Structural Basis for the Oxidation of Protein-bound Sulfur by the Sulfur Cycle Molybdoenzyme-Enzyme Sulfane Dehydrogenase SoxCD*§

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The sulfur cycle enzyme sulfane dehydrogenase SoxCD is an essential component of the sulfur oxidation (Sox) enzyme system of Paracoccus pantotrophus. SoxCD catalyzes a six-electron oxidation reaction within the Sox cycle. SoxCD is an α2β2 heterotetrameric complex of the molybdenum cofactor-containing SoxC protein and the diheme c-type cytochrome SoxD with the heme domains D1 and D2. SoxCD1, the heme-2 domain D2 and is catalytically as active as SoxCD. The crystal structure of SoxCD1 was solved at 1.33 Å. The substrate of SoxCD is the outer (sulfane) sulfur of Cys-110 persulfide located at the C-terminal peptide swinging arm of SoxY of the SoxYZ carrier complex. The SoxCD substrate funnel toward the molybdopterin is narrow and partially shielded by side-chain residues of SoxD. For access of the sulfane-sulfur of SoxY-Cys-110 persulfide we propose that (i) the blockage by SoxD-Arg-98 is opened via interaction with the C terminus of SoxY and (ii) the C-terminal peptide VTIGGCGG of SoxY provides interactions with the entrance path such that the cysteine-bound persulfide is optimally positioned near the molybdenum atom. The subsequent oxidation reactions of the sulfane-sulfur are initiated by the nucleophilic attack of the persulfide anion on the molybdenum atom that is, in turn, reduced. The close proximity of heme-1 to the molybdopterin allows easy acceptance of the electrons. Because SoxYZ, SoxXA, and SoxB are already structurally characterized, with SoxCD, the structures of all key enzymes of the Sox cycle are known with atomic resolution.

Reduced inorganic sulfur compounds like hydrogen sulfide, sulfur, or thiosulfate are attractive prokaryotic energy sources, and their oxidation to sulfuric acid is one of the major reactions of the global sulfur cycle as shown for thiosulfate (Equation 1).

\[
\text{S-SO}_3^- + 5 \text{H}_2\text{O} \rightarrow 2 \text{SO}_4^{2-} + 8 \text{e}^- + 10 \text{H}^+ \quad \text{(Eq. 1)}
\]

Oxidation of inorganic sulfur compounds to sulfate is mainly mediated by various specialized aerobic chemotrophic and anaerobic phototrophic prokaryotes, bacteria and archaea (1–3). Two different modes for bacteria have been proposed recently; one as present in e.g. the anaerobic phototrophic sulfur oxidizing bacterium Allochromatium vinosum involves the reverse acting dissimilatory sulfate dehydrogenase (DsrAB) (Equation 2) which is with 13 other proteins encoded by the dsr operon (4). The product sulfite is subsequently oxidized to sulfate by adenosine 5’-phosphosulfate reductase or sulfite:acceptor oxidoreductase (5).

\[
\text{HS}^- + 3 \text{H}_2\text{O} \rightarrow \text{HSO}_3^- + 6 \text{e}^- + 6 \text{H}^+ \quad \text{(Eq. 2)}
\]

The other mode as present in e.g. the aerobic facultative chemotrophic bacterium Paracoccus pantotrophus (6) involves sulfane dehydrogenase SoxCD, which is together with 14 other proteins encoded by the sox operon in this strain. SoxCD is an α2β2 heterotetrameric complex of the molybdoprotein SoxC and the hybrid di-heme cytochrome c like protein SoxD. This sulfane dehydrogenase (formerly designated sulfuran dehydrogenase (7)) is a key enzyme of the sulfur-oxidizing (Sox)3 enzyme system and catalyzes the oxidation of protein-bound sulfane-sulfur (oxidation state −1) to sulfone (oxidation state +5) in a six-electron transfer reaction (8, 9) (Equation 3).

\[
\text{SoxZY-S}^- + 3 \text{H}_2\text{O} + 6 \text{Cyt c}_{\text{ox}} \rightarrow \text{SoxZY-SO}_3^- + 6 \text{Cyt c}_{\text{red}} + 6 \text{H}^+ \quad \text{(Eq. 3)}
\]

The current model of the Sox reaction cycle involves sequential activity of four different periplasmic proteins SoxXA, SoxB, SoxYZ, and SoxCD (Fig. 1) (2, 9). The four proteins oxi-

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** The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Fig. 1.

The atomic coordinates and structure factors (code 2XTS) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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complete the cycle. Besides thiosulfate, the Sox enzyme system also oxidizes hydrogen sulfide, sulfur, and sulfite. With thiosulfate the core system SoxYZ, SoxXA, and SoxB, i.e. the system without SoxCD, yields just 2 mol of electrons/mol of sulfur substrate (14). Also, the core system without SoxCD oxidizes thiosulfate at 25% the rate as compared with SoxCD. This material balance identified (i) the turnover number of the Sox system to be identical with and without SoxCD, and (ii) the molybdoprotein-cytochrome c complex SoxCD catalyzes a unique six-electron transfer reaction (Equation 2).

Six electron-transfer reactions are rare in nature. The few examples are the reverse-acting sulfite reductase RdsrAB containing various [4Fe4S] centers (15) and the pentaheme cytochrome c nitrite reductase (16).

The core Sox system without SoxCD oxidizes sulfite, and this is the first example for a molybdoprotein-free sulfite oxidation (8). Thus, SoxCD is catalytically distinct from sulfite dehydrogenases. However, the 26.5% identity with respect to the primary sequence of SoxC to the molybdoprotein SorA of the sulfite dehydrogenase SorAB of Starkeya novella (9) suggested a relation to sulfite dehydrogenase. Although the heme-1 domain of SoxD is conserved, the heme-2 domain is missing in some chemo- and phototrophic bacteria. Deletion of the heme-2 domain leads to a SoxCDS, which is identically active in the in vitro assay as SoxCD (7).

The proteins of the core Sox system from various sources have been structurally characterized earlier; SoxAX of the phototroph Rhodovulum sulfidophilum (10), SoxXA and SoxYZ of P. pantotrophus (11, 12), and a SoxB homologue from Thermus aquaticus (13). The structure of SoxCD presented here delivers the missing link for a structural interpretation of the complete enzymatic reaction cycle. The structure of the sulfite dehydrogenase SorAB from S. novella (17) and associated kinetic analysis (18–21) provided the first insight into the molecular mechanism of the intramolecular electron transfer between the molybdenum and the heme prosthetic group of the bound c-type cytochrome subunit upon oxidation of a sulfur compound. Comparison of the SoxCD, structure with SorAB and further related enzymes like chicken sulfite oxidase CSO (22) highlights structural similarities as these molybdooenzymes have in common their catalysis of hydroxylation reactions with a sulfur atom as substrate coupled with dehydrogenation. SoxCD, however, differs significantly from these enzymes as (i) SoxCD performs a six-electron transfer reaction instead of a two-electron transfer reaction, and (ii) its sulfur substrate is covalently bound to a sulfur carrier protein (SoxYZ).

The structure enables further insight into the common features and determinants for the electron transfer between the molybdenum and heme prosthetic group. Because a six-electron transfer of a molybdooenzyme within one enzymatic conversion is so far unique, SoxCD will be of broader interest for enzymology and protein chemistry.

**EXPERIMENTAL PROCEDURES**

**Production and Purification of SoxCD**—SoxCD from P. pantotrophus is a complex of the molybdenum containing protein SoxC, and the di-heme c-type cytochrome protein
SoxD. SoxCD, is the construct in which the second heme-2 domain of SoxD is deleted (7). The SoxCD complex was purified from P. pantotrophus strain GB17 as described previously (2, 23). SoxCD was enriched from the cell extract by differential centrifugation and ammonium sulfate fractionation. Soluble proteins precipitated between 44 and 65% ammonium sulfate saturation were redissolved and purified to homogeneity using anion exchange chromatography on Q-Sepharose, gel filtration on Sephadex G-200, and anion exchange on Resource Q. The activity of the SoxCD complex was examined from the reconstituted thiosulfate-oxidizing enzyme system using horse heart cytochrome c as final electron acceptor as described before (23).

Crystallization—Crystallization experiments were carried out using the hanging-drop method at room temperature. SoxCD crystals were prepared by mixing equal amounts of protein- and reservoir solution (4% (w/v) MPEG 5000, 40 mM Tris-HCl (pH 8), 20 mM calcium acetate, and 10 mM Co(III)-hexamine chloride), and the drops were equilibrated against the reservoir solution. Rod-shaped crystals appeared within 5–10 days and diffracted x-rays beyond 1.5 Å resolution. The crystals belonged to a trigonal space group with cell dimensions a = 123 Å and c = 76 Å. Before data collection the crystals were cryo-protected in a solution containing 4% (w/v) MPEG 5000, 40 mM Tris-HCl (pH 8), 20 mM calcium acetate, 10 mM Co(III)-hexamine chloride, and 10% (v/v) glycerol and flash-cooled in liquid nitrogen.

Data Collection and SAD Phasing—X-ray data were collected on beamline ID23-1 (24) at the European Synchrotron Radiation Facility, Grenoble, France. To obtain experimental phases, a data set was collected at the K-edge of iron. The data set was indexed, integrated, and scaled using the XDS package (25). The structure was solved using the SAD protocol of Auto-Rickshaw, the EMBL-Hamburg automated crystal structure determination platform (26). The input diffraction data were prepared and converted for use in Auto-Rickshaw using programs of the CCP4 suite (27). Structure factors were calculated using the program SHELXC (28). Initial phases were calculated using the program SHELXC (28). Based on an initial analysis of the data, the maximum resolution for substructure determination and initial phase calculation was set to 3.2 Å. All of the heavy atoms requested were found using the program SHELXD (29). The correct enantiomorph space group for the substructure was determined using the programs ABS (30) and SHELXE (31). Initial phases were calculated after density modification using the program SHELXE (31). The 2-fold non-crystallographic symmetry operator was found using the program RESOLVE (32). Density modification, phase extension to 2.4 Å, and non-crystallographic symmetry averaging was performed using the program DM (33). At this stage, around one-third of the protein complex was built using the program ARP/wARP (34, 35). This model together with sequence information was used as a starting model for the subsequent MRSA (SAD combined with molecular replacement) protocol of Auto-Rickshaw (36). The resulting electron density map was improved further by density modification using DM (33). The resulting phases together with the SoxCd sequence were used as input for model building with PHENIX autobuild (37). Before further refinement with REFMAC5 (38), the water molecules were manually removed from the Phenix-generated autobuild model. This resulted in the first SoxCD model at 2.4 Å. A high resolution data set was collected from a second crystal up to a resolution of 1.33 Å. After integration using XDS, the data were scaled by XSDELC and converted into CCP4 format using XDSCONV keeping the same reflections marked for Rmerge assignment as in the SAD dataset. The amplitudes from the high resolution data set and phases calculated from the 2.4 Å model were used as input for ARP/wARP (35), also providing the SoxCD sequence. The resulting model was examined, coatomers were built, and water molecules were added using Coot (39) followed by refinement using REFMAC5 until no further interpretable features in the electron density map were visible. The refinement statistics are summarized in Table 1. Final model analysis, imaging, and ray tracing were performed using the program PyMOL (40). The search for structurally related proteins in the protein data bank (PDB) (41) was performed using the DALI server (42). The peptide model was manually built using PyMOL with structure idealization performed with REFMAC5.

### Table 1

| Statistics for data collection, processing, and structure refinement |
|---------------------------------------------------------------|
| **Data collection and processing**                             |
| Data set           | SAD-peak          | High resolution |
| X-ray source       | ID23-1 (ESRF, Grenoble, France) |
| Detector           | ADXQ quantum X31e |
| Wavelength (Å)     | 1.74              | 0.98           |
| Temperature (K)    | 100 K             | 100 K          |
| Crystal-to-detector distance (mm) | 180              | 180           |
| Oscillation range (degree) | 1.0              | 0.5           |
| Total oscillation range (degree) | 360              | 60           |
| Space group        | P3(1)             | P3(1)          |
| Cell dimensions (Å) | a = 123.19, c = 76.42 | a = 122.97, c = 76.39 |
| Resolution limit (Å) | 50-2.37 (3.0-2.37) | 50-1.33 (1.4-1.33) |
| Completeness (%)   | 92.4 (88.2)       | 92.7 (79.2)    |
| No. observations (overall / unique) | 550,606/48,615 | 507,433/273,185 |
| Average redundancy | 11.3 (11.2)       | 11.8 (2.6)     |
| (I/σ(I))| 31.4 (19.5)       | 11.8 (2.6)     |
| Rmerge | 2.0 (5.9)         | 4.1 (25.2)     |
| B-factor from Wilson plot (Å²) | 27.0             | 9.7           |

**Refinement statistics**

| Resolution limit (Å) | 50.33          |
| Number of unique reflections | 273,185 |
| Completeness of data (%) | 96.8 |
| Rmerge (%) | 9.8 |
| Rfree (%) | 11.2 |
| No. of non-H atoms | 10,432 |
| Protein | 9,473 |
| Solvent | 959 |
| Ramachandran plot (%) | 97.1/2.8/0.1 |
| Coordinate error | 0.169 |
| r.m.s.d. from ideal values | |
| Bond lengths (Å) | 0.028 |
| Bond angles (degree) | 2.368 |
| Mean B-factor (Å²) | 10.7/13.2/10.9/12.8 |
| Protein chain A/B/C/D | |
| Backbone | 9.5/12.0/9.4/11.8 |
| Side chain | 11.8/14.5/11.6/13.9 |
| Solvent | 19.6 |
| PDB entry code | 2X7S |
RESULTS

Overall Structure—The structure represents the SoxCD₁ construct in which the heme-2 domain has been deleted on the cDNA level, as it was observed that only the first heme domain is essential for sulfur oxidation activity (7). As determined by analytical size exclusion chromatography and multiple angle laser light scattering, SoxCD₁ forms a heterotetrameric complex of around 130 kDa (2 × 43.9-kDa SoxC and 2 × 24.1-kDa SoxD₁) (data not shown). The crystal structure of SoxCD₁ resembles a tight α₂β₂ complex with internal 2-fold symmetry. It is composed of two molybdenum-containing SoxC domains forming the core of the complex and...
two heme-containing SoxD\(_1\) domains sitting at the exterior of the complex (Fig. 2). The two SoxD\(_1\) subunits are positioned at the edge of the SoxC dimer and do not contact each other. The C-terminal peptide of the SoxD\(_1\) subunit displays an extended loop with two short \(\alpha\)-helical and one short \(\beta\)-stranded conformation that embraces its closest SoxC subunit. SoxD\(_1\) represents a cytochrome-c-like-fold with 8 helices and 5 strands. SoxC contains 6 \(\alpha\)-helices and 23 \(\beta\)-strands organized in five \(\beta\)-sheets with the molybdenum binding site in the center and a C-terminal immunoglobulin-like fold. The two SoxC subunits of the tetramer form a large interior interface (3939.3 Å\(^2\) interface area, determined using SURFNET (43)) in a head-to-tail arrangement. The N-terminal part of SoxC (residues 42 to 75) builds additional contacts with its non-crystallographic symmetry mate (Fig. 2).

**Molybdenum Binding Site**—Due to the high resolution of the final electron density map, the type of the molybdenum-containing prosthetic group (Fig. 3A) could be unambiguously identified as the sulfite oxidase-type molybdenum cofactor (44, 45) as also reported for chicken sulfite oxidase CSO (22), the sulfur dehydrogenase SorAB from *S. novella* (17), the sulfite oxidase from *Arabidopsis thaliana* (46), and the sulfite oxidase from *Escherichia coli* (47) (Fig. 4A). As proposed in the literature (45), the molybdenum-containing prosthetic group will be subsequently referred to as molybdenum cofactor. Molybdenum cofactor binding domains are highly similar in their central architecture and form a distinct family (48). Eight residues are highly conserved and function as a coordination sphere (Fig. 5 and supplemental Table 1). Among these eight residues, one cysteine residue (Cys-160 in SoxC) is found in all molybdenum cofactor-containing structures as one ligand for the molybdenum ion (Figs. 4A and 5). The molybdenum ion in SoxC displays a distorted square pyramidal coordination geometry where two thiolates from the molybdopterin heterocycle, the thiol group from cysteine residue Cys-160, and one water/hydroxyl oxygen are the equatorial ligands, and the terminal oxo group occupies the axial position (Fig. 3A and 5). The C-terminal Arg-114 of SoxC is a highly conserved residue in the vicinity of the molybdenum cofactor (Fig. 4A). Arg-114 is proposed to coordinate and thereby to polarize a water molecule before the concerted transfer of an oxygen atom onto the sulfur atom (presented to the active site by the SoxYZ complex) and transfer of two electrons from the sulfur onto the molybdenum cofactor (20). This first oxidation step is completed by a stepwise transfer of the two electrons from the molybdenum cofactor to the heme group of

[FIGURE 3. Stereographic view of the cofactors of SoxCD, superimposed with the surrounding electron density distribution. The contour level of the \(2F_{\text{obs}} - F_{\text{calc}}\) electron density map is 3.0σ. Shown are molybdenum (A) and heme (B) cofactors with coordinating residues. The protein environment in panels A and B is displayed as tube representation in light gray.]

Crystal Structure of Sulfane Dehydrogenase SoxCD
Subsequently these electrons are transferred to the iron of an interacting cytochrome subunit. His-116 of SoxC is located between the molybdenum cofactor and heme-1 of SoxD1 and might be involved in the transfer of the electrons from the molybdenum to the heme iron (Fig. 6A).

**Heme-1 Site**—Heme-1 displays typical properties of c-type cytochromes (49); the porphyrin ring system is covalently attached to two cysteine residues (Cys-70 and Cys-73). Its central iron is axially coordinated by the SD sulfur of methionine (Met-121) and the NE2 nitrogen of histidine (His-74) (Fig. 6B).
Crystal Structure of Sulfane Dehydrogenase SoxCD

3B). Heme-1 of SoxD1 shows distinct deviation from ideal planarity (Fig. 3A and supplemental Fig. 1) and differs significantly from other high resolution heme c structures, e.g. the cytochrome c3 from Desulfovibrio vulgaris (50).

Structural Comparison of SoxCD1 Subunits —The closest structural homologues of SoxC are the central domains of (i) sulfite oxidase CSO from chicken liver (PDB entry 1SOX (22)), (ii) the molybdenum cofactor binding domain of maize nitrate reductase SorAB from S. novella (PDB entry 2BLF (17)), and (iii) the molybdenum cofactor binding domain of maize nitrate reductase (PDB entry 2BII (51)) (Fig. 4A). As cytochrome c-type proteins have a ubiquitous fold, a large number of homologous structures to SoxD1 is found within the protein data base with the closest homologues 1CED, a cytochrome c6 from Monoraphidium braunii (52), 2VO8, a cytochrome c6 from Phormidium laminosum (53), and 2ZBO, a cytochrome c6 from Hizikia fusiformis (Fig. 4B). Within the homologous structures, the heme binding domain SorB of chicken sulfite oxidase and of SorAB from S. novella are found as well. These two protein complexes, SoxC and SorAB, are the only two examples of structures known containing a molybdenum and a heme cofactor binding domain in different subunits. Although chicken liver sulfite oxidase CSO is a homodimeric enzyme with the molybdenum and heme cofactor binding domain within one protein chain, sulfite dehydrogenase SorAB is a heterodimer formed between the molybdenum cofactor binding protein SorA and the c-type cytochrome SorB.

Subunit Interaction —Whereas the molybdenum and heme cofactor containing subunits of SoxCD1 and SorAB individually align well to each other, the relative positions of the subunits in both heterodimers differ (Fig. 6C). This also leads to a different orientation of the two cofactors in each complex. Nevertheless, the position of the heme propionate at carbon atom C13 relative to the molybdenum cofactor appears to be almost identical in both structures (Fig. 6B). As the distances between the molybdenum and the heme cofactor are very similar in SoxCD1 (Fig. 6A) and SorAB (17), it is assumed that in both cases a physiologically active complex is formed. In contrast, in chicken sulfite oxidase CSO the heme iron is 32.3 Å away from the molybdenum and, thus, the structure is interpreted as an inactive complex (22).

DISCUSSION

Overall Structure —The SoxCD1 structure presented is based on a truncated version of the protein SoxCD in which the second heme-2 domain of SoxD was deleted on cDNA level. Functional SoxCD forms a heterotetramer in solution and in the crystal structure. Within the SoxCD2 tetramer the largest interface area is observed between two SoxC subunits (3939.3 Å2), whereas the interface between SoxC and SoxD is around 1910 Å2 (Fig. 2). Based on statistical analysis of known protein-protein complex structures, the interface area between subunits for stable complexes requires around 1500 Å2 and larger (54). In comparison, the interface between the molybdenum cofactor domains of the homologous chicken sulfite oxidase is only 1410.1 Å2. The heme-1 domain of SoxD1 contains a PCM2XDCI motif, which is present in many but not all sulfur-oxidizing bacteria. Interestingly, the two cysteine residues within this motif form the only disulfide bridge observed in the SoxCD2 protomer. Being around 50 Å away from the heme-1 iron of SoxD1, it is unlikely that the disulfide is involved in the reaction mechanism of the enzyme but might form an important interface for other associated proteins.
Cofactor Sites—The structures known so far of molybdenum-dependent sulfur-oxidizing enzymes harbor the same molybdenum cofactor assigned as the sulfite oxidase-type (44, 45). Based on the high resolution structure, the same type of cofactor, molybdopterin, was identified for the sulfane dehydrogenase SoxCD1. Heme-1 displays a nonplanar distortion induced through the cysteine thioether linkages between the porphyrin pyrrole groups and the polypeptide. A similar, however weaker distortion is reported for the yeast iso-1-cytochrome c (55). It is proposed that the redox potential of the heme group can be modulated by such geometric variation (55).

SoxYZ-bound Sulfur Compounds Are the Substrates for SoxCD—The reaction of SoxCD differs significantly from that of its close structural relative SorAB from S. novella. Whereas SorAB solely oxidizes sulfite to sulfate, SoxCD cannot oxidize free sulfite. To display catalytic activity, SoxCD requires the presentation of the sulfur compound covalently bound to SoxYZ (7). SoxYZ carries the intermediates of thiosulfate-oxidation on the Cys-110 of the C-terminal peptide “swinging arm” $^{105}$VTGCGG$^{112}$ of SoxY (12). Whereas the substrate for SoxCD, SoxZY-Cys-110-persulfide, and the final product, SoxZY-Cys-110-$S$-sulfonate, have been identified (7), the intermediates SoxZY-Cys-110-$S$-sulfenate and SoxZY-Cys-110-$S$-sulfinate (Fig. 1B) are hypothetical. The protein-bound substrate for SoxCD is a fundamental difference to SorAB and can be rationalized by analysis of the substrate binding pocket next to the molybdenum cofactor.

SoxCD Substrate Binding Pocket—The SoxCD1 structure exhibits a positively charged entrance path leading like a fun-
nel from the protein surface to the molybdenum atom (Fig. 7, A and B). According to the assumption that the molybdenum cofactor is the first electron acceptor and the interaction partner of the substrate, it is most likely that the positively charged pocket (Fig. 7, C and D) plays a key role in the reaction mechanism. The entrance funnel toward the molybdenum cofactor is formed in part by residues from SoxD. Interestingly, the entrance is partially closed by the side chain of

FIGURE 7. Substrate binding pocket. A–D, a representation of the calculated electrostatic surface potential is shown. A, shown is a side view of the heterotetrameric SoxCD. B, shown is a side view of the dimeric sulfite dehydrogenase SorAB from S. novella. C, a close-up view of the entrance path toward the molybdenum cofactor binding site in SoxCD shows the closing of the entrance path by SoxD-Arg-98. D, shown is close-up view of the entrance path toward the molybdenum cofactor within SorAB. The calculations of the electrostatic potential were performed with Delphi (64, 65). The molecular surface is color-ramped according to the electrostatic potential, with red indicating negative potential and blue indicating positive potential; fully saturated colors indicate a potential of $\pm 7 \text{kT}/e$ (assuming an ionic strength of 150 mM, a protein interior dielectric of 4.0, and a solvent dielectric of 80.0). The rendered surface representation was prepared with PyMOL (40). The displayed molecules for panel A and B are not on the same scale. The displayed entrance path in panel C and D are on the same scale. E, stereo representation of the putative substrate binding pocket of SoxCD is shown. The main-chain trace of the protein is displayed in ribbon representation in light gray. The molybdenum- and heme-cofactor are displayed as ball-and-stick with carbon atoms colored in dark gray. The peptide-109VTIGCGG-COOH, representing the C-terminal swinging arm of SoxY, modeled into the binding pocket is represented as ball-and-stick. Carbon atoms of the peptide and those of the amino acid residues of the protein are colored in green. Three-letter codes are used to identify amino acids. The peptide is superimposed with the surface representation of the binding cavity as determined with the program CAVER (66) and displayed in PyMOL (40). The cavity is displayed as transparent surface and colored in light blue.
SoxD$_1$-Arg-98. This putative binding pocket is large enough to accommodate the C-terminal swinging arm of SoxY with the cysteine side chain pointing toward the equatorial oxygen of the molybdenum cofactor (Fig. 7E). Modeling studies show that a C-terminal VTIGGCGG$^{112}$ peptide of *P. pantotrophus* SoxY with a cysteine-linked sulfane (SoxY-Cys-110-S\(^{-}\)),

FIGURE 8. Proposed reaction scheme for the oxidation steps of SoxCD. The initial step of the cycle is based on the modeled peptide-$^{105}$VTIGGCGG-COOH as shown in Fig. 7.
sulfenate (SoxY-Cys-110-SO\textsuperscript{2–}), sulfinate (SoxY-Cys-110-SO\textsubscript{3}\textsuperscript{2–}), and sulfonate (SoxY-Cys-110-SO\textsubscript{4}\textsuperscript{2–}) (Fig. 1) can easily be accommodated within the SoxC binding site next to the molybdenum cofactor (Fig. 7E). In the modeling studies the C-terminal peptide reaches into the active site such that the side chain of cysteine SoxY-Cys-110 is positioned close to the axial oxo-group of the molybdenum atom. Interestingly, the C terminus of the C-terminal peptide is positioned such that it can form an ion pair with the side chain of SoxD-Arg-98. A similar stabilization of the C terminus is discussed for the interaction of SoxB and SoxY (13). The side chain of SoxD-Arg-98 is stretched over the entrance path such that it might block smaller negatively charged molecules like sulfite or thiosulfate from access to the active site. It is conceivable that due to interaction with the substrate-loaded SoxYZ, this blockage is released, and the sulfur-compound can be positioned within the active site. This scenario might explain the fact that SoxCD can only catalyze the reaction of SoxY-Cys persulfide but not free sulfite or thiosulfate (Fig. 8). After three individual oxidation steps (transfer of each two electrons onto the molybdenum and one oxygen onto the sulfur), the final product SoxY-Cys-110-SO\textsubscript{3} is produced, and no further oxidation can take place. Due to its size, this final product does not fit well in the active site any longer, and the transient complex formed between SoxCD and SoxYZ is released as SoxZY-Cys-110-SO\textsubscript{3} to enable another round of reaction.

Reaction Mechanism of SoxCD—A reaction mechanism for bacterial sulfite oxidases has been proposed based on electron paramagnetic resonance spectroscopy (19, 57, 58). The initial step in this mechanism is the coordination of one oxygen atom of sulfite to molybdenum followed by a nucleophilic attack of the free electron pair of the sulfur onto the axial oxo-group. For the initial step of oxidation in SoxCD, the electrons from the terminal sulfur atom of the SoxY-Cys-110-persulfide anion have to perform the nucleophilic attack onto the molybdenum atom with subsequent transfer of one Mo=O oxygen onto the terminal sulfur atom. We propose that the swinging arm sequence \textsuperscript{105}VT1GGCGG-COOH serves to localize the sulfane-sulfur of the SoxY-Cys-110-persulfide in close proximity to the equatorial Mo=O oxygen (Fig. 8). For the second and third step of oxygen transfer, an intermediate cis-dioxomolybdenum(VI) (44) can be proposed such that the Mo=O–S linkage is not released by the attack of hydroxide from the solvent. The Mo=O–S linkage remains as long as further pairs of electrons can be transferred from the terminal sulfur atom onto the molybdenum atom. After transfer of two electrons monooxomolybdenum(IV), Mo(IV)O, is subsequently reoxidized to Mo(VI) by two one-electron transfer steps onto heme-1 coupled with deprotonation of a molybdenum-bound water molecule. After the addition of the third oxygen to the sulfur atom and electron transfer, the product SoxY-Cys-110-S-sulfonate is displaced by hydroxide, and the oxidized cofactor Mo(VI)O\textsubscript{2} is regenerated. As SoxY-Cys-110-S-thiosulfate cannot be further oxidized, it dissociates from the active site binding pocket. Regarding individual rate constants, it can be assumed that the transfer of electrons from the molybdenum cofactor to the heme cofactor, binding of hydroxide ion to the molybdenum ion and subsequent attack of the molybdenum bound oxygen by the electron pairs of the terminal sulfur atom is much faster compared with the release of the oxidized sulfur intermediate from the active site binding pocket. Thus, as long as the sulfur compound has free electrons on the terminal sulfur atom, the oxidation takes place, and only after the third and last possible transfer of an electron pair is the fully oxidized sulfur compound completely released.

The distance of the reaction centers in SoxCD is very similar to the distance in the active state of SorAB. Interestingly, the relative position of the molybdenum- and heme cofactor-harbor ing subunits differs between SoxCD and SorAB (Fig. 6C). The propionate moiety at the carbon atom C13 of the heme groups, however, are almost identically positioned (Fig. 6B). This suggests that the propionate facilitates the electron transfer between the molybdenum cofactor and the heme as it exhibits the closest distance between the two (Fig. 6). It has been shown previously that electrons can be tunneled in proteins between redox centers up to distances of 14 Å (59) at very fast rates. Between the molybdenum cofactor and the heme cofactor, the residue His-116 of SoxC is located with a distance of 5.6 Å to the molybdenum, 2.7 Å to O4 of the aromatic ring system of the molybdenum cofactor, and 3.2 Å to the heme propionate. These residues can serve as an alternative path for the electron transfer (Fig. 6).

In 1982 Kelly (60) stated, “The reactions about which least are known are those effecting thiosulfate cleavage and those converting sulfur to sulfite.” The reaction for thiosulfate cleavage has been proposed earlier (9, 13). Considering the oxidation reaction of sulfur to sulfite, the structure of the molybdenoprotein–cytochrome c complex SoxCD\textsubscript{1} provides detailed insight into the molecular basics for its unique six-electron transfer that requires the substrate to be bound covalently to its specific carrier protein. With SoxCD\textsubscript{1}, all enzyme structures of the core reaction cycle for sulfur oxidation are determined.

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Crystal Structure of Sulfane Dehydrogenase SoxCD
