Reduction of Cysteine Sulfinic Acid in Peroxiredoxin by Sulphiredoxin Proceeds Directly through a Sulphiredoxin Phosphoryl Ester Intermediate*

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Sulphiredoxin (Srx) catalyzes a novel enzymatic reaction, the reduction of protein cysteine sulfenic acid, Cys-SO$_2^-$ (1). This reaction is unique to the typical 2-Cys peroxiredoxins (Prx) and plays a role in peroxide-mediated signaling by regulating the activity of Prxs. Two mechanistic schemes have been proposed that differ regarding the first step of the reaction. This step involves either the direct transfer of the γ-phosphate of ATP to the Prx molecule or through Srx acting as a phosphorylated intermediary. In an effort to clarify this step of the Srx reaction, we have determined the 1.8 Å resolution crystal structure of Srx in complex with ATP and Mg$^{2+}$. This structure reveals the role of the Mg$^{2+}$ ion to position the γ-phosphate toward solvent, thus preventing an in-line attack by the catalytic residue Cys-99 of Srx. A model of the quaternary complex is consistent with this proposal. Furthermore, phosphorylation studies on several site-directed mutants of Srx and Prx, including the Prx-Asp mimic of the Prx-SO$_2^-$ species, support a mechanism where phosphorylation of Prx-SO$_2^-$ is the first chemical step.

The ubiquitous peroxiredoxins (Prxs) are thiol-dependent peroxidases that reduce H$_2$O$_2$, peroxyirnitrile, and alkyl hydroperoxides to water, nitrite, and alcohols, respectively (1). Besides this detoxification role, Prxs can regulate hydrogen peroxide-mediated cell signaling in response to receptor stimulation (2). Peroxiredoxins (Prxs) that can be divided into three classes depending on the reactivity of Prxs. Two mechanistic schemes have been proposed that differ regarding the first step of the reaction. This step involves either the direct transfer of the γ-phosphate of ATP to the Prx molecule or through Srx acting as a phosphorylated intermediary. In an effort to clarify this step of the Srx reaction, we have determined the 1.8 Å resolution crystal structure of Srx in complex with ATP and Mg$^{2+}$. This structure reveals the role of the Mg$^{2+}$ ion to position the γ-phosphate toward solvent, thus preventing an in-line attack by the catalytic residue Cys-99 of Srx. A model of the quaternary complex is consistent with this proposal. Furthermore, phosphorylation studies on several site-directed mutants of Srx and Prx, including the Prx-Asp mimic of the Prx-SO$_2^-$ species, support a mechanism where phosphorylation of Prx-SO$_2^-$ is the first chemical step.

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**EXPERIMENTAL PROCEDURES**

**Protein Preparation and Modification**—An engineered truncation of hSrx (residues 32–137) was expressed from a pET19 (Novagen) vector derivative containing a PreScission protease (GE Healthcare) cleavage site between Srx and the N-terminal His tag. The protein was expressed in C41(DE3) *Escherichia coli* cells and purified using nickel affinity and size-exclusion chromatography after the removal of the His tag (20). His-tagged hPrxII was purified in a similar manner as described previously (20, 22). Mutant forms of hSrx (C99S and C99A) and hPrxII (C70S, C172S, and C51D) were generated using the QuikChange site-directed mutagenesis kit from Stratagene, and the proteins were purified as described for the wild-type proteins. Oxidation of PrxII to the sulfenic acid form was performed with excess diithiothreitol and H$_2$O$_2$ as described previously (20). A PD10 desalting column (GE Healthcare) was used to remove excess diithiothreitol and H$_2$O$_2$.

**Crystallization**—Human SrxET-C99S was crystallized by the vapor diffusion method. Equal volumes of protein (16.6 mg/ml in 20 mM HEPES, pH 7.5, 100 mM NaCl) and well solutions (1 mM potassium phosphate, pH 8.8, 8–13% (±)-2-methyl-2,4-pentanediol (MPD)) were mixed and incubated at 20 °C for a week as sitting drops. The crystal drop solution was slowly changed over the course of a day to a phosphate-free solution containing 1 M sodium malonate, pH 7.5, and 10% MPD. The ligands, 90 mM ATP and 5 mM Mg$_2^{2+}$ in the same solution, were slowly added to the crystal drop over an 8-h period and allowed to incubate overnight. A cryoprotectant consisting of the ATP soak solution with 20% ethylene glycol was added to the crystal drop over 2 h before flash-freezing the crystal in nitrogen gas at 100 K.

**Data Collection and Structure Determination**—A single wavelength (0.979 Å) data set was collected on beamline X4A at NSLS, Brookhaven National Laboratory. Diffraction intensities were integrated using HKL2000 and scaled to 1.8 Å resolution. Cross-validation was performed with 4.9% of the reflections that were set aside. The space group of the crystal was P3$_2$1 with unit cell dimensions $a = b = 68.5, c = 51.1$. Initial phases for the ATP-bound Srx complex were obtained by molecular replacement using PHASER and the apo-Srx structure (Protein Data Bank 1WX3) as the search model (20). The molecular replacement solution was refined with CNS using alternating cycles of simulated annealing and positional and B-factor refinement (23). Model building was performed with COOT (24). Water molecules were visually confirmed within an $F_o - F_i$ map contoured at 3σ and added with COOT. The final cycles of refinement were performed with REFMAC5 (25). The structure was validated using the MOLPROBITY server, which reported 100% of the residues in the Ramachandran favored regions (26). The data collection and refinement statistics for the structure are summarized in Table 1. All structural figures were prepared with the program PyMol (DeLano Scientific).

**γ$^{32}$P Phosphorylation**—Proteins (Srx variants at 2 μM and PrxII variants at 1 μM) and 1 μM ATP spiked with $[^{32}$P]ATP (500,000 dpm) were incubated at 37 °C in a reaction mixture containing 25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM MgCl$_2$. The reactions were quenched by the addition of 5× nonreducing, SDS-PAGE sample-loading buffer containing EDTA (final concentrations in sample, 12 mM Tris, pH 6.8, 5 mM EDTA, 5% glycerol, 0.4% SDS, 0.002% bromphenol blue). The samples were kept on ice until loaded onto 15% SDS-polyacrylamide gels that were also run on ice. The gels were wrapped in plastic wrap and compressed against a phosphorimaging plate and stored overnight at −80 °C. The gels were visualized using a STORM 840 analyzer (GE Healthcare). The data presented are representative of at least three experiments.

**RESULTS AND DISCUSSION**

Crystal Structure of Srx in Complex with ATP-Mg$^{2+}$—To understand the structural basis underlying the first step of the repair mechanism, we set out to determine the crystal structure of hSrx (residues 32–137) in complex with the substrate ATP and Mg$^{2+}$. Potential ATP hydrolysis was prevented by mutat-

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**FIGURE 1. Comparison of the proposed sulfenic acid reduction mechanisms of Srx.** Path 1 represents the mechanism originally proposed by Biteau et al. (10). Path 2 incorporates modifications to the reaction pathway as suggested by Jeong et al. (21). Step 1 involves the formation of the sulfenic acid phosphoryl ester intermediate. In Step 2 of the reaction, the addition of a thiol group leads to the formation of different thiosulfinate intermediates. This intermediate is subsequently resolved by additional thiols in Step 3 of the thiolytic cycle. RSH could represent GSH or Trx.
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TABLE 1
Crystalllographic data and refinement statistics

<table>
<thead>
<tr>
<th>Data collection</th>
<th>ATP-Mg(^{2+})-SrxET-C99S*</th>
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<tbody>
<tr>
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<tr>
<td>Cell dimensions</td>
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<tr>
<td>α, β, γ (°)</td>
<td>68.5, 68.5, 51.1</td>
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<tr>
<td>Wavelength (Å)</td>
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<td>Resolution*</td>
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<tr>
<td>R(<em>{work})/R(</em>{free}) (%)</td>
<td>3.8 (29.8)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.8 (100)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>10.5 (10.5)</td>
</tr>
</tbody>
</table>

Refinement

| Resolution | 38.7 to 1.8 |
| R\(_{work}\)/R\(_{free}\) (%) | 20.1/23.1 |
| No. of atoms | Protein: 829; Ligands: 33; Water: 68 |
| Bond lengths (Å) | 0.018 |
| Bond angles (°) | 1.77 |
| Average B-factor (Å\(^2\)) | Protein: 33.2; Ligands: 31.6; Solvent: 37.6 |
| Ramachandran analysis | 100% |
| Favored regions | 100% |

* Numbers in parentheses are for the highest resolution shell.

Sulfiredoxin-dependent repair of eukaryotic, 2-Cys Prxs has been suggested to proceed via two different mechanisms (Fig. 1) (10, 21). In the originally proposed reaction mechanism (Fig. 1, Path 1), Prx-SO\(_2^-\) is phosphorylated to generate a transient sulfenic acid phosphoryl ester (Prx-SO\(_2\)PO\(_3^-\)) intermediate (10). While testing this mechanism, Jeong et al. (21) observed that Ser-99 within the enzymatically inactive, C99S hSrx mutant was phosphorylated. This led the authors to propose that Cys-99 attacks the γ-phosphate of ATP resulting in the generation of a thiophosphate (Srx-S-PO\(_3^-\)) intermediate. In the Srx-ATP-Mg\(^{2+}\) complex (Fig. 2), there was no indication of Ser-99 phosphorylation even though the crystal was soaked with an excess of ATP overnight. The OH group of Ser-99 within this mutant is 4.4 Å away from the phosphorous atom of the γ-phosphate of ATP. Moreover, as a consequence of its interaction with the Mg\(^{2+}\) ion, the γ-phosphate projects into solvent parallel to Ser-99. This observation suggests that Cys-99 of the wild-type enzyme would not be able to perform an in-line nucleophilic attack on the ATP molecule, the preferred mode of attack for kinases and other ATP-dependent enzymes (Ref. 27 and references cited therein). The importance of Mg\(^{2+}\) for the correct positioning of the γ-phosphate is supported further by the loss of activity upon the addition of EDTA (10).

Model of ATP-Mg\(^{2+}\) Bound to the Srx-Prx Complex—As shown in Fig. 3A, Cys51-SPO\(_2\) (Csd51) of the hPrxII active site exists in a salt bridge to Arg-127 and is covered by the GGLG and YF structural motifs. These interactions prevent access to Csd51 and illustrate the need for dramatic structural changes to occur before Srx can access and repair Csd51 (4, 22, 28). In the rearrangement process one of the oxygen atoms of Csd51 could...
perform an in-line attack on the ATP molecule bound with the Srx active site. To further explore the structural basis for the first step of the repair mechanism, we superimposed the Srx-ATP-Mg$^{2+}$ complex onto the structure of the hSrx-hPrxI complex stabilized via a disulfide bond between the active site Cys residues (supplemental Fig. S1) (22). The phosphorous atom of the $\gamma$-phosphate of ATP is located 3.1 Å from the S-$\gamma$ atom of Prx-Cys-S$_2$H and 3.7 Å from the S-$\gamma$ atom of Srx-Cys-99. From this superposition we generated an enzyme-substrate model (Fig. 3B) by disrupting the intermolecular disulfide bond and converting the peroxidatic Cys to the sulfenic acid form. As a result the active site loop was adjusted slightly to accommodate the changes. In this model one oxygen atom of Prx-Cys-S$_2$PO$_2^-$ is located 1.7 Å from the $\gamma$-phosphate atom. The geometric relationship between the sulfenic acid moiety and ATP suggests that the oxygen atom of Cys-S$_2$PO$_2^-$ performs a direct in-line attack on the $\gamma$-phosphate. In contrast, Cys-99 of Srx points away from the $\gamma$-phosphate, making phosphate transfer to this residue unlikely.

Phosphorylated Species in Srx and Prx Variants—To test the enzyme substrate model and to distinguish between the two proposed repair mechanisms, we investigated the phosphorylation status of Srx and Prx. Previous attempts to identify phosphorylated wild-type proteins were unsuccessful suggesting that the phosphoprotein intermediate(s) were labile (10, 21). Because the reaction is thiol-dependent and the presence of additional thiols may inadvertently break down the first reaction intermediate, the reactions were performed without the addition of GSH or Trx. Moreover, all but the active site cysteine residues of PrxII were mutated to serine (C70S and C172S, referred to as hPrxII-C2S). Srx contains only one Cys residue. Furthermore, we envisioned that a reduced temperature would increase the stability of the species during SDS-PAGE analysis.

In another approach to potentially stabilize the Prx-S$_2$PO$_2PO_3^-$ intermediate, we generated reduction inactive variants of hSrx. With the C99S mutant, the putative thiol attack by Srx to form the thiosulfinate intermediate (Fig. 1, Path 1, Step 2) would be prevented. We were, however, unable to see phosphorylation of hPrxII-C2S with this Srx mutant (Fig. 4A). Instead, we observed phosphorylation of the C99S mutant as previously described by Jeong et al. (21). In their studies Srx C99S, but not Srx C99A, was phosphorylated, which led the authors to conclude that Srx was phosphorylated at Ser-99 to form a conventional, stable phosphoserine adduct. Unlike this previous study, we also observed phosphorylation of Srx C99A and to some extent wild-type Srx. Moreover, in the absence of PrxII-SO$_2^-$, the Srx variants were phosphorylated suggesting that the phosphorylation of Srx may be nonspecific to some degree (Fig. 4B). In the original mechanism proposed for Srx (Fig. 1), one oxygen atom on the sulfenic acid moiety functions as the nucleophile in the attack on the $\gamma$-phosphate of ATP, resulting in the formation of a sulfenic acid phosphoryl ester intermediate (10). This mechanism is reminiscent of the ATP-dependent activation of a carboxyl group by a variety of enzymes, including glutamine synthetase (GS) and peptide ligases (29). In GS the phosphorylation of one oxygen of the carboxyl group of Glu generates a detectable acyl phosphate intermediate, which in turn is attacked by an ammonium ion (or amine in other enzymes with similar reactions) to generate inorganic phosphate (P$_i$) and Gln. By analogy, we envisioned that the substitution of the sulfenic acid moiety in PrxII-SO$_2^-$ with a carboxyl group (Prx-CO$_2^-$), i.e.
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The reaction, we incubated the PrxII-C2S-C51D mutant with the C99S and C99A Srx variants (Fig. 4A). In both cases phosphorylation of PrxII occurred as well as SrxC99S and SrxC99A. In all cases the level of phosphorylation in Prx was greater than the Srx variants.

To further test the validity of the two reaction mechanisms, we monitored the rate of phosphorylation in Prx versus Srx. In the presence of hPrxII-C2S-SO2-3, phosphorylation of SrxC99S was observed after 3 min and increased with time (Fig. 4B, 1st panel). In contrast, formation of phosphoaaspartate in the PrxII-C2S-C51D mutant was seen within 1 min of incubation with all Srx variants (Fig. 4B, 2nd to 4th panels). Phosphorylation of Srx C99S in the presence of PrxII-C2S-C51D was delayed for 10 min. These data support that PrxII-C51D is the preferred substrate for phosphorylation. The same pattern was observed with the C99A Srx mutant. These observations are in agreement with phosphorylation of Prx being the first step in the reaction mechanism. Moreover, the phosphorylation of Prx appears to be independent of Cys-99 within Srx.

Structural Models of the Reaction Mechanism—We have investigated the enzymatic mechanism of sulfenic acid reduction by Srx. Our studies provide support for the first step of the reaction mechanism proposed by Biteau et al. (10). This is demonstrated by phosphorylation studies of Srx and Prx (Fig. 4), which show that the acid form of the active site residue of Prx could be phosphorylated before the phosphorylation of Srx could be detected. Furthermore, the crystal structure of Srx in complex with ATP and Mg2+ (Fig. 2) shows that Prx-SO2- is geometrically favored to attack ATP over Cys-99 in Srx. This arrangement contrasts with the phosphatase PTP1B, for example, where the incoming phosphotyrosine is attacked by Cys-215 in an in-line fashion, leading to a phospho-Cys intermediate (Cys-S-PO2-3) (32).

Our recent crystal structure of Srx in complex with Prx (22) and the Srx-ATP-Mg2+ complex presented here provides a structural model of how the repair may occur. Srx accesses the active site of Prx by displacing the YF motif of Prx (Fig. 3A). The binding of the conserved Phe-50 of Prx into a hydrophobic surface pocket of Srx is also thought to result in the unfolding of the Prx active site helix. As a result Csd51 of PrxII reorients to approach the γ-phosphate of ATP for an in-line attack (Fig. 3B). Surprisingly, these conformational changes are dependent on the unfolding and packing of the C-terminal tail of Prx onto the “backside” surface of Srx far from the Prx and Srx active sites (22). This latter interaction is essential for repair to occur.

The second step of the reaction, the thiol-dependent attack on the sulfenic phosphoryl ester intermediate, is still unproven (Fig. 1, Path A and Step 2). An external thiol such as GSH could access the phosphoryl ester from the ATP-binding pocket. Alternatively, GSH could enter ~120° from the ATP-binding site and attack the sulfenic phosphoryl ester moiety from the backside. A more compelling scenario is the direct attack by Cys-99 of Srx on the Prx intermediate. In our quaternary model Csd51 is positioned 1.7 Å from the γ-phosphorous atom of ATP. Once the Prx-SO2-P=O3- species has been formed, Cys-99 is in close proximity (~3 Å) and in a favorable geometric position to attack the S-γ atom of Prx-SO2-P=O3- resulting in the formation of a Prx-Srx thiol sulfinate intermediate (Fig. 3C).

FIGURE 4. Detection of phospho-protein intermediates of Srx and Prx. A, analysis of Srx and Prx variants within a 30 min reaction at 37 °C containing [γ-32P]-ATP. The reactions were quenched with nonreducing sample buffer containing EDTA and separated on 15% SDS-polyacrylamide gels on ice as described under “Experimental Procedures.” The positions and variants of Srx and Prx are indicated. The PrxII protein used in these experiments contains the background mutations C70S and C172S, and PrxII are indicated. The PrxII protein used in these experiments contains the background mutations C70S and C172S, i.e. hPrxII-C2S, to prevent the inadvertent breakdown of phosphorylated intermediates. B, reaction mixtures described in (A) were incubated for the indicated time before analysis. The control samples that did not contain either Srx or Prx were incubated for 120 min. The image in panel 2 was electronically separated only to facilitate alignment of the samples for comparison.
This intermediate could be resolved by the external GSH, resulting in the formation of a mixed Srx-GSH species (Fig. 1, Path 1, Step 3) or glutathione disulfide (Path 2, Step 3). Additional experiments are clearly needed to clarify the reaction beyond Step 1. The identification and temporal appearance of the thiosulfinate intermediate(s), Srx-S–S–G and potentially other species would further help to resolve the unique reaction mechanism of Srx and the possible cross talk between redox-regulated signaling pathways involving Prxs and GSH.

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REFERENCES
