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

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Among many proteins with cysteine sulfinic acid (Cys-SO₂H) 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 Prxl, their ATPase activity, and their ability to reduce sulfenic Prxl. Our results suggest that three surface-exposed cysteine residues, corresponding to Arg⁵⁰, Asp⁷⁹, and Asp⁷⁹ of rat Srx, are critical for substrate recognition. The presence of the sulfinic form (but not the reduced form) of Prxl induces the conserved cysteine of Srx to take the γ-phosphate of ATP and then immediately transfers the phosphate to the sulfinic moiety of Prxl to generate a sulfinic acid phosphoryl ester (Prx-Cys-S=-O)OPO₃2-. This ester is reductively cleaved by a thiol molecule (RSH) such as GSH, thioredoxin, and dithioretil 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) 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 (Prxl to PrxVI), which are classified into three subtypes (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). Prxl to PrxIV, which belong to the 2-Cys Prx subgroup, exist as homodimers and possess two conserved cysteine residues (Cys⁵¹ and Cys⁷⁷² in Prxl). In the catalytic cycle of 2-Cys Prx enzymes, the NH₂-terminal Cys-SH (Cys⁵¹ in Prxl) is first converted to cysteine sulfinic acid (Cys-SOH) by a peroxide. The unstable sulfenic intermediate (7) then reacts with the COOH-terminal conserved Cys-SH (Cys⁷⁷² in Prxl) 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-SO₂H) (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 sulfenylated 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 H₂O₂ 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 H₂O₂ (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 H₂O₂ 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 phosphate 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 sulfenic 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 sulfenic 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 sulfenic 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 Prxl and their ability to reduce the sulfinic form of Prxl. 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 Prxl.
and that the resulting sulfenic 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 sulfenic 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.; MgCl₂ was from Alfa Aesar; ATP was from Calbiochem; GSH-Sepharose resin and [γ⁻³²P]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 Prxl were prepared as described previously (25). Rabbit antisera specific for human Srx (26), for the hypoxidized cysteine-containing Prx enzymes (27), and for Prxl (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 through a GSH-Sepharose column.

Preparation of Sulfinic PrxI(C172S)—Human PrxI(C172S), in which Cys₁⁷² 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 H₂O₂ (1 mM H₂O₂ was added three times at 30-min intervals). The sulfenic oxidation state of Cys₁⁷² of hyperoxidized Prxl was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (9).

Polyethyleneimine-Cellulose TLC of Hydrolyzed Phosphate—Separation of P₁ 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-hydroxycinnamic 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 Cys⁹⁹ in hSrx and Cys⁹⁸ 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 sulfenic Prx by yeast Srx is dependent on ATP and Mg²⁺, Biteur et al. (12) proposed that the first step of the Srx reaction involves phosphorylation of sulfenic acid (Prx-Cys-SO₂H) to yield a sulfenic acid phospho- ester (Prx-Cys-S=[O-]PO₃²⁻), 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 H₂O₂ results in the formation by Srx of a DTT-reducible covalent complex with Prx, they also suggested that the activated sulfenic acid phosphoester 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 sulfenic acid phosphoryl ester intermediate by incubating Prxl-Cys-SO₂H4 with both [γ⁻³²P]ATP and purified hSrx under reducing conditions and then subjecting either the reaction mixture or Prxl immunoprecipitated from the reaction mixture to SDS-PAGE and autoradiography. No ³²P radioactivity associated with Prxl was detected by either approach (Fig. 1A 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 sulfenic acid phosphoryl ester intermediate, we therefore repeated the experiment with a Cys⁹⁹→Ser mutant of hSrx (hSrx(C⁹⁹S)). Again, we failed to detect ³²P-labeled Prxl (Fig. 1A). Instead, ³²P radioactivity was found associated with hSrx(C⁹⁹S). This phosphorylation of the Srx mutant was observed in the presence of sulfenic Prxl but not in the presence of reduced Prxl. 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. 1B). To determine whether Ser⁹⁹ was the site of phosphorylation in hSrx(C⁹⁹S), we subjected a Cys⁹⁹→Ala mutant of hSrx (hSrx(C⁹⁹A)) to the phosphorylation reaction. Phosphorylation of hSrx(C⁹⁹A) was not detected (Fig. 1C). To confirm that Ser⁹⁹ was the site of phosphorylation in hSrx(C⁹⁹S), we digested the ³²P-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 ³²P-labeled peptide corresponded to residues 86–101 (H³⁹⁹GAGGDFYYSFGSRR), consistent with the conclusion that Ser⁹⁹ is the site of phosphorylation.

While our work was in progress, Jónsson 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 Gly⁹₈. Cys⁹⁹ 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 Cys⁹⁹ in the transfer of the γ-phosphate from ATP to sulfenic Prx (phosphotransferase reaction) and suggested that the sulfenic anion directly extracts the γ-phosphate from ATP.

To determine whether the substitution of Cys⁹⁹ 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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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 ($\approx 6 \mu M$) 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 Cys99 of hSrx exists predominantly as a thiolate anion as a result of its interaction with the guanidine group of Arg51 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 Ser99–OH in hSrx(C99S) is also likely increased, although not as readily as that of Cys99–SH in the wild-type protein, through interaction with Arg51. The increased nucleophilicity of Ser99–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 Prxl. Although Srx binds to both reduced and sulfinic forms of Prxl, Prxl 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 Ser99–O$^-$ or Cys99–S$^-$ anions from Arg51 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 Cys99 would be expected to be faster than that of Ser99. 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. 1A is probably attributable not only to the known instability of thiophosphate com-

![FIGURE 1. Phosphorylation of hSrx(C99S). A, reaction mixtures containing 100 mM Tris-Cl (pH 7.4), 1 mM MgCl$_2$, 0.1 mM ATP, 70 nM [$^{32}$P]ATP (0.4 mCi/ml), and 10 μM sulfinic Prxl (Prx-SO$_2$H), 10 μM reduced Prxl (Prx-SH), or no Prxl (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 Prxl(32P-Prx) or Prx and hSrx(32P-Srx or Srx) are indicated. B, the sulfinic Prx-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 Prxl 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.]

![FIGURE 2. Effects of ATP on the fluorescence emission spectra of wild-type (WT) and C995 mutant forms of hSrx. A, fluorescence emission spectra for a 1-ml reaction mixture containing 20 mM Tris-Cl (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 $\frac{\Delta F}{\Delta F_{max}}$ (where $\Delta F$ represents the change in fluorescence intensity obtained for a specific ATP concentration and $\Delta F_{max}$ is the maximal change in fluorescence intensity at saturation) against ATP concentration.]}
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pounds but also to the presence of the sulfinic anion that is positioned closely to initiate nucleophilic attack on the thiophosphate. Alternatively, Cys99–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 suggesting that the conserved cysteine is essential for the production of Pi. Other thiols were able to substitute for GSH, with replacement by Trx or DTT affecting neither the amplitude nor the rate of Pi formation (Fig. 3C). A substantial level of Pi 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 Cys99–SH of hSrx in ATP hydrolysis. In the presence of 20 μM sulfinic Prxl, the final level of Pi formed was similar to the corresponding concentration of Prxl (Fig. 3D). Furthermore, in reaction mixtures containing 10, 20, or 40 μM sulfinic Prxl, the final level of Pi formed was much greater than 20 μM hSrx concentration (Fig. 3F). However, when GSH was omitted from the reaction mixture containing 20 μM sulfinic Prxl, 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 hSrx (Fig. 3F).

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 Prxl to form hSrx-Cys99–SPO3–, the high energy thiophosphate of which is then used to phosphorylate the sulfinic moiety of Prxl. Although thiolate is susceptible to hydrolysis, hSrx-Cys99–SPO3– is unlikely to be the direct source of the Pi measured in the

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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 MgCl2, 250 μM [γ-32P]ATP (6.7 μCi/ml), 5 μM hSrx, and 20 μM sulfinic Prxl (circles) or reduced Prxl (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 Pi 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 Prxl 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 Prxl 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 Trxl (diamonds), or 10 mM GSSG (upright triangles). For the reaction mixture containing Trxl, 250 mM Trxl 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 Prxl and GSH and that the concentration of hSrx was 2 μM (triangles), 5 μM (circles), or 10 μM (squares). E, ATP hydrolysis was measured as in A with the exception that all reaction mixtures contained sulfinic Prxl and GSH and that the concentration of sulfinic Prxl was 10 μM (triangles), 20 μM (circles), or 40 μM (squares). F, ATP hydrolysis was measured as in A with the exception that all reaction mixtures contained sulfinic Prxl and no GSH and that the concentration of hSrx was 2 μM (triangles), 5 μM (circles), or 10 μM (squares). Data in all panels are means of two independent experiments.
experiments shown in Fig. 3 because such a pathway is not able to account for the observation that more Pi was produced in the absence of GSH than in its presence or to explain why the maximal level of PiC generated in the presence of GSH was similar to the concentration of sulfinic PrxI. Prx-Cys-S(\(\text{O}\))S-R (see Fig. 8). Our results suggest that the chemical nature of the linkage is imprecise, however, given that it was based on immunoblot intensities obtained with antibodies specific for sulfinic Prxl.

We considered the possibility that the COOH-terminal conserved cysteine (Cys\(^{172}\)) of Prxl provides the thiolate anion that replaces the phosphate moiety of Prx-Cys-S(\(\text{O}\))OPO\(^{3-}\). Prxl 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 (Prxl(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 Prxl(C172S) and wild-type Prxl (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\(_2\)O\(_2\)-treated yeast cells. On the basis of this observation, these researchers concluded that sulfinic Prx and Srx form a disulfide–S-monoxy linked and proposed that Prx1 possesses thioltransferase activity. The chemical nature of the linkage was not characterized, however. To obtain insight into this linkage, we incubated sulfinic Prxl 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 Prxl and 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 Prxl (25 kDa) and hSrx (12 kDa) (Fig. 5A). Similar analysis of the reaction mixture on a reducing gel revealed only these latter two bands (Fig. 5B). The sizes and relative intensities of the bands detected by both anti-Prxl and anti-hSrx suggest that they correspond to complexes of (Prxl\(_1\))(hSrx\(_2\), (Prxl\(_1\), hSrx\(_1\), Prxl-hSrx\(_2\)), and Prxl-hSrx, respectively. The complex corresponding to the 35-kDa Prxl-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-monoxy-linked complex of Cys\(^{51}\)-containing Prxl (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 Da in the latter but not in the former. The disappearance of the signal at 4,068.0 Da from the untreated sample was accompanied by the appearance of two new signals at 2,349.9 and 1,722.3 Da in the DTT-treated sample. Given that theoretical mass values for the Cys\(^{172}\)-containing Prxl peptide (residues 169–190) and the Cys\(^{99}\)-containing hSrx peptide (residues 86–101) are 2,349.2 and 1,721.7 Da, respectively, and that the theoretical mass for the two peptides joined by a disulfide is 4,068.9 Da, we concluded that the 35-kDa Prxl-hSrx complex observed in Fig. 5A is joined through a disulfide between Cys\(^{172}\) of Prxl and Cys\(^{99}\) of hSrx, not through a disulfide–S-monoxy between Cys\(^{51}\) of Prxl and Cys\(^{99}\) of hSrx. We further analyzed a mixture of reduced Prxl and hSrx as in Fig. 5A and detected a ladder of complexes containing Prxl and hSrx similar to that observed in Fig. 5A; the Prxl and hSrx molecules were found to be connected through a disulfide either between Cys\(^{52}\) of Prxl and Cys\(^{99}\) of hSrx or between Cys\(^{172}\) of Prxl and Cys\(^{99}\) of hSrx (data not shown). 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 Prxl 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 against time (lower panel); data are means of values from two independent experiments.

![Figure 4. Reduction of the sulfinic forms of wild-type (WT) Prxl and Prxl(C172S) by hSrx.](image-url) 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 \(\mu\)M hSrx, and 5 \(\mu\)M sulfinic wild-type or C172S forms of Prxl were incubated for the indicated times at 30 °C and then subjected to immunoblot analysis with antibodies specific for sulfinic Prxl (upper panels). The concentration of sulfinic Prxl 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.
not shown). These results suggest that sulfiredoxin 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 Arg50, Asp57, Lys60, Asp79, His99, and Arg100 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(H92N)), and Met (rSrx(R100M)), respectively, to generate mutant rSrx proteins fused with GST. In addition, Asp66 and Ser74, which are conserved among mammalian Srx enzymes but not among those from other species (26), were changed to Ala, and the catalytic Cys98 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 sulfiredoxin reductase activity (Fig. 6).

We have previously shown that reduced and sulfiredoxin forms of Prxl bind to rSrx with similar affinities (26). To examine the interaction of GST-rSrx proteins with Prxl, we incubated the GST fusion proteins with either recombinant Prxl or HeLa cell lysates, precipitated the fusion proteins with GSH-Sepharose, and subjected the precipitates to immunoblot analysis with antibodies to Prxl and to GST (Fig. 6A). Similar results were obtained with recombinant Prxl and HeLa cell lysates. Mutation of Arg50 or Asp79 of rSrx completely blocked association with Prxl; Prxl 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 Prxl 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. 6B). Mutation of Arg50, Cys98, His99, or Arg100 abolished both the ATPase and reductase activities, whereas mutation of Asp66 or Ser74 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 5. Formation of DTT-sensitive complexes between sulfiredoxin Prxl and hSrx. Sulfiredoxin Prxl 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 MgCl2, 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 Prxl (lanes 1) or to hSrx (lanes 2). The positions of molecular size standards are indicated in kilodaltons.

FIGURE 6. Characterization of rSrx mutants. A, the effect of rSrx mutations on the interaction with Prxl. 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 Prxl (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 mg/ml N-acetoxybenzenesulfonfyl fluoride, aprotinin (10 μg/ml), leupeptin (10 μg/ml), and 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 Prxl (upper panels) or to GST (lower panels). The intensity of Prxl 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 sulfiredoxin Prxl and 10 mM GSH as described in the legend for Fig. 3C. The amount of sulfiredoxin Prxl remaining was determined from the intensity of the sulfiredoxin Prxl band normalized by that of the Prxl 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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as quantitative as is the detection of $^{32}$P, 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 ratelimiting step in the catalytic cycle.

The x-ray structure of hSrx (30) suggests that Arg$^{50}$, Asp$^{79}$, 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 transfer the $\gamma$-phosphate of 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.

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$^{79}$, and Asp$^{79}$ in rSrx. ATP binds, independently of Prxl, to a pocket of Srx, in which the $\alpha$- and $\beta$-phosphates of ATP interact with Lys$^{60}$, His$^{99}$, and Arg$^{100}$ of rSrx. The thiol of Cys$^{98}$ of rSrx is deprotonated as a result of its ionic interaction with the guanidine group of Arg$^{50}$, and the resulting thiol anion transfers the $\gamma$-phosphate of ATP, probably through formation of a transient thioephosphate intermediate, to the sulfinic acid moiety of Prxl, 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)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).
Acknowledgments—We thank Sun Mi Lee and Sunjoo Park for technical assistance.

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