a concentration of 2.5 mM. It has been demonstrated for 2-Cys Prxs that this inactivation coincides with the over-oxidation of CysP into sulfinic (SO2H) or sulfonic (SO3H) acids. As PtGpx5 is purified in an oxidized form, the molecular mass of PtGpx5 was analysed by MS, after incubating the protein in the presence of a mixture of DTT and \( \text{H}_2\text{O}_2 \) at different concentrations for a period of 5 min. In the presence of DTT, only one peak with a molecular mass of 19281 Da was detected. This is consistent with the theoretical mass of PtGpx5 with a cleaved N-terminus.
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Figure 2 Kinetics of peroxynitrile reduction by PtGpx5

Peroxynitrile (1.2 μM) in 10 mM NaOH was rapidly mixed with HRP (5 μM) in the absence or presence of increasing concentrations of reduced PtGpx5 in 100 mM sodium phosphate buffer (pH 7) plus 0.1 mM DTPA. The final pH measured at the outlet was 7.4 ± 0.1. The time courses of HRP compound I formation were followed at the Soret band. The inset shows the experimental traces corresponding to HRP compound I formation without PtGpx5 (control) and with 7.3 μM reduced PtGpx5. Experimental data were fitted to single exponentials from which observed rate constants of HRP compound I formation were determined. The latter were plotted against PtGpx5 concentrations.

Figure 3 Inhibition of the peroxidase activity of PtGpx5 by increasing H2O2 concentration

Peroxidase activity was measured under steady-state conditions using the Trx reduction system by measuring the rate of NAPDH oxidation between 30 s and 1 min after the addition of Tpxs. Results are represented as a percentage of the maximal activity (100%) obtained at 50 μM H2O2 for At2-Cys Prx (open bars) and at 500 μM H2O2 for PtGpx5 (closed bars).

methionine residue. Following H2O2 incubation, two other protein peaks (19 313 and 19 330 Da), presenting mass increments of ∼32 or ∼49 Da, were detected, probably indicating the presence of two or three additional oxygen atoms, and suggesting that one or several cysteine residues have been over-oxidized.

In order to identify whether the cysteine residues are indeed the target of oxidation, and which of the two cysteine residues involved in the catalytic mechanism is the most sensitive, the redox state of the Cys44 and Cys92 was analysed using the C73/92S and C44/73S variants respectively, by incubating 50 μM reduced proteins with different peroxide concentrations (250 μM, 500 μM and 5 mM) for 1 min. Subsequently, the proteins were incubated with NBD-Cl, a compound which reacts with thiol groups and sulfenic acids, but not with sulfonic or sulfonic forms. The covalent attachment of NBD-Cl generates an absorption band which peaked at 415 nm upon reaction with thiol groups, whereas it peaked at 350 nm upon reaction with sulfenic acids. Following reaction with NBD-Cl, the absorption spectra of both reduced mutant proteins exhibited the expected absorption band at 415 nm (Figure 4). From the absorbance, the levels of NBD-Cl adducts detected are consistent with the full alkylation of all cysteine residues. Treatment of the C73/92S mutant with 250 μM H2O2 led to the appearance of the absorption band specific to the reaction of NBD-Cl with sulfenic acids, indicating that Cys44 or Cys92 is readily oxidized to a sulfenic form. The decrease or the disappearance of this signal upon treatment with 500 μM and 5 mM H2O2 respectively, probably indicates that Cys92 is over-oxidized to sulfonic or sulfonic acid forms. Consistently, MS analysis of the C73/92S variant treated with 5 mM DTT, 50 μM H2O2 and 1 mM H2O2 identified protein species with molecular masses of 19 249 Da, 19 266 Da (+17 Da) and 19 281 Da (+32 Da) respectively. For the C44/73S variant,
no oxidation has been observed on CysR, whatever the H₂O₂ concentration used.

pKₐ of the catalytic cysteine residues and midpoint redox potential of the catalytic disulfide

The catalytic efficiency of thiol-dependent oxidoreductases largely depends on the reactivity of the catalytic cysteine residue which is usually made more acidic by the protein environment and on the midpoint redox potential (Eₘ) of the catalytic disulfide, if any. In order to investigate the redox properties of PtGpx5, both parameters have been determined. The midpoint redox potential (Eₘ) of the catalytic disulfide was determined with DTT at various dithiol/disulfide ratios. After prolonged incubation, mBBr, a fluorescent thiol probe, can react with free thiol groups. Titration data were fitted by non-linear regression to the Nernst equation setting the value of n as 2, as expected for a disulfide/dithiol two-electron transfer process (Figure 5A). The average Eₘ of three independent experiments was −286 ± 5 mV at pH 7.0.

The pKₐ of the thiol groups of Cys₄⁴ and Cys₉₂ has been measured using a thiol-cleavable fluorophore, named PDT-bimane, on the basis of the fact that the thiolate deprotonated form of cysteine only weakly reacts with this compound [25]. From the plot of the Iₜ/₀ of half-maximal release of pyridyl-2-thiolate against pH, the pKₐ of Cys₄⁴ (CysP) is 5.23 ± 0.02 and the pKₐ of Cys₉₂ (CysR) is 7.66 ± 0.09 (Figure 5B). Residues forming the catalytic tetrad in Gpxs have been shown to be essential components for Gpx reactivity and to contribute to the low pKₐ value of CysP [9,13,15]. Amino acid sequence comparisons indicate that, beyond CysP and among the three other residues, the residue in position 79 of PtGpx5 or in the equivalent position in other Gpxs is the most variable; most SeCys-Gpxs or Cys-Gpxs possess a glutamine, whereas PtGpx5 has a glutamate. Hence, we have explored whether this variation could influence protein reactivity by measuring first both the pKₐ of CysP and the redox potential of the catalytic disulfide in the E79Q variant. However, both parameters were not significantly altered with a pKₐ of Cys₉₂ of 5.32 ± 0.06 and an Eₘ value of −281 ± 5 mV (Figures 5A and 5B). With regard to peroxide reduction, the Eₗₐ₉/qₗ₉ₐ substitution only slightly affected the apparent affinity and turnover number for H₂O₂ and COOH compared with the WT enzyme, whereas it had a dramatic effect on the apparent affinity for t-BOOH and thus on catalytic efficiency (Table 1). This suggests that Glu79 is important for peroxide recognition, maybe playing some role in determining substrate preference.

The unwinding of α₂-helix is promoted by the oxidation of CysP

From the structural comparison of the reduced and oxidized form of PtGpx5, it has been proposed that the observed helix-coil transition could be linked to a helical instability conferred by the thiolate form of CysP and by two negatively charged residues (Asp⁸⁹ and Asp⁹⁹) located on the same face of three helical turns [18]. Thus we hypothesized that the mutation of one of these residues could block the unwinding of α₂-helix. However, the substitution of the Asp⁹⁹ by a residue with an opposite charge (D89K variant) had no effect on the kinetic parameters, probably indicating that none of the different steps of the catalytic mechanism are appreciably affected (Table 1).

Next, to investigate what causes this structural rearrangement, the secondary structure content of PtGpx5 and of some of the cysteineic mutants was analysed using CD. Figure 6(A) shows the spectra of PtGpx5 WT either in the reduced or in the oxidized state, generated by adding a slight excess of H₂O₂. Although the α-helical and β-sheet contents deduced from these spectra do not correspond to the percentage obtained from the three-dimensional structure, the strong modification of the spectrum for the oxidized form at 222 nm, confirmed the variation in α-helical content of PtGpx5 depending on its redox state (Figure 6A). Recently, the same difficulties for correlating secondary structure contents between CD spectra and three-dimensional structure have been encountered for a Gpx-like tryparaedoxin peroxidase [30]. Hence, since for unknown reasons the percentage of secondary structures cannot be determined accurately, only the qualitative aspects will be considered in these experiments. Similar experiments have been performed with the C73/92S and C44/73S variants (Figures 6B and 6C). Although similar modifications have been obtained in the CD spectra of the C73/92S variant in the oxidized forms, there was no change in the variant retaining only Cys⁹₂ and where Cys⁴⁴ is absent. These data clearly show that it is the change of the redox state of the catalytic residue and presumably the formation of the sulfenic acid, but not substrate binding, which governs the unwinding of α₂-helix.
B. Selles and others

Figure 6 Redox-dependent conformational changes
CD was used to evaluate the variations in α-helix content in PtGpx5 WT (A), C73/92S (B) or C44/73S (C). Spectra were recorded in 10 mM phosphate buffer (pH 7.1) with 25 μM pre-reduced protein either maintained reduced by 150 μM of DTT (broken lines) or oxidized by 75 μM H₂O₂ (continuous lines).

Dimerization is not required for Gpx5 activity
In either reduced or oxidized crystal structures of PtGpx5, Tyr¹⁵¹ is found at the interface between the two subunits of the dimer, in a position possibly contributing to its stabilization. Thus the oligomerization state of a Y151R variant was evaluated by analytical gel filtration in comparison with the WT enzyme. From the theoretical mass of 19.2 kDa, the results indicated that the WT enzyme is essentially in a dimeric form (∼95%), whereas a minor form corresponded to a small fraction of tetramers (∼5%) (results not shown). Unlike PtGpx5 WT and other variants tested, the majority (∼95%) of the Y151R variant eluted at an apparent mass corresponding to a monomer, whereas a minor form (∼5%) still corresponds to a dimer. With regard to the kinetic parameters, this mutation altered the affinity and the catalytic efficiency for the reduction of COOH compared with WT Gpx5 (0.739 mM and 5 × 10⁻⁴ M⁻¹ · s⁻¹ compared with 0.081 mM and 7.8 × 10⁻³ M⁻¹ · s⁻¹), and it also affected to a lesser extent the apparent affinity for t-BOOH (Table 1). On the other hand, no significant difference was observed between WT or Y151R proteins with regard to kinetic parameters for H₂O₂, and the apparent affinity for Trxh1, with Kₘ values for the latter of 5.2 μM and 3.7 μM respectively. Overall, these data point out to two important properties of Gpx5, the dimeric form is not required for its interaction with Trxh1, but it might be important for the reduction of some complex or large peroxides, such as those with an aromatic cycle.

Role of Phe⁹⁰ and Cys⁹² in the interaction with Trx
From molecular docking experiments, we previously built two complex models for the interaction between Trxh1 and PtGpx5 with a transient intermolecular disulfide bond formed between the catalytic Cys⁹⁰ of poplar Trxh1 and either Cys⁴⁴ (Cysₚ, complex 1) or Cys⁹² (Cysᵣ, complex 2) of the oxidized PtGpx5 [18]. Although both complexes were found to be structurally possible, complex 2 proved to be more stable from the calculated free energy. In addition, in both cases, the aromatic ring of Phe⁹⁰ was found to interact perpendicularly with its edge pointing towards the tryptophan residue of the active site Trxh1 (W⁹⁰CPPC₄₂ active site). An F90E variant, substituting the aromatic side chain by a negatively charged residue has been constructed and analysed. The apparent affinity of the F90E mutant for Trxh1 is substantially modified with a Kₘ value of 25.4 μM compared with the value of 5.2 μM obtained with PtGpx5 WT (Table 1). In order to identify which of Cysₚ or Cysᵣ of PtGpx5 forms the transient disulfide with Cys₉⁰ of Trxh1, we have formed heterocomplexes between a Trxh1 C42S variant and an oxidized PtGpx5 (Figure 7). In order to separate the PtGpx5–Trxh1 adduct from the monomeric and oligomeric forms of both partners, the mixture was separated on non-reducing SDS/PAGE and the band corresponding to this adduct (present in lane 6 in Figure 7) was analysed by MS after in-gel digestion. The gel band was cut into small pieces and divided into two separate pools. One pool was first treated with DTT to reduce all disulfide bonds within the protein adduct followed by IAM treatment to block all cysteine residues (carbamidomethylation), whereas the other pool was not treated with DTT but only IAM, meaning that only free cysteine residues should be carbamidomethylated and cysteine residues involved in the covalent adduct remain unaffected. The tryptic peptide of PtGpx5 containing Cys⁴⁴ was present in both samples, whereas the tryptic peptide of PtGpx5 containing Cys⁹² and the one of Trxh1 containing Cys₉⁰ were present only in the first sample. For reasons presumably related to peptide separation or detection, these two latter peptides supposed to be covalently linked in

Lanes 1 and 4, Trxh1 C42S; lanes 2 and 5, PtGpx5; and lanes 3 and 6, mixture of oxidized PtGpx5 and Trxh1 C42S. Lanes 1–3 correspond to reducing conditions and lanes 4–6 correspond to non-reducing conditions. The monomers and heterodimer are indicated by arrows. The molecular mass in kDa is indicated on the left-hand side.
sample 2 were not detected. Hence peptides extracted from this second pool and originally treated with IAM only were further analysed by MS after reduction and alkylation. In this case, both peptides absent in the non-reduced sample were now detected, confirming that the covalent adduct is formed between Cys\(^{39}\) of Trxh1 and Cys\(^{86}\) of PtGpx5. The peptide spectra with their corresponding sequences are shown in Supplementary Figure S3 (at http://www.BiochemJ.org/bj/442/bj4420369add.htm).

**DISCUSSION**

Plant Cys-Gpxs belong to multigenic families composed of five to eight members. Although PtGpx5 is predicted to be secreted, thus presumably residing in the apoplast, there are other predicted or confirmed paralogues in other subcellular compartments, e.g. cytosol, plastids and mitochondria. In all of these compartments, there is evidence for the presence of H\(_{2}\)O\(_{2}\) and potentially of peroxynitrite, taking into account the presence of sources of superoxide anion, the diffusion capability of NO and the fast reaction between both radicals. Thus peroxynitrite-decomposing systems are likely to be essential in plants to prevent deleterious effects or to tightly control signalling processes involving protein tyrosine nitration or cysteine oxidation/nitrosation. In the present study, we have demonstrated, using steady-state and pre-steady-state activity measurements, that PtGpx5 possesses catalytic efficiencies in the 10\(^{6}\), 10\(^{5}\), 10\(^{4}\) and 10\(^{3}\) M\(^{-1}\) s\(^{-1}\) ranges toward peroxynitrite, H\(_{2}\)O\(_{2}\), COOH and t-BOOH respectively. To our knowledge, this is the first demonstration that Cys-Gpxs are competent for peroxynitrite reduction. Particularly in plants, the results of the present study indicate that Gpxs may constitute an efficient system for peroxynitrite reduction in potentially all compartments and, in plastids, it could represent an alternative to two Prxs (2-Cys Prx and Prx IIE) previously shown to possess peroxynitrite reductase activity [7,8]. The value of catalytic efficiency for the reduction of peroxynitrite is in the same range as that previously described for some Prxs, whereas the value for the reduction of H\(_{2}\)O\(_{2}\) is similar to human PrxV, an atypical 2-Cys Prx, to DmGpx (Dm is *Drosophila melanogaster*) and to *Mycobacterium tuberculosis* AhpE, but lower than that reported for some 2-Cys Prxs (yeast Tsa1 and 2, *Salmonella typhimurium* AhpC) and for XIPrxQ\(^{\beta}\) (Xf is *Xylella fastidiosa*) [14,20,31–34].

**Dimerization is important for organic peroxide reduction, but not essential**

The dimeric organization of PtGpx5 contrasts with the homotetrameric organization of SeCys-Gpxs (mammalian Gpx1, Gpx2 or Gpx3) or the monomeric nature of mammalian Gpx4, DmGpx and TbPxII and TbPxIII (Tb is *Trypanosoma brucei*) [14,15,35,36]. It was previously proposed that a monomeric state is required for its interaction with Trxs [14]. The Trx-dependent recycling of the dimeric PtGpx5 indicates that this assumption is also valid for plant dimeric Cys-Gpxs. However, contrary to *Escherichia coli* Tpx for example, PtGpx5 is not an obligate homodimer since a monomeric Y151R variant exhibited the same affinity for Trxh1 as the WT enzyme [17]. This variant has nevertheless an impaired activity especially with complex substrates presenting a benzene cycle (COOH) or a ramified carbon chain (t-BOOH), but not H\(_{2}\)O\(_{2}\), which could indicate that the native dimeric organization of plant Cys-Gpxs might be more essential for peroxide than for Trx recognition. Quite similarly, a single amino acid substitution affecting the decameric organization of *S. typhimurium* AhpC showed that the dimeric form is less efficient for peroxide reduction [33].

**Reactivity of Cys\(_{39}\) and conservation of the catalytic tetrad between SeCys- and Cys-Gpxs**

The hallmark of most SeCys- or Cys-Gpxs is the presence of the so-called catalytic tetrad formed by Cys\(^{34}\), Glu\(^{19}\), Trp\(^{13}\) and Asn\(^{14}\) (PtGpx5 numbering, Supplementary Figure S4 at http://www.BiochemJ.org/bj/442/bj4420369add.htm). These residues are thought to modulate Cys\(_{39}\) reactivity by stabilizing the deprotonated form of Cys\(_{39}\), thus decreasing its pKa and stabilizing the transition state of the reaction [9]. However, in some enzymes, their mutation does not completely abolish enzymatic activity and in some three-dimensional structures they are simply not located in the active site [15,26,37,38]. Instead, in TbPxIII, three conserved lysine residues seem important for substrate binding [37].

Except for the replacement of the glutamine residue by a glutamate in PtGpx5, as well as in some specific plant orthologues, the residues forming the catalytic tetrad are conserved in plant Cys-Gpxs (Supplementary Figure S4). In human Gpx4, the substitution of this Glu\(^{14}\) by a glutamate residue decreased the activity of a Gpx4 SeCys-to-Cys variant by a factor of 5 [36]. Accordingly, the opposite substitution in PtGpx5 resulted in an increased catalytic efficiency from a factor of 2 for H\(_{2}\)O\(_{2}\) to a factor of 50 for t-BOOH (Table 1).

In the case of t-BOOH, the E79Q substitution enhances the apparent substrate affinity, decreasing the Km by a factor of 30, but it does not modify both the midpoint redox potential and the pKa of Cys\(_{39}\). This suggests that the nature of this residue is important for the accessibility of some organic hydroperoxides inside the active-site pocket. The pKa value measured for Cys\(_{39}\) in PtGpx5 (5.23) is in the range of those determined for other Tpxs such as *S. typhimurium* AhpC, yeast Tsa1 and Tsa2, XIPrxQ\(^{\beta}\) or yeast Orp1/Gpx3 [13,20,31,34,39,40]. However, it is worth mentioning that decreasing the pKa of the thiol group below 6.4, where 90% of the enzyme would be deprotonated anyway, would not necessarily be favourable, since thioclates with lower pKa are less nucleophilic than others [41]. Instead, the specialized and efficient peroxidase activity of Prxs is thought to rely on transition state stabilization [42].

**The formation of the sulfenic acid is responsible for the PtGpx5 conformational rearrangement**

Following an aerobic purification, PtGpx5 is found in an oxidized state and a reduced enzyme is very rapidly re-oxidized without any oxidative treatment indicating that, in the absence of a reducing system, the enzyme is more stable in the oxidized state. However, an unresolved question in the case of PtGpx5 was to know whether the binding of the substrate is sufficient to promote the unwinding of α2-helix, or whether the formation of the sulfenic acid is required. In the present analysis, we have demonstrated, using CD, that the oxidation of Cys\(_{39}\) triggered this helix-to-coil transition, as observed for XIPrxQ\(^{\beta}\) [31].

Some differences in structural behaviour have been reported for a number of Cys-Gpxs. The structures of the oxidized and reduced *T. brucei* PxIII, determined by NMR, are very similar [r.m.s.d. (root mean square deviation) of 0.59 Å (1 Å = 0.1 nm)] suggesting that there is no structural rearrangement during the catalytic act [37]. However, the measurement of the secondary structure of WT PxIII by CD is contradictory as it showed that reduction increased the overall helicity of the protein [30]. In the same study, it was also suggested, from structural analyses of a C76S mutated version of *T. brucei* PxIII, that this conserved cysteine residue (equivalent to Cys\(^{39}\) in PtGpx5), albeit not affecting catalytic activity, might be important for the occurrence of these conformational changes [30]. Indeed, in this mutant, no drastic
conformational change seems to be required for CysP–CysR disulfide formation. However, in the case of PtGpx5, the C73S variant still exhibits redox-dependent conformational changes as assessed by gel-shift migration and from its fluorescence properties (results not shown).

Strikingly, the resolution of the three-dimensional structure of *T. brucei* PrxII, which is nearly identical with the PrxIII isoform indicated on the contrary that, in the reduced state, the cysteine residues are very distant, as in PtGpx5 [35]. This apparent discrepancy has been tentatively explained by the existence of a certain structural plasticity in these enzymes. This is illustrated by the unstructured aspect of the active site in a reduced TcGpx1 (Tc is *Trypanosoma cruzi*) enzyme, which led to the proposal that the reduced forms of Cys-Gpxs can adopt multiple conformations that exchange on the microsecond to millisecond timescale [38].

In view of all these observations and the present data, we propose the following sequence of structural events. (i) In some Gpxs, such as TbTxIII or TcGpx1, we hypothesize that substrate binding promotes the transition from a reduced unstructured form to a catalytically competent reduced form in which the α2-helix is present. In the case of TbTxII or PtGpx5 this competent, more ‘stable’ form might have been favoured by the crystallization conditions. Then, the oxidation of CysP to a sulfenic acid, concomitant to peroxide reduction, triggers α2-helix unwinding, allowing the conformational rearrangement to occur and the formation of the intramolecular disulfide bridge.

**The Trx-dependent regeneration of Cys-Gpxs**

Previous studies on Tpx–Trx interactions showed that in the case of the Cys-Gpx from *D. melanogaster* and of *T. brucei* PrxIII, the regeneration of the internal disulfide bridge proceeds via the formation an intermediate heterocomplex formed between the two partners and involving the catalytic cysteine residue of Trx and CysR of Tpx. In contrast, in the case of poplar Prx II or *M. tuberculosis* thioredoxin peroxidase, the transient complex also involved the catalytic cysteine residue of Trx, but CysP of Tpx [43,44]. Using a pre-reduced Trx mutated on the second active site cysteine residue, stable dead-end intermediates have been obtained by reaction with an oxidized WT PtGpx5. MS analysis of the heterocomplex indicated that, in the case of this plant Cys-Gpx, the disulfide is formed between CysS or CysR of PtGpx5 and the catalytic cysteine residue of TrxH1. The mutational analysis of Phe90 confirmed its implication for Trx recognition. This suggests that the catalytic cysteine residue of Trxh1. The mutational analysis of Phe90 confirmed its implication for Trx recognition. This study demonstrated the ability to regulate gene expression in response to H2O2 [45]. In *Schizosaccharomyces pombe*, a similar mechanism probably occurs for the TpxI-dependent activation of Pap1 at low H2O2 levels [49]. However, at higher peroxide concentrations, the inactivation of Tpx1 by oxidation of its catalytic cysteine residue to a sulfenic acid prevents such a redox relay [49]. Whether this regulation also occurs for ScGpx3 (Sc is *S. cerevisiae*) is not known. In this context, the observation of irreversible cysteine oxidation forms on a plant Cys-Gpx could be physiologically relevant and could provide an additional potential mediator for H2O2 signalling pathways, although no similar API-dependent regulation mechanism has so far been described in plants. Instead, it has been shown that *A. thaliana* Gpx3, by physically interacting with a protein phosphatase 2C named ABI2 ([ABA (abscisic acid) INSENSITIVE 2], can regulate the redox state and thus the activity of ABI2, probably relaying the H2O2 signal coupled to ABA signalling in response to drought stress [50].

A crucial point for such regulation, especially for Tpxs using a recycling cysteine residue in their catalytic mechanism, is the lifetime of the sulfenic acid moiety, as it is usually considered as a short-lived species. All enzymes implicated so far are of the 2-Cys Prx or Cys-Gpx type. Interestingly, the formation of the inter- or intra-molecular disulfide bridge formed during the regeneration of these enzymes is accompanied by a structural rearrangement, whose rate might explain, at least partly, the stability of the sulfenic acid in these proteins. Incidentally, it is also very probably responsible of the CysP over-oxidation observed when the H2O2 concentration increases [51]. Alternatively, sulfenic acids can be stabilized in specific protein environments. The chemical trapping of the sulfenic acid intermediate in a WT Tpx has only been achieved recently for yeast Gpx3, further supporting the view that the protein context is important and that not all Tpxs can act as peroxide sensors [47].

Besides their roles as peroxidase or peroxide sensor components, Prxs also undergo a functional change upon over-oxidation of their catalytic cysteine residue, e.g. they acquire a molecular chaperone activity subsequent to the formation of high-molecular-mass complexes. In plants, Tpx over-oxidation has been observed both for 2-Cys Prx and 1-Cys Prx [29,52]. Concerning the 2-Cys Prx, it has been shown that the redox state of the protein affected its subcellular localization, with the reduced or over-oxidized decameric forms being attached to the thylakoids and the oxidized dimeric form being released in the stroma for Trx reduction [29]. Overall, all of these observations support the view that the ability of Cys-Gpxs to adopt different redox states (reduced, oxidized or over-oxidized) in response to different oxidative conditions could dramatically modify their biochemical properties and thus their physiological role and interaction network.

**Physiological relevance of the sulfenic, sulfinic and sulfonic acid forms detected on PtGpx5**

Besides their peroxidase activity, it has been shown that some Tpxs display peroxide signalling functions. This has been well illustrated recently by showing that yeast cells lacking the eight Tpxs (five Prxs and three Cys-Gpxs) have lost their ability to regulate gene expression in response to H2O2 [45]. For instance, the Tpx-dependent perception of peroxide by AP1 (activator protein 1) transcription factors in several yeast species has been very well documented. In *Saccharomyces cerevisiae*, the detailed characterization of the Orp1/Gxp3-dependent activation of Yap1 occurs through a redox relay consisting of the conversion of the sulfenic acid generated on these Tpxs from their reaction with H2O2, into an intramolecular disulfide on AP1 transcription factors which would mask the nuclear export signal, thus promoting its nuclear activity [13,46–48]. In *Schizosaccharomyces pombe*, a similar mechanism probably occurs for the Tpx1-dependent activation of Pap1 at low H2O2 levels [49]. However, at higher peroxide concentrations, the inactivation of Tpx1 by oxidation of its catalytic cysteine residue to a sulfenic acid prevents such a redox relay [49]. Whether this regulation also occurs for ScGpx3 (Sc is *S. cerevisiae*) is not known. In this context, the observation of irreversible cysteine oxidation forms on a plant Cys-Gpx could be physiologically relevant and could provide an additional potential mediator for H2O2 signalling pathways, although no similar API-dependent regulation mechanism has so far been described in plants. Instead, it has been shown that *A. thaliana* Gpx3, by physically interacting with a protein phosphatase 2C named ABI2 ([ABA (abscisic acid) INSENSITIVE 2], can regulate the redox state and thus the activity of ABI2, probably relaying the H2O2 signal coupled to ABA signalling in response to drought stress [50].

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**AUTHOR CONTRIBUTION**

Benjamin Selles carried out most of the experimental work as a part of his Ph.D. thesis, Martin Hugo and Madia Trujillo were involved in enzymatic assays and analysis of kinetic parameters. Vaibhav Srivastava assisted with the MS analysis under the supervision of Gunnar Wingele. Nicolas Rouhier was the principal investigator on the project and designed the experiments together with Jean-Pierre Jacquot, Madia Trujillo and Rafael Rada, who brought their respective expertise.

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SUPPLEMENTARY ONLINE DATA
Hydroperoxide and peroxynitrite reductase activity of poplar thioredoxin-dependent glutathione peroxidase 5: kinetics, catalytic mechanism and oxidative inactivation

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Figure S1 Kinetics of H2O2-mediated PtGpx5 oxidation by a competition approach
The time course of HRP compound I formation by the reaction of H2O2 (0.65 μM) with HRP (5 μM) in the absence (continuous line) or presence of reduced PtGpx5 (28.5 μM) (broken line), in 100 mM sodium phosphate buffer plus 0.1 mM DTPA (pH 7.4 and 25 °C) was followed at the Soret band.

Figure S2 Rapid reduction of peroxynitrite by reduced PtGpx5
Peroxynitrite (20 μM) in 10 mM NaOH was rapidly mixed with 100 mM sodium phosphate buffer plus 0.1 mM DTPA (pH 7.2) without further addition (continuous line, control) or reduced PtGpx5 (10 μM) in the same buffer (broken line), at 25 °C. Peroxynitrite decay was measured at 310 nm. The final pH measured at the outlet was 7.4 ± 0.1.

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Figure S3  Trypsin-digested peptide analysis of the PtGpx5–Trxh1 heterodimer

The band corresponding to the PtGpx5–Trxh1 heterodimer was cut from SDS/PAGE and in-gel digested after reduction by DTT and alkylation by IAM (A), after IAM alkylation only (B) or after IAM alkylation and another round of DTT reduction and IAM alkylation (C).
Catalytic mechanism of poplar Gpx5

Figure S4 Amino acid sequence alignment of plant Cys-Gpxs compared with characterized SeCys- and Cys-Gpxs

Alignment was made with ClustalW. Amino acids strictly and partially conserved have a black and grey background respectively. The residue numbering corresponds to PtGpx5. The residues forming the catalytic tetrad are indicated by a star and those mutated in the present study in PtGpx5 are indicated by an arrow. The N-terminal part of this alignment has been manually adjusted. Pt, Populus trichocarpa (PtGpx1, UniProtKB gene name POPTR_0006s28120; PtGpx2, UniProtKB gene name POPTR_0007s02160; PtGpx3, UniProtKB gene name POPTR_0003s12620; PtGpx4, UniProtKB gene name POPTR_0014s13490; and PtGpx5, UniProtKB gene name POPTR_0001s09280); At, Arabidopsis thaliana (AtGpx1, AT2G25080; AtGpx2, AT2G31570; AtGpx3, AT2G43350; AtGpx4, AT2G48150; AtGpx5, AT3G63080; AtGpx6, AT4G11600; AtGpx7, AT4G31870; AtGpx8, AT1G63460); Rc, Ricinus communis (RcGpx, GenBank® accession number XP_002509791.1); Vv, Vitis vinifera (VvGpx, GenBank® accession number XP_002272936.1); Hs, Homo sapiens (HsGpx1, GenBank® accession number NP_000572.2; HsGpx2, GenBank® accession number NP_002074; HsGpx3, GenBank® accession number NP_002075.2; HsGpx4, GenBank® accession number AAH46163; and HsGpx5, GenBank® accession number NP_001500.1); Dm, Drosophila melanogaster (DmPHGpx, GenBank® accession number NP_728869); Bt, Bos taurus (BtGpx1, GenBank® accession number NP_776501.1); Tb, Trypanosoma cruzi (TbGpx1, GenBank® accession number CAC85914.1); Tb, Trypanosoma brucei (TbTrpxI, GenBank® accession number XP_845737.1; TbTrpxII, GenBank® accession number XP_845738.1); Sc, Saccharomyces cerevisiae (ScGpx1, GenBank® accession number NP_012899.1; ScGpx2, GenBank® accession number NP_009803.1; and ScGpx3, GenBank® accession number NP_012303.1).
**Table S1  Primers used in the present study**

The primers used for cloning possess NcoI or BamHI restriction sites (underlined). Codons carrying the mutations are indicated in bold in the mutagenic primers. for, forward; rev, reverse.

<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide sequence (5′→3′)</th>
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<tbody>
<tr>
<td>PtGpx5 for</td>
<td>CCCCCCATGGCAACCCAAACCTCA</td>
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<tr>
<td>PtGpx5 rev</td>
<td>CCCCCGATCCCTCATGAGATCTGCCAGCAGTTCC</td>
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<tr>
<td>PtGpx5 C44S for</td>
<td>AACGTTGGCTCAAGAACCCAAACCTCA</td>
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<tr>
<td>PtGpx5 C44S rev</td>
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