Direct Evidence for the Formation of a Complex between 1-Cysteine Peroxiredoxin and Glutathione *S*-Transferase π with Activity Changes in Both Enzymes†

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Received October 11, 2005; Revised Manuscript Received November 11, 2005

ABSTRACT: Glutathione *S*-tranferase π (GST π) has been shown to reactivate oxidized 1-cysteine peroxiredoxin (1-Cys Prx, Prx VI, Prdx6, and AOP2). We now demonstrate that a heterodimer complex is formed between 1-Cys Prx with a C-terminal His_6 tag and GST π upon incubation of the two proteins at pH 8.0 in buffer containing 20% 1,6-hexanediol to dissociate the homodimers, followed by dialysis against buffer containing 2.5 mM glutathione (GSH) but lacking 1,6-hexanediol. The heterodimer can be purified by chromatography on nickel-nitriloacetic acid agarose in the presence of GSH. N-Terminal sequencing showed that equimolar amounts of the two proteins are present in the isolated complex. In the heterodimer, 1-Cys Prx is fully active toward either H_2O_2 or phospholipid hydroperoxide, while the GST π activity is ∼25% of that of the GST π homodimer. In contrast, the 1-Cys Prx homodimer lacks peroxidase activity even in the presence of free GSH. The heterodimer is also formed in the presence of *S*-methylglutathione, but no 1-Cys Prx activity is found under these conditions. The yield of heterodimer is decreased in the absence of 1,6-hexanediol or GSH. Rapid glutathionylation of 1-Cys Prx in the heterodimer is detected by immunoblotting. Subsequently, a disulfide-linked dimer is observed on SDS−PAGE, and the free cysteine content is decreased by 2 per heterodimer. The involvement of particular binding sites in heterodimer formation was tested by site-directed mutagenesis of the two proteins. For 1-Cys Prx, neither Cys^{47} nor Ser^{32} is required for heterodimer formation but Cys^{47} is essential for 1-Cys Prx activation. For GST π, Cys^{47} and Tyr^{7} (at or near the GSH-binding site) are needed for heterodimer formation but three other cysteines are not. We conclude that reactivation of oxidized 1-Cys Prx by GST π occurs by heterodimerization of 1-Cys Prx and GST π harboring bound GSH, followed by glutathionylation of 1-Cys Prx and then formation of an intersubunit disulfide. Finally, the GSH-mediated reduction of the disulfide regenerates the reduced active-site sulfhydryl of 1-Cys Prx.

Reactive oxygen species (ROS)[1] have been implicated in diverse environmental stresses in plants and animals and appear to be common participants in many degenerative conditions in eukaryotic cells. The peroxidation of lipids, the cross-linking and inactivation of proteins, and mutations in DNA are typical consequences of ROS (*1*). Peroxides, byproducts of oxidative stress and ROS, have been implicated in cellular aging and cellular signaling (*2*). Given the now appreciated role of peroxides in physiology, the need to study antioxidant proteins is evident.

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are mainly expressed in the cytosol and are ubiquitous in nature. GST functions in xenobiotic biotransformation (*3*), drug metabolism (*4*), protection against an oxidative stress-induced peroxidation of lipids in the nucleus (*5*), and isomerization of protaglandins (*6*). Human GST π (hGSTP1-1) is one of a family of GSTs. Several crystal structures of GST π have shown it to consist of two subunits that combine to form a homodimer with a subunit molecular weight of 23.5 kDa (*7*−*10*). This GST class is particularly important because of its presence in various human cancer and precancerous tissues. GST π is broadly distributed among mammalian tissues, with a notable absence in the liver, suggesting that it has functions in living systems in addition to detoxification.

Peroxiredoxins (Prx, EC 1.11.1), an important family of antioxidant proteins, are nonseleno peroxidases catalyzing the degradation of peroxides to either water or alcohol, depending upon the substrate. Structurally, Prx proteins have a thioredoxin fold with a conserved Cys residue present in their N-terminal region, which is thought to be involved in the peroxidase activity (*11*). Prx proteins are classified into two major groups, the 2-Cys Prx and 1-Cys Prx (*11*). The

† This work was supported by NIH Grant R01-CA66561 (to R.F.C.), NIH fellowship 1F31 GM75387 (to L.A.R.), and NIH Grant P01-HL79063 (to A.B.F.).

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[1] Abbreviations: ROS, reactive oxygen species; GST, glutathione *S*-transferase; GST π, π-class glutathione *S*-transferase; hGSTP1-1, human π-class glutathione *S*-transferase, isozyme 1-1; GST M1-1, μ-class glutathione *S*-transferase, isozyme 3-3; GST A1-1, α-class glutathione *S*-transferase, isozyme 1-1; 1-Cys Prx, 1-cysteine peroxiredoxin; 2-Cys Prx, 2-cysteine peroxiredoxin; WT, wild-type enzyme; Ni−NTA, nickel−nitriloacetic acid agarose; GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; mBBr, monobromobimane; BITC, benzyl isothiocyanate; DTNB, 5,5′-dithiobis(2-nitrobenzoate); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; PLPCOOH, 1-palmitoyl-2-linolenoylhydroperoxide-*sn*-glycero-3-phosphocholine.

10.1021/bi0520737 CCC: $33.50
Published on Web 12/15/2005

2-Cys Prx possesses an additional Cys in its C-terminal domain, while 1-Cys Prx has only the single conserved peroxidatic Cys (*12*). For 2-Cys Prx, the reaction mechanism begins by attack of the N-terminal domain Cys on an oxygen of the organic peroxide substrate to yield the alcohol product and Cys sulfenic acid. This step is followed by the formation of a disulfide bond with the C-terminal domain Cys residue of the second subunit of the same molecule, which can be reduced by thioredoxin to regenerate the reduced active-site thiol (*12*). However, for 1-Cys Prx, the reaction mechanism is different because this class lacks the C-terminal domain cysteine necessary for the formation of the intersubunit disulfide seen in the oxidation of the 2-Cys Prx (*13*). The oxidized 1-Cys Prx intermediate must react with another thiol compound to regenerate the sulfhydryl cysteine of active 1-Cys Prx. The identity of that SH compound was until recently unknown, as was the mechanism of regeneration of the active-site thiol. Structurally, 1-Cys Prx exists as a homodimer with a subunit molecular weight of 25 kDa containing 224 amino acids/subunit, including the conserved Cys-47−sulfenic acid (*13*).

A recent study by Manevich et al. raised the possibility of an entirely new function for GST π: a carrier of glutathione (GSH) capable of reducing the inactive Cys-47−sulfenic acid form of 1-Cys Prx and regenerating an active 1-Cys Prx (*14*). They proposed the heterodimerization between the two proteins; however, they did not provide *direct* evidence for the existence of the complex. In this study, we have isolated and characterized a physical complex between 1-Cys Prx and GST π. Our experiments suggest that the mechanism accounting for activation of 1-Cys Prx by GST π involves glutathionylation of 1-Cys Prx, followed by the formation of an intermolecular disulfide between the two protein subunits and then by the GSH-dependent reduction of the disulfide to regenerate the reduced active-site thiol.

EXPERIMENTAL PROCEDURES

Materials. Reduced GSH, 1-chloro-2,4-dinitrobenzene (CDNB), *S*-hexylglutathione Sepharose, *S*-hexylglutathione, *S*-methylglutathione, oxidized GSH, mercaptosuccinic acid, benzyl isothiocyanate, Sephadex G-25, imidazole, and chemicals for the preparation of buffers were obtained from Sigma Chemical Co. Nickel−nitrilotriacetic acid agarose (Ni−NTA) was purchased from Qiagen, Inc. Monobromobimane (mBBr) was obtained from Molecular Probes. 1,6-Hexanediol was supplied by ACROS Organics. Oligonucleotides for mutagenesis were obtained from Biosynthesis, Inc. Reagents used for mutagenesis were from Stratagene. The kit used for plasmid extraction was from Qiagen, Inc. Centriplus YM-10 concentrators were from Millipore. All chemicals were of reagent grade.

Expression and Purification of Wild-Type and Mutant GST P1-1 and 1-Cys Prx. The full-length cDNA for human GST P1-1 was encoded in a pUC120 plasmid, as described by Manoharan et al. (*15*), and was a gift from W. E. Fahl (University of Wisconsin, Madison, WI). Site-directed mutagenesis was performed using the Stratagene QuikChange kit. The following oligonucleotides and their complements were used to incorporate the mutations (position of the mutation underlined): C14S, 5′-GGC CGC AGC GCG GCC CTG CGC ATG CTG; C47S, 5′-GCC TCC AGC CTA TAC GGG CAG CTC CCC; C101S, 5′-GAG GAC CTC CGC AGC AAA TAC ACC; C167S, 5′-GCC CCT GGC AGC CTG GAT GCG TTC CCC; Y8F, 5′-CCC TAC ACC GTG GTC TTT TTC CCA GTT CGA; Y108S, 5′-TCC CTC ATC AGC ACC AAC TAT GAG GCG GGC. Y103S was constructed as described previously (*16*). Mutations were confirmed by DNA sequencing (forward sequencing primer, 5′-CCG CCC TAC ACC GTG GTC TAT TTC CCA GTT, and reverse sequencing primer, 5′-CTG TTT CCC GTT GCC ATT GAT GGG GAG GTT), which was carried out at the University of Delaware Center for Agricultural Biotechnology using an ABI Prism model 377 DNA sequencer (PE Biosystems). The full-length cDNA for human 1-Cys Prx was encoded in a pET-21b (*17*). C47S and S32A mutants for 1-Cys Prx were constructed as described previously (*17*). For the expression of GST, the WT and mutant plasmids were transformed into *Escherichia coli* JM105 and the cells grown and induced for expression of GST (*16*). For the expression of 1-Cys Prx, WT and mutant plasmids were transformed in *E. coli* BL21 (DE3) cells (*17*). Because GST P1-1 does not have a His tag, purification of wild-type and mutant proteins was performed using a *S*-hexylglutathione agarose affinity column, as described previously (*16*). In all cases, GST P1-1 was eluted using a buffer containing 2.5 mM *S*-hexylglutathione. 1-Cys Prx contains a His_6 tag at the C terminus; thus, purification of wild-type and mutant proteins was performed using a Ni−NTA column, as described previously (*17*). All of the enzymes were purified to homogeneity, yielding a single peptide by N-terminal sequencing on an Applied Biosystems gas-phase sequencer (Model Procise) equipped with an on-line microgradient Delivery System (Model 140C) and a computer (Model 610 Macintosh). Each of the enzymes exhibited a single band by SDS−PAGE (*18*). All purified GSTs were stored in aliquots at −80 °C, and all purified 1-Cys Prx proteins were stored at 4 °C in aliquots both stored in 50 mM Tris-HCl buffer at pH 8.0.

Standard Assay for GST Activity. Enzymatic activity toward CDNB was measured in a total volume of 1.0 mL using a Hewlett−Packard 8453 spectrophotometer by monitoring the formation of the conjugate of CDNB (3 mM) and GSH (2.5 mM) at 340 nm ($\Delta\epsilon = 9600\ M^{-1}\ cm^{-1}$) in 0.1 M potassium phosphate buffer (pH 6.5), containing 1 mM EDTA at 25 °C, according to the method of Habig et al. (*18*). All measurements were corrected for the spontaneous nonenzymatic rate of formation of the conjugate of GSH and CDNB.

Standard Assay for Peroxidase Activity of 1-Cys Prx. A standard GSH reductase/GSH/NADPH-coupled GSH peroxidase assay was used at pH 8.0 at 25 °C, as described previously (*20*). Either H_2O_2 (250 μM) or 1-palmitoyl-2-linolenoylhydroperoxide-*sn*-glycero-3-phosphocholine (PLP-COOH) (250 μM) was used as a substrate. The disappearance of NADPH fluorescence was continuously recorded at 460 nm (excitation at 340 nm).

Formation of GST P1-1/1-Cys Prx Complexes Under Standard Conditions. Heterodimers were generated in which one subunit was a non-His-tagged GST π (wild-type or mutant) and the other subunit was a His-tagged 1-Cys Prx (wild type or mutant). Because these are dimeric proteins, to dissociate them into monomers, the enzymes were

incubated together in 20% 1,6-hexanediol in 50 mM Tris-HCl at pH 8.0 for 2 h at 25 °C (*21*). A total of 1 mg of each enzyme in 1 mL of buffer was used. This mixture was then dialyzed overnight against 50 mM Tris-HCl buffer containing 2.5 mM reduced GSH at pH 8.0 and 4 °C to remove 1,6-hexanediol and allow the reformation of dimers. The mixture of two homodimers and one heterodimer was then loaded onto a Ni−NTA column (1.5 mL) equilibrated in 50 mM Tris-HCl buffer at pH 8.0 containing 2.5 mM reduced GSH at 4 °C. The enzymes separate because 1-Cys Prx is a homodimer that has a His tag on each subunit and binds tightly to the Ni−NTA column, while the GST homodimer has no His tag and does not bind to the column. The heterodimer (in which only one of the two subunits has a His tag) binds less tightly to the Ni−NTA column than does the 1-Cys Prx homodimer, in which both subunits contain a His tag. The column was washed with 50 mM Tris-HCl buffer at pH 8.0, containing 2.5 mM GSH, to remove unbound wild-type GST P1-1 homodimer. The heterodimer and 1-Cys Prx homodimer were separated using a linear gradient from 50 mM Tris-HCl at pH 8.0 containing 2.5 mM reduced GSH to the same buffer with the addition of 0.3 M imidazole (20 mL of each buffer). The column eluate was monitored at $A_{280\ nm}$. Fractions of 1 mL were collected, and the GST activity was determined under standard conditions. The three peaks were pooled separately and concentrated to approximately 1 mL using the Centriplus YM-10 centrifugal filter device (molecular-weight cutoff = 10 kDa). To remove the imidazole, the heterodimer and the 1-Cys Prx homodimer were dialyzed against 50 mM Tris-HCl buffer at pH 8.0 containing 2.5 mM reduced GSH. The activity of the recovered WT GST P1-1 homodimer, as well as the purified heterodimer, was determined under standard conditions. The $A_{280\ nm}$ was used to determine the protein concentration. All three enzymes were stored at 4 °C in 50 mM Tris-HCl buffer at pH 8.5 containing 2.5 mM reduced GSH. The N-terminal sequences of the recovered homodimers and purified heterodimers were determined to confirm the composition and purity of the enzymes.

Determination of Kinetic Parameters. For GST π and the heterodimer, the apparent K_m value of GSH was determined at 25 °C by varying GSH concentrations (0.01−10 mM) while keeping a constant CDNB concentration (3 mM) under the conditions of the standard assay. Similarly, the apparent K_m value for CDNB was determined from a range of concentrations of CDNB (0.01−4.0 mM) at a constant GSH concentration (2.5 mM) in 0.1 M potassium phosphate buffer (pH 6.5), containing 1 mM EDTA. For benzyl isothiocyanate (BITC), the apparent K_m value was determined from a range of concentrations (4−400 μM) at a constant GSH concentration (450 μM) (*16*). For the determination of the K_m value of mBBr, the GSH concentration was maintained at 600 μM, whereas mBBr varied from 10 to 200 μM (*22*). Data were analyzed by fitting directly to the Michaelis−Menten equation using a nonlinear curve-fitting program (SigmaPlot from SPSS).

For 1-Cys Prx [separated from GST π using a GST-trap column (*14*)] and the heterodimer obtained from the Ni−NTA column, the apparent K_m value was determined using the standard GSH reductase/GSH/NADPH-coupled GSH-peroxidase assay with a constant (0.66 mM) concentration of GSH (*17*) and varied concentrations (from 5 to 1200 μM) of PLPCOOH or H_2O_2, respectively. The data represents an average of three independent experiments. Standard errors are presented.

Sulfhydryl Determinations. The two homodimers (GST P1-1 and 1-Cys Prx) and the heterodimer recovered from the Ni−NTA column contained 2.5 mM GSH in the storage buffer, which interferes with the sulfhydryl determination. In the preparation of the enzymes for the 5,5′-dithiobis(2-nitrobenzoate) (DTNB) assay, excess GSH was separated from the proteins by the gel-centrifugation method of Penefsky (*23*), in which aliquots of the enzymes were applied to a 5-mL column of Sephadex G-25 equilibrated with 50 mM Tris-HCl buffer at pH 8.0 and centrifuged. The protein concentration in the filtrate was determined using the $A_{280\ nm}$ ($\epsilon^{1\%}_{280\ nm} = 1.16$). Aliquots of each enzyme (0.2 mL of ∼1 mg/mL in 50 mM Tris-HCl buffer at pH 8.0) were added to 0.6 mL of 500 mM Tris-HCl buffer at pH 8.0, and sodium dodecyl sulfate [0.1 mL, 10% (w/v)] was added to denature the protein. The absorbance of the solution was read at 412 nm against a protein-free buffer blank that was identical with the test solution. A freshly prepared 10 mM DTNB solution (0.1 mL) was added to the reaction mixture and the protein-free buffer blank. The reaction was complete after 5 min. The concentration of free −SH groups in the enzyme was calculated from the change in absorbance, using a molar extinction coefficient of 13 600 M^{-1} cm^{-1} at 412 nm for thionitrobenzoate (*24*).

Factors That Influence GST π/1-Cys Prx Complex Formation. Wild-type GST π and wild-type 1-Cys Prx complexes were formed under standard conditions (2 h of incubation with 20% 1,6-hexanediol at 25 °C) as described above and separated using Ni−NTA chromatography. The conditions were the same for all experiments, except that dialysis and column buffers included either no GSH, 2.5 mM *S*-methylglutathione, 2.5 mM oxidized GSH, or 100 μM mercaptosuccinate and 2.5 mM reduced GSH. Additionally, a complex was formed under standard conditions between wild-type GST π and wild-type 1-Cys Prx that had been pretreated with 40 μM H_2O_2 for 1 h. Heterodimers were also formed in the absence of 1,6-hexanediol during the 2 h incubation, in the absence or presence of 2.5 mM GSH, and separated with a Ni−NTA column. The activity of the recovered WT GST P1-1 homodimer, as well as the purified heterodimer, was determined under standard conditions as described previously. The $A_{280\ nm}$ was used to determine the protein concentration. The N-terminal sequences of the recovered homodimers and purified heterodimers were determined to confirm the purity of the enzymes. Purified wild-type α- (*21*) and μ-GST (*25*, *26*) were incubated with wild-type 1-Cys Prx for 2 h in the presence of 20% 1,6-hexanediol at 25 °C. The proteins were subsequently dialyzed in 50 mM Tris-HCl buffer at pH 8.0 containing 2.5 mM GSH and separated with a Ni−NTA column equilibrated with 50 mM Tris-HCl buffer at pH 8.0 containing 2.5 mM GSH.

Detection of Glutathionylation with Immunoblot Analysis. An equimolar mixture of wild-type 1-Cys Prx and GST π, containing 2.5 mM GSH, was incubated for 1 min at room temperature. After that, GSH was eliminated from the mixture by size-exclusion chromatography using a Micro Bio-Spin 6 column (Bio-Rad, Hercules, CA). The proteins from the reaction mixture (3 μg/lane) were separated with SDS−PAGE [12% Tris-glycine, 1 mm thickness using the

Nu-PAGE system (Invitrogen, Carlsbad, CA)]. The gel was stained using SimplyBlueSafeStain (Invitrogen). After electrophoretic resolution, the proteins were electroblotted to Immobilon P membranes (Millipore, Bradford, MA) using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) at 10 V for 30 min. The membrane was probed with a monoclonal anti-SSG antibody (Virogen, Watertown, MA) according to the instructions of the manufacturer. The blot was developed using anti-mouse IRDye 700DX (red) secondary antibody (Rockland, Gilbertsville, PA) following the the instructions of the manufacturer. Finally, the immunoblot was imaged with an Odyssey dual-color Infrared Excited Imaging System (LI-COR, Lincoln, NE).

Protein−Protein Docking of the GST π and 1-Cys Prx Complex. We have used a two-stage procedure called ZDOCKpro 1.0. The ZDOCKpro package is based on the ZDOCK and RDOCK programs within Insight II molecular-modeling software (Molecular Simulations, Inc.). The Protein Data Bank structures used were PDB 1PRX (1-Cys Prx) and 19GS (GST π). In the initial stage, GST π was selected as the protein receptor and 1-Cys Prx was selected as the protein ligand and submitted to the ZDOCK program, which treats both proteins as rigid bodies exploring all six rotational and translational degrees of freedom. Contact information was limited to only account for one subunit of each protein. An angular step of 6° was used, which results in 54 000 poses. In the refinement stage, RDOCK, the best pose of near-native structures obtained in the initial stage is refined to optimize the model.

RESULTS

Production of Wild-Type GST P1-1/Wild-Type 1-Cys Prx Heterodimer. Purified human 1-Cys Prx, His-tagged at its C-terminal end (1 mg), was incubated with human GST π (1 mg), which lacks a His tag, in 50 mM Tris-HCl buffer at pH 8.0. Because both enzymes exist as homodimers, 20% 1,6-hexanediol was used to promote dissociation of the two proteins to their respective monomers. The mixture was subsequently dialyzed against buffer containing 2.5 mM *reduced* GSH but lacking 1,6-hexanediol to allow for random reassociation of the monomers. Three dimeric species were expected: WT GST P1-1 homodimer containing no His tag, WT GST P1-1/WT 1-Cys Prx heterodimer with only one His tag per enzyme dimer, and WT 1-Cys Prx containing two His tags per dimer.

The two homodimers and heterodimer were separated using a Ni−NTA column containing GSH in all buffers, as described in the Experimental Procedures. For each dimer mixture, separation on the Ni−NTA column yielded three peaks, as illustrated in Figure 1 for the separation of WT GST P1-1 homodimer (eluting first, as peak I), WT GST P1-1/WT 1-Cys Prx heterodimer (peak II), and WT 1-Cys Prx homodimer (peak III). The separation is effective because the GST homodimer has no His tag, does not bind to the Ni−NTA column, and elutes in the starting buffer. In contrast, the 1-Cys Prx homodimer has two His tags and thus is bound tightly to the column. The heterodimer, with only one His tag, elutes between the two homodimers. The two proteins containing the His tags were separated using an imidazole gradient. The composition of the three peaks was confirmed in each case by N-terminal sequencing, as

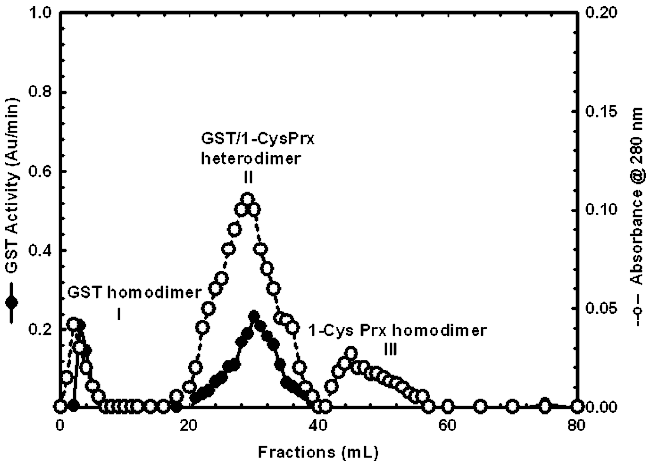

FIGURE 1: Separation of dimeric species of GST π and 1-Cys Prx using a Ni−NTA column as monitored by GST activity (●, 20 μL assayed) and absorbance at 280 nm (○). The column was initially (fractions 1−15) eluted with 2.5 mM GSH in 50 mM Tris-HCl at pH 8.0 (buffer A). At fraction 15, a linear gradient was started from buffer A (20 mL) to 0.3 M imidazole in buffer A (20 mL). After the gradient, elution was continued with 0.3 M imidazole in buffer A.

Table 1: N-Terminal Sequencing of WT GST P1-1/WT 1-Cys Prx Heterodimer[a]

	GST P1-1		1-Cys Prx	
cycle	amino acid	amount (pmol)	amino acid	amount (pmol)
1	Pro	23	Pro	23
2	Tyr	25	Gly	20
3	Thr	24	Gly	20
4	Val	27	Leu	35
5	Pro	26	Leu	34
6	Pro	25	Leu	33
7	Tyr	22	Gly	19
8	Thr	22	Asp	21
9	Val	22	Val	22
10	Val	27	Ala	28
11	Tyr	19	Pro	21
12	Phe	22	Asn	21
	average	24	average	25
		molar ratio = 1.03		

[a] The picomoles of heterodimer shown here do not represent the total amount of heterodimer recovered from the column shown in Figure 1 because only an aliquot of purified heterodimer was subjected to N-terminal sequencing.

illustrated in Table 1 for the WT GST P1-1/WT 1-Cys Prx heterodimer. Two amino acids are observed at every cycle (except for those positions at which the two proteins have the same amino acid). The two proteins are present in equimolar amounts, confirming the presence and purity of the heterodimer. These results are typical of those obtained for all heterodimers studied. To further identify the elution positions of GST P1-1 and 1-Cys Prx homodimers, control studies were conducted in which GST P1-1 and 1-Cys Prx were treated individually with 20% 1,6-hexanediol followed by dialysis with buffer containing GSH. After treatment, each enzyme was loaded onto the Ni−NTA column. For GST P1-1 and 1-Cys Prx, elution from the Ni−NTA column yielded only peak I and peak III, respectively.

The purified heterodimer was stored in 50 mM Tris-HCl buffer at pH 8.0 containing 2.5 mM GSH at 4 °C. However, it was important to establish if the heterodimer is stable under the conditions of purification and storage. To address this

Table 2: Wild-Type Dimeric Enzymes Recovered from the Ni−NTA Column and Their Relative Specific Activities

enzyme	GST activity[a] [μmol min^{-1} (mg of GST present)$^{-1}$]	1-Cys Prx activity H_2O_2 [μmol min^{-1} (mg of 1-Cys Prx present)$^{-1}$]	1-Cys Prx activity PLPCOOH [μmol min^{-1} (mg of 1-Cys Prx present)$^{-1}$]
WT GST P1-1 homodimer	60	0	0
WT 1-Cys Prx homodimer	0	0	0
WT GST/Prx heterodimer	16	**4.9**	**5.0**

[a] Enzyme activity for GST P1-1 was measured with respect to CDNB as the electrophilic substrate.

issue, two aliquots of the heterodimer from peak II (Figure 1), which had been purified and stored at 4 °C, were reapplied to the Ni−NTA column. One aliquot of the heterodimer was reapplied to the column under the same conditions as in Figure 1 containing 2.5 mM GSH in all buffers, while the other aliquot was dialyzed against buffer to remove GSH and also reapplied to the Ni−NTA column but in the absence of GSH. Any dissociation of the heterodimer into its monomeric subunits could have resulted in new peaks, peaks I and III, corresponding to the GST homodimer and 1-Cys Prx homodimer, respectively. In the presence of GSH, the only peak obtained was peak II, corresponding to the heterodimer. Furthermore, the specific activity of GST did not change. In contrast, in the absence of GSH, the heterodimer partially dissociated and rearranged into the respective homodimers as demonstrated by the reappearance of peaks I and III and, while some heterodimer remained, these results demonstrate that the heterodimer is stabilized by the presence of GSH.

Activity of Recovered Proteins. The dimers recovered from the Ni−NTA column were evaluated for GST activity using CDNB as the substrate (Table 2). The specific activity of the wild-type GST homodimer after recovery from the Ni−NTA column was the same as the initial specific activity before mixing in 1,6-hexanediol, indicating that treatment with 1,6-hexanediol does not irreversibly affect GST P1-1. Correspondingly, purified 1-Cys Prx homodimer was inactive before mixing with hexanediol, indicating that purified 1-Cys Prx exists in the Cys−sulfenic acid form. After recovery from the Ni−NTA column, the 1-Cys Prx homodimer remained inactive, clearly indicating that GSH alone cannot reactivate 1-Cys Prx. In contrast, the heterodimer exhibited ~25% of the GST activity of the GST P1-1 homodimer but *recovered full 1-Cys Prx activity* when assayed by hydrogen peroxide or phospholipid peroxide (*17*). As a control, we determined that GST P1-1 does not have any peroxidase activity. Similarly, we ascertained that 1-Cys Prx does not exhibit GST activity. These data demonstrate that heterodimerization of 1-Cys Prx with GST π in the presence of GSH results in reactivation of 1-Cys Prx, while inhibiting GST activity toward CDNB as a substrate.

Factors That Influence GST π/1-Cys Prx Complex Formation. To evaluate the factors that influence complex formation between GST π and 1-Cys Prx, various conditions were tested for incubation of GST π and 1-Cys Prx, after which the samples were applied to the Ni−NTA column and eluted as illustrated in Figure 1. To facilitate comparisons, the amount of protein in each of the three protein peaks is expressed as total absorbance units at 280 nm (a measure of the area under each peak). Table 3 records these results.

Table 3: Comparison of Recoveries of Wild-Type GST π Homodimer, Wild-Type 1-Cys Prx Homodimer, and Heterodimer Protein Peaks from the Ni−NTA Column under Various Conditions

conditions	total absorbance$_{280\ nm}$ units: peak I (GST homodimer)	peak II (heterodimer)	peak III (1-Cys Prx homodimer)
with hexanediol treatment[a]			
reduced GSH (2.5 mM)	0.12	**1.08**	0.21
no GSH	0.88	**0.25**	0.41
S-methylglutathione (2.5 mM)	0.17	**1.06**	0.38
oxidized GSH (2.5 mM)	0.97	**0.0**	0.37
mercaptosuccinate (100 μM) and GSH (2.5 mM)	0.59	**0.61**	0.39
H_2O_2-treated 1-Cys Prx[b]	0.59	**0.38**	0.51
no hexanediol treatment[a]			
reduced GSH (2.5 mM)	1.08	**0.27**	0.18
no GSH	1.16	**0.11**	0.31

[a] Enzymes used were WT GST P1-1 (1 mg) mixed with WT 1-Cys Prx (1 mg). [b] GSH (2.5 mM) was present during the dialysis and dimer separation on the Ni−NTA column.

It is possible that the enzymes need to be completely dissociated into their respective monomers before heterodimers can be generated. Therefore, 20% 1,6-hexanediol, a reagent previously used to promote heterodimer formation among GSTs (*21*, *27*), was used to separate GST π (1 mg) and 1-Cys Prx (1 mg) into monomers followed by dialysis against 50 mM Tris-HCl buffer at pH 8.0 in the absence and presence of 2.5 mM GSH. The mixtures were separated using a Ni−NTA column, as described previously. In the presence of GSH, the amount of heterodimer reached 1.08 total absorbance units (line 1 in Table 3). However, in the absence of GSH, there was a 4−5-fold decrease in the amount of heterodimer obtained (line 2 in Table 3). These results suggest that monomers of GST π and 1-Cys Prx have a higher affinity for each other when GSH is present in the medium.

To test if the free −SH group of GSH is important for heterodimer formation, *S*-methylglutathione was substituted for GSH. *S*-Methylglutathione is a derivative of GSH, in which the −SH is blocked by the small methyl group; *S*-methylglutathione is known to be a competitive inhibitor with respect to GSH (*25*). With 2.5 mM *S*-methylglutathione in every buffer, the amount of heterodimer obtained is similar to the amount obtained with reduced GSH (compare lines 1 and 3 in Table 3). These data suggest that a reduced thiol in the GSH tripeptide is not required for heterodimer formation. In the presence of oxidized GSH, which also lacks a free −SH group, no visible heterodimer is formed possibly because of the large size of the GSSG molecule (line 4 in Table 3).

To determine if the active site of 1-Cys Prx is directly involved in heterodimer formation, we attempted to generate heterodimers in which the active site of the 1-Cys Prx subunit was occupied by mercaptosuccinate, a competitive inhibitor of the peroxidase site (*17*, *28*). Thus, 100 μM mercaptosuccinate was added together with 2.5 mM GSH. The amount of heterodimer obtained was approximately 0.61 total $Abs_{280\ nm}$ units (line 5 in Table 3), a decrease compared with the experiment with 2.5 mM GSH present alone (line 1 in Table 3). Additionally, we generated a heterodimer in which

Table 4: Specific Activities of Wild-Type GST π Homodimer, Wild-Type 1-Cys Prx Homodimer, and Heterodimer Protein Peaks from the Ni−NTA Column under Various Conditions

conditions	GST P1-1 activity[b]	1-Cys Prx activity[a]	GST activity of heterodimer[b]	peroxidase activity of heterodimer[a]	
				H_2O_2	PLPCOOH
with hexanediol treatment					
reduced GSH	60	0.0	16	4.9	5.0
S-methylglutathione	56	0.0	57	0.2	0.2
mercaptosuccinate and GSH	67	0.0	52	0.0	0.0
H_2O_2-treated 1-Cys Prx	66	0.0	59	0.9	0.2
no hexanediol treatment					
reduced GSH	60	0.0	19	4.9	5.0

[a] Peroxidase activity was measured with respect to hydrogen peroxide (H_2O_2) and phospholipid peroxide (PLPCOOH) [units = μmol min^{-1} (mg of 1-Cys Prx present)$^{-1}$]. [b] GST activity was measured with respect to CDNB as the electrophilic substrate [units = μmol min^{-1} (mg of GST present)$^{-1}$].

the active site of the 1-Cys Prx subunit had previously been hyperoxidized by H_2O_2, prior to mixing with GST π. The amount of heterodimer formed (line 6 in Table 3) was reduced to ~35% of that formed with untreated GST π (line 1 in Table 3). These results indicate that the active site of the 1-Cys Prx subunit may be partially involved in complex formation.

Manevich et al. reported that incubation of inactive 1-Cys Prx with reduced GSH-saturated GST π resulted in heterodimer formation and activation of 1-Cys Prx (*14*). To test whether reduced GSH by itself is sufficient to yield heterodimer, the incubation of GST π and 1-Cys Prx was carried out in the absence of 1,6-hexanediol. As shown in lines 7 and 8 of Table 3, in the absence of GSH, a small amount of heterodimer was obtained (0.11 total $Abs_{280\ nm}$). This heterodimer peak was increased in the presence of GSH, yielding 0.27 total $Abs_{280\ nm}$ units. These results indicate that in the absence of 1,6-hexanediol, GSH can influence heterodimer formation. However, because the amount of peak II is smaller in the absence of 1,6-hexanediol (compare lines 1 and 2 with lines 7 and 8 in Table 3), it is apparent that GSH alone is not sufficient to produce high amounts of the complex.

We investigated whether 1-Cys Prx distinguishes among the various classes of GSTs in forming heterodimers. As representative of the α class, we used recombinant rat GST A1-1, prepared as described in ref *21*; and for the μ class, we used recombinant rat GST M1-1, purified as described in ref *26*. Our results show that 1-Cys Prx does not readily form a heterodimer with other GST classes when treated with 20% 1,6-hexanediol, followed by dialysis and chromatography on the Ni−NTA column in the presence of 2.5 mM GSH. For GST A1-1 (α class) no detectable amount of complex (peak II on the Ni−NTA column) was identified. GST M1-1 (μ class) formed a small amount of heterodimer with 1-Cys Prx. The total $Abs_{280\ nm}$ was 0.19 as compared with 1.08 peak II for GST π (line 1 in Table 3). Furthermore, the peroxidase specific activity was only ~20% of that of the GST P1-1/1-Cys Prx complex (line 3 in Table 2). These observations probably reflect differences in affinity between the 1-Cys Prx and GST isozymes.

The proteins recovered from the Ni−NTA column were assayed for GST activity using CDNB as the substrate (Table 4). The specific activity of the wild-type GST homodimer after recovery from the Ni−NTA column under the different conditions examined was the same as the initial specific activity, indicating that conditions tested did not permanently affect GST P1-1. Likewise, purified 1-Cys Prx homodimer was inactive before any of the treatments, confirming that pure 1-Cys Prx exists in the Cys−sulfenic acid form. After recovery from the Ni−NTA column, 1-Cys Prx homodimer remained inactive, clearly demonstrating that none of the conditions tested was able to reactivate 1-Cys Prx. For heterodimers treated with 20% 1,6-hexanediol, we found that, when buffers on the Ni−NTA column contained reduced GSH, the heterodimer recovered exhibited ~25% of the GST activity of the GST P1-1 homodimer but recovered full 1-Cys Prx activity when assayed by hydrogen peroxide or phospholipid hydroperoxide, as discussed previously (line 1 in Table 4). In contrast, when buffers on the Ni−NTA contained *S*-methylglutathione, the heterodimer displayed similar activity to that of the GST P1-1 homodimer; moreover, the heterodimer did not recover 1-Cys Prx activity when assayed by hydrogen peroxide or phospholipid hydroperoxide (line 2 in Table 4). A similar effect was also apparent with the complex that was isolated from the Ni−NTA column with buffers containing reduced GSH and mercaptosuccinate (line 3 in Table 4) and with the complex formed between GST π and hyperoxidized 1-Cys Prx (line 4 in Table 4). These results demonstrate that, while reduced GSH is not critical for complex formation, it *is* necessary to *reactivate* 1-Cys Prx. Additionally, these data suggest that inhibition of GST activity of the heterodimer is an indicator of reactivation of 1-Cys Prx because no reactivation was observed for any of the complexes that exhibited full GST activity.

Furthermore, activities were measured for the heterodimer obtained without 1,6-hexanediol treatment but in the presence of reduced GSH. The results are comparable to the heterodimer produced with 1,6-hexanediol treatment, indicating that hexanediol does not influence the activity of the heterodimer (lines 1 and 5 in Table 4); it only enhances the amount of heterodimer complex formed (Table 3). Activities could not be measured for heterodimers produced in the absence of GSH because of the instability of the complex when GSH is not present in the storage mixture.

Kinetic Properties of Wild-Type GST P1-1/Wild-Type 1-Cys Prx Heterodimer. Because the GST specific activity of the heterodimer is ~25% of that of the GST homodimer, the association of GST with 1-Cys Prx must have an effect on the active site of GST P1-1. The kinetic parameters of the heterodimer were determined with various substrates of GST P1-1 to evaluate which of the known substrate sites of GST π are affected by the heterodimerization with 1-Cys Prx. Table 5 shows the K_m value for GSH and the K_m and

Table 5: Kinetic Parameters for the GSH Conjugation to Different Substrates by WT GST P1-1 Homodimer and WT GST P1-1/WT 1-Cys Prx Heterodimer

	WT GST P1-1 homodimer		WT GST/WT Prx heterodimer	
substrate	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)	V_{max} [μmol min^{-1} (mg of GST present)$^{-1}$]
CDNB	650 ± 39	65 ± 6	613 ± 36	19 ± 1
BITC	68 ± 5	70 ± 4	77 ± 2	14 ± 0
mBBr	42 ± 2	21 ± 3	60 ± 2	22 ± 2
GSH[a]	51 ± 1	59 ± 2	187 ± 12	16 ± 0

[a] GSH kinetics measured using CDNB as the electrophilic substrate.

V_{max} values for each of the following xenobiotic substrates: CDNB, mBBr, and BITC. We have previously shown that these substrates occupy distinct sites of GST π (*16*). For wild-type hGSTP1-1 homodimer, the V_{max} for mBBr is $\sim^1/_3$ of that for the other substrates, whereas the K_m for CDNB is about 10 times that of the other substrates (Table 5). For the GST P1-1/1-Cys Prx heterodimer, the K_m values of the three xenobiotic substrates do not change appreciably. In contrast, the K_m for GSH is ~3.5 times that of the homodimer, indicating that the heterodimer has a weakened affinity for GSH. The V_{max} of the heterodimer was approximately 14−19 μmol min^{-1} mg^{-1}, indicating that the association of the two proteins partially inhibits the catalytic activity of the GST subunit with respect to most substrates. In contrast, the V_{max} of the heterodimer with respect to mBBr is not affected by the association of GST π with 1-Cys Prx.

The kinetic parameters of the complex were also determined for 1-Cys Prx substrates. For the wild-type 1-Cys Prx homodimer, the K_m for H_2O_2 is 180 μM and remains essentially unchanged for the heterodimer (K_m = 148 μM). Similarly, the K_m for PLPCOOH of the wild-type 1-Cys Prx homodimer is 120 μM and that of the heterodimer is 156 μM. These data clearly indicate that the known substrate-binding sites of 1-Cys Prx are not affected by the heterodimerization.

Sulfhydryl Content of Wild-Type GST P1-1/Wild-Type 1-Cys Prx Heterodimer. Human GST π contains four cysteines per subunit: Cys-14, Cys-47, and Cys-101 identified as part of the monobromobimane site (*22*) and Cys-169. Human 1-Cys Prx contains a nonconserved cysteine, at position 91, per subunit, in addition to the conserved Cys-47. Thus, heterodimerization of π GST with 1-Cys Prx would be expected to give six sulfhydryl groups per dimer if all are reduced. We therefore determined the free cysteine content of the individual enzymes and of the heterodimer complex after the rapid and complete removal of GSH, as described in the Experimental Procedures. The free sulfhydryls were determined by the reaction of each protein sample with DTNB ′under denaturing conditions (*24*). After complete removal of GSH, the intact GST P1-1 dimer exhibits an average of 7.6 cysteine residues, close to the expected number of 8. The average enzyme dimer of 1-Cys Prx has 2.4 reduced cysteines, a decrease of 1.6 from the expected 4.0 per dimer if all of the cysteines were reduced; these results are consistent with Cys-47 of 1-Cys Prx being present in an oxidized form. For the heterodimer, the average number of cysteines is 3.8 per dimer. The lower than expected value

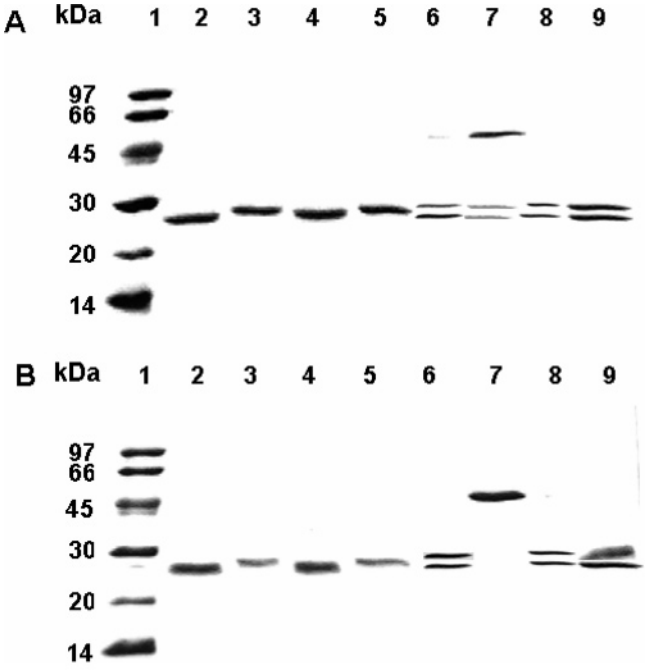

FIGURE 2: Analysis of wild-type homodimers and heterodimer proteins by nonreducing and reducing SDS−PAGE. Isolated dimers (10 μg of each sample) were each mixed with 20 μL of nonreducing sample buffer [0.5 M Tris-HCl at pH 6.8, 4% (w/v) SDS, and 30% (v/v) glycerol] or reducing sample buffer (nonreducing sample buffer and 0.1 mM DTT), heated at 95 °C for 5 min, subjected to SDS−PAGE on a 15% gel, and stained with Coomassie Brilliant Blue. (A) Electrophoresis conducted immediately after isolation of the heterodimer complex or (B) electrophoresis conducted after 24 h of storage at 4 °C of the heterodimer complex. Lane 1, protein standards (with molecular weights given in kilodaltons); lane 2, GST π homodimer (−DTT); lane 3, 1-Cys Prx homodimer (−DTT); lane 4, GST π homodimer (+DTT); lane 5, 1-Cys Prx homodimer (+DTT); lane 6, wild-type heterodimer in the presence of *S*-methylglutathione (−DTT); lane 7, wild-type heterodimer in the presence of GSH (−DTT); lane 8, wild-type heterodimer in the presence of *S*-methylglutathione (+DTT); and lane 9, wild-type heterodimer in the presence of GSH (+DTT).

(by about 2 cysteines per heterodimer) may be caused by the formation of a disulfide bond between the two subunits.

To evaluate whether the two subunits in the complex form an intersubunit disulfide bond, as suggested by the DTNB assay, the GST P1-1 and 1-Cys Prx homodimers and the heterodimer recovered from the Ni−NTA column were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE) either with or without pretreatment of the protein sample with dithiothreitol (DTT) (Figure 2). Electrophoresis was conducted either immediately after isolation of the heterodimer complex (Figure 2A) or after 24 h of storage at 4 °C of the heterodimer complex (Figure 2B). Figure 2 shows that the homodimers of GST P1-1 and 1-Cys Prx were detected at molecular sizes corresponding to the monomeric form independent of the time and the absence or presence of DTT (lanes 2−5 in parts A and B of Figure 2). However, for the heterodimer treated with GSH, in the absence of DTT at time 0 (lane 7 in Figure 2A), we detected a higher molecular-weight band corresponding to ~50 kDa, indicative of an SDS-resistant dimer, along with two residual lower molecular-weight bands corresponding, respectively, to the two monomers (lane 7 in Figure 2A). The 50-kDa band is eliminated when the heterodimer sample is pretreated with DTT (lane 9 in Figure

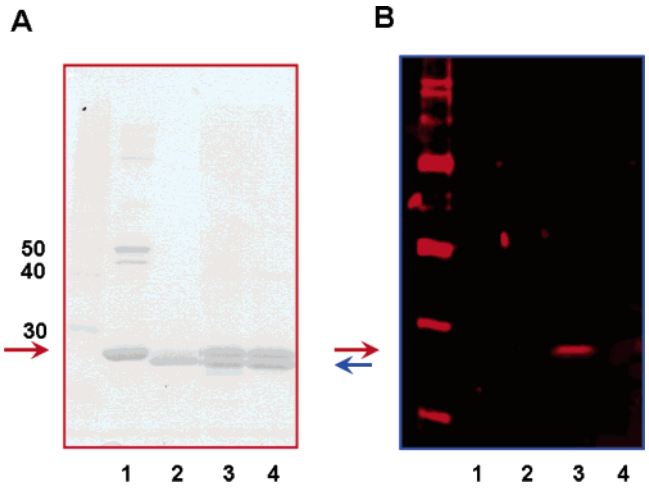

FIGURE 3: Detection of glutathionylation using immunoblot analysis. Proteins were separated by SDS−PAGE and transferred to the Immobilon P membrane, and glutathionylation was detected with monoclonal anti-SSG antibody. (A) SDS−PAGE. (B) Immunoblot. Lane 1, WT 1-Cys Prx (control); lane 2, WT GST π (control); lane 3, mixture of 1-Cys Prx and GST π after 1 min of incubation with excess GSH; and lane 4, same as lane 3 after reduction with DTT. The molecular-weight markers are shown on the left, and the presumed positions of 1-Cys Prx (red arrow) and GST π (blue arrow) are indicated.

2A). This effect of DTT suggests the formation of an intermolecular disulfide bridge. Moreover, for the heterodimer in the absence of DTT at 24 h (lane 7 in Figure 2B), only the ~50-kDa molecular-weight band is evident, indicating that the heterodimers completely formed a disulfide linkage prior to the electrophoresis run; again the dimer is converted to two monomers by incubation with DTT (lane 9 in Figure 2B). In contrast, the heterodimer sample that was treated with *S*-methylglutathione did not exhibit an SDS-resistant dimer independent of the time and absence or presence of DTT (lanes 6 and 8 in parts A and B of Figure 2), suggesting that reduced GSH is necessary to produce an intermolecular disulfide within the heterodimer. These data demonstrate that a disulfide is slowly formed between the two subunits of the heterodimer complex only when the heterodimer is incubated with reduced GSH.

Detection of Glutathionylation Using Immunoblot Analysis. SDS−PAGE and Western blot analysis were used to detect the subunit location of the GST π-mediated glutathionylation in the complex. Figure 3A shows bands in an SDS−PAGE gel corresponding to 1-Cys Prx (lane 1), GST π (lane 2), GST π/1-Cys Prx heterodimer incubated with GSH for 1 min (excess GSH was removed using size-exclusion chromatography) (lane 3), and GST π/1-Cys Prx heterodimer incubated with GSH for 1 min but under reducing conditions (lane 4). The gel from Figure 3A was immunoblotted against an antibody for GSH to identify if glutathionylation occurs in either the GST π subunit or the 1-Cys Prx subunit of the complex (Figure 3B). The resulting blot shows that the *1-Cys Prx subunit* (lane 3 in Figure 3B) rather than the GST π subunit *is glutathionylated*.

Properties of Mutant−Wild-Type Interactions. Mutant proteins, with replacement of critical amino acids (one at a time) were selected to test which binding sites of 1-Cys Prx and GST π are needed to form a complex. For 1-Cys Prx, the active-site cysteine 47 was mutated to serine to evaluate if glutathionylation of this cysteine is required for heterodimer formation. Additionally, Ala replaced Ser-32 because it has been found that this residue is essential for phospholipid binding but not for peroxidase activity (*17*). For GST π, the GSH site was disrupted by mutating Tyr-7 to Phe to remove the hydroxyl group, which functions to stabilize the thiol of GSH (*29*). In addition, the CDNB site of GST π was altered by replacing Tyr-108 with Ser because it has been demonstrated that a major determinant in the binding of CDNB is the aromatic ring of Tyr-108 (*30*). Other sites that were mutated include the two xenobiotic sites that we have identified, the mBBr site, and the BITC site (*16*, *22*). We have shown that Cys-101 is part of the mBBr site; therefore, this residue was replaced by Ser. Additionally, we have shown that Tyr-103 belongs to the BITC site; thus, this site was impaired by changing this residue to Ser, because we have shown that removal of the aromatic ring greatly decreases binding. We also replaced the additional three cysteines (Cys-14, Cys-47, and Cys-167) by Ser to assess whether glutathionylation of one of these cysteines is required for heterodimer formation.

Table 6: Enzyme Pairs Used To Form Heterodimers and the Resulting Three Species Recovered from the Ni−NTA Column

enzymes[a]		total absorbance$_{280\ nm}$ units		
GST P1-1	1-Cys Prx	peak I	peak II (heterodimer)	peak III
wild type	wild type	0.12	**1.08**	0.21
wild type	C47S	0.18	**1.22**	0.35
wild type	S32A	0.48	**0.99**	0.26
Y103S	wild type	0.31	**0.99**	0.47
Y108S	wild type	0.11	**0.84**	0.21
C101S	wild type	0.18	**1.31**	0.19
C14S	wild type	0.11	**1.01**	0.29
C167S	wild type	0.10	**1.19**	0.46
C47S	wild type	1.19	**0.0**	0.54
Y7F	wild type (no hexanediol treatment)	1.21	**0.0**	0.47

[a] Wild-type and most mutant proteins (1 mg of each) were treated with 20% 1,6-hexanediol followed by dialysis against buffer containing 2.5 mM GSH and loaded onto a Ni−NTA column as described in the Experimental Procedures. Only the Y7F-GST mutant was incubated with 1-Cys Prx in the absence of hexanediol.

All mutants were constructed, expressed, and purified to homogeneity. In each case, heterodimer formation was evaluated by incubation of the two proteins, in which one was wild-type and the other was a mutant, and the protein mixture was applied to a Ni−NTA column as described in the Experimental Procedures. Table 6 summarizes the recoveries of the protein in the three peaks from the Ni−NTA column. Both C47S and S32A mutants of 1-Cys Prx formed heterodimers with wild-type GST π, indicating these residues do not contribute to complex formation (lines 2 and 3 in Table 6).

All three mutants of GST π (Y103S, Y108F, and C101S), representative of the three known xenobiotic substrate sites, formed complexes with wild-type 1-Cys Prx in amounts that did not differ appreciably from that of the wild-type heterodimer, suggesting these residues do not mediate complex formation between the two subunits (lines 4−6 in Table 6). We attempted to form a complex between wild-type 1-Cys Prx and Y7F-GST π under the standard conditions used for the other proteins, but the mutant precipitated in the presence of 15, 17, and 20% 1,6-hexanediol. Therefore, Y7F-GST π

Table 7: Specific GST and Peroxidase Activities of Wild-Type and Mutant Enzymes and the Heterodimer Complexes

					peroxidase activity of heterodimer[a]	
GST P1-1	activity[b]	1-Cys Prx	activity[a]	GST activity of heterodimer[b]	H_2O_2	PLPCOOH
wild type	62	wild type	0.0	16	4.9	5.0
wild type	60	C47S	0.0	62	0.0	0.0
wild type	60	S32A	0.0	18	4.8	0.0
Y103S	89	wild type	0.0	44	4.8	4.9
Y108S	37	wild type	0.0	9	4.6	4.7
C101S	88	wild type	0.0	22	4.1	4.0
C14S	45	wild type	0.0	10	3.0	2.9
C167S	66	wild type	0.0	19	4.9	5.0

[a] Peroxidase activity was measured with respect to hydrogen peroxide (H_2O_2) and phospholipid peroxide (PLPCOOH) [units = μmol min^{-1} (mg of 1-Cys Prx present)$^{-1}$]. [b] GST activity was measured with respect to CDNB as the electrophilic substrate [units = μmol min^{-1} (mg of GST present)$^{-1}$].

and 1-Cys Prx were incubated in the absence of hexanediol. No heterodimer was generated under the conditions tested (line 10 in Table 6), indicating the importance for complex formation of an intact GST π GSH site. These results contrast with the observation of the heterodimer ($Abs_{280\ nm}$ units = 0.27, line 7 in Table 3) when the two wild-type enzymes were incubated in the absence of hexanediol.

We considered the cysteine residues of GST π to determine whether they are involved in heterodimerization. C14S, C101S, and C167S mutants of GST π formed heterodimers with wild-type 1-Cys Prx in amounts comparable to the wild-type heterodimer (lines 6−8 in Table 6). In contrast, incubation of C47S GST π with wild-type 1-Cys Prx (line 9 in Table 6) did not yield any visible heterodimer, indicating that this residue is essential for the generation of a complex.

All recovered heterodimers were subsequently assayed for GST and peroxidase activities, as recorded in Table 7. For the mutations of the 1-Cys Prx subunit, we found that, as expected, the WT GST/C47S 1-Cys Prx heterodimer did not exhibit any peroxidase activity. Additionally, inhibition of the GST activity was not seen, confirming the observation that inhibition of the GST activity correlates with activation of 1-Cys Prx (line 2 in table 7). In contrast, the WT GST/S32A 1-Cys Prx heterodimer exhibited peroxidase activity when assayed with H_2O_2 but not with the phospholipid peroxide (PLPCOOH), and GST activity was markedly decreased (line 3 in Table 7).

For mutations of the GST π subunit, the heterodimers with amino acid replacements in the GST xenobiotic substrate sites displayed peroxidase activity with respect to both hydrogen peroxide and phospholipid peroxide and exhibited lower GST activity (lines 4−6 in Table 7). Therefore, substitutions at these GST π residues do not affect the activation of 1-Cys Prx. Heterodimers with cysteine to serine mutations at positions 14, 101, and 167 displayed peroxidase activity, although that of the C14S GST P1-1/1-Cys Prx complex was lower than that of the wild-type heterodimer (lines 6−8 in Table 7). Because no heterodimers were produced when the GSH-binding site of GST π was altered (C47S and Y7F), no activity data appear in Table 7.

To determine the residues responsible for the formation of the intersubunit disulfide bond between the two enzymes, mutant heterodimers recovered from the Ni−NTA column were subjected to SDS−PAGE, in the absence or presence of DTT (Figure 4). In the absence of DTT, all heterodimers, *except* WT GST P1-1/C47S 1-Cys Prx, were detected at molecular sizes corresponding to an SDS-resistant dimer

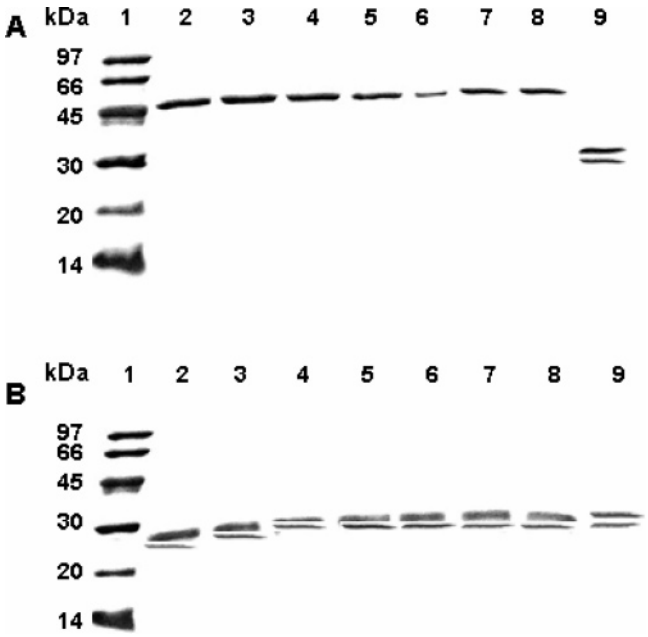

FIGURE 4: Analysis of wild-type and mutant heterodimer proteins by nonreducing and reducing SDS−PAGE. Purified heterodimers (10 μg of each sample) were each mixed with 20 μL of (A) nonreducing sample buffer [0.5 M Tris-HCl at pH 6.8, 4% (w/v) SDS, and 30% (v/v) glycerol] and (B) reducing sample buffer (sample buffer and 0.1 mM DTT), heated at 95 °C for 5 min, subjected to SDS−PAGE on a 15% gel, and stained with Coomassie Brilliant Blue. Lane 1, protein standards (with molecular weights given in kilodaltons); lane 2, WT GST π/WT 1-Cys Prx heterodimer; lane 3, Y103S GST π/WT 1-Cys Prx heterodimer; lane 4, Y108S GST π/WT 1-Cys Prx heterodimer; lane 5, C14S GST π/WT 1-Cys Prx heterodimer; lane 6, C101S GST π/WT 1-Cys Prx heterodimer; lane 7, C167S GST π/WT 1-Cys Prx heterodimer; lane 8, WT GST π/S32A 1-Cys Prx heterodimer; and lane 9, WT GST π/C47S 1-Cys Prx heterodimer.

(lanes 2−8 in Figure 4A). This result suggests that GST π residues Tyr-103, Tyr-108, Cys-101, Cys-14, and Cys-167 are not involved in the linkage formed between the two protein subunits. Similarly, Ser-32 of 1-Cys Prx is not involved in forming a link with the GST π subunit. In contrast, the WT GST P1-1/C47S 1-Cys Prx heterodimer migrated as two monomers (lane 9 in Figure 4A), suggesting that Cys-47 of 1-Cys Prx is directly involved in the formation of an intersubunit linkage between GST π and 1-Cys Prx, either immediately after isolation of the heterodimer complex or after 24 h of storage at 4 °C of the heterodimer complex. In the presence of DTT, all heterodimer proteins (wild-type or mutants) were detected at molecular weights correspond-

ing to the two monomers (Figure 4B), confirming that the two subunits are linked by a disulfide bond. These data demonstrate that Cys-47 of 1-Cys Prx is one of the cysteines involved in the formation of the disulfide between the two subunits, which is part of the mechanism of activation of 1-Cys Prx.

DISCUSSION

Various proteins function in the form of a complex, and it is necessary to understand the mechanism of the protein−protein interaction to clarify the function of these proteins. Manevich et al. demonstrated that GST π activates the antioxidant enzyme 1-Cys Prx by formation of a heterodimer (*14*); however, the chemical and structural basis of complex formation between GST π and 1-Cys Prx was not examined in the original study. In the present study, we have isolated a heterodimeric complex between 1-Cys Prx with a C-terminal His_6 tag and GST π. The salient observations are the following: the heterodimer was formed by incubation of inactive, oxidized 1-Cys Prx homodimer with the active GST homodimer in the presence of GSH. The purified complex exhibited GST activity that is ~25% of that of the GST π homodimer, while 1-Cys Prx was fully active toward either H_2O_2 or phospholipid peroxide. The heterodimer also forms in the presence of *S*-methylglutathione, but no 1-Cys Prx activity was found under these conditions. Glutathionylation of 1-Cys Prx in the heterodimer was detected by immunoblot analysis. Subsequently, a disulfide-linked dimer was observed on SDS−PAGE, and the reduced cysteine content was decreased by 2 per complex.

Thus, in our *in vitro* study, we have successfully formed, isolated, and characterized a pure heterodimer of GST π and 1-Cys Prx. In the heterodimer, the 1-Cys Prx subunit affects the GST subunit by decreasing V_{max}, as well as by increasing the K_m of GSH ~4-fold. This may result from a change in conformation of the GSH site of GST, making it less accessible to the solvent. In contrast, when assayed for peroxidase activity, the heterodimer is fully active [5.0 μmol min^{-1} (mg of 1-Cys Prx present)$^{-1}$], while the isolated 1-Cys Prx homodimer has no peroxidase activity. After 1-Cys Prx reacts with a peroxide substrate, the critical cysteine of the enzyme is converted to the corresponding sulfenic acid and, until that amino acid is restored to cysteine, the enzyme cannot react with an additional peroxide substrate (*11*). We conclude that reactivation of oxidized (sulfenic acid) 1-Cys Prx requires its heterodimerization with GST π. GSH bound to GST is used as an electron donor to reduce the oxidized 1-Cys Prx. However, we must emphasize that *free* GSH does not activate 1-Cys Prx; this observation may be explained by the fact that Cys−sulfenic acid of 1-Cys Prx is buried, as in the crystal structure (*13*).

Our experiments lead to the conclusion that GSH serves other roles in addition to donating reducing equivalents in the catalytic cycle of the heterodimer. GSH stabilizes the complex because, in its absence, the heterodimer dissociates when subjected to a second round of the Ni−NTA column. In contrast, the heterodimer does not dissociate when subjected to a second round of the Ni−NTA column but with added GSH in the column buffers. GSH also increases the affinity of the 1-Cys Prx subunit toward the GST π subunit when the two proteins are in their monomeric forms. This conclusion is based on the observation that there is an increase in the amount of heterodimer generated when GSH is present.

Although physiological reactivation of 1-Cys Prx may require heterodimerization with GST π, dissociating reagents, such as 1,6-hexanediol, are not present in living systems. Therefore, there must be other factors that influence the formation of the complex biologically. We evaluated whether the complex still forms in the absence of hexanediol but with GSH added to the incubation mixture and found that fully active heterodimer *is* formed, but its amount is decreased ~4-fold when compared to the amount of complex obtained with 1,6-hexanediol. A complex is also formed in the absence of both 1,6-hexanediol and GSH in the incubation mixture, but the amount was only half of that with added GSH. Physiologically, complex formation may result from a monomer−dimer equilibrium among the two proteins, which is driven toward the complex by preferential binding of GSH to the complex; this process may be facilitated by the high concentration of GSH (1−10 mM) inside cells (*31*). Alternatively, physiological formation of heterodimers may occur during synthesis of the two proteins before homodimers actually form. It is interesting that the GST π monomer has been reported to form a complex with another protein, Jun N-terminal kinase (*32*, *33*). Thus, complex formation between GST π and other proteins may be a more general function of GST π.

There are two general processes involved in the activation of 1-Cys Prx: formation of the heterodimer and reaction of GSH with the Cys−sulfenic acid. Heterodimer formation is necessary but not sufficient for peroxidase activation. We have shown that reduced GSH promotes both heterodimer formation and reactivation of 1-Cys Prx. In contrast, when *S*-methylglutathione was included in the incubation solution as well as in the buffers used for chromatography on the Ni−NTA column, the amount of heterodimer isolated was equivalent to the amount obtained with reduced GSH; however, the heterodimer did not exhibit any peroxidase activity and, interestingly, no inhibition toward GST activity. Furthermore, when the peroxidase active site of 1-Cys Prx is perturbed by either its occupation by mercaptosuccinate or by hyperoxidation, a complex still forms but in a decreased amount. However, this complex is also inactive with respect to peroxidase activity but exhibits full GST activity. On the basis of these results, it can be concluded that the lack of peroxidase activity correlates with the lack of GST inhibition. The addition of oxidized GSH to the protein mixture yielded no heterodimer, possibly because the larger disulfide-linked molecule with its six amino acid residues, when bound to a GST subunit, sterically hinders the approach of the 1-Cys Prx subunit.

In addition to the GST π, α and μ classes of GST are prevalent in mammalian tissues. We have also tested both pure α- and pure μ-GST with 1-Cys Prx, but only the μ-GST formed a heterodimer with some peroxidase reactivation. Crystal structure studies of the different classes of GSTs have shown that the π and μ isozymes are the most similar in their crystal structures (*8*). Moreover, Pettigrew and Colman reported that GST heterodimers can form between π-GST and μ-GST but not with α-GST (*34*). Therefore, it is possible that the subunit interface region through which the GST subunit interacts with Prx is similar for both μ- and π-GST.

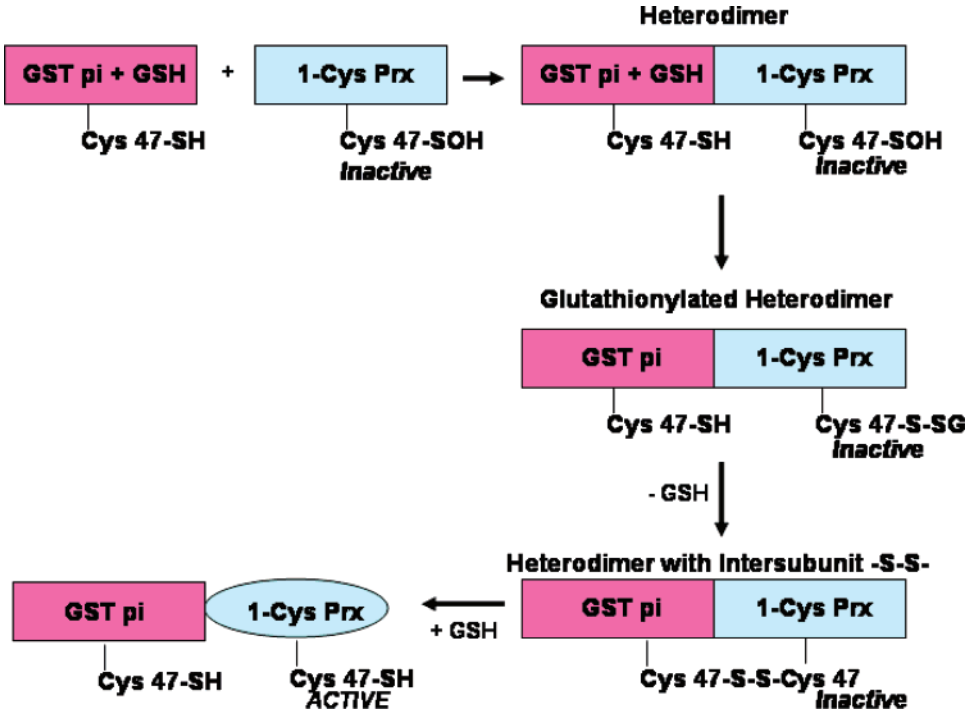

FIGURE 5: Scheme of activation of oxidized 1-Cys Prx by GST π and GSH. Formation of heterodimer of GST π and GSH (shown in pink) with the oxidized 1-Cys Prx (shown in cyan) leads to Cys-47 glutathionylation of 1-Cys Prx, followed by the formation of an intersubunit disulfide. Finally, the GSH-mediated reduction of the disulfide regenerates the reduced active-site cysteine 47 of 1-Cys Prx.

Because the heterodimerization of GST π with 1-Cys Prx and the activation of 1-Cys Prx is a new function for GST π, we mutated known binding/active sites for the two enzymes to ascertain whether this new role makes use of these sites. For the GST π subunit, our studies clearly indicate that disruption of residues at or near the GSH site affect the production of the complex. The mutation of Tyr-7, an important residue in the function of the GSH site of GST π (*35*), is critical in heterodimer formation because disruption of this site did not yield any heterodimer. Additionally, Cys-47 of π-GST, a residue that has been shown to contribute to the binding of GSH (*36*), is also an essential residue for heterodimer formation because its mutation to serine did not yield heterodimer. It is notable that our mutagenesis experiments demonstrate that the three other cysteines of GST π are not needed for heterodimer formation or for reactivation of 1-Cys Prx. Of the 1-Cys Prx sites, Cys-47 is critical for peroxidase activity, while Ser-32 is essential for the hydrolase function (*17*). Neither of these known binding/active sites is necessary for the production of the complex, as demonstrated by the high yields of the heterodimer after mutation of Cys-47 to Ser and Ser-32 to Ala. However, the WT GST π/C47S Prx complex lacked peroxidase activity, confirming the postulate that heterodimer formation and 1-Cys Prx activation are two separate processes. Similarly, the WT GST π/S32A Prx heterodimer exhibited peroxidase activity with respect to H_2O_2 as the substrate but lacked peroxidase activity with respect to phospholipid peroxides, consistent with its previously designated function in the binding of phospholipids (*17*).

The process of activation of 1-Cys Prx has been proposed to involve regeneration of cysteine from the corresponding cysteine sulfenic acid form. However, the mechanism by which this conversion occurs has not previously been determined. On the basis of our studies, we propose that the mechanism of reactivation initially requires heterodimer formation between GST π and 1-Cys Prx. The formation of the complex allows GSH to have access to the oxidized cysteine 47 of 1-Cys Prx, facilitating its glutathionylation. Glutathionylation of 1-Cys Prx changes the conformation of the heterodimer, permitting a disulfide to form between Cys-47 of GST π and Cys-47 of 1-Cys Prx, followed by reduction of the disulfide by GSH with regeneration of the peroxidatic cysteine 47 of 1-Cys Prx. The steps involved in the activation of oxidized 1-Cys Prx by GST and GSH are shown schematically in Figure 5. The chemistry of sulfenic acids has been reviewed (refs *37*−*39* and references therein), including the reaction of cysteine sulfenic acid with a mercaptan to form a disulfide.

Experimental evidence for the first step in this mechanism comes from the observation that the pure 1-Cys Prx dimer is catalytically inactive; therefore, heterodimerization with GST π must be the first step in reactivation. Glutathionylation of 1-Cys Prx, as the second step in the mechanism, was indicated by the immunological detection of glutathionylated 1-Cys Prx after 1 min of incubation of 1-Cys Prx with GST π and GSH. Glutathionylation must precede disulfide formation because the heterodimer sample in the presence of *S*-methylglutathione, which cannot glutathionylate 1-Cys Prx, fails to produce a disulfide as indicated by the lack of a higher molecular-weight band in SDS−PAGE (lanes 6 and 8 in parts A and B of Figure 2). If disulfide formation occurs before glutathionylation, a band corresponding to a dimer would have been observed for the heterodimer formed in the presence of *S*-methylglutathione. Finally, the last step in the mechanism is the regeneration of the active-site cysteine of 1-Cys Prx through reduction of the disulfide with GSH. From this proposed mechanism, we can conclude that the GST π subunit functions as a substitute for the second Prx subunit that participates in the 2-Cys Prx mechanism (*11*).

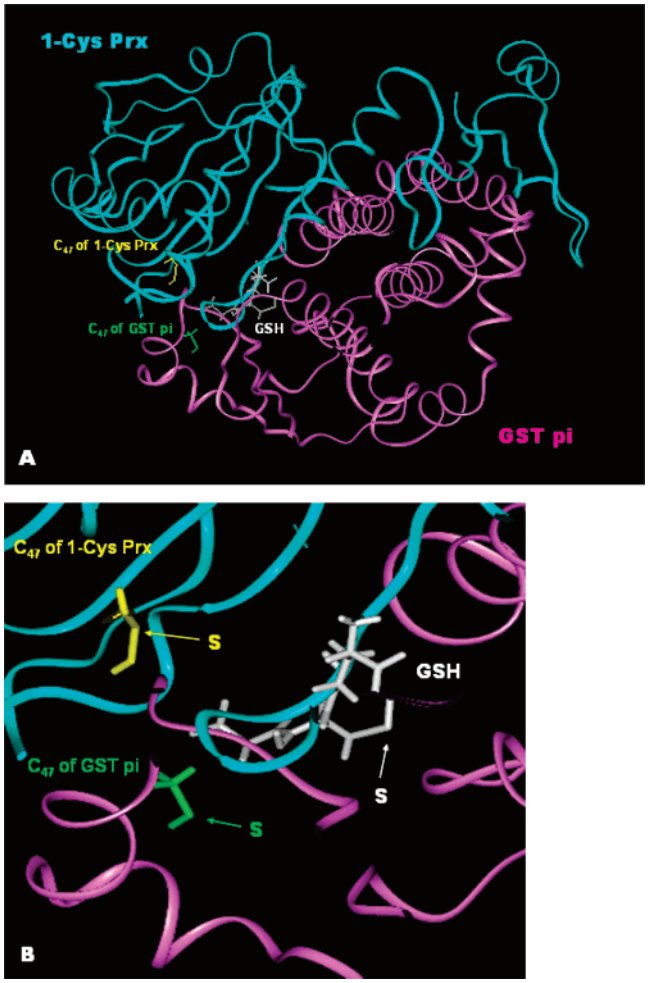

FIGURE 6: Model of the heterodimer of GST π and 1-Cys Prx docked "in silico" using Insight II and ZDOCKpro. (A) Ribbon representation of 1-Cys Prx (PDB 1PRX) complexed with GST π (PDB 19GS). The backbone of the subunit B of 1-Cys Prx is cyan, and the backbone of the subunit A of GST π is pink. GSH (in white) bound to GST π and Cys-47 of GST π (highlighted in green) are not far from the critical Cys-47 sulfenic acid of 1-Cys Prx (highlighted in yellow). (B) Close-up view of A. The white arrow indicates the −SH of GSH; the green arrow points to the sulfur atom of Cys-47 of GST; and the yellow arrow points to the sulfur atom of Cys-47 sulfenic acid of 1-Cys Prx.

Crystal structures are available in the Protein Data Bank for both 1-Cys Prx and for GST π. Using one subunit of 1-Cys Prx and one subunit of GST π, we have docked the two monomers to form an energy-minimized heterodimer "in silico" as shown in Figure 6. Figure 6A illustrates a plausible arrangement of the monomers of the two enzymes in a stable heterodimer. The modeled complex suggests that the GSH-binding site is at the interface of the heterodimer; thus, the complex provides a protected environment for the bound GSH molecule. Figure 6B shows a closeup view of the area of interaction between GSH and the two cysteine 47 residues. GSH (in white), bound to GST π, is approximately 15 Å from the critical Cys-47 sulfenic acid of 1-Cys Prx. To glutathionylate 1-Cys Prx, GSH would have to move, from its present location, closer to Cys-47 of 1-Cys Prx. This movement could result from a conformational change of the two subunits upon forming the heterodimer; alternatively, the enzyme-bound GSH could migrate (tunnel) from the GSH site on GST π to the active site of 1-Cys Prx. The two cysteine 47 residues of the two proteins are 9.0 Å apart in the structure shown in Figure 6. After glutathionylation of 1-Cys Prx, the heterodimer may undergo a conformational change, bringing the two cysteine 47 residues closer together to facilitate the formation of a disulfide bond. However, this hypothesis requires further experimentation.

In summary, we have purified and characterized a heterodimer between 1-Cys Prx and GST π. We have determined the optimum conditions for heterodimer formation and stabilization and identified the binding sites important for complex formation. Finally, we have proposed a feasible mechanism to account for the reactivation of the oxidized 1-Cys Prx by GST π.

ACKNOWLEDGMENT

We thank Dr. Stephanie A. Misquitta for providing GST A1-1 and for her help and suggestions at the start of this project, Jennifer L. Hearne for providing GST M1-1, and Dr. Yu Chu Huang for N-terminal sequencing of the proteins.

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BI0520737