Redox Regulation of a Soybean Tyrosine-Specific Protein Phosphatase†

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ABSTRACT: Plant protein tyrosine phosphatases (PTPs) are important in regulating cellular responses to redox change through their reversible inactivation under oxidative conditions. Studies on the soybean (*Glycine max*) *Gm*PTP have shown that, compared with its mammalian counterparts, the plant enzyme is relatively insensitive to inactivation by H_2O_2 but hypersensitive ($k_{inact} = 559\ M^{-1}\ s^{-1}$) to S-glutathionylation (thiolation) promoted by the presence of oxidized glutathione (GSSG). Through a combination of chemical and mutational modification studies, three of the seven cysteine residues of *Gm*PTP have been identified by mass spectrometry as being able to inactivate the enzyme when thiolated by GSSG or alkylated with iodoacetamide. Conserved Cys 266 was shown to be essential for catalysis but surprisingly resistant to S-modification, whereas the regulatory Cys 78 and Cys 176 were readily thiolated and/or alkylated. Mutagenesis of these cysteines showed that all three residues were in proximity of each other, regulating each's reactivity to S-modifying agents. Through a combination of protein modification and kinetic experiments, we conclude that the inactivation of *Gm*PTP by GSSG is regulated at two levels. Cys 176 appears to be required to promote the formation of the reduced form of Cys 266, which is otherwise unreactive. When thiolated, Cys 176 immediately inactivates the enzyme, and this is followed by the thiolation of Cys 78, which undergoes a slow disulfide exchange with Cys 266 giving rise to a Cys 78−Cys 266 disulfide. We speculate that this two-tiered protection is required for regulation of *Gm*PTP under highly oxidizing conditions.

In animals, protein tyrosine phosphatases (PTPs)[1] are a superfamily of enzymes with integral roles in controlling the phosphorylation of signaling proteins that regulate cell growth, proliferation, and mobility as well as central metabolism (*1*). A conserved mechanism for regulating their phosphatase activity is the oxidative inactivation of a conserved cysteine which acts as a nucleophile and intermediate phosphate acceptor in the catalytic mechanism (*2*). Oxidation of this reactive cysteine to the respective sulfenic acid derivative by hydrogen peroxide or reactive oxygen species effectively inactivates the enzyme, and this then can initiate signaling cascades. The oxidation of the sulfenic acid can be reversed directly by reduction by glutathione (GSH, γ-Glu-Cys-Gly) or thioredoxin. However, there is a danger that under strongly oxidizing conditions the PTP can be rendered permanently inactive by the conversion of the sulfenic acid to the irreversible sulfinic and sulfonic derivatives (*1*). It is therefore important for this catalytic cysteine to be protected from irreversible oxidation while allowing the inactive sulfenic form to be generated to promote signaling. In mammalian PTPs, three protective mechanisms have been described. In the human enzyme PTP1B, the sulfenic form of the catalytic Cys 215 can react with glutathione to form a mixed disulfide (*3*). Structural studies have also shown that the partially oxidized cysteine can be stabilized through the formation of a sulfenyl amide, in which the sulfur atom is covalently bonded to the N atom of the adjacent Ser 216 (*4*, *5*). In the phosphatase and tensin homologue (PTEN), Cdc25 phosphatases, and low-molecular weight (LMW) PTPs, a further mechanism is employed in which the sulfenic catalytic cysteine forms an intramolecular disulfide, with a closely oriented regulatory cysteine (*6*−*8*). In all cases, these modifications result in a transiently inactivated phosphatase that can be reactivated in the presence of GSH and/or thioredoxin.

In plants, the importance of PTPs in regulating signaling events has only recently been uncovered (*9*). Tyrosine phosphorylation in plants was ignored originally due to the apparent absence of classic tyrosine kinases, though recent bioinformatics studies in the model species *Arabidopsis thaliana* have now challenged this view (*10*). It is now known that tyrosine phosphorylation/dephosphorylation is involved in multiple developmental processes (*11*), with PTPs assigned roles in processes as diverse as pollen development (*12*) and stomatal opening (*13*). Specific roles identified include the deactivation of mitogen-activated protein kinases in *Arabidopsis* (*14*−*16*). As is the case in animals, the *Arabidopsis* PTP1 which regulates the activity of MAP kinases was found to be reversibly inhibited by H_2O_2 (*17*). Similarly, a protein phosphatase 2C from *Arabidopsis* was also shown to be

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[1] Abbreviations: DTT, 1,4-dithiothreitol; ESI-TOF MS, electrospray ionization time-of-flight mass spectrometry; *Gm*PTP, *Glycine max* (soybean) protein tyrosine phosphatase; GSH, glutathione (reduced); GSSG, oxidized glutathione; GSNO, *S*-nitrosoglutathione; IPTG, isopropyl β-D-thiogalactopyranoside; PAO, phenylarsine oxide; pNPP, 4-nitrophenyl phosphate; PTP, protein tyrosine phosphatase.

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sensitive to inhibition by H_2O_2 and phenylarsine oxide (PAO), a compound known to bind to vicinal cysteines which are capable of forming disulfides (*18*). The mechanism by which the *At*PTP was redox regulated has not been reported, and as plant cells are subject to large-scale changes in redox potential and ROS generation as a result of, for example, photosynthesis and pathogen attack, we were interested in the protective mechanisms adopted by plant PTPs to prevent irreversible oxidation of the catalytic cysteine. We were particularly interested in studying the regulation of plant PTPs by protein S-glutathionylation (thiolation), as although this post-translational modification is well known to regulate many proteins in animal cells (*19*), very little is known about the respective reactions in plants. Our results suggest that plants have evolved yet another novel mechanism for preventing the irreversible oxidative inactivation of PTP enzyme activity.

MATERIALS AND METHODS

Phosphatase Assays. PTP activity was measured in 100 mM bis-tris-HCl (pH 6.1) containing 2.5 mM 4-nitrophenyl phosphate (*p*NPP) at 30 °C, by following the increase in absorbance at 400 nm due to the liberation of 4-nitrophenol ($\epsilon = 0.22$ mM^{-1} cm^{-1} at pH 6.1). Inhibitor treatments were added to the assays, and the enzyme rate was then measured immediately.

*PTP Mutagenesis and Synthesis. Gm*PTP was cloned as a His-tagged protein in pET24a as described previously (*20*). Cysteine 176 was changed to serine by site-directed mutagenesis of pET-*Gm*PTP using the QuikChange kit (Stratagene) and oligonucleotides caagatggcaaatctggagattattttc and gaaaataatctccagattttgccatcttg. This gave the construct pET-*Gm*PTP-C176S, expressing the enzyme *Gm*PTP-C176S. This mutant and the parent *Gm*PTP were further mutagenised to change Cys 266 to serine by PCR using the mutagenic primer atagtggtgcactgcagtgcaggtattggt and an external primer, utilizing the *Apa*LI restriction site. The mutated fragment was then subcloned into pET-*Gm*PTP and pET-*Gm*PTP-C176S, to give the constructs pET-*Gm*PTP-C266S (expressing *Gm*PTP-C266S) and pET-*Gm*PTP-C176/266S (expressing *Gm*PTP-C176/266S), respectively. Finally, Cys 78 was mutated by PCR using the mutagenic primer caaattgacgtcgtcatacgcgacagtacttcttctcctcatctcgc and an external primer, utilizing the *Aat*II restriction site. The mutated fragment was subcloned into pET-*Gm*PTP and pET-*Gm*PTP-C176S, to give pET-*Gm*PTP-C78S (expressing *Gm*PTP-C78S) and *Gm*PTP-C78/176S (expressing *Gm*PTP-C78/176S), respectively. Each construct was transformed into *Escherichia coli* strain BL21(DE3) and grown in LB broth at 37 °C to mid-log phase. Expression was induced by addition of IPTG (1 mM), and cells were harvested by centrifugation 3 h later and the recombinant *Gm*PTPs affinity purified by Ni^{2+}–iminodiacetic acid chromatography (*20*).

PTP Modification. Freshly purified protein was fully reduced in 10 mM DTT. Samples were desalted by gel filtration (Pharmacia HiTrap 5 mL desalting column) into 20 mM Tris-HCl (pH 7.8) and then immediately treated with one of the following for 20 min at 4 °C: (a) 2 mM DTT, (b) 2 mM oxidized glutathione (GSSG), (c) 2 mM GSSG followed by 10 mM DTT, (d) 2 mM *S*-nitrosoglutathione (GSNO), and (e) 10 mM iodoacetamide. GSNO was prepared by the method of Hart (*21*). Following treatment, samples were desalted into either 2 mM Tris-HCl (pH 7.8) or 10 mM ammonium acetate and prepared for MS analysis by addition of acetonitrile to 50% (v/v) and formic acid to 0.1% (v/v).

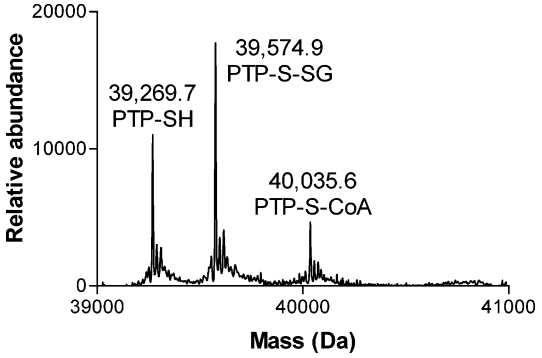

FIGURE 1: Deconvoluted mass spectrum of recombinant *Gm*PTP-His purified by Ni^{2+} chromatography from *E. coli*, showing masses consistent with an unmodified protein (PTP-SH) and adducts thiolated with glutathione (PTP-S-SG; 305.3 Da) and coenzyme A (PTP-S-CoA; 765.5 Da).

Mass Spectrometry. Protein samples were injected directly into a Micromass LCT time-of-flight (TOF) mass spectrometer, using electrospray ionization (ESI) at a flow rate of 0.1 mL/min. In positive ion mode, mass spectrometry (MS) data were collected in the mass range of 500−2000 Da and analyzed using the supplied MassLynx software, with multiply charged peaks deconvoluted using the MaxEnt1 plug-in after calibration with horse heart myoglobin. Trypsin digests were performed in 10 mM ammonium acetate (pH 7.2) for 16 h at 37 °C, using sequencing-grade modified trypsin (Promega), and analyzed by MS as described for the parent polypeptides.

Homology Modeling. The *Gm*PTP structure was predicted using the SWISS-MODEL automated homology modeling server (http://swissmodel.expasy.org/) using the full-length sequence. Similarity to PTP structures in the database extends from residue 74 to cover the catalytic domain as the amino-terminal region has previously been shown to be restricted to plant PTPs (*20*). The most similar PTPs have 35−40% sequence identity and BLASTP *e* values ranging from 10^{-34} to 10^{-39} which are sufficiently high for homology modeling. The model was built using the following PDB coordinates for PTPs: 1FPRA, 1GWZ, 2SHPA, 2SHPB, and 1C83A. Reactive cysteines were depicted using PyMOL.

RESULTS

Identification of Reactive Cysteines in PTP. Previous work determined that recombinant *Gm*PTP required the addition of a reducing agent to activate the enzyme following initial extraction and purification from the bacterial lysate (*20*). This suggested that like other PTPs, the enzyme had an essential catalytic cysteine residue that was sensitive to oxidation. To determine why reducing agents activated recombinant *Gm*PTP, the freshly isolated pure protein was analyzed by ESI-TOF MS (Figure 1). Following deconvolution, three polypeptide species could be identified, corresponding to native *Gm*PTP (assuming cleavage of the N-terminal methionine residue = 39 269.7 Da), a major adduct (*Gm*PTP + 305.3 Da), and a lesser adduct (*Gm*PTP + 765.5 Da). Following treatment of *Gm*PTP with DTT, the only observed mass species cor-

Table 1: Effect of Thiolation and S-Alkylation on *Gm*PTP and Mutant Derivatives[a]

		observed mass following treatment (Da)			
enzyme	enzyme activity (nkat mg^{-1})	unmodified	with GSSG	with GSNO	with iodoacetamide
PTP	5960	39 269.0	39 574.3 (83%) [+1 SG] 40 186.5 (17%) [+3 SG]	40 185.3 [+3 SG]	39 327.7 (65%) [+1 IA] 39 441.1 (35%) [+3 IA]
PTP-C266S	0	39 254.1	39 865.3 [+2 SG]	39 865.3 [+2 SG]	39 312.8 (15%) [+1 IA] 39 368.5 (85%) [+2 IA]
PTP-C176S	1370	39 253.5	39 253.0 (45%) [+0 SG] 39 558.7 (25%) [+1 SG] 39 864.6 (30%) [+2 SG]	39 558.5 (14%) [+1 SG] 39 865.0 (86%) [+2 SG]	39 254.2 (65%) [+0 IA] 39 368.8 (35%) [+2 IA]
PTP-C78S	4170	39 252.5	39 251.5 (15%) [+0 SG] 39 558.2 (85%) [+1 SG]	39 559.2 (60%) [+1 SG] 39 864.9 (40%) [+2 SG]	39 311.4 (70%) [+1 IA] 39 368.8 (30%) [+2 IA]
PTP-C78/176S	540	39 236.4	39 237.0 [+0 SG]	39 237.6 (15%) [+0 SG] 39 542.0 (85%) [+1 SG]	39 237.6 (75%) [+0 IA] 39 298.5 (25%) [+1 IA]
PTP-C176/266S	0	39 238.9	39 544.5 [+1 SG]	39 544.6 (67%) [+1 SG] 39 849.9 (33%) [+2 SG]	39 239.0 (20%) [+0 IA] 39 294.3 (80%) [+1 IA

[a] In each case, proteins were fully reduced with DTT and then assayed for phosphatase activity prior to either thiolation with GSSG or GSNO or alkylation with iodoacetamide. Polypeptide masses were determined by ESI-MS, with species corresponding to more than 10% of the total signal shown. Relative intensities are given in parentheses, and the deduced identities of the species are given in brackets.

responded to parent *Gm*PTP, demonstrating that the two higher-molecular mass species were mixed disulfide adducts of the parent protein. On the basis of our recent studies with recombinant dehydroascorbate reductases (*22*), the presence of a 305.3 Da adduct was consistent with a single cysteine of *Gm*PTP having undergone S-glutathionylation. Interestingly, modification with glutathione could not account for the 765.5 Da species, but instead, it most likely corresponded to a mixed disulfide between *Gm*PTP and coenzyme A (CoA). Formation of the putative *Gm*PTP−CoA mixed disulfide is likely to be solely a result of the high level of CoA present in *E. coli* (approaching the concentration of GSH), and it is not unsurprising that both CoA and GSH can form mixed disulfides with reactive cysteines in proteins such as PTP.

Reduced *Gm*PTP was then treated with chemicals known to react with cysteinyl residues and the formation of covalent adducts monitored by MS (Table 1). When reduced *Gm*PTP was treated with GSSG, two adducts were identified, corresponding to *Gm*PTP S-modified with one or three glutathione molecules. GSNO was also used as a more aggressive thiolating agent, as this glutathione derivative spontaneously degrades to the respective reactive sulfenic acid and other reactive species (*23*, *24*). When exposed to GSNO, all the protein became triply glutathionylated. Alkylation with iodoacetamide also demonstrated that three cysteines were available for modification, with one of these being particularly reactive. As *Gm*PTP contains a total of seven cysteines (Figure 2), it could be concluded that the other four residues were either present as intramolecular disulfides or not solvent-exposed. Alignments of *Gm*PTP with related plant PTP sequences demonstrated that six of the cysteines were conserved in soybean, pea, and *A. thaliana* (Figure 2). Further alignments with mammalian PTP sequences identified Cys 266 as the essential catalytic residue. This was confirmed in *Gm*PTP by mutating Cys 266 to serine; this completely abolished enzyme activity (Table 1). The status of Cys 266 as one of the redox reactive cysteines was then confirmed by subjecting the C266S mutant to S-glutathionylation and S-alkylation. In both cases, the mutant was almost quantitatively converted to the respective doubly derivatized species (Table 1).

To identify the other two reactive cysteines, samples of PTP were treated with and without a thiolation treatment with GSSG or an alkylation treatment with iodoacetamide and digested with trypsin, and then the digests were analyzed by ESI MS. As compared with the untreated PTP, the thiolated protein digest contained two novel mass ions. One peptide (*m*/*z* 1761.5 Da) corresponded to the tryptic fragment containing Cys 176 forming a mixed disulfide with glutathione (Figure 2). The other (*m*/*z* 3641 Da) could be best explained as being the tryptic fragments containing the catalytic Cys 266 and Cys 78, respectively, covalently coupled together through a disulfide bond rather than being S-glutathionylated. The alkylated digest contained three novel mass ions, with masses consistent with fragments containing alkylated Cys 78 (*m*/*z* 1325.7 Da), Cys 176 (1513.7 Da), and Cys 266 (*m*/*z* 2304.4 Da). There were no mass ions consistent with any of the remaining cysteines being alkylated or S-glutathionylated.

The reactivity of Cys 176 was confirmed by mutagenesis. The *Gm*PTP-C176S mutant retained 23% enzyme activity and could only undergo two thiolation or alkylation events, confirming Cys 176 as one of the three reactive cysteines but in this instance a noncatalytically essential one. Unlike the studies with the *Gm*PTP-C266S mutant which showed that the other cysteines were similarly reactive to thiolation and alkylation treatments, the identical derivatizations carried out on the *Gm*PTP-C176S enzyme showed that one of the remaining cysteines was more reactive than the other (Table 1). The identification of Cys 176 and Cys 266 then allowed for the double mutant *Gm*PTP-C176/266S to be generated. When the enzyme was treated with GSSG or iodoacetamide, only one cysteine became derivatized. However, when GSNO was used, a further cysteine became partially modified. From this it was concluded that the structure of the double mutant was distorted such that when exposed to the reactive GSNO a normally inaccessible cysteine was available for mixed disulfide formation.

To confirm that Cys 78 was the third reactive cysteine, it was mutated to the serine and the resulting *Gm*PTP-C78S analyzed. The mutant *Gm*PTP-C78S retained activity, confirming that Cys 78 was not a catalytic residue. Thiolation and alkylation analysis demonstrated that as with the C176S mutant, only two reactive cysteines were present, and on the basis of the relative abundance of the adducts, the reactivity of these two cysteines was not equivalent, with one undergo-

FIGURE 2: Alignment of PTPs from soybean, pea, and *Arabidopsis*. Cysteine residues are shown in inverted type, with the corresponding residue number above. For *Gm*PTP, trypsin-digested fragments containing cysteine residues are underlined, with expected masses as indicated (the value marked with an asterisk is a mass for a recombinant, His-tagged protein). Amino acid residues conserved between sequences are denoted with an asterisk. Predicted chloroplast transit peptides are shown in lowercase letters.

ing derivatization much more readily than the other (Table 1).

Once the two redox reactive cysteines which were not essential for catalysis had been established, it was then of interest to complete their individual characterization by creating the other double mutant, *Gm*PTP-C78/176S, which left only the catalytic Cys 266 active. Under standard assay conditions, the mutant retained only 9% of the activity of the wild-type enzyme, as expected on the basis of the loss of activity seen with the individually mutated residues. The thiol modification studies showed that the removal of Cys 78 and Cys 176 left the catalytic residue surprisingly resistant to thiolation by GSSG or alkylation. In fact, aggressive GSNO treatment was required to S-glutathionylate Cys 266 in the absence of these other cysteines.

Redox Regulation of PTP Activity in the Wild-Type and Mutant Enzymes. When the S-glutathionylated and S-alkylated *Gm*PTPs generated in Table 1 were assayed, all were found to be inactive (data not shown). However, these modification studies were carried out under nonphysiological conditions to achieve rigorous derivatization. To study the kinetics of inactivation, *Gm*PTP and the Cys-to-Ser mutants were exposed to chemical oxidants under milder conditions. For illustration, the loss of phosphatase activity of *Gm*PTP and the Cys-to-Ser mutants in the presence of PAO is shown in Figure 3. In all cases, adding 0.1 mM GSSG or 0.5 mM PAO to the enzymes gave a loss of activities over time which closely fitted an exponential decay curve, consistent with inactivation through covalent modification of one or more reactive cysteine residues. Pseudo-second-order rate constants were calculated for inactivation due to modification of the most reactive cysteine residue of each enzyme (Table 2). For inhibition by GSSG, *Gm*PTP and *Gm*PTP-C78S had similar rate constants, *Gm*PTP-C176S exhibited much slower inhibition, and *Gm*PTP-C78/176S was not significantly inhibited. Treatment with PAO gave different results: *Gm*PTP-C78S was more sensitive to inhibition than unmodified *Gm*PTP, while the inactivation of *Gm*PTP-C176S was relatively slow. As determined with thiolation, *Gm*PTP-C78/176S was unaffected by PAO. Inhibition experiments with H_2O_2 showed that although the enzyme was inactivated by this treatment (showing 45% inhibition after a 15 min pretreatment with 10 μM H_2O_2), unlike other thiolation treatments this inhibition was unchanged in the C176S mutant and was therefore not subject to the same complex regulatory mechanism. Further experiments with H_2O_2 were not therefore performed.

To confirm the link between thiolation and regulation of *Gm*PTP activity as well as to study the dynamics of this reaction, the enzyme was exposed to GSSG and then alkylated with iodoacetamide at timed intervals, prior to MS analysis and determination of residual phosphatase activity after reducing any thiolated cysteines with DTT (Table 3). At time zero, the majority of the reactive cysteinyl residues were available for alkylation, resulting in virtually all activity being abolished following treatment with iodoacetamide. After a 0.5 min exposure to GSSG, the protein *Gm*PTP was recovered as a mixture of either a single mixed disulfide with glutathione or a doubly glutathionylated species with a single alkylation. The only explanation for this pattern of derivatization was that in the singly thiolated species the other two cysteinyl residues must be protected from alkylation by

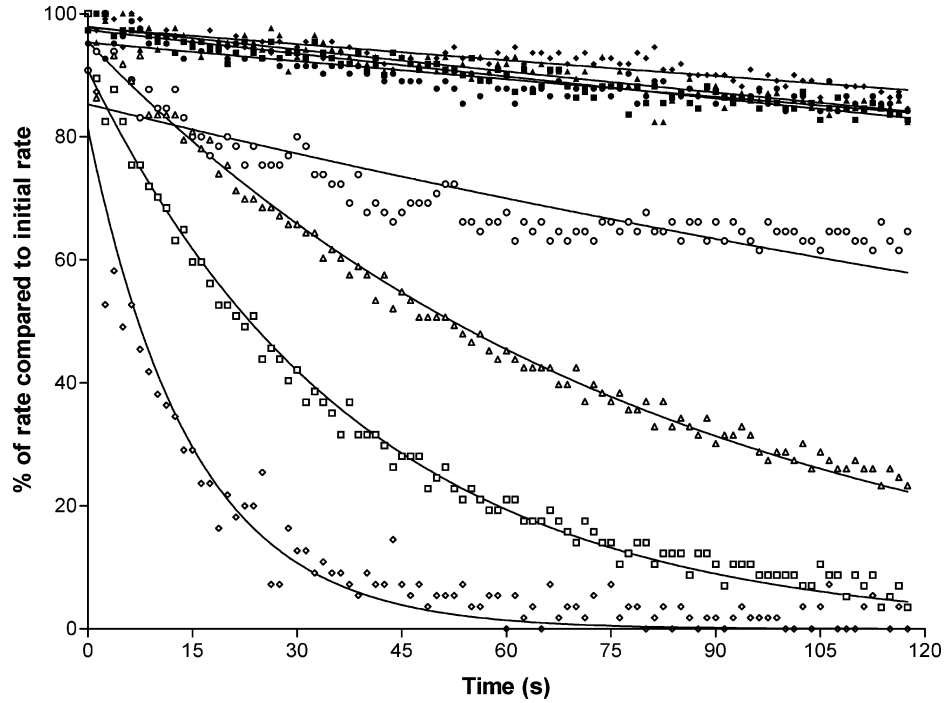

FIGURE 3: Inhibition kinetics of wild-type and mutated *Gm*PTPs in the presence of 0.5 mM phenylarsine oxide. Filled symbols represent data for reactions without PAO; open symbols represent data for reactions with 0.5 mM PAO added at 0 s: (■) *Gm*PTP, (◆) *Gm*PTP-C78S, (▲) *Gm*PTP-C176S, and (●) *Gm*PTP-C78/176S. Curves indicate best fits to an exponential decay. In each case, 100% activity for each of the enzymes is quoted in Table 1.

Table 2: Inactivation of Wild-Type *Gm*PTP and Mutant Enzyme Activities by GSSG and PAO[a]

	pseudo-second-order rate constant for enzyme inactivation [k_{inact} (M^{-1} s^{-1})]	
enzyme	0.1 mM GSSG	0.5 mM PAO
*Gm*PTP	559	74
*Gm*PTP-C78S	485	194
*Gm*PTP-C176S	172	36
*Gm*PTP-C78/176S	<50	<10

[a] Inhibition kinetics closely followed an exponential decay curve, with the values shown here giving the enzyme half-life.

Table 3: Inactivation of *Gm*PTP by Iodoacetamide with an Increasing Level of Exposure to GSSG[a]

thiolation time (min)	+IA	+SG	+3IA	+2IA + SG	+IA + 2SG	activity
0	32%	−	42%	25%	−	4%
0.5	−	59%	−	−	41%	41%
1	−	64%	−	−	36%	43%
5	−	72%	−	−	28%	52%
10	−	78%	−	−	22%	63%

[a] Freshly prepared *Gm*PTP (5960 nkat/mg of protein) was treated with 1 mM GSSG for up to 10 min and then alkylated with 10 mM iodoacetamide to derivatize any remaining available thiols. Desalted protein preparations were then analyzed by ESI-MS to give the relative abundance of the observed adducts shown. Modifications were inferred from mass spectrometric analysis. Samples thiolated for 0 min were reacted with iodoacetamide 20 min before addition of GSSG. Enzyme activity of the derivatized *Gm*PTP was determined after treatment with 20 mM DTT to reduce any thiolated cysteines.

forming a disulfide bond with each other. In the case of the doubly thiolated species, an unbound cysteine was available for alkylation. Over a 10 min incubation, the proportion of *Gm*PTP recovered in the monothiolated form steadily increased, suggesting that more of the intramolecular disulfide was forming. This was associated with a corresponding increase in the activity of the enzyme following reduction of the disulfide with DTT.

DISCUSSION

Our results demonstrate that *Gm*PTP is highly sensitive to inhibition by S-glutathionylation promoted by the presence of oxidized glutathione. Whereas in this study we have demonstrated that *Gm*PTP forms mixed disulfides with glutathione, *in planta* this reaction would proceed with homoglutathione (Glu-Cys-β-Ala), the dominant thiol in soybean (*25*). Since the reactivities of the thiols in glutathione and homoglutathione and their usage in redox reactions appear to be indistinguishable (*26*, *27*), we would expect identical regulation of *Gm*PTP with oxidized homoglutathione as determined with GSSG. The sensitivity to inhibition of *Gm*PTP by GSSG is very high compared to those of mammalian PTPs. For example, for the inhibition of human PTP1b, a k_{inact} of 0.012 M^{-1} s^{-1} was determined with GSSG, and with the strong sulfydryl oxidant diamide in the presence of GSH, k_{inact} = 0.305 M^{-1} s^{-1} (*3*). The differential sensitivity of plant and animal PTPs to GSSG may suggest a fundamental difference in the way in which *Gm*PTP needs to use oxidants in redox signaling. Plant cells are known to undergo major changes in the GSH:GSSG ratio under oxidative conditions which initiate signaling events (*29*). The selective sensitivity of the plant PTPs to GSSG may reflect an adaptation to regulation by thiol redox balance rather than direct ROS and H_2O_2 generation. This requirement may relate to the subcellular compartment in which *Gm*PTP operates. The *Gm*PTP peptide sequence contains an N-terminal extension not found in the animal PTPs to which no function could be assigned (*20*). Further analysis has identified that the 33 N-terminal amino acid residues form a putative chloroplast transit peptide. Similarly, the PTP from

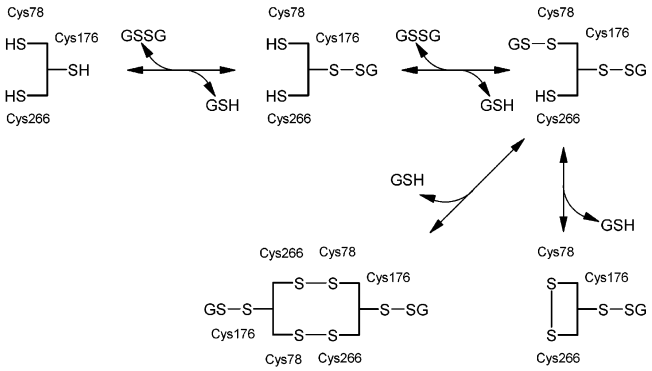

FIGURE 4: Graphical representation of the proposed model for oxidation of *Gm*PTP by GSSG. GSSG rapidly glutathionylates cysteines 176 and 78 of *Gm*PTP. A slow rearrangement follows, with an intramolecular disulfide forming between Cys 78 and the active site Cys 266. An alternative disulfide architecture is also shown, giving rise to a disulfide-linked dimer.

pea (GenBank entry AJ005589) has a putative 54-residue chloroplast targeting peptide; kidney bean PTP (GenBank entry AY603965) has a putative 31-residue target peptide, and the *Arabidopsis* PTP (GenBank entry AF0055635) has a putative 23-residue target peptide (*30*). In each case, TargetP prediction scores for chloroplastic targeting are higher than that indicating the 95% confidence limit for correct prediction. The presence of such leader sequences in all four plant PTPs strongly suggests that these enzymes are localized in the chloroplast. Significantly, the chloroplast is subject to constant H_2O_2 generation due to photooxidative reactions, and an enzyme that was highly sensitive to this oxidant would have limited usefulness in redox signaling in the chloroplast. Instead, we propose that the enzyme would be modulated by changes in the GSH:GSSG redox state brought about by specific changes in ambient light and the rate of photosynthesis (*31*). Since the physiological substrates for plant PTPs have yet to be determined, the exact significance of the inactivation of the phosphatase activity in the presence of an increased level of GSSG can only be surmised. One potential function may be to regulate the distribution of light energy between the two photosystems, with light-harvesting complex II being recently shown to undergo regulatory phopshorylation of tyrosine and threonine residues during the transition to light state 2 (*32*).

Whatever the functional role of *Gm*PTP, our studies on the regulation of its activity through regulatory cysteinyl residues shed new light on the direct control of signaling enzymes through changes in redox state. Thus, whereas the regulation of *Gm*PTP shares some common features with its mammalian counterparts, others appear to be unique in utilizing a regulatory network of cysteines. On the basis of the data that were generated, we can propose a model for describing the reactivity of the three accessible cysteine residues, and how their modification affects enzyme activity (Figure 4). The evidence points to Cys 176 being the most reactive residue undergoing rapid thiolation in the presence of GSSG, with this modification leading to the inactivation of the enzyme. On the basis of the inhibition studies with the Cys-to-Ser mutants, we then propose that Cys 78 must be located near the active site and can also undergo S-glutathionylation, albeit less readily than Cys 176. Once thiolated, Cys 78 then appears to be able to undergo a disulfide exchange with the catalytic Cys 266, releasing GSH and forming an intramolecular disulfide. Evidence for the model can be summarized as follows. (1) Triple thiol modification of *Gm*PTP showed the presence of three reactive cysteines. (2) The identities of these cysteines as cysteines 78, 176, and 266 were confirmed as mutation of each reduced the number of reactive cysteines. (3) All three identified cysteine residues are catalytically important as mutation of each reduced or eliminated enzyme activity. (4) Since *Gm*PTP was either triply or singly modified, this was good evidence for an intramolecular disulfide and evidence against intermolecular disulfide (dimerization) as no doubly modified forms were found. (5) The GSSG treatment time course (Table 3) showed initially three reactive cysteines and rapid thiolation. Over time, two cysteines lost reactivity, again good evidence for a slow-forming intramolecular disulfide. (6) The C266S mutant was quantitatively doubly modified (i.e., no disulfides), so Cys 266 must form one-half of the proposed disulfide. (7) The C176S mutant was mostly unmodified by GSSG, so the disulfide must be present between Cys 78 and Cys 266 (otherwise, Cys 78 should be quantitatively modified). (8) Iodoacetamide modification data for the C176S mutant showed that either Cys 78 and Cys 266 were both available for modification ($2 \times$ IA addition) or neither was available (no IA addition). Again this is good evidence for a Cys 78−Cys 266 disulfide. (9) The remaining data are consistent with the model.

Surprisingly, it appears that the active site Cys 266 is the least reactive available cysteine, even though as a catalytic residue it needs to be highly reactive to promote the formation of the reactive thiolate anion at a relatively low pH. The lack of reactivity of Cys 266 was demonstrated by the inability of GSSG to thiolate the *Gm*PTP-C78/176S double mutant, being modified only by the much more reactive GSNO. The apparent lack of reactivity of Cys 266 may have a number of causes. It may be that Cys 266 is relatively inaccessible to thiol-modifying reagents, because of the surrounding PTP structure imposing steric and/or charge constraints. Alternatively, Cys 266 may have a relatively high pK_a, in which case it would be predominantly un-ionized and relatively unreactive. This in turn would require Cys 266 to only become ionized on substrate binding. A further possibility for explaining the lack of reactivity of Cys 266 is that the basal state of the catalytic sulfur atom is locked in the form of a sulfenyl amide as described for human PTP1B (*4*, *5*). Significantly, like human PTP1B, *Gm*PTP contains a serine (Ser 267) next to the catalytic cysteine that would be available to make the analogous derivative. The active site in its sulfenyl amide form is not catalytically active but is primed for ready reactivation by reduction by thiols. Our results suggest that in the wild-type enzyme the active form of Cys 266 is promoted by the close presence of the reactive Cys 176. This conclusion is based on the low activity seen with *Gm*PTP-C176S. Cys 78 may also play a supporting role, though the respective mutant retained much of its activity. We could therefore envisage a situation where Cys 176 in its reduced state promotes the activation of Cys 266 and that this supporting role is lost following thiolation. Cys 78 appears to provide a second tier of control, with the available evidence pointing to its thiolated derivative undergoing disulfide exchange with Cys 266 to prevent its oxidation. The *Gm*PTP would therefore be regulated by a hybrid "push−pull" regulation using both

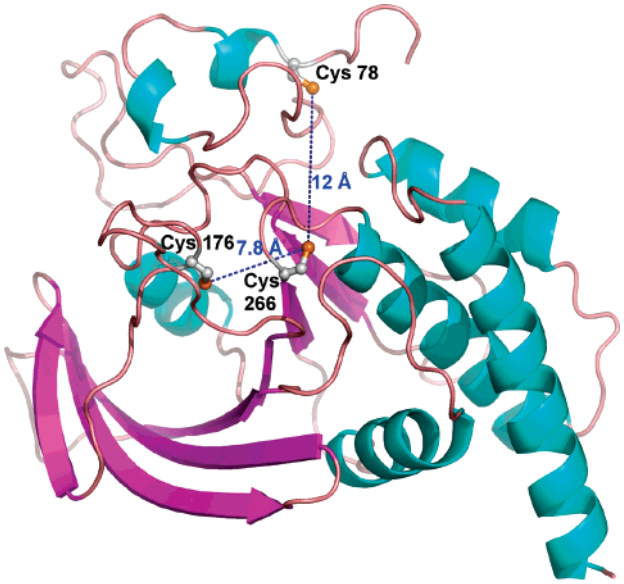

FIGURE 5: Homology-based structural model of *Gm*PTP, showing the active site cysteine (Cys 266) and the two remaining reactive cysteines (Cys 78 and Cys 266). Predicted distances between Cys 266 and the reactive cysteines are given.

sulfenyl amide and disulfide covalent bonds to regulate the PTP activity.

Homology modeling (Figure 5) of *Gm*PTP using related PTP structures confirmed that Cys 266 is the active site cysteine, and showed Cys 176 (analogous to Cys 121 in human PTP1B) is adjacent to Cys 266 (7.8 Å apart). It was possible to align Cys 78 with Cys 32 from human PTP1B (which is located ~16 Å from the active site cysteine), to give a predicted distance between Cys 78 and Cys 266 of 12 Å. However, the N-terminal region of *Gm*PTP is very different from that of PTPs with known structures, and the structure of *Gm*PTP is likely to be very different in this region; therefore, the structure of *Gm*PTP would need to be determined to locate Cys 78 with any degree of certainty. It was particularly intriguing that in unmodified *Gm*PTP, Cys 78 somehow protects Cys 176 and Cys 266 from being cross-linked by PAO, since in *Gm*PTP-C78S this protection was lost. Structural studies are therefore now required to unravel how these three reactive cysteinyl residues interact. Following from these structural studies, it will then be of interest to determine the physiological significance of the redox regulation of *Gm*PTP *in planta*.

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REFERENCES

1. Tonks, N. K. (2003) PTP1B: From the sidelines to the front lines! *FEBS Lett. 546*, 140−148.
2. Denu, J. M., and Dixon, J. E. (1998) Protein tyrosine phosphatases: Mechanisms of catalysis and regulation, *Curr. Opin. Chem. Biol. 2*, 633−641.
3. Barrett, W. C., DeGnore, J. P., Keng, Y.-F., Zhang, Z.-Y., Yim, M. B., and Chock, P. B. (1999) Roles of superoxide radical anion in signal transduction mediated by reversible regulation of protein-tyrosine phosphatase 1B, *J. Biol. Chem. 274*, 34543−34546.
4. Salmeen, A., Andersen, J. N., Myers, M. P., Meng, T. C., Hinks, J. A., Tonks, N. K., and Barford, D. (2003) Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate, *Nature 423*, 769−773.
5. van Montfort, R. L. M., Congreve, M., Tisi, D., Carr, R., and Jhoti, H. (2003) Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B, *Nature 423*, 773−777.
6. Caselli, A., Marzocchini, R., Camici, G., Manao, G., Moneti, G., Pieraccini, G., and Ramponi, G. (1998) The inactivation mechanism of low molecular weight phosphotyrosine-protein phosphatase by H_2O_2, *J. Biol. Chem. 273*, 32554−32560.
7. Lee, S.-R., Yang, K.-S., Kwon, J., Lee, C., Jeong, W., and Rhee, S. G. (2002) Reversible inactivation of the tumor suppressor PTEN by H_2O_2, *J. Biol. Chem. 277*, 20336−20342.
8. Savitsky, P. A., and Finkel, T. (2002) Redox regulation of Cdc25C, *J. Biol. Chem. 277*, 20535−20540.
9. Luan, S. (2002) Tyrosine phosphorylation in plant cell signaling, *Proc. Natl. Acad. Sci. U.S.A. 99*, 11567−11569.
10. Carpi, A., Di Maira, G., Vedovato, M., Rossi, V., Naccari, T., Floriduz, M., Terzi, M., and Filippini, F. (2002) Comparative proteome bioinformatics: Identification of a whole complement of putative protein tyrosine kinases in the model flowering plant *Arabidopsis thaliana*, *Proteomics 2*, 1494−1503.
11. Sheng, L. (2003) Protein phosphatases in plants, *Annu. Rev. Plant Biol. 54*, 63−92.
12. Gupta, R., Ting, J. T. L., Sokolov, L. N., Johnson, S. A., and Luan, S. (2002) A tumor suppressor homolog, AtPTEN1, is essential for pollen development in *Arabidopsis*, *Plant Cell 14*, 2495−2507.
13. MacRobbie, E. A. C. (2002) Evidence for a role for protein tyrosine phosphatase in the control of ion release from the guard cell vacuole in stomatal closure, *Proc. Natl. Acad. Sci. U.S.A. 99*, 11963−11968.
14. Xu, Q., Fu, H. H., Gupta, R., and Luan, S. (1998) Molecular characterization of a tyrosine-specific protein phosphatase encoded by a stress-responsive gene in *Arabidopsis*, *Plant Cell 10*, 849−857.
15. Huang, H. J., Lin, Y. M., Huang, D. D., Takahashi, T., and Sugiyama, M. (2003) Protein tyrosine phosphorylation during phytohormone-stimulated cell proliferation in *Arabidopsis* hypocotyls, *Plant Cell Physiol. 44*, 770−775.
16. Ulm, R., Ichimura, K., Mizoguchi, T., Peck, S. C., Zhu, T., Wang, X., Shinozaki, K., and Paszkowski, J. (2002) Distinct regulation of salinity and genotoxic stress responses by *Arabidopsis* MAP kinase phosphatase 1, *EMBO J. 21*, 6483−6493.
17. Gupta, R., and Luan, S. (2003) Redox control of protein tyrosine phosphatases and mitogen-activated protein kinases in plants, *Plant Physiol. 132*, 1149−1152.
18. Meinhard, M., and Grill, E. (2001) Hydrogen peroxide is a regulator of ABI1, a protein phosphatase 2C from *Arabidopsis*, *FEBS Lett. 508*, 443−446.
19. Klatt, P., and Lamas, S. (2000) Regulation of protein function by *S*-glutathiolation in response to oxidative and nitrosative stress, *Eur. J. Biochem. 267*, 4928−4944.
20. Fordham-Skelton, A. P., Skipsey, M., Eveans, I. M., Edwards, R., and Gatehouse, J. A. (1999) Higher plant tyrosine-specific protein phosphatases (PTPs) contain novel amino-terminal domains: Expression during embryogenesis, *Plant Mol. Biol. 39*, 593−605.
21. Hart, T. W. (1985) Some observations concerning the *S*-nitroso and *S*-phenylsulphonyl derivatives of L-cysteine and glutathione, *Tetrahedron Lett. 26*, 2013−2016.
22. Dixon, D. P., Davis, B. G., and Edwards, R. (2002) Functional divergence in the glutathione transferase superfamily in plants: Identification of two classes with putative functions in redox homeostasis in *Arabidopsis thaliana*, *J. Biol. Chem. 277*, 30859−30869.
23. Huang, K. P., and Huang, F. L. (2002) Glutathionylation of proteins by glutathione disulfide S-oxide, *Biochem. Pharmacol. 64*, 1049−1056.
24. Ji, Y., Akerboom, T. P. M., Sies, H., and Thomas, J. A. (1999) S-Nitrosylation and S-glutathiolation of protein sulfhydryls by *S*-nitroso glutathione, *Arch. Biochem. Biophys. 362*, 67−78.
25. Klapheck, S. (1988) Homoglutathione: Isolation, quantification and occurrence in legumes, *Physiol. Plant. 74*, 727−732.
26. Zopes, H., Klapheck, S., and Bergmann, L. (1993) The function of homoglutathione and hydroxymethylglutathione for the scavenging of hydrogen-peroxide, *Plant Cell Physiol. 34*, 515−521.

27. Moran, J. F., Iturbe-Ormaetxe, I., Matamoros, M. A., Rubio, M. C., Clemente, M. R., Brewin, N. J., and Becana, M. (2000) Glutathione and homoglutathione synthetases of legume nodules. Cloning, expression, and subcellular localization, *Plant Physiol. 124*, 1381−1392.
28. Denu, J. M., and Tanner, K. G. (1998) Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: Evidence for a sulfenic acid intermediate and implications for redox regulation, *Biochemistry 37*, 5633−5642.
29. May, M. J., Vernoux, T., Leaver, C., Van Montagu, M., and Inzé, D. (1998) Glutathione homeostasis in plants: Implications for environmental sensing and plant development, *J. Exp. Bot. 49*, 649−667.
30. Emanuelsson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence, *J. Mol. Biol. 300*, 1005−1016.
31. Noctor, G., Arisi, A.-C. M., Jouanin, L., Kunert, K. J., Rennenberg, H., and Foyer, C. H. (1998) Glutathione: Biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants, *J. Exp. Bot. 49*, 623−647.
32. Forsberg, J., and Allen, J. F. (2001) Protein tyrosine phosphorylation in the transition to light state 2 of chloroplast thylakoids, *Photosynth. Res. 68*, 71−79.

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