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Molecular Mechanism of the Reduction of Cysteine Sulfinic Acid of Peroxiredoxin to Cysteine by Mammalian Sulfiredoxin*

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Woojin Jeong[‡§1], **Sung Jun Park**[‡1], **Tong-Shin Chang**[‡§], **Duck-Yeon Lee**[‡], **and Sue Goo Rhee**[‡§2]

From the [‡]*Laboratory of Cell Signaling, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892 and the* [§]*Center for Cell Signaling Research and Division of Molecular Life Sciences, Ewha Womans University, Seoul 120-750, Korea*

Among many proteins with cysteine sulfinic acid (Cys-SO_2H) residues, the sulfinic forms of certain peroxiredoxins (Prxs) are selectively reduced by sulfiredoxin (Srx) in the presence of ATP. All Srx enzymes contain a conserved cysteine residue. To elucidate the mechanism of the Srx-catalyzed reaction, we generated various mutants of Srx and examined their interaction with PrxI, their ATPase activity, and their ability to reduce sulfinic PrxI. Our results suggest that three surface-exposed amino acid residues, corresponding to Arg^{50}, Asp^{57}, and Asp^{79} of rat Srx, are critical for substrate recognition. The presence of the sulfinic form (but not the reduced form) of PrxI induces the conserved cysteine of Srx to take the γ-phosphate of ATP and then immediately transfers the phosphate to the sulfinic moiety of PrxI to generate a sulfinic acid phosphoryl ester (Prx-Cys-S(=O)OPO_3^{2-}). This ester is reductively cleaved by a thiol molecule (RSH) such as GSH, thioredoxin, and dithiothreitol to produce a disulfide-*S*-monoxide (Prx-Cys-S(=O)–S-R). The disulfide-*S*-monoxide is further reduced through the oxidation of three thiol equivalents to complete the catalytic cycle and regenerate Prx-Cys-SH.

Members of the peroxiredoxin (Prx)[3] family of enzymes catalyze the reduction of hydroperoxides with the use of reducing equivalents provided by thiol-containing proteins (1–5). Mammalian cells express six isoforms of Prx (PrxI to PrxVI), which are classified into three subgroups (2-Cys, atypical 2-Cys, and 1-Cys) on the basis of the number and position of cysteine residues that participate in catalysis (2, 6). PrxI to PrxIV, which belong to the 2-Cys Prx subgroup, exist as homodimers and possess two conserved cysteine residues (Cys^{51} and Cys^{172} in PrxI). In the catalytic cycle of 2-Cys Prx enzymes, the NH_2-terminal Cys-SH (Cys^{51} in PrxI) is first converted to cysteine sulfenic acid (Cys-SOH) by a peroxide. The unstable sulfenic intermediate (7) then reacts with the COOH-terminal conserved Cys-SH (Cys^{172} in PrxI) of the other subunit in the homodimer to form a disulfide, which is subsequently reduced by a thiol-containing reducing equivalent, such as thioredoxin (Trx), to complete the catalytic cycle (2–5, 8). As a result of the slow rate of its conversion to a disulfide, the sulfenic intermediate is occasionally oxidized further to cysteine sulfinic acid (Cys-SO_2H) (8–10). Given that cysteine sulfinic acid is not reduced by biological reductants such as ascorbic acid, glutathione, and Trx, its formation in 2-Cys Prx isoforms results in the inactivation of peroxidase function. However, studies of the fate of such overoxidized Prx enzymes led to the unexpected finding that the formation of the sulfinic acid form is a reversible step in mammalian cells (11). The enzyme responsible for the reduction of sulfinylated Prx was subsequently identified in yeast and named sulfiredoxin (Srx) (12).

Prx enzymes are thought to relieve cells from oxidative stress by removing the low levels of hydroperoxides produced as a result of normal cellular metabolism (6, 13, 14). However, cells also produce H_2O_2 for signaling purposes in response to stimulation via various cell surface receptors (15). Given that prokaryotic Prx enzymes do not undergo oxidative inactivation and that prokaryotes do not contain Srx, the reversible overoxidation of mammalian 2-Cys Prx has been suggested to represent an adaptation of eukaryotic cells to accommodate the intracellular messenger function of H_2O_2 (15, 16). This notion is supported by the recent observation that, in *Schizosaccharomyces pombe*, the overoxidation of Prx represents a redox switch that regulates the function of Prx as an H_2O_2 sensor and a redox transducer in the activation of the transcription factor Pap1 (17, 18).

Oxidation of cysteine to sulfinic acid is not restricted to Prx enzymes. Critical cysteine residues of many other proteins, including glyceraldehyde-3-phosphate dehydrogenase (19), carbonic anhydrase III (20), metalloproteinases (21), protein tyrosine phosphatase 1B (22), and the Parkinson disease-associated protein DJ-1 (23), also undergo this modification. Nevertheless, reduction by Srx appears to be a highly selective process. Among the three subtypes of Prx isoforms, only the sulfinic forms of members of the 2-Cys Prx subgroup, not those of members of the atypical 2-Cys or 1-Cys subgroups, were found to be reduced by Srx, and Srx did not act on the sulfinic forms of glyceraldehyde-3-phosphate dehydrogenase or DJ-1 (24). Srx may thus exist solely to support the reversible sulfinic modification of specific Prx enzymes.

The *in vitro* reduction of sulfinic acid requires harsh reaction conditions, and the reduction of sulfinic 2-Cys Prx isoforms is the first known biological example of such a reaction. We have now studied the mechanism of the Srx-catalyzed reaction. We generated Srx mutants by altering conserved amino acid residues and examined both the interaction of the mutant proteins with PrxI and their ability to reduce the sulfinic form of PrxI. In particular, examination of ATP hydrolysis catalyzed by Srx under various reaction conditions provided important insight into the reaction mechanism. Our results suggest that the conserved cysteine of Srx mediates the phosphorylation of the sulfinic moiety of PrxI

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[1] Both authors equally contributed to this work.

[2] To whom correspondence should be addressed: Ewha Womans University, Science Bldg. C, Rm. 211, 11-Daehyun-dong, Seodaemoon-gu, Seoul 120-750, Korea. Tel.: 82-02-3277-2948; Fax: 82-02-3277-3760; E-mail: rheesg@ewha.ac.kr.

[3] The abbreviations used are: Prx, peroxiredoxin; Trx, thioredoxin; Srx, sulfiredoxin; rSrx, rat Srx; hSrx, human Srx; ACTH, adrenocorticotropic hormone; DTT, dithiothreitol; GST, glutathione S-transferase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

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and that the resulting sulfinic acid phosphoryl ester is reduced to Cys-SH after oxidation of four thiol equivalents. Contrary to the proposal made with yeast Srx (12), the conserved cysteine was not necessary for the reduction of Prx I sulfinic acid phosphoryl ester.

EXPERIMENTAL PROCEDURES

Materials—NADPH, GSH, GSSG, and dithiothreitol (DTT) were obtained from Sigma; creatine phosphate and creatine kinase were from USB Corp.; $MgCl_2$ was from Alfa Aeser; ATP was from Calbiochem; GSH-Sepharose resin and [γ-^{32}P]ATP were from Amersham Biosciences; and polyethyleneimine-cellulose thin layers on plastic sheets were from J. T. Baker. A site-directed mutagenesis kit was obtained from Stratagene, and a monoclonal antibody to glutathione *S*-transferase (GST) was from Santa Cruz Biotechnology. Human Trx1, rat Trx reductase, and human PrxI were prepared as described previously (25). Rabbit antisera specific for human Srx (26), for the hyperoxidized cysteine-containing Prx enzymes (27), and for PrxI (28)were generated as described previously.

Preparation of Recombinant Srx Proteins—Escherichia coli expression plasmids encoding GST fusion proteins of human Srx (GST-hSrx) or rat Srx (GST-rSrx) were described previously (26). Mutant Srx proteins were generated by standard PCR-mediated site-directed mutagenesis with cDNA clones encoding GST-hSrx or GST-rSrx as the template. *E. coli* BL21 cells harboring the plasmids for GST-Srx fusion proteins were cultured at 37 °C in LB medium supplemented with ampicillin (100 μg/ml). After the addition of isopropyl-1-thio-β-D-galactopyranoside (0.1 mM), the cultures were incubated for 3 h at 25 °C, and the cells were then lysed. The GST-Srx proteins were isolated from cell lysates by chromatography on a column of GSH-Sepharose and then dialyzed against 20 mM Tris-HCl (pH 7.4). The GST moiety was removed from each fusion protein by digestion with thrombin for hSrx or with factor Xa for rSrx, and the released GST was separated from the Srx proteins by passage of the digest through a GSH-Sepharose column.

Preparation of Sulfinic PrxI(C172S)—Human PrxI(C172S), in which Cys^{172} is replaced by serine, was generated by standard PCR-mediated site-directed mutagenesis and purified as described (26). The purified protein was oxidized by incubation in the presence of 10 mM DTT and H_2O_2 (1 mM H_2O_2 was added three times at 30-min intervals). The sulfinic oxidation state of Cys^{51} of hyperoxidized PrxI was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (9).

Polyethyleneimine-Cellulose TLC of Hydrolyzed Phosphate—Separation of P_i by TLC on polyethyleneimine-cellulose was performed as previously described (29). In brief, portions (2 μl) of reaction mixture were applied 3 cm from the lower edge of the thin-layer plate, and ascending chromatography was carried out with 0.75 M potassium phosphate (pH 3.4) in a closed glass chamber for ~90 min at room temperature.

Mass Spectrometry—Axima-CFR plus mass spectrometer (Shimadzu Corp., Kratos Analytical) equipped with 337 nm nitrogen was used. All mass spectra were obtained in the positive ion mode with pulsed extraction value of 2,000, and linear acceleration voltage was 25 kV. 1 μl of sample was mixed with 1 μl of the matrix solution containing α-cyano-4-hydroxycinna-mic acid in acetonitrile/water (1:1, v/v) including 0.1% trifluoroacetic acid. 1 μl of mixture was loaded on the stainless plate and allowed to dry in air. External calibration was performed with a mixture of bradykinin fragment 1–7 (m/z 757.3997), angiotensin II (human, m/z 1,046.5423), P14R (synthetic peptide, m/z 1,533.8582), and ACTH fragment 18–39 (human, m/z 2,465.1989).

RESULTS AND DISCUSSION

Phosphate-Carrier Function of the Conserved Cysteine of Srx—Srx enzymes of species ranging from yeast to mammals all contain a conserved cysteine, which is Cys^{99} in hSrx and Cys^{98} in rSrx (12). Substitution of serine for the conserved cysteine abolishes the reductase activity of Srx (12, 26). On the basis of the observation that the reduction of sulfinic Prx by yeast Srx is dependent on ATP and Mg^{2+}, Biteau *et al.* (12) proposed that the first step of the Srx reaction involves phosphorylation of sulfinic acid (Prx-Cys-SO_2H) to yield a sulfinic acid phosphoryl ester (Prx-Cys-S(=O)OPO_3^{2-}), which is reminiscent of the activation of a carboxylic group by phosphorylation in a variety of enzyme systems. In addition, on the basis of the observation that treatment of yeast cells with H_2O_2 results in the formation by Srx of a DTT-reducible covalent complex with Prx, they also suggested that the activated sulfinic acid phosphoryl ester might react with the conserved cysteine of Srx to produce a disulfide-*S*-monoxide (Prx-Cys-S(=O)–S-Cys-Srx), also known as thiosulfinate, which might then undergo reductive cleavage by biological thiols such as Trx to regenerate Prx-Cys-SH and Srx-Cys-SH (12). Srx was thus proposed to be a bifunctional enzyme that acts both as a specific phosphotransferase and as a thioltransferase (12, 30).

We attempted to detect the proposed sulfinic acid phosphoryl ester intermediate by incubating PrxI-Cys-SO_2H with both [γ-^{32}P]ATP and purified hSrx under reducing conditions and then subjecting either the reaction mixture or PrxI immunoprecipitated from the reaction mixture to SDS-PAGE and autoradiography. No ^{32}P radioactivity associated with PrxI was detected by either approach (Fig. 1*A* and data not shown). We considered the possibility that the failure to detect the intermediate was due to its rapid reaction with Srx-Cys-SH to form Prx-Cys-S(=O)–S-Cys-Srx. To promote accumulation of the sulfinic acid phosphoryl ester intermediate, we therefore repeated the experiment with a $Cys^{99} \rightarrow$ Ser mutant of hSrx (hSrx(C99S)). Again, we failed to detect ^{32}P-labeled PrxI (Fig. 1*A*). Instead, ^{32}P radioactivity was found associated with hSrx(C99S). This phosphorylation of the Srx mutant was observed in the presence of sulfinic PrxI but not in the presence of reduced PrxI. It was also a slow process, with <1% of hSrx(C99S) having undergone phosphorylation after incubation for 4 h, at which time the reaction was still progressing (Fig. 1*B*). To determine whether Ser^{99} was the site of phosphorylation in hSrx(C99S), we subjected a $Cys^{99} \rightarrow$ Ala mutant of hSrx (hSrx(C99A)) to the phosphorylation reaction. Phosphorylation of hSrx(C99A) was not detected (Fig. 1*C*). To confirm that Ser^{99} was the site of phosphorylation in hSrx(C99S), we digested the ^{32}P-labeled mutant protein with trypsin and subjected the resulting peptides to reversed-phase high-performance liquid chromatography. A single peak of radioactivity was obtained (data not shown). Mass spectrometry analysis revealed that the ^{32}P-labeled peptide corresponded to residues 86–101 (^{86}GAQGGDYFYSF-GGSHR^{101}), consistent with the conclusion that Ser^{99} is the site of phosphorylation.

While our work was in progress, Jönsson *et al.* (30) determined the crystal structure of hSrx complexed with ADP and, on the basis of the structure, proposed a mode of ATP binding in which the γ-phosphate oxygen interacts with the backbone nitrogen atom of Gly^{98}. Cys^{99} was found to be positioned close to the γ-phosphate, but no direct interaction was apparent. These researchers thus did not propose a specific role for Cys^{99} in the transfer of the γ-phosphate from ATP to sulfinic Prx (phosphotransferase reaction) and suggested that the sulfinic anion directly extracts the γ-phosphate from ATP.

To determine whether the substitution of Cys^{99} by serine affects the interaction between ATP and hSrx, we studied the binding of ATP to hSrx by monitoring of fluorescence emission spectra (Fig. 2). The extent

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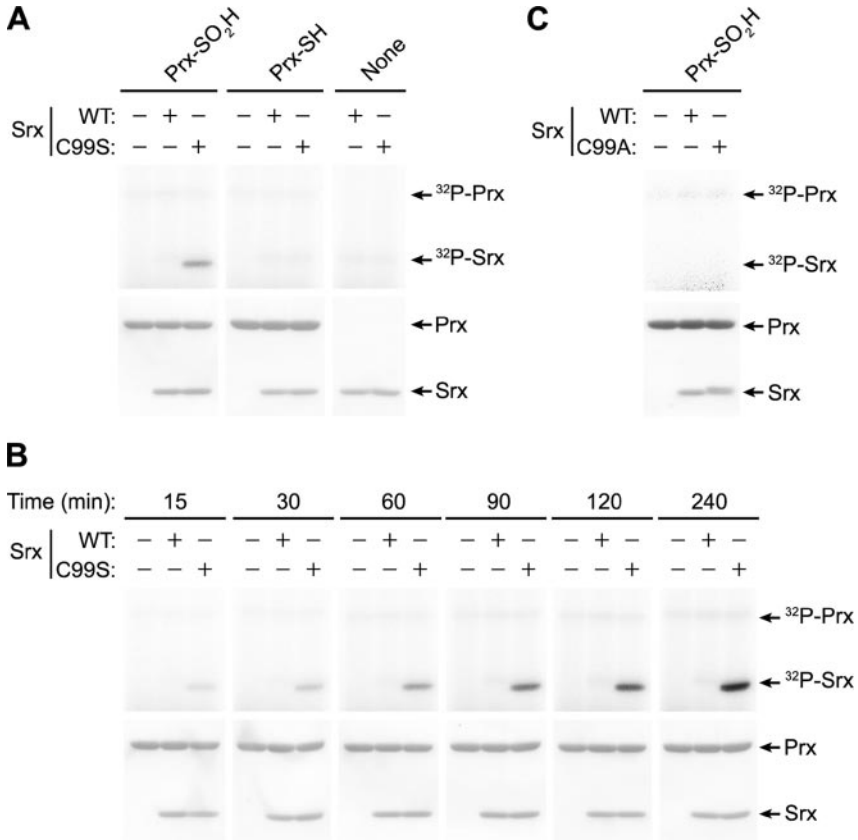

FIGURE 1. **Phosphorylation of hSrx(C99S).** *A*, reaction mixtures containing 100 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 0.1 mM ATP, 70 nM [γ-^{32}P]ATP (0.4 mCi/ml), and 10 μM sulfinic PrxI (Prx-SO_2H), 10 μM reduced PrxI (Prx-SH), or no PrxI (*None*) were incubated for 30 min at room temperature with 10 μM wild-type hSrx (*WT*) or 10 μM hSrx(C99S). Proteins were then separated by SDS-PAGE on a 16% gel, transferred to a nitrocellulose membrane, and visualized by autoradiography (*upper panels*) or staining with Ponceau S (*lower panels*). The positions of PrxI (*^{32}P-Prx* or *Prx*) and hSrx (*^{32}P-Srx* or *Srx*) are indicated. *B*, the sulfinic PrxI-containing reaction mixtures described in *A* were incubated for the indicated times before analysis. *C*, reaction mixtures similar to those in *A* but containing sulfinic PrxI and either wild-type hSrx or hSrx(C99A) were incubated for 30 min before analysis. Data in all panels are representative of at least three separate experiments.

of the decrease in fluorescence intensity induced by ATP was similar for wild-type and the C99S mutant of hSrx, and the dissociation constant (K_d) estimated from titration data were also virtually identical (~6 μM) for both forms of hSrx. These results suggested that, as indicated by the crystal structure, the conserved cysteine of hSrx does not interact with ATP, as well as that the environment surrounding the bound ATP molecule is similar for wild-type and C99S forms of the enzyme. The x-ray structure indicates that the thiol group of Cys^{99} of hSrx exists predominantly as a thiolate anion as a result of its interaction with the guanidine group of Arg^{51} and that the nucleotide binding motif of hSrx resembles that of protein tyrosine phosphatases, in which the active site cysteine serves as the phosphate carrier (30, 31). The nucleophilicity of Ser^{99}–OH in hSrx(C99S) is also likely increased, although not as readily as that of Cys^{99}–SH in the wild-type protein, through interaction with Arg^{51}. The increased nucleophilicity of Ser^{99}–OH is probably still not sufficient, however, to extract the γ-phosphate of ATP given that phosphorylation of hSrx(C99S) required the presence of sulfinic PrxI. Although Srx binds to both reduced and sulfinic forms of PrxI (26), reduced PrxI was not able to support the phosphorylation of hSrx(C99S), suggesting a critical role for the sulfinic moiety. It may be necessary for the sulfinic anion to displace the Ser^{99}–O^- or Cys^{99}–S^- anions from Arg^{51} for hSrx to initiate a nucleophilic attack on the γ-phosphate of ATP. Given that thiolate anions are generated more readily and are more efficient nucleophiles than are oxy anions, phosphorylation of Cys^{99} would be expected to be faster than that of Ser^{99}. Protein thiophosphate intermediate has previously been detected with protein tyrosine phosphatases that contain an essential cysteine, which serves as the phosphate carrier during catalysis (31). The thiophosphate intermediate was apparent at the earliest time point (10 s) examined and diminished rapidly. Our failure to detect phosphorylated wild-type hSrx in Fig. 1*A* is probably attributable not only to the known instability of thiophosphate com-

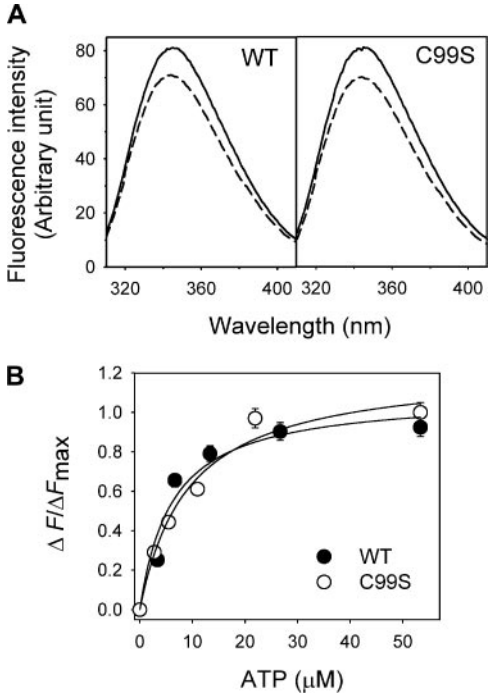

FIGURE 2. **Effects of ATP on the fluorescence emission spectra of wild-type (*WT*) and C99S mutant forms of hSrx.** *A*, fluorescence emission spectra for a 1-ml reaction mixture containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 5 μM wild-type hSrx or hSrx(C99S) at 25 °C were determined before (*solid line*) and after (*dashed line*) the addition of 5 μl of 1 mM ATP. The excitation wavelength was 292 nm. *B*, the dissociation constant for the interaction between ATP and either wild-type hSrx or hSrx(C99S) was determined from experiments similar to that shown in *A* by plotting the values of $\Delta F/\Delta F_{max}$ (where ΔF represents the change in fluorescence intensity obtained for a specific ATP concentration and ΔF_{max} is the maximal change in fluorescence intensity at saturation) against ATP concentration.

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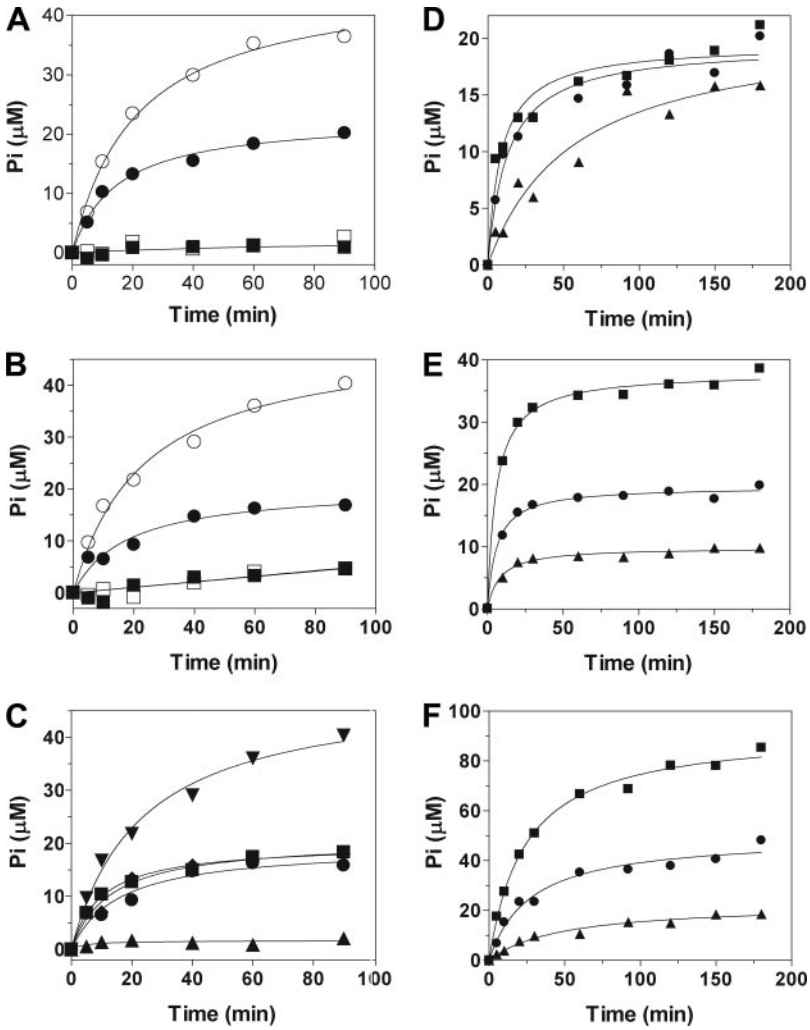

FIGURE 3. **ATP hydrolysis catalyzed by hSrx under various reaction conditions.** *A*, reaction mixtures containing 100 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 250 μM [γ-^{32}P]ATP (6.7 μCi/ml), 5 μM hSrx, and 20 μM sulfinic PrxI (*circles*) or reduced PrxI (*squares*), in the presence (*closed symbols*) or absence (*open symbols*) of 10 mM GSH, were incubated at 30 °C. At the indicated times, portions (2 μl) of reaction mixture were subjected to TLC on polyethyleneimine-cellulose. The chromatogram was allowed to dry in air, radioactive material was visualized by autoradiography, and the radioactivity associated with P_i was measured by using a liquid scintillation counter. *B*, ATP hydrolysis was measured as in *A* with the exception that all reaction mixtures contained sulfinic PrxI and that wild-type hSrx (*circles*) or hSrx(C99A) (*squares*) was used in the presence (*closed symbols*) or absence (*open symbols*) of GSH. *C*, ATP hydrolysis was measured as in *A* with the exception that all reaction mixtures contained sulfinic PrxI and that the reactions were performed in the absence of thiol equivalents (*inverted triangles*) or in the presence of 10 mM GSH (*circles*), 10 mM DTT (*squares*), 30 μM Trx1 (*diamonds*), or 10 mM GSSG (*upright triangles*). For the reaction mixture containing Trx1, 250 nM Trx reductase and 500 μM NADPH were also included. *D*, ATP hydrolysis was measured as in *A* with the exception that all reaction mixtures contained sulfinic PrxI and GSH and that the concentration of hSrx was 2 (*triangles*), 5 (*circles*), or 10 μM (*squares*). *E*, ATP hydrolysis was measured as in *A* with the exception that all reaction mixtures contained GSH and that the concentration of sulfinic PrxI was 10 (*triangles*), 20 (*circles*), or 40 μM (*squares*). *F*, ATP hydrolysis was measured as in *A* with the exception that all reaction mixtures contained sulfinic PrxI and no GSH and that the concentration of hSrx was 2 (*triangles*), 5 (*circles*), or 10 μM (*squares*). Data in all panels are means of two independent experiments.

pounds but also to the presence of the sulfinic anion that is positioned closely to initiate nucleophilic attack on the thiophophate. Alternatively, Cys^{99}–S^- anion may not form the thiophosphate intermediate but is necessary to activate the γ-phosphate of ATP before phosphorylation of sulfinic Prx. The OH group of sulfinic acid is a poor leaving group in a reaction involving nucleophilic attack of a thiol at sulfur. Phosphorylation of the OH group changes it to a good leaving group that can be easily replaced by a thiol. This is reminiscent of the phosphorylation of OH group in various reactions involving carboxylic acid activation.

ATP Hydrolysis by Srx in the Presence of Sulfinic PrxI—We next measured the Srx-dependent release of phosphate from [γ-^{32}P]ATP in reaction mixtures also containing reduced or sulfinic PrxI either with or without GSH. ^{32}P-labeled P_i was produced in the presence of sulfinic PrxI but not in the presence of reduced PrxI (Fig. 3*A*). The requirement for sulfinic PrxI likely reflects the unique role of the sulfinic moiety in transfer of the γ-phosphate of ATP to the sulfur atom of Cys^{99}–SH in hSrx, as observed in the transfer of the γ-phosphate to the oxygen atom of Ser^{99}–OH in hSrx(C99S). The sulfinic requirement also suggests that the observed ATP hydrolysis was not due to nonspecific ATP-hydrolyzing activity contaminating the preparations of hSrx or PrxI. The hydrolysis of ATP increased gradually with time in both the absence and presence of GSH (Fig. 3*A*). In the presence of GSH, it reached a plateau at ~20 μM P_i; the concentration of sulfinic PrxI in the reaction mixture was also 20 μM. In the absence of GSH, the extent of ATP hydrolysis progressed further. Substitution of alanine for Cys^{99} in hSrx blocked ATP hydrolysis in both the absence and the presence of GSH (Fig. 3*B*), suggesting that the conserved cysteine is essential for the production of P_i. Other thiols were able to substitute for GSH, with replacement by Trx or DTT affecting neither the amplitude nor the rate of P_i formation (Fig. 3*C*). A substantial level of P_i formation was not observed in the presence of GSSG given that Srx becomes inactivated as a result of glutathionylation of its active site cysteine (data not shown), again indicating the critical role of Cys^{99}–SH of hSrx in ATP hydrolysis. In the presence of 20 μM sulfinic PrxI, 250 μM [γ-^{32}P]ATP, and 10 mM GSH, the rate of ATP hydrolysis was dependent on hSrx concentration (2, 5, 10 μM), but the final amount of P_i released (~20 μM) was independent of hSrx concentration (Fig. 3*D*). Furthermore, in reaction mixtures containing 10, 20, or 40 μM sulfinic PrxI, the final level of P_i formed was similar to the corresponding concentration of PrxI (Fig. 3*E*). However, when GSH was omitted from the reaction mixture containing 20 μM sulfinic PrxI, the final extent of ATP hydrolysis was dependent on hSrx concentration and was much greater than 20 μM in the presence of 5 or 10 μM Srx (Fig. 3*F*).

These results are consistent with the following sequence of events (see Fig. 8). The thiol of hSrx is transiently phosphorylated by ATP in the presence of sulfinic PrxI to form hSrx-Cys^{99}–SPO_3^{2-}, the high energy thiophosphate of which is then used to phosphorylate the sulfinic moiety of PrxI. Although thiophosphate is suceptible to hydrolysis, hSrx-Cys^{99}–SPO_3^{2-} is unlikely to be the direct source of the P_i measured in the

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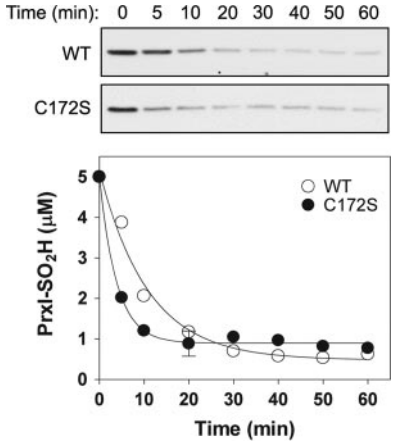

FIGURE 4. **Reduction of the sulfinic forms of wild-type (*WT*) PrxI and PrxI(C172S) by hSrx.** Reaction mixtures containing 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM ATP, 5 mM creatine phosphate, creatine kinase (6.5 units/ml), 10 mM GSH, 1 μM hSrx, and 5 μM sulfinic wild-type or C172S forms of PrxI were incubated for the indicated times at 30 °C and then subjected to immunoblot analysis with antibodies specific for sulfinic PrxI (*upper panels*). The concentration of sulfinic PrxI remaining in each reaction mixture was determined from the corresponding immunoblot band intensity and then plotted against time (*lower panel*); data are means of values from two independent experiments.

experiments shown in Fig. 3 because such a pathway is not able to account for the observation that more P_i was produced in the absence of GSH than in its presence or to explain why the maximal level of P_i generated in the presence of GSH was similar to the concentration of sulfinic PrxI regardless of the amount of hSrx present. Srx-Cys^{99}–SPO_3^{2-} appears to yield its phosphate rapidly to sulfinic PrxI, and the resulting Prx-Cys-S(=O)OPO_3^{2-} is likely the direct source of the measured P_i. This mechanism is supported by the observation that the amount of P_i produced in the presence of GSH is equivalent to that of sulfinic PrxI. Prx-Cys-S(=O)OPO_3^{2-} is reductively cleaved by GSH to yield Prx-Cys-S(=O)–SG and P_i. Prx-Cys-S(=O)–SG is then further reduced by GSH to Prx-Cys-SH. The production of P_i in the presence of GSH is thus limited by the amount of sulfinic PrxI. In the absence of GSH, however, Prx-Cys-S(=O)OPO_3^{2-} is hydrolyzed to regenerate sulfinic Prx, resulting in a futile cycle of phosphorylation and dephosphorylation that consumes ATP continuously and explains why the amount of P_i produced is much higher than that of sulfinic PrxI present. Nevertheless, even in the absence of GSH, P_i formation did not increase linearly and was hyperbolically saturated (Fig. 3, *A* and *F*). This observation likely reflects the inactivation of hSrx as the result of disulfide formation via its active site cysteine with PrxI (see below).

The thiolate anion that attacks Prx-Cys-S(=O)OPO_3^{2-} has been proposed to be Cys^{99}–S^- of hSrx, not GSH (12, 30). Srx was thus proposed to possess a thioltransferase function for which the conserved cysteine is essential. According to this mechanism, however, the amount of P_i produced in the absence of GSH should be equal to that of hSrx. Our observation (Fig. 3*C*) that the rate of P_i production was unchanged when GSH was replaced with a protein thiol (Trx) or a nonphysiological thiol (DTT) suggests that the sulfinic acid phosphoryl ester moiety of Prx-Cys-S(=O)OPO_3^{2-} is freely accessible to any thiol in the solution and that its reductive cleavage does not require a specific thioltransferase activity associated with Srx. Nucleophilic substitution of phosphate by a thiolate anion (R-S^-) produces a disulfide-*S*-monoxide (Prx-Cys-S(=O)–S-R). Disulfide-*S*-monoxide undergoes a sequential reduction reaction in the presence of thiols. Three thiol equivalents are required to regenerate Prx-Cys-SH from Prx-Cys-S(=O)–S-R (see Fig. 8). Our results suggest that the conserved cysteine of Srx is essential for sulfinic reductase activity because of its role in the phosphotransferase reaction and that it does not play a role in the thiol transfer reaction.

We evaluated the rate of ATP hydrolysis in the presence of GSH from the data shown in Fig. 3. The turnover rate calculated from the initial reaction rates was in the range of 0.2–0.5 min^{-1} at 30 °C, which is similar to the turnover rate of 0.18 min^{-1} estimated for the reduction of sulfinic PrxI by hSrx at 30 °C (26). This similarity indicates that phosphorylation of the sulfinic moiety might be the rate-limiting step in the catalytic cycle. Measurement of the sulfinic reduction rate was relatively imprecise, however, given that it was based on immunoblot intensities obtained with antibodies specific for sulfinic PrxI.

We considered the possibility that the COOH-terminal conserved cysteine (Cys^{172}) of PrxI provides the thiolate anion that replaces the phosphate moiety of Prx-Cys-S(=O)OPO_3^{2-}. PrxI is an obligate homodimer, and its active site consists of Cys^{51} and Cys^{172} contributed by the two respective subunits (2, 32–34). We generated the sulfinic form of a Cys^{172} → Ser mutant (PrxI(C172S)) and subjected it to reduction by hSrx, with the reaction being monitored by immunoblot analysis with the sulfinic-specific antibodies. No substantial difference in the rate of reduction was apparent between PrxI(C172S) and wild-type PrxI (Fig. 4), suggesting that Cys^{172}–SH does not play a role in sulfinic reduction.

Analysis of the DTT-sensitive Linkage between Prx and Srx Molecules—Biteau *et al.* (12) showed that Prx and Srx form oligomers that are connected by a DTT-sensitive linkage in H_2O_2-treated yeast cells. On the basis of this observation, these researchers concluded that sulfinic Prx and Srx form a disulfide-*S*-monoxide linkage and proposed that Srx possesses thioltransferase activity. The chemical nature of the linkage was not characterized, however. To obtain insight into this linkage, we incubated sulfinic PrxI and hSrx in a sulfinic reduction reaction mixture lacking GSH. Analysis of the reaction mixture by nonreducing SDS-PAGE followed by immunoblot analysis with antibodies to PrxI and to hSrx revealed four bands, corresponding to molecular sizes of 70, 60, 48, and 35 kDa, that were detected by both types of antibodies, in addition to the bands corresponding to PrxI (25 kDa) and hSrx (12 kDa) (Fig. 5*A*). Similar analysis of the reaction mixture on a reducing gel revealed only these latter two bands (Fig. 5*B*). The sizes and relative intensities of the bands detected by both anti-PrxI and anti-hSrx suggest that they correspond to complexes of $(PrxI)_2$-$(hSrx)_2$, $(PrxI)_2$-hSrx, PrxI-$(hSrx)_2$, and PrxI-hSrx, respectively. The complex corresponding to the 35-kDa PrxI-hSrx band was digested with trypsin, and the resulting peptides were analyzed by MALDI-TOF mass spectrometry (data not shown). We did not detect a signal at a mass value of 4,771.2 Da, corresponding to a disulfide-*S*-monoxide-linked complex of Cys^{51}-containing PrxI (residues 38–62) and Cys^{99}-containing hSrx (residues 86–101) peptides. Comparison by MALDI-TOF mass spectrometry of samples treated with DTT or left untreated revealed a signal at a mass value of 4,068.0 mu in the latter but not in the former. The disappearance of the signal at 4,068.0 mu from the untreated sample was accompanied by the appearance of two new signals at 2,349.9 and 1,722.3 mu in the DTT-treated sample. Given that theoretical mass values for the Cys^{172}-containing PrxI peptide (residues 169–190) and the Cys^{99}-containing hSrx peptide (residues 86–101) are 2,349.2 and 1,721.7 mu, respectively, and that the theoretical mass for the two peptides joined by a disulfide is 4,068.9 mu, we concluded that the 35-kDa PrxI-hSrx complex observed in Fig. 5*A* is joined through a disulfide between Cys^{172} of PrxI and Cys^{99} of hSrx, not through a disulfide-*S*-monoxide between Cys^{52} of PrxI and Cys^{99} of hSrx. We further analyzed a mixture of reduced PrxI and hSrx as in Fig. 5*A* and detected a ladder of complexes containing PrxI and hSrx similar to that observed in Fig. 5*A*; the PrxI and hSrx molecules were found to be connected through a disulfide either between Cys^{52} of PrxI and Cys^{99} of hSrx or between Cys^{172} of PrxI and Cys^{99} of hSrx (data

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not shown). These results suggest that sulfinic PrxI and Srx do not form a complex linked through a disulfide-*S*-monoxide during its reduction reaction and that Srx does not possess thioltransferase activity.

Characterization of Mutant Srx Proteins—Six charged amino acids are fully conserved among Srx proteins from cyanobacteria, yeast, plants, *Drosophila*, and mammals. These residues are Arg^{50}, Asp^{57}, Lys^{60}, Asp^{79}, His^{99}, and Arg^{100} in rSrx (the residue numbers are higher by one in hSrx), and they were individually changed to Met (rSrx(R50M)), Asn (rSrx(D57N)), Arg (rSrx(K60R)), Asn (rSrx(D79N)), Asn (rSrx(H99N)), and Met (rSrx(R100M)), respectively, to generate mutant rSrx proteins fused with GST. In addition, Asp^{66} and Ser^{74}, which are conserved among mammalian Srx enzymes but not among those from other species (26), were changed to Ala, and the catalytic Cys^{98} was changed to Ser to generate GST fusion proteins of rSrx(D66A), rSrx(S74A), and rSrx(C98S), respectively. We evaluated these mutant enzymes for their interaction with PrxI, ATPase activity, and sulfinic reductase activity (Fig. 6).

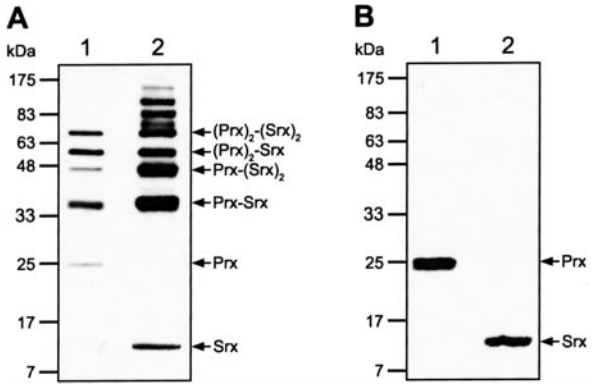

FIGURE 5. **Formation of DTT-sensitive complexes between sulfinic PrxI and hSrx.** Sulfinic PrxI and hSrx, each at 5 μM, were incubated for 10 min at 30 °C in a reaction mixture containing 100 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 10 mM ATP. Free thiol groups were then blocked by incubation with 50 mM iodoacetamide for 30 min in the presence of 100 mM Tris-HCl (pH 8.8), 1% SDS, and 1 mM EDTA. Proteins were resolved by nonreducing (*A*) or reducing (*B*) SDS-PAGE on 14% gels and were visualized by immunoblot analysis with antibodies to PrxI (*lanes 1*) or to hSrx (*lanes 2*). The positions of molecular size standards are indicated in kilodaltons.

We have previously shown that reduced and sulfinic forms of PrxI bind to rSrx with similar affinities (26). To examine the interaction of GST-rSrx proteins with PrxI, we incubated the GST fusion proteins with either recombinant PrxI or HeLa cell lysates, precipitated the fusion proteins with GSH-Sepharose, and subjected the precipitates to immunoblot analysis with antibodies to PrxI and to GST (Fig. 6*A*). Similar results were obtained with recombinant PrxI and HeLa cell lysates. Mutation of Arg^{50} or Asp^{79} of rSrx completely blocked association with PrxI; PrxI associated to a markedly reduced extent with rSrx(D57N) and to a greater extent with rSrx(C98S) than with the wild-type protein; and the binding of PrxI to the other mutants was similar to that apparent with wild-type rSrx.

The ATPase and reductase activities of the rSrx mutants were measured after cleavage of the GST moiety from the corresponding fusion proteins (Fig. 6*B*). Mutation of Arg^{50}, Cys^{98}, His^{99}, or Arg^{100} abolished both the ATPase and reductase activities, whereas mutation of Asp^{66} or Ser^{74} resulted in a partial reduction (~30–50%) in both activities. A low level of ATPase activity (~10% of that of wild-type rSrx) and no measurable reductase activity were also observed with the rSrx(K60R) and rSrx(D79N) mutants. Both the ATPase and reductase activities of rSrx(D57N) were similar to those of wild-type rSrx. Although the immunoblot assay used for measurement of the reductase activity is not

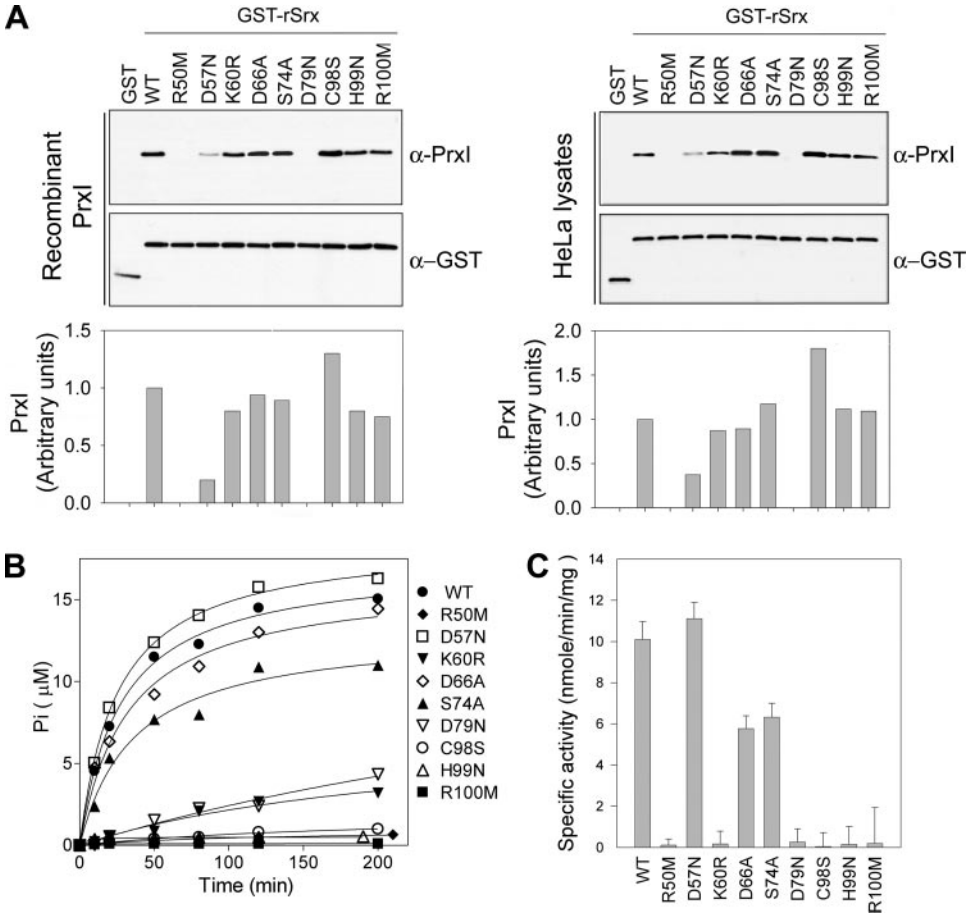

FIGURE 6. **Characterization of rSrx mutants.** *A*, the effect of rSrx mutations on the interaction with PrxI. GST or GST-rSrx fusion proteins (5 μg of each) were incubated for 2 h at 4 °C with either 1 μg of purified recombinant PrxI (*left panels*) or 0.5 mg of HeLa cell lysate (*right panels*) in 1 ml of binding buffer (50 mM Hepes-NaOH (pH 7.0), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM 4-(2-aminoethyl)benzene-sulfonyl fluoride, aprotinin (10 μg/ml), leupeptin (10 μg/ml)) containing 0.5 mg of bovine serum albumin. Cell lysates were prepared by lysing cells in the binding buffer. Proteins precipitated from the binding mixtures with GSH-Sepharose resin were subjected to immunoblot analysis with antibodies to PrxI or to GST (*upper panels*). The intensity of PrxI bands was normalized by that of the corresponding GST-rSrx bands, and means of the normalized values from two independent experiments are shown in arbitrary units (*lower panels*). *WT*, wild type. *B*, the effect of rSrx mutations on ATPase activity. ATP hydrolysis by the various rSrx mutants (5 μM) was measured in the presence of 20 μM sulfinic PrxI and 10 mM GSH as described in the legend for Fig. 3. *C*, the effect of rSrx mutations on the reduction of sulfinic PrxI. A reaction mixture containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 5 mM creatine phosphate, creatine kinase (6.5 units/ml), 10 mM GSH, 5 μM sulfinic PrxI, and 1 μM wild-type or mutant rSrx proteins was incubated at 30 °C. Portions of the reaction mixture were collected at the indicated times and subjected to immunoblot analysis with antibodies specific for sulfinic PrxI or for PrxI. The amount of sulfinic PrxI remaining was determined from the intensity of the sulfinic PrxI band normalized by that of the PrxI band and was then plotted against time to determine the specific activity as described (26). Data are means ± S.E. of values from four experiments.

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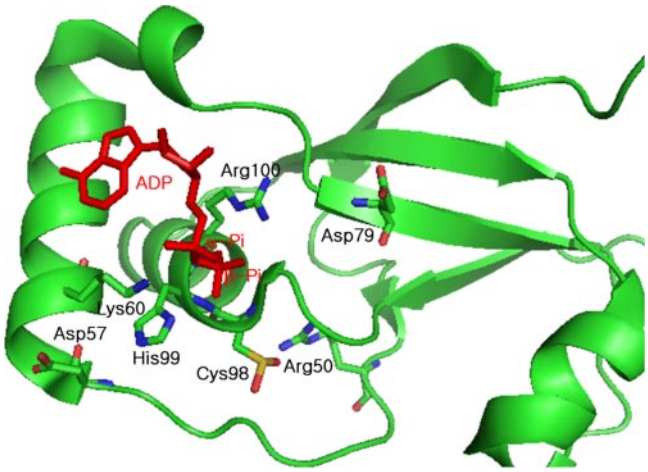

FIGURE 7. **Structure of ADP-complexed rSrx.** Arg[50], Asp[57], Lys[60], Asp[79], Cys[98], His[99], and Arg[100] are shown as *sticks*. ADP (*red*) is also indicated with its α-and β-phosphates (α-and β-P_i). This figure is deduced from the x-ray data of ADP-complexed hSrx (30).

as quantitative as is the detection of $^{32}P_i$ by TLC used for the ATPase assay, the observation that the two enzymatic activities were closely related suggests that phosphorylation of sulfinic PrxI is likely the rate-limiting step in the catalytic cycle.

The x-ray structure of hSrx (30) suggests that Arg[50], Asp[57], and Asp[79] of rSrx are positioned at the solvent interface (Fig. 7). These residues may therefore be expected to play a critical role in the interaction of Srx with Prx. Indeed, we have now shown that rSrx(R50M) and rSrx(D79N) do not bind to PrxI and, consequently, exhibit no or little ATPase or reductase activity. Structural analysis of hSrx indicates that Arg[50] of rSrx interacts with the thiol of Cys[98] via its guanidine group (Fig. 7). Mutation of Arg[50] would be expected to disrupt this interaction. Nevertheless, the inability of rSrx(R50M) to bind to PrxI is not likely related to loss of the Arg[50]–Cys[98] interaction because rSrx(C98S) bound to PrxI to a greater extent than did wild-type rSrx. The rSrx(D57N) mutant bound only weakly to PrxI, but its ATPase and reductase activities were similar to those of the wild-type protein. These results suggest that the phosphorylation reaction that follows binding is so slow that, so long as the enzyme and substrate molecules interact, the affinity with which they do so has no effect on its rate. Although PrxI appeared to bind normally to rSrx(H99N) and rSrx(R100M), these two rSrx mutants exhibited no enzymic activity. Structural analysis of the ADP-bound form of hSrx reveals that the β-phosphate of ADP interacts with His[99] and Arg[100] of rSrx (Fig. 7). When we performed fluorometric titration experiments similar to that shown in Fig. 2, the extent of the decrease in fluorescence intensity induced by ATP was negligible (not shown), suggesting that rSrx(H99N) and rSrx(R100M) are catalytically inactive because they do not bind ATP. Lys[60] interacts with one of the oxygen atoms of the α-phosphate of ADP (30). However, substitution of Arg for Lys[60] in rSrx(K60R) reduced the ATP affinity significantly. Although the fluorescence of wild-type rSrx is maximally decreased by ATP at 52 μM (K_d = 6 μM) (Fig. 2*B*), the fluorescence change observed with rSrx (K60R) in the presence of the same concentration of ATP was about 70% that observed with wild type, and the fluorescence level still decreased upon further addition of ATP (not shown). The ATPase activity of rSrx(K60R) was ~10% of that of wild-type rSrx (Fig. 6*B*). The weak ATP binding and low ATPase activities of rSrx(K60R) suggest that the guanido group of Arg still can interact with the α-phosphate of ATP. Mutation of Asp[66] and Ser[74], which are conserved only among mammalian Srx proteins, had no effect on PrxI binding but reduced ATPase and reductase activities. Examination of the hSrx structure did not reveal an obvious explanation for the observations made with Asp[66] and Ser[74] mutants.

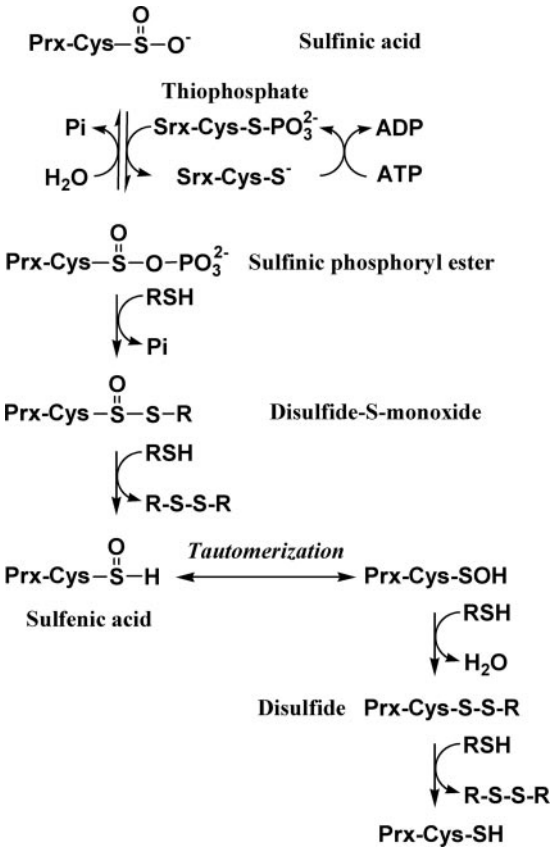

FIGURE 8. **Proposed mechanism for reduction of the sulfinic form of 2-Cys Prx by Srx.** The conserved cysteine of Srx serves as a phosphate carrier from ATP to sulfinic Prx. The resulting sulfinic phosphoryl ester of Prx is converted to a disulfide-*S*-monoxide by a thiol in solution (but not by the conserved cysteine of Srx or the COOH-terminal conserved cysteine of Prx). The disulfide-*S*-monoxide reacts with three equivalents of thiols sequentially to yield reduced Prx. RSH represents a thiol equivalent such as GSH, DTT, or Trx.

In summary, the following mechanism is proposed for the reduction of sulfinic Prx by Srx on the basis of data presented here and reported previously (Fig. 8). Srx specifically recognizes members of the 2-Cys Prx subgroup through contacts with several of its surface-exposed amino acid residues, including Arg[50], Asp[57], and Asp[79] in rSrx. ATP binds, independently of PrxI, to a pocket of Srx, in which the α- and β-phosphates of ATP interact with Lys[60], His[99], and Arg[100] of rSrx. The thiol group of Cys[98] of rSrx is deprotonated as a result of its ionic interaction with the guanidine group of Arg[50], and the resulting thiolate anion transfers the γ-phosphate of ATP, probably through formation of a transient thiophosphate intermediate, to the sulfinic acid moiety of PrxI, thereby yielding a sulfinic acid phosphoryl ester (Prx-Cys-S(=O)OPO$_3^{2-}$). The sulfinic acid moiety is critical for the phosphotransferase reaction because it promotes the extraction of the phosphate from ATP by the thiolate anion of Cys[98] in addition to serving as the acceptor of the phosphate. The sulfinic acid phosphoryl ester is then reductively cleaved by thiols such as GSH, DTT, or Trx to produce a disulfide-*S*-monoxide (Prx-Cys-S(=O)–S-R, where RSH indicates the thiol donor molecule). The disulfide-*S*-monoxide is further reduced, after oxidation of three thiol equivalents, to Prx-Cys-SH. Our proposed reaction mechanism indicates that the conserved cysteine of Srx is critical for sulfinic reductase activity as a result of its phosphate-carrier function, not because of a thioltransferase function, as proposed previously (12, 31).

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Molecular Mechanism of the Reduction of Cysteine Sulfinic Acid of Peroxiredoxin to Cysteine by Mammalian Sulfiredoxin

Woojin Jeong, Sung Jun Park, Tong-Shin Chang, Duck-Yeon Lee and Sue Goo Rhee

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