The SoxYZ Complex Carries Sulfur Cycle Intermediates on a Peptide Swinging Arm

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The bacterial Sox (sulfur oxidizing) system allows the utilization of inorganic sulfur compounds in energy metabolism. Central to this process is the SoxYZ complex that carries the pathway intermediates on a cysteine residue near the C terminus of SoxY. Crystal structures have been determined for Paracoccus pantotrophus SoxYZ with the carrier cysteine in the underivatized state, conjugated to the polysulfide mimic β-mercaptopethanol, and as the sulfonate adduct pathway intermediate. The carrier cysteine is located on a peptide swinging arm and is bracketed on either side by diglycine dipeptides acting as molecular universal joints. This structure provides a novel solution to the requirement that the cysteine-bound intermediates be able to access and orient themselves within the active sites of multiple partner enzymes. Adjacent to the swinging arm there is a conserved, deep, apolar pocket into which the β-mercaptopenthaln adduct extends. This pocket would be well suited to a role in protecting labile pathway intermediates from adventitious reactions.

Oxidation of inorganic sulfur species to sulfate by bacteria is a vital part of the global sulfur cycle and has applied importance in agriculture, waste treatment, biocorrosion, and bio-mining. The bacteria use the electrons that they derive from the sulfur species as the reductant in carbon dioxide fixation or as the electron donor for respiratory energy metabolism.

The Sox (sulfur oxidizing) system appears to be the most widely distributed sulfur oxidation pathway and is found in both photosynthetic and non-photosynthetic sulfur-oxidizing Eubacteria. The Sox pathway has been most extensively studied in the α-Proteobacterium Paracoccus pantotrophus. The current model for the oxidation of thiosulfate (S\text{2−}-SO\text{4}−) to sulfate by the Sox pathway in this organism is shown in Fig. 1A. It involves the four periplasmic protein complexes SoxYZ, SoxAX, SoxB, and SoxCD (1, 4, 5). Intermediates in the pathway are covalently bound to a cysteine residue located within a characteristic GGCGG pentapeptide at the C terminus of the SoxY subunit of the SoxYZ complex (6). All but the extreme C-terminal glycine residue are conserved in SoxY orthologs and are presumably essential (Fig. 1, B and C). The first step in the pathway is oxidative linkage of thiosulfate to SoxYZ to form a cysteine S-thiosulfonate (thiocysteine sulfate) derivative. This reaction is catalyzed by the triheme-containing SoxAX complex. Crystal structures of SoxAX reveal unprecedented cysteine persulfide coordination to the heme group at the active site of the enzyme (7, 8). In subsequent steps the S-thiosulfonate species is degraded by a combination of the hydrolase SoxB, which removes terminal sulfone (SO\text{4}−) groups as sulfate, and the molydopterin-containing SoxCD complex, which oxidizes terminal sulfane (S−) groups to the corresponding sulfone. The eight electrons extracted in the two oxidative steps of the Sox pathway are transferred to a small c-type cytochrome for delivery to the electron transfer chain.

As well as its role in thiosulfate metabolism the Sox pathway has been reported to be involved in the oxidation of other reduced sulfur species including sulfide (HS−), elemental sulfur (S\text{8}), sulfite (HSO\text{3}−), and tetrasulfate (O\text{3S-S-S-SO}\text{3}−) (9–11). The modular nature of the Sox system would allow such species to be fed into the pathway as an appropriate intermediate (illustrative examples are shown in Fig. 1D). In some cases this would result in formation of adducts containing long chains of sulfur atoms before the terminal sulfane or sulfone groups. An additional important consequence of the modular arrangement of the Sox system is that the reactions are not necessarily ordered. It is, therefore, conceivable that instead of a sulfane intermediate being oxidized by SoxCD it could be oxidatively conjugated to a thiosulfate molecule by SoxAX (Fig. 1E). This would be a further mechanism by which longer chain sulfur species might accumulate on SoxY. Such species have been experimentally observed (6). Whatever the origins of long chain sulfur species, they can be broken down by repeated iterations of the SoxCD and SoxB reactions.

Many bacteria with a Sox system lack the SoxCD component found in the prototypical Sox pathway of α-Proteobacteria (1, 12, 13). In these organisms it is thought that the Sox system feeds sulfane groups derived from thiosulfate into other sulfur...
oxidation pathways. In some cases this involves incorporation of the sulfane atoms into distinct zero-valent storage forms of sulfur located in periplasmic sulfur globules (purple sulfur bacteria) or deposited extracellularly (green sulfur bacteria) (13–15).

The SoxYZ complex is the pivotal component of the Sox system because it participates in every reaction of the pathway. Consideration of the molecular tasks it must undertake reveals the remarkable nature of this protein. The SoxYZ complex has to carry sulfur compounds containing either a terminal sulfane or sulfone group, and that may contain variable numbers of zero-valent sulfur atoms. In addition, it must interact with multiple partner proteins, namely SoxAX, SoxB, and SoxCD (or alternatives in organisms lacking this complex), and possibly other proteins coded at sox loci such as the flavocytochrome SoxEF and thioredoxins. SoxYZ is unique among known sulfur carrier proteins in that it both carries multiple types of adduct and exhibits specific interactions with multiple structurally and functionally unrelated partner proteins. To understand the molecular basis for this multitasking behavior we have determined the crystal structure of the SoxYZ complex from P. pantotrophus together with two mechanically relevant derivatives. These structures reveal that the SoxYZ complex carries the Sox pathway intermediates on an unusual swinging arm structure in which the carrier cysteine is bracketed by two molecular universal joints. The structure also identifies an apolar pocket that may protect labile pathway intermediates.

**EXPERIMENTAL PROCEDURES**

Preparation of Plasmids for Heterologous Coexpression of SoxYZ

A plasmid suitable for heterologous co-expression of P. pantotrophus LMD82.5<sup>T</sup> SoxYZ proteins in Escherichia coli was constructed. PCR was used to amplify the sox<sup>Y</sup> gene without the signal peptide coding region. The electron released in the reactions are accepted by a small <i>c</i>-type cytochrome. In organisms lacking a SoxCD complex it is thought that the sulfur atom of the sulfane intermediate (star) is fed into other sulfur oxidation pathways or into storage forms of sulfur. The cysteine residue of SoxY is shown in its ionized state. However, the reactive form of this residue in the SoxAX-catalyzed reaction is not clear and it may be protonated.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Oxidation of reduced inorganic sulfur compounds by the Sox system. A, Friedrich-Kelly model for the oxidation of thiosulfate by Sox systems that include a SoxCD complex (1). The electrons released in the reaction are accepted by a small <i>c</i>-type cytochrome. In organisms lacking a SoxCD complex it is thought that the sulfur atom of the sulfane intermediate (star) is fed into other sulfur oxidation pathways or into storage forms of sulfur. The cysteine residue of SoxY is shown in its ionized state. However, the reactive form of this residue in the SoxAX-catalyzed reaction is not clear and it may be protonated. B and C, primary sequence alignments of SoxY (B) and SoxZ (C) subunits from various sulfur-oxidizing bacteria. Sequences are those of the SoxY and SoxZ proteins from P. pantotrophus (Pp), Rhodovulum sulfidophilum (Rs), Thermus thermophilus (Tt), Chlorobium tepidum (Ct), Allochromatium vinosum (Av), and Aquifex aeolicus (Aa). Residue numbering corresponds to the native, mature P. pantotrophus sequences. ▲ indicates the substrate-carrying cysteine residue in SoxY. Completely conserved residues are highlighted. D, examples of how sulfur species other than thiosulfate could feed into the Sox pathway at the appropriate intermediate state via either enzymatic (top) or non-enzymatic (bottom) conjugation to SoxY. E, scheme showing how long chain sulfur compounds could be assembled on the SoxY carrier cysteine during thiosulfate oxidation if SoxAX is able to act upon sulfane intermediates (starred species in A). This reaction could occur if the sulfane adduct interacts in a random order with partner proteins rather than being directed to react with SoxCD. It could also occur if the organism lacks a SoxCD complex.
Crystal Structure of *P. pantotrophus* SoxYZ

cate copy of the optimized *E. coli* ribosome binding site sequence of pQE80L directly in front of the gene. The *P. pantotrophus* SoxYZ expression plasmid was labeled pVS005. The primers used in this process are given in supplementary Table S1.

**Purification of Heterologously Expressed SoxYZ Proteins—** *P. pantotrophus* SoxYZ was expressed in *E. coli* strain BL21 (16) from plasmid pVS005. The cells were cultured aerobically at 37 °C in 1 liter of LB medium supplemented with 100 μg ml⁻¹ ampicillin. Expression of the *soxYZ* genes was induced at an A₆₀₀nm = 0.8 by addition of 0.1 mM isopropyl 1-thio-β-D-galactopyranoside. The cells were cultured for a further 5 h at 30 °C and then harvested by centrifugation at 9,000 × g at 4 °C for 20 min. The pellet was resuspended in 10 ml of 50 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, 5% glycerol, 10 mM imidazole (resuspension buffer) together with a tablet of EDTA-free Complete protease inhibitors (Roche), a pinch of lysozyme (Sigma), and a pinch of DNase I (Sigma). The suspension was passed 3 times through a French press at 8000 p.s.i. The lysate was centrifuged at 200,000 × g at 4 °C for 30 min and the supernatant loaded onto a 5-ml Ni(II)-loaded chelating Sepharose Fast Flow column (GE Healthcare) equilibrated with resuspension buffer. The column was washed with resuspension buffer and then resuspension buffer containing 25 mM imidazole until the A₂₈₀nm of the column eluant fell to a low and constant value. The SoxYZ protein was then eluted using a 40-ml linear 25–185 mM imidazole gradient in resuspension buffer. The SoxYZ peak from the Ni(II) affinity column was dialyzed overnight at 4 °C against 30 mM Bicine, pH 9.0, 5 mM DTT (Bicine buffer). The dialysate was loaded onto a 5-ml Q-Sepharose Fast Flow column (GE Healthcare) pre-equilibrated with the Bicine buffer. This was followed by a 40-ml linear 0 to 110 mM NaCl gradient in Bicine buffer and then isotropic elution of SoxYZ in Bicine buffer containing 110 mM NaCl. Fractions containing SoxYZ were pooled, dialyzed against 10 mM Tris-HCl, pH 8.0, 5 mM DTT at 4 °C and concentrated by ultrafiltration. Mass spectrometry demonstrated that both subunits had the masses expected of the native recombinant proteins (supplemental Fig. S2).

*P. pantotrophus* SoxYZ bearing a β-mercaptoethanol adduct on the SoxY carrier cysteine was prepared in the same way as native SoxYZ except that 5 mM β-mercaptoethanol was added and DTT omitted from all purification buffers. N-terminal sequencing of the purified SoxYZ-β-mercaptoethanol adduct protein gave the expected sequence for SoxY (MRGSHHH-HHH) but showed that SoxZ (ADDAKPRVKV) had its initiation methionine processed. Electrospray mass spectrometry analysis of this protein (supplemental Fig. S5A) showed a SoxZ peak at the expected mass of 11,719 Da and a SoxY peak at 76 Da greater than the expected mass of 12,375 Da corresponding to a single β-mercaptoethanol adduct. After dialysis against 5 mM DTT the mass of SoxZ was unchanged but the SoxY mass moved to its predicted value (supplemental Fig. S5B). This confirmed that the β-mercaptoethanol adduct was linked to SoxY via a disulfide bridge.

For preparation of selenomethionine-labeled *P. pantotrophus* SoxYZ plasmid pVS005 was transformed into the *E. coli* methionine auxotroph B834 (16). The strain was cultured aerobically at 37 °C in LB medium containing 100 μg ml⁻¹ ampicillin until the A₆₀₀nm reached 0.2. The culture was then centrifuged at 2,000 × g for 10 min at 4 °C, the pellet was washed twice with 15 ml of SelenoMet medium (Molecular Dimensions), and then resuspended in 1 liter of SelenoMet medium containing 100 μg ml⁻¹ ampicillin. The resulting culture was grown aerobically at 37 °C until the A₆₀₀nm reached 0.8 at which point 100 μM isopropyl 1-thio-β-D-galactopyranoside was added and growth was continued for a further 16 h. Purification of selenomethionine SoxYZ used the same protocol as employed for the SoxYZ-β-mercaptoethanol adduct. Mass spectrometry gave mass peaks at 12,592 and 11,766 Da corresponding to a full incorporation of selenomethionine in both the SoxX (three methionines) and SoxZ (one methionine) proteins with retention of β-mercaptoethanol derivatization of the SoxY carrier cysteine.

The SoxX cysteine-S-sulfonate derivative of *P. pantotrophus* SoxYZ, (SoxYZ-SO₃⁻) was produced by overnight dialysis of native SoxYZ against 50 mM potassium tetrathionate, 10 mM Tris-HCl, pH 8.0. The derivatized protein was then purified by size exclusion chromatography. The nature of the modification to the SoxX cysteine residue was verified by mass spectrometry of the derivatized protein before and after treatment with 5 mM DTT (supplemental Fig. S6).

**Crystallization and Crystal Structure Determinations—** The selenomethionyl SoxYZ-β-mercaptoethanol derivative was concentrated to 10 mg/ml prior to crystallization. Single crystals were obtained by vapor diffusion at 20 °C using PEG 3350 as precipitant and could be cryoprotected by addition of 25% (v/v) ethylene glycol. These crystals contained four copies of the heterodimer in the asymmetric unit. The crystal structure was solved by Se-MAD using heavy atom sites located by SOLVE (17). Refinement at 1.98-Å resolution resulted in a final model lacking only residues 35 to 41 in two copies of SoxZ (these residues lie at the extremity of the Z-loop), the C-terminal Gly-Gly dipeptide of two copies of SoxY, and the penultimate glycine residue alone in a further copy. Crystals of native *P. pantotrophus* SoxYZ were grown under similar conditions to those found for the β-mercaptoethanol derivative and were cryoprotected by addition of 15% (v/v) ethylene glycol. They were found to be essentially isomorphous with those of the selenomethionine protein and the structure was solved by molecular replacement using a monomer of selenomethionyl β-mercaptoethanol SoxYZ as search model. Refinement at 1.4-Å resolution resulted in a model in which all four copies of SoxY in the asymmetric unit lack the C-terminal Gly-Gly dipeptide. The carrier cysteine residue of SoxX, Cys¹¹⁰, is also missing in two of these copies. An additional redundant sulfur single wavelength anomalous dispersion dataset was taken from an isomorphous single crystal of native SoxYZ using X-rays of wavelength λ = 2.07 Å. Anomalous difference Fourier maps calculated using this data were used to help position the Sy atom of the carrier cysteine residue in the two visible copies of this residue in SoxY.

Crystals of the SoxYZ S-sulfonate derivative grew in vapor diffusion hanging drops at 20 °C. The protein concentration and crystallization condition used were similar to those employed for native SoxYZ and for the β-mercaptoethanol
derivative. For this form of the protein, however, the crystals were of space group P1 with four copies of the heterodimer in the asymmetric unit. Crystals were cryoprotected by addition of mother liquor containing 5% (v/v) glycerol and 15% (v/v) ethylene glycol. The structure was solved by molecular replacement using a monomer of native SoxYZ lacking the C-terminal peptide as a search model and refined at 2.35-Å resolution.

Data collection and refinement statistics for all the structures reported here are presented in Table 1. Full details of the structure solution methods and refinement protocols employed are given in the supplemental data.

Protein Structure Analysis—Analysis of the SoxYZ protein-protein interface used the Protein-Protein Interaction Server (18) (available at www.biochem.ucl.ac.uk/bsm/PP/server/index.html). Protein structure superposition was performed with DALI (19).

RESULTS

The Crystal Structure of SoxYZ—Recombinant hexahistidine-tagged *P. pantotrophus* SoxYZ complex was expressed in the cytoplasm of *E. coli* and purified in the presence of dithiothreitol. Mass spectrometric analysis of the purified complex showed that both subunits had their expected molecular masses (supplemental Fig. S2). SoxZ possessed 108 amino acids (having lost its N-terminal methionine residue) and SoxY 124 amino acids (the predicted C-terminal 112 amino acids of SoxY plus the sequence Met-Arg-Gly-Ser followed by a hexahistidine tag and a Gly-Ser dipeptide introduced as a cloning artifact at the N terminus). No post-translational modification of the single cysteine residues in each of the subunits was observed. The complex was crystallized and a structure determined at 1.4-Å resolution. The final structural model has an overall crystallographic R-factor of 16.8% (R_{free} 19.4%) (Table 1). The four copies of SoxYZ found in the crystallographic asymmetric unit are very similar, with a root mean square deviation calculated for the Ca atoms of 0.66 ± 0.36 Å.

SoxY and SoxZ both possess an immunoglobulin-like β-sandwich fold consisting of 7 β-strands (labeled β1 to β7) organized in a typical Greek key topology and arranged in stacked three-stranded and four-stranded antiparallel sheets (Fig. 2A). The folds of SoxY and SoxZ are clearly homologous despite the absence of significant sequence similarity between the subunits. Structural alignment of the two subunits gives a root mean square deviation of 1.8 Å for 84 aligned residues of which only 13 are identical (Fig. 2B). These identical residues appear to be conserved for structural reasons. In addition to the core strands of the immunoglobulin-like fold, an additional β-strand (βA) is found in the loop joining strands β1 and β2 in both subunits. The βA strand forms a short parallel sheet by hydrogen bonding to a further strand (labeled βB) that forms a continuation of strand β7. This interaction is present in all subunits of the four heterodimers found in the asymmetric unit except in one copy of SoxX in which strand βB does not form. This variation is apparently due to lattice contact differences between molecules in the crystal and suggests that the βA–βB interaction is susceptible to disruption.

The main differences between the structures of the two subunits of SoxYZ are the presence of an α-helix at the N terminus of SoxY, an extended loop linking strands β2 and β3 in SoxZ (hereafter termed the Z-loop), and the conserved GGCGG carrier pentapeptide (residues 108–112) containing the carrier cysteine that forms an extension of the C terminus of SoxY relative to SoxZ (Fig. 2, A and B). Analysis of residue average temperature factors shows that the Z-loop and the C-terminal pentapeptide of SoxY bearing the carrier cysteine are among the most mobile regions of the SoxYZ complex (supplemental Fig. S1). Indeed, no electron density is observed for the extreme C-terminal Gly-Gly dipeptide of SoxY (Fig. 2C) and the residues of a β-turn at the extremity of the Z loop are visible in only two copies of the complex.

The side chain of the single cysteine residue in SoxZ contributes to the hydrophobic core of the subunit and is completely inaccessible to solvent (Fig. 2A). Previous studies have identified a cross-link between this residue and the carrier cysteine of SoxY and a role for this linkage in the Sox mechanism has been suggested (22). The structure reveals that this residue could not plausibly be involved in a disulfide linkage to SoxY in the native protein.

Overall, 20 hydrogen bonds and a single salt bridge contribute to the stabilization of the SoxY heterodimer interface. These contacts result in the burial of 1121 ± 18 Å² of solvent accessible surface area, representing around 18% of the surface area of isolated SoxZ and 20% of the surface area of isolated SoxY. The interface is also well packed as evidenced by a calculated shape complementarity statistic (23) of 0.74. The heterodimer interface thus has the general characteristics of that from a permanent complex (18). In the crystal, SoxYZ forms a higher order (SoxYZ)_2 heterotetramer structure in which SoxYZ heterodimers interact primarily through the SoxY N-terminal helix (supplemental Fig. S3). This tetramer interaction is probably not of physiological importance because it is not observed in analytical ultracentrifugation measurements (carried out either in the crystallization buffer or at neutral pH), which confirm that SoxYZ forms a stable heterodimer in solution (supplemental Fig. S4). The SoxZ subunit of SoxYZ has the highest degree of structural similarity to the unpublished SoxZ protein from *Thermus thermophilus* (root mean square deviation of 1.9 Å) (PDB entry 1V8H). The closest structural neighbor to SoxY is the superoxide reductase from *Pyrococcus furiosus* (root mean square deviation of 2.2 Å) (PDB entry 1DO6) (24). The intersubunit packing of this homodimeric protein is similar to, but looser than, that of SoxYZ.

The SoxY GGCGG Carrier Pentapeptide and Its Environment—In the structure of native *P. pantotrophus* SoxYZ, the conserved GGCGG carrier pentapeptide immediately follows the β8 strand of SoxY and lies along the top of the immunoglobulin β-sandwich fold (Fig. 2A). A Fourier map calculated using highly redundant anomalous difference data measured near the sulfur K-edge was used to aid structural interpretation in the region of the SoxY cysteine residue. A peak at 2σ above the mean difference density contributed to the positioning of the γ-sulfur atom (Fig. 2C). However, additional contiguous and unbranched electron density is apparent immediately adjacent to this atom that does not appear to originate from the native protein. As mass spectrometric analysis of the protein used for crystallization gave the expected native molecular mass of the SoxY, an extended loop linking strands β2 and β3 in SoxZ (hereafter termed the Z-loop), and the conserved GGCGG carrier pentapeptide (residues 108–112) containing the carrier cysteine that forms an extension of the C terminus of SoxY relative to SoxZ (Fig. 2, A and B). Analysis of residue average temperature factors shows that the Z-loop and the C-terminal pentapeptide of SoxY bearing the carrier cysteine are among the most mobile regions of the SoxYZ complex (supplemental Fig. S1). Indeed, no electron density is observed for the extreme C-terminal Gly-Gly dipeptide of SoxY (Fig. 2C) and the residues of a β-turn at the extremity of the Z loop are visible in only two copies of the complex.

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Crystal Structure of P. pantotrophus SoxYZ

### TABLE 1
Data collection and refinement statistics

Dataset nomenclature is as follows, SoxYZ-β-mercaptoethanol: SeMet-SoxYZ containing S-(2-hydroxyethylthio)Cys110Y. SoxYZ-S-sulfonate: SoxYZ containing Cys110Y-S-sulfonate. Numbers in parentheses refer to data in the highest resolution bin.

| Dataset       | SoxYZ-β-mercaptoethanol (MAD data collection) | Native SoxYZ | SoxYZ-S-sulfonate |
|---------------|---------------------------------------------|-------------|------------------|
| Beamline      | ESRF                                        | ESRF 14.1   | SRS 10.1         |
| Space group   | C2                                           | C2          | P1               |
| Cell parameters |                                             |             |                  |
| a, b, c (Å)   | 206.6, 54.7, 77.9                           | 206.8, 54.7, 77.7 | 40.2, 54.5, 104.9 |
| α, β, γ (°)   | 90, 98.6, 90                                | 90, 98.7, 90 | 103.1, 95.2, 90.0 |
| Wavelength (Å) |                                            |             |                  |
| λ1            | 0.979                                        | 0.873       | 0.394            |
| λ2            | 0.873                                        | 0.934       | 1.488            |
| Resolution (Å) | 50-2.21 (2.29-2.21)                         | 50-1.98 (2.03-1.98) | 50-1.40 (1.44-1.40) |
| Completeness (%) | 99.9 (99.3)                               | 98.7 (95.4) | 96.4 (91.8)      |
| R cryst (%)   | 5.3 (14.0)                                  | 7.0 (27.0)  | 4.2 (39.5)       |
| Rmerge (%)    | 5.6 (9.3)                                   | 5.2 (9.3)   | 10.5 (14.4)      |
| Independent reflections | 43,104 (3,953) | 59,443 (3,653) | 164,923 (11,854) |
| Multiplicity  | 43,104 (3,953) | 59,443 (3,653) | 164,923 (11,854) |
| Overall temperature factor (Å²) | 26.9 | 19.7 | 15.9 |
| FOM (MAD phasing) | 0.39 (0.23) | 0.39 (0.23) | 0.39 (0.23) |
| FOM (solvent flattened) | 0.73 (0.47) | 0.73 (0.47) | 0.73 (0.47) |

Refinement statistics

- SoxYZ heterodimers per AU
  - R free
    - Total atoms: 7,282
    - Water molecules: 895
  - R factor:
    - Overall: 21.1 (28.5)
    - Water molecules: 17.3 (20.3)
  - Ramachandran analysis:
    - Most favored: 91.6 (90.9)
    - Additional allowed: 8.4 (9.1)
    - Generously allowed: 0.0 (0.0)
  - Root mean square deviations:
    - Bond lengths (Å): 0.013
    - Angles (°): 1.35
    - Planes (Å²): 0.005
  - Mean atomic B-value (Å²): 22.6

* R cryst = \(\frac{\sum |F_o| - |F_c|}{\sum |F_o|}\), where \(F_o\) and \(F_c\) are the measured and calculated structure factors.
* Rmerge = \(\frac{\sum |F_o| - |F_c|}{\sum |F_o|}\) (reported for both MAD datasets to a maximum resolution of 2.2 Å).
* FOM (MAD phasing) = 0.73 (0.47)
* FOM (solvent flattened) = 0.73 (0.47)
* AU refers to the crystallographic asymmetric unit.

Masses of the two Sox proteins interpretation of this density feature must remain speculative (mass spectrometric analysis of protein recovered from crystals was unsuccessful). After testing many possible interpretations, in the final refined structure this additional electron density has been modeled as a molecule of the cryoprotectant used in the x-ray experiment, ethylene glycol (Fig. 2C). This interpretation is plausible but not unambiguous. Moreover, in this model the refined cysteine γ-sulfur to ethylene glycol hydroxyl oxygen non-bonded distance is short (around 2.4 Å). This is indicative at least of reduced occupancy of the bound molecule and, by implication, the same must be true of the residues of the C-terminal peptide of SoxY in the refined structure reported in this paper.

A single hydrogen bond between the α-amino group of Gly109Y and the carbonyl of SoxY Leu17 serves to restrain the conformation of the SoxY C-terminal peptide (Fig. 2C). Nevertheless, the atomic temperature factors for the modeled portion of the SoxY C-terminal peptide are higher than the molecular average suggesting it to be a relatively mobile part of the structure (supplementary Fig. S1). The carrier cysteine side chain (Cys110Y) is located at the entrance to a deep pocket generated by the packing of the SoxY and SoxZ subunits. This pocket is the only one found in the vicinity of the GCGGG peptide and is the largest detected in the protein. It is roughly 10 Å deep and 6 Å wide at its mouth, tapering slightly toward the bottom and has a volume of 146 Å³. The inner surface of the pocket is formed by the side chains of residues contributed by both subunits. Non-polar atoms predominate, contributing around 66% of the 166 Å² solvent accessible surface of its lining. The residues making major contributions to the surface of the pocket are Ser32, Glu33, Pro67, and Ala68 from SoxZ; and Asn54, Pro55, Glu56, Arg58, and Ala80 from SoxY. Only one of these, Asn54 of SoxY, is invariant (Fig. 1, B and C). The side chain of this residue hydrogen bonds to the main chain of Arg76 and Ala80 of SoxY and forms one side of the pocket. Located within the pocket are two molecules of the cryoprotectant (discussed above) as well as three water molecules that are bound tightly at the bottom of the pocket and have temperature factors that are comparable with protein main chain atoms in their vicinity.

Crystal Structure of a SoxYZ-β-Mercaptoethanol Adduct Has Implications for the Stabilization of Polysulfanated Pathway Intermediates—Mass spectrometry of P. pantotrophus SoxYZ protein purified in the presence of β-mercaptoethanol displayed mass peaks corresponding to SoxZ and SoxY + 76 Da (supplemental Fig. S5). This additional mass of SoxY corresponds to that of β-mercaptoethanol. The extra mass was removed if the protein was subsequently incubated with DTT suggesting that the modifying species was disulfide-linked to

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masses of the two Sox proteins interpretation of this density feature must remain speculative (mass spectrometric analysis of protein recovered from crystals was unsuccessful). After testing many possible interpretations, in the final refined structure this additional electron density has been modeled as a molecule of the cryoprotectant used in the x-ray experiment, ethylene glycol (Fig. 2C). This interpretation is plausible but not unambiguous. Moreover, in this model the refined cysteine γ-sulfur to ethylene glycol hydroxyl oxygen non-bonded distance is short (around 2.4 Å). This is indicative at least of reduced occupancy of the bound molecule and, by implication, the same must be true of the residues of the C-terminal peptide of SoxY in the refined structure reported in this paper.

A single hydrogen bond between the α-amino group of Gly109Y and the carbonyl of SoxY Leu17 serves to restrain the conformation of the SoxY C-terminal peptide (Fig. 2C). Nevertheless, the atomic temperature factors for the modeled portion of the SoxY C-terminal peptide are higher than the molecular average suggesting it to be a relatively mobile part of the structure (supplemental Fig. S1). The carrier cysteine side chain (Cys110Y) is located at the entrance to a deep pocket generated by the packing of the SoxY and SoxZ subunits. This pocket is the only one found in the vicinity of the GCGGG peptide and is the largest detected in the protein. It is roughly 10 Å deep and 6 Å wide at its mouth, tapering slightly toward the bottom and has a volume of 146 Å³. The inner surface of the pocket is formed by the side chains of residues contributed by both subunits. Non-polar atoms predominate, contributing around 66% of the 166 Å² solvent accessible surface of its lining. The residues making major contributions to the surface of the pocket are Ser32, Gln33, Pro67, and Ala68 from SoxZ; and Asn54, Pro55, Glu56, Arg58, and Ala80 from SoxY. Only one of these, Asn54 of SoxY, is invariant (Fig. 1, B and C). The side chain of this residue hydrogen bonds to the main chain of Arg76 and Ala80 of SoxY and forms one side of the pocket. Located within the pocket are two molecules of the cryoprotectant (discussed above) as well as three water molecules that are bound tightly at the bottom of the pocket and have temperature factors that are comparable with protein main chain atoms in their vicinity.
the SoxY cysteine residue. This was verified in the x-ray crystal structure of this protein refined at 1.98-Å resolution where the cysteine residue in each molecular copy of SoxY was found modified to S-(2-hydroxyethylthio)cysteine. As expected, no modification was apparent to the single, solvent-inaccessible cysteine residue found in SoxZ. The crystallographic unit cell...
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was essentially isomorphous with that of the native protein and, apart from the residues of the SoxY C-terminal peptide, no significant differences in conformation to that of the native protein structure were evident. The S-(2-hydroxyethylthio) group has some physical similarity to polysulfide chains in that both form short, linear, hydrophobic chains with a polar terminus. As such, the structure of the β-mercaptoethanol derivative of SoxYZ may provide insight into the way that the protein carries labile polysulfonated intermediates of the Sox pathway.

In contrast to the situation observed for the native protein, the electron density for the β-mercaptoethanol-modified SoxY C-terminal GGCGG peptide was structured beyond the cysteine residue and for one copy of the heterodimer a structural model for the complete peptide could be built (Fig. 3A and B). In each case, the average atomic temperature factor for the modified cysteine residue is comparable with that of other protein atoms in its vicinity. Strikingly, the modified SoxY S-(2-hydroxyethylthio)cysteine side chain penetrates deep into the large surface pocket identified in the structure of the native protein (Fig. 3C) displacing the molecule of cryoprotectant. Most of the interactions of the β-mercaptoethanol moiety with pocket residues involve non-polar contacts. These occur with the apolar parts of the side chains of Gln34 and Ala48 in SoxZ and with Asn54, Pro55, and Arg78 in SoxY. The hydroxyl group at the end of the derivatized side chain makes a single hydrogen bond with the carbonyl oxygen of SoxY Glu56 (Fig. 3B). The three ordered water molecules observed in the pocket in the structure of the native protein are conserved but do not form hydrogen bonding interactions with the side chain adduct. No further water molecules are found in the vicinity. Outside the pocket only three direct interactions with the protein serve to stabilize the conformation of the main chain of the GGCGG peptide. These are hydrogen bonds between the α-nitrogen of Gly109 and the carbonyl of SoxY Leu79 (as in the native structure), between the carbonyl of Cys110 and the side chain of SoxZ Ser32, and between the SoxY C-terminal (Gly111) carboxyl group and the α-nitrogen atom of SoxZ Lys48 (Fig. 3B). These interactions are presumed to be important to help stabilize the conformation of the SoxY C-terminal peptide in this modified form. It is noteworthy that the only interaction involving an amino acid side chain is with the semiconserved SoxZ residue Ser32 (Figs. 1C and 3B).

To test the hypothesis that the pocket is of the size, shape, and character necessary to bind cysteine residues bearing pathway intermediates, in silico models of SoxYZ complexes carrying various putative intermediates were generated based on the crystal structure of the β-mercaptoethanol adduct. The largest entity that could be accommodated in the pocket was that of cysteine-S-trisulfide. We attempted to identify similar surface pockets in other protein structures using pVS0AR (25) but no significant hits were found. In this context it is also notable that there is no symmetry-related pocket on the opposite face of the SoxYZ β-sandwich, nor is there a substantive pocket in P. furiosus superoxide reductase, which has similar subunit packing to SoxYZ (above). We used homology modeling methods and our structure of the native P. pantotrophus protein to build models of SoxYZ from the various representative sulfur bacteria whose sequences are shown in Fig. 1. Analysis of the resulting models revealed that the pocket does not change significantly in volume or character among related proteins (see supplemental Table S2) adding indirect evidence for a functional role for this structural feature.

Crystal Structure of a Sulfonated SoxYZ Pathway Intermediate—To further investigate how pathway intermediates interact with the SoxYZ complex we used treatment with tetra-thionate to generate the intermediate in which the carrier cysteine is S-sulfonated (Fig. 1A). Mass spectrometric analysis confirmed that the modification, which was again reversible by treatment with DTT, was restricted to the carrier cysteine in the SoxY subunit (supplemental Fig. S6). A change of space group from that of the native protein occurred on crystallization (Table 1). The x-ray crystal structure of the modified protein was solved by molecular replacement using the native protein structure and refined at 2.35-Å resolution. Inspection of the electron density maps revealed that the cysteine residue in each SoxY subunit was indeed modified to the cysteine-S-sulfonate derivative. Again, no modification was apparent to the single, solvent-inaccessible SoxZ cysteine residue.

No major changes in overall protein structure relative to that of native SoxYZ are observed. However, the four copies of SoxYZ in the asymmetric unit in the P1 cell show two sets of two distinctly different arrangements of the SoxY C-terminal polypeptide, demonstrating the intrinsic flexibility of this region (Fig. 3D). In the first of these, the S-sulfonate group lies adjacent to the mouth of the presumptive binding pocket (Fig. 3E). In this conformer, as was the case for the native protein structure, no significant electron density was apparent for the SoxY C-terminal diglycine dipeptide. The sulfonate group now makes the side chain hydrogen bonding contact with SoxZ Ser32 that stabilized the C-terminal diglycine dipeptide in the structure of the β-mercaptoethanol derivative. The three well ordered water molecules are conserved in the pocket, as is the ethylene glycol cryoprotectant molecule observed in the native protein structure. In the second conformer, a rearrangement of the SoxY C-terminal peptide has occurred in which residues 107 and 108 now form a 2-residue bulge in the βB strand (Fig. 3F). The residues of the extreme C-terminal Gly111-Gly112 dipeptide now occupy the position where the preceding Gly109-Cys110 dipeptide was located in the native structure maintaining a regular pattern of hydrogen bonding interactions with the latter part of the βA strand. A hydrogen bond between the carbonyl of residue SoxY Thr106 and the amino group of residue Gly109 stabilizes the bulge structure. This conformation is also stabilized by interaction of the S-sulfonate group with the amide nitrogen atom of Gly108 and through a non-bonded contact with a neighboring SoxYZ protein in the crystal lattice. The conformation of the C-terminal peptide thus appears sensitive to crystal lattice packing forces. The observation of multiple conformers for the SoxY C-terminal carrier peptide in this structure suggests that the SoxYZ complex does not provide a specific binding site for this pathway intermediate. It appears that S-sulfonate cysteine is sufficiently stable that it does not need to be protected by the protein.
FIGURE 3. Crystal structures of SoxYZ modified at the carrier cysteine residue. A, overall architecture of the SoxYZ-β-mercaptoethanol adduct. Residues of the C-terminal pentapeptide of SoxY are shown in ball and stick representation. Structural elements are colored as in Fig. 2A. B, the environment of the SoxY C-terminal peptide in the SoxYZ-β-mercaptoethanol adduct. The 2Fo – Fc electron density map around SoxY cysteine 110 is shown at 1.98 Å resolution and contoured at the 1.0σ level. The three ordered water molecules (W1–W3) seen in the large pocket identified in the structure of the native protein are conserved. Hydrogen bonds are indicated by dashed lines. C, a close up of the electrostatic potential at the solvent-accessible molecular surface in the vicinity of the SoxY C-terminal peptide in the SoxYZ-β-mercaptoethanol adduct (note that the surface displayed excludes that for the SoxY terminal pentapeptide). Color coding is as follows: red, acidic; white, neutral; blue, basic. D, a superposition of the two observed conformations of the SoxY C-terminal peptide observed in the crystallographic asymmetric unit of the SoxY cysteine-S-sulfonate pathway intermediate. The S-sulfonated side chains of SoxY Cys110 are shown in ball and stick representation. In the first arrangement (yellow, labeled 1), the sulfonate group is poised at the lip of the interface pocket. The two extreme C-terminal glycine residues are not evident in the electron density for this arrangement. In the alternative conformation (blue, labeled 2), the flexible C-terminal polypeptide has rearranged to generate a two-residue bulge and the sulfonate group interacts with the amide nitrogen atom of Gly108Y. In this conformation, the strands βA and βB are absent but the complete SoxY C-terminal pentapeptide is ordered. E, a view of the 2Fo – Fc electron density map contoured at the 1.0σ level in the vicinity of the S-sulfonate group lying in the first arrangement. An ethylene glycol molecule occupies a similar position in the pocket to that observed in the structure of the native protein. F, the second arrangement of the cysteine-S-sulfonate group. This conformation is stabilized by an intramolecular hydrogen bond generating a two-residue bulge and by an intermolecular non-bonded interaction between the Cys110 side chain and residue Ala15 of a lattice neighbor copy of SoxY (shown in light blue). There is also a hydrogen bond between the carbonyl of Val105Y and the amino group of the same lattice neighbor Ala15Y residue (not shown). The residues forming the pocket rearrange slightly due to a small shift in the Z-loop and the pocket increases in volume.
DISCUSSION

The multiple structures of SoxYZ reported here reveal that the carrier cysteine residue is located on a swinging arm at the C terminus of the SoxY subunit. The flexibility of this arm is attested by the high crystallographic B-factors (supplemental Fig. S1) and the weakness of the electron density for the residues involved, as well as by the observation that the arm adopts different conformers both in different molecules of the crystallographic unit and when the carrier cysteine is conjugated to different molecules (Fig. 4A). Locating the carrier cysteine on a swinging arm allows delivery of the Sox pathway intermediates into the active sites of the multiple, structurally unrelated partner enzymes that include SoxAX, SoxB, and SoxCD. Locating the mobile peptide at the C terminus of SoxY rather than in an internal loop reduces the volume of polypeptide that would have to be inserted into the active sites of the partner enzymes. Residues of SoxY from Gly108 onwards protrude from the end of the ββ strand and are potentially part of the swinging arm. Moreover, the native and S-sulfonate pathway intermediate structures described in this paper clearly show that the βA–βB interaction is susceptible to disruption. It is reasonable to suppose that the βB strand can be similarly unwound when SoxYZ is in complex with its partner proteins and that the full swinging arm may thus constitute the eight residues from Val106 onwards. The crystal structure of P. pantotrophus SoxAX (8) reveals a tapering active site access channel roughly 10 Å deep and 12 Å wide at its mouth. Such a distance should be readily traversed by a swinging arm of this size.

The carrier cysteine of the swinging arm is bracketed by two pairs of glycine residues with the last of these glycine residues being the C-terminal amino acid of SoxY. The terminal glycine residue is absent in the SoxY proteins of a few organisms, but otherwise the glycine residues that surround the carrier cysteine are invariant and presumably essential (Fig. 1B). What is the function of this unusual conserved sequence? Glycine residues lack a side chain. Therefore, the C-terminal pentapeptide of SoxY will experience minimal steric clashes or bonding interactions when it is inserted into the active sites of the partner enzymes. The carrier cysteine is, thus, positioned in the most neutral amino acid context possible. The fact that glycine lacks a side chain also allows the polypeptide backbone a greater degree of conformational flexibility than any other amino acid. We suggest that each glycine dipeptide has the properties of a molecular universal joint. This first glycine pair would, thus, allow the sulfur intermediate attached to the carrier cysteine residue to adopt diverse conformations relative to SoxY. This conformational flexibility would enable the sulfur species to adopt different positions required for catalysis in the active site of each of the partner enzymes. What is the function of the second glycine pair? If these residues were not present the carrier cysteine would be the last amino acid of SoxY and would possess a main chain carboxylic acid group. There would be the danger that the charged terminal carboxylate would engage in strong bonding interactions in the active sites of the partner proteins thereby interfering with the correct positioning of the pathway intermediate bound to the cysteine side chain. Indeed, the SoxA active site is known to contain multiple conserved basic residues (7, 8) that would interact strongly with a carboxyl group. Similarly the SoxB active site appears to use positive charges in binding the substrate sulfonate group.9 We propose that SoxY uses the second glycine dipeptide primarily as a spacer so that the peptide C terminus does not interfere with catalysis. There is also the intriguing possibility that the dipeptide acts as a second universal joint to allow correct positioning of the cysteine conjugate should the terminal carboxylate become immobilized. In summary, it appears that SoxY has found an optimal engineering solution to the need to correctly position pathway intermediates in the anion-selective active sites of multiple, structurally diverse partner enzymes. This strategy, in which SoxY is designed to be a universal partner, would be an advantage during the evolution of sulfur oxidation pathways because it minimizes the structural changes necessary for an enzyme to successfully interact with the intermediate-carrying protein.

Two classes of proteins that are structurally distinct from SoxYZ and that may carry sulfur intermediates on an external mobile arm have been characterized. Proteins of the DsrC family have a flexible C-terminal 7-amino acid tail in which an invariant cysteine is the penultimate residue (26). Some homologues have a single glycine residue directly before the carrier cysteine but otherwise glycine is not found in the tail. DsrC proteins are associated with the sulfite reductases of both sulfur oxidation and sulfate reduction pathways (27, 28). Another member of the family, TusE, has recently been shown to play a role in the biosynthesis of thiouridine at tRNA wobble positions with in vitro studies suggesting that the tail cysteine of TusE carries a sulfane group (29). The second distinct structural class of proteins that carry sulfur species on an external arm includes the MoaD protein involved in molybdenum cofactor biosynthesis (30) and the ThiS protein that participates in thiamine biosynthesis (31). These proteins have a 6-amino acid tail at the C terminus of the protein. The tail carries a sulfur atom by conjugation to the C-terminal carboxylic acid group. In a possible mechanistic parallel to SoxY the C-terminal amino acids of MoaD and ThiS are a pair of glycine residues. Certainly the tail of the MoaD protein adopts very different conformations when inserted into the active sites of different partner enzymes (32) and the terminal glycine pair has been shown to move during the reaction cycle at one active site (30).

It is clear that the SoxY swinging arm allows captured sulfur intermediates significant conformational mobility. However, this still leaves the question of why the intermediates are linked to a carrier protein in the first place. In other words what is the purpose of the SoxYZ protein? A possibility is that formation of an adduct between the sulfur substrate and a cysteine is a mechanistic necessity for the Sox reactions. We suggest that conjugation of thiosulfate to cysteine is required to activate thiosulfate for hydrolytic cleavage by SoxB. Isolated thiosulfate is stable to hydrolysis to sulfide and sulfate even though the reaction is exergonic under biological conditions (33). The reason for this behavior is that breaking the sulfur-sulfur bond

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5 V. Sauvê, S. M. Lea, and B. C. Berks, unpublished data.
would require electron movement onto the already negatively charged sulfane sulfur atom. By contrast, when thiosulfate is conjugated to the SoxY carrier cysteine the inner sulfur atom is neutral, rather than negatively charged, and the developing negative charge on this atom during bond cleavage is stabilized by the electron withdrawing \( \gamma \) sulfur atom of the carrier cysteine. These changes make the sulfone a good leaving group and hydrolysis of the thiosulfate moiety becomes straightforward.

A second possible role for SoxYZ is that of protecting (chaperoning) labile intermediates until they can be processed by the next enzyme in the pathway. Particularly vulnerable are intermediates containing reactive sulfane or zero-valent sulfur atoms (34, 35). We suggest that the interface pocket could bind and protect persulfurated and polysulfanated SoxY C-terminal peptides. This proposal is supported by the observation that the \( S-(2\text{-hydroxyethylthio}) \) cysteine adduct, which arguably has some of the features of a \( S \)-polysulfanated cysteine intermediate, is located in this pocket (Fig. 3B). The pocket is missing in structurally similar dimers such as the \( P.\) furiosus superoxide dismutase and these lack a Z-loop. The generally apolar nature of the residues lining the pocket provides a hydrophobic environment that would exclude adventitious polar reactants present in the surrounding environment and that would preferentially stabilize (poly)sulfanated cysteine residues bound therein in the less reactive neutral thiol form (36). Sequestration in this pocket would provide a mechanism for the stabilization of polysulfide and sulfane SoxY pathway intermediates that is not obviously the same as that found in other sulfur carrier proteins and sulfur transferases such as rhodanese (37), the Sud protein from \( W.\) succinogenes (38), and the sulfur oxygenase reductase (39).

The paradigm swinging arms are those found in multifunctional enzymes and multienzyme complexes, such as the 2-oxo acid dehydrogenases, where it is considered that the arm allows substrate channeling (see, for example, Ref. 40). While there is no evidence that SoxYZ ever forms a tight complex with other Sox components, it is still possible that linking pathway intermediates to a carrier protein enhances Sox reactions through more transient protein-protein interactions between SoxYZ and its partner enzymes. Which regions of the SoxYZ molecule might be involved in interactions with Sox enzymes? Analysis of invariant and the semi-conserved residues contributing to the heterodimer molecular surface reveals two conserved surface patches (Fig. 4B). These lie in the vicinity of the SoxY swinging arm and at the base and other portions of the Z-loop that are adjacent to SoxY suggesting that this region may act as a common adaptor for recognition of Sox enzymes. If this is the

FIGURE 4. Conformational flexibility and surface conservation in SoxYZ.
A, a superposition of the 12 molecules of SoxYZ in the asymmetric units of the native and derivatized proteins reported in this study. The conformationally variable regions correspond to the most flexible regions seen in supplemental Fig. S1 (i.e. those with highest temperature factors), namely the SoxY \( \alpha 1-\beta 1 \) loop and the Z-loop (both light blue) and the SoxY C-terminal octapeptide (magenta). The SoxYZ complex is displayed in a similar orientation to that in Fig. 2A. B, three views of a ConSurf (41) color-coded surface representation of SoxYZ showing the invariant and the semi-conserved residues as shown in Fig. 1A. B and C. The normalized conservation scores calculated by ConSurf are a relative measure of evolutionary conservation at each residue position. The highest scores (8 and 9 on the ConSurf scale) represent the most conserved residue positions and are shown colored blue for SoxY and red for SoxZ. The surface displayed excludes that for the SoxY terminal pentapeptide that is shown in stick representation.
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case, the nature of the modification at the carrier cysteine residue may itself act as the specificity determinant for the recognition process. The Z-loop could have the function of increasing the contact area between SoxYZ and its partner proteins. The length and flexibility of the loop might enable it to pack onto partners of different structure.

In summary, the structure of SoxYZ provides insights into the adaptation of a simple, commonly observed protein fold for the purpose of generating a novel class of sulfur carrier proteins and helps explain the unique and pivotal role of SoxYZ in the oxidation of inorganic sulfur compounds.

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