Structural basis for the oxidation of thiosulfate by a sulfur cycle enzyme

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Reduced inorganic sulfur compounds are utilized by many bacteria as electron donors to photosynthetic or respiratory electron transport chains. This metabolism is a key component of the biogeochemical sulfur cycle. The SoxAX protein is a heterodimeric *c*-type cytochrome involved in thiosulfate oxidation. The crystal structures of SoxAX from the photosynthetic bacterium *Rhodovulum sulfidophilum* have been solved at 1.75 Å resolution in the oxidized state and at 1.5 Å resolution in the dithionite-reduced state, providing the first structural insights into the enzymatic oxidation of thiosulfate. The SoxAX active site contains a haem with unprecedented cysteine persulfide (cysteine sulfane) coordination. This unusual post-translational modification is also seen in sulfurtransferases such as rhodanese. Intriguingly, this enzyme shares further active site characteristics with SoxAX such as an adjacent conserved arginine residue and a strongly positive electrostatic potential. These similarities have allowed us to suggest a catalytic mechanism for enzymatic thiosulfate oxidation. The atomic coordinates and experimental structure factors have been deposited in the PDB with the accession codes 1H31, 1H32 and 1H33.

Keywords: cysteine persulfide haem ligand/SoxAX complex/sulfur cycle/thiosulfate oxidation/X-ray crystal structure

Introduction

Sulfide and thiosulfate are the most abundant reduced inorganic sulfur species in the environment and are converted to sulfate, primarily by bacterial action, in the oxidative half of the sulfur cycle. Photosynthetic sulfur bacteria use the sulfur compounds as a source of electrons for reductive carbon dioxide fixation during anoxic autotrophic growth (Brune, 1989), while chemolithoautotrophic sulfur bacteria use the electrons derived from oxidation of inorganic sulfur species both in carbon dioxide fixation and as respiratory electron donors (Wood, 1988). The Sox mechanism, also referred to as TOMES (thiosulfate-oxidizing multi-enzyme system), is the best

studied and most widely distributed pathway for the oxidation of thiosulfate to sulfate (Kelly *et al.*, 1997; Friedrich *et al.*, 2001). In some organisms, the Sox pathway is also employed for the oxidation of sulfide, sulfur, sulfite and tetrathionate (Mukhopadhyaya *et al.*, 2000; Appia-Ayme *et al.*, 2001; Rother *et al.*, 2001). The enzymes of the Sox pathway are located in the bacterial periplasm and use a small, monohaem, *c*-type cytochrome as the direct electron acceptor. The current model for the Sox mechanism as it applies to thiosulfate oxidation is depicted in Figure 1. The central player is the SoxYZ complex which carries the pathway intermediates covalently bound to a cysteine residue located in a conserved Gly-Gly-Cys-Gly-Gly motif at the extreme C-terminus of the SoxY protein (Lu *et al.*, 1985; Appia-Ayme *et al.*, 2001; Friedrich *et al.*, 2001; Quentmeier and Friedrich, 2001). The initial reaction in the pathway is the oxidative formation of a disulfide linkage between the sulfane sulfur of thiosulfate and the SoxY cysteine. This reaction is catalysed by the haemoprotein SoxAX complex. The terminal sulfone group of the resultant adduct is released as sulfate by SoxB in a hydrolytic reaction. The sulfane atom is then oxidized to a sulfone by the SoxCD complex and released from SoxY as sulfate by the activity of SoxB.

The SoxAX complex isolated from the photosynthetic marine bacterium *Rhodovulum sulfidophilum* is a heterodimeric *c*-type cytochrome in which the SoxA subunit contains two, and the SoxX subunit one, covalently bound haem (Appia-Ayme *et al.*, 2001; Cheesman *et al.*, 2001). The electron acceptor from *R.sulfidophilum* SoxAX is cytochrome *c*₂. Sequence alignments of the *R.sulfidophilum* SoxA and SoxX sequences with those of other sulfur-oxidizing bacteria are shown in Figure 2. Here we report the X-ray crystal structures of the oxidized form of the *R.sulfidophilum* SoxAX complex refined at 1.75 Å resolution and the reduced form of the enzyme refined at 1.5 Å resolution. These high resolution structures combined with spectroscopic data and chemical analysis reveal the novel arrangement of a cysteine persulfide residue coordinated to the catalytic *c*-haem of the SoxA subunit and provide insights into the mechanism of this key oxidative enzyme of the global sulfur cycle.

Results and discussion

The architecture of the SoxAX complex

The crystal structure of the SoxAX heterodimer with four molecules in the asymmetric unit of a *P*2₁ cell was solved at 2.5 Å resolution by Fe-multiple wavelength anomalous dispersion (MAD). The structure subsequently was refined at 1.75 Å resolution using data collected from a crystal of space group *P*2₁2₁2 with a single molecular copy in the asymmetric unit (Table I). The heterodimer seen in the crystal corresponds to the oligomeric state observed in

Fig. 1. The Friedrich–Kelly model for the Sox mechanism of thiosulfate oxidation in *R.sulfidophilum*. SoxAX catalyses a two-electron oxidation in which the sulfane sulfur of thiosulfate is linked to the antepenultimate cysteine residue of SoxY to produce SoxY–thiocysteine-S-sulfate. The figure is modified from Friedrich *et al.* (2001). Open circles represent sulfur atoms, whilst oxygen atoms are shown as filled circles.

solution by analytical ultracentrifugation studies (Appia-Ayme *et al.*, 2001). SoxAX is an elongated molecule of approximate dimensions $34 \times 36 \times 75 \text{ \AA}^3$ (Figure 3A and B). The SoxA subunit is organized into two domains each binding a *c*-type haem with histidine–cysteine axial coordination. These are designated haems 1 and 2, labelled in the order in which the corresponding CXXCH ligand motifs appear in the SoxA sequence. Post-translational modification of the cysteine ligand to haem 2 is apparent and is discussed further below. The single domain SoxX subunit binds a *c*-type haem with histidine–methionine axial ligands (haem 3) and forms the interface with the C-terminal domain of SoxA. The haem ligands observed in the structure are in agreement with those inferred from previous spectroscopic characterization of oxidized protein samples (Cheesman *et al.*, 2001). The geometrical arrangement of the haem groups is presented in Table II. The use of cysteinate as a haem iron ligand is unusual, with only cytochromes P450, the CO sensor protein CooA and cystathionine β -synthase previously having been reported to have such a ligand (Ojha *et al.*, 2000). Indeed, SoxA is the first *c*-type cytochrome with cysteine haem iron coordination and only the second structurally characterized haemoprotein containing a haem with a cysteine–histidine ligand set, cystathionine β -synthase being the other (Meier *et al.*, 2001).

The haem 2–haem 3 interplanar angle and distance of closest approach together with the haem solvent-accessible areas are consistent with haem 3 being the route for electron transfer away from the complex (Table II). The solvent-exposed edge of haem 3, the presumed locus for cytochrome c_2 binding, lies on the opposite face of SoxX from haem 2. The edge–edge separation of haems 1 and 2, in contrast to that between haems 2 and 3, is sufficiently long to preclude efficient electron transfer in the absence of a chain of cofactors (Page *et al.*, 1999). An extended groove runs across the molecule at the subunit interface. This leads to a solvent-filled channel lined with basic residues, at the bottom of which the haem 2 iron atom and modified cysteine ligand lie exposed to solvent

(Figure 3C). These observations, together with the post-translational modification to haem 2, led us to identify this haem as the catalytic active site.

The folds of the SoxA and SoxX subunits

The SoxX subunit has a *c*-type cytochrome fold as defined in the SCOP database (Holm and Sander, 1993). Yeast mitochondrial cytochrome *c* (PDB entry 1yc, 108 residues) was found to be most similar, with 82 structurally aligned residues and an r.m.s.d. of 2.3 Å. The SoxX subunit can thus be classified as a member of the monodomain cytochrome *c* family (Murzin *et al.*, 1995). Inspection of the SoxA subunit reveals a pronounced internal pseudo-dyad encompassing residues 51–150 and 151–250 for domains that we term SoxA_N and SoxA_C, respectively. Alignment of these domains gave an overall r.m.s.d. of 1.8 Å for 93 structurally conserved residues for which the percentage residue identity was 18%. The haem groups in each domain are also well conserved, having an r.m.s.d. of 1.2 Å in this alignment (excluding propionate groups). The folds of these domains are clearly highly homologous and, as was the case for SoxX, each of these A-subunit domains has a *c*-type cytochrome fold. Again taking the mitochondrial cytochrome *c* for comparison, the r.m.s.ds are 2.7 and 3.1 Å for the SoxA_N and SoxA_C domains (both 69 aligned residues), respectively. The structure, in conjunction with sequence alignment, suggests that SoxA, apart from the N-terminal 50 amino acids, has evolved from duplication of a cytochrome *c* gene.

The di-haem SoxA subunit

Like SoxA, proteins of the two-domain cytochrome *c* family possess a pseudo 2-fold symmetry axis relating the two haem domains (Murzin *et al.*, 1995). However, fold homology searches failed to align the SoxA subunit with members of this family (Chen *et al.*, 1994; Fülöp *et al.*, 1995; Kadziola and Larsen, 1997). As such, SoxA represents the first example of a new family of two-domain cytochromes *c* in which the packing of the two domains differs radically from the previously characterized examples (Figure 3D). The novel domain arrangement seen in SoxA may arise because a combination of the C-terminal (residues 251–261) and N-terminal polypeptides (residues 1–50) of SoxA block the face of the C-terminal domain that forms the domain interface in the other two-domain cytochrome *c* proteins. The result is that in SoxA the two domains pack with their C-terminal α_4 helices arranged about the local pseudo 2-fold axis relating the SoxA_N and SoxA_C domains. A major consequence of this unusual packing is to give a distance of closest approach of the haems in SoxA (24 Å) that is much greater than that seen in the other two-domain cytochrome *c* proteins (<14 Å). The result is that effective electron transfer between the SoxA haems is precluded.

It is notable that the haem 1 consensus Cys-Xaa-Xaa-Cys-His haem-binding motif is absent from the SoxA proteins of *Rhodospseudomonas palustris* and of *Chlorobium* species (Figure 2A). In place of haem 1, an intradomain disulfide bridge is found between the remaining (second) cysteine of the haem-binding motif and the conserved cysteine residue that is a ligand to that haem in *R.sulfidophilum* SoxA (Klarskov *et al.*, 1998). However, the α -carbon atoms of these cysteines in the SoxA_N

Fig. 2. Amino acid sequences of SoxA and SoxX subunits from different sulfur-oxidizing bacteria. (A and B) Primary sequence alignments and haem coordination in the *R.sulfidophilum* SoxA and SoxX subunits, respectively. Sequences are those of the SoxAX proteins from *R.sulfidophilum* (Appia-Ayme *et al.*, 2001) (*Rs*), *Paracoccus denitrificans* (Friedrich *et al.*, 2000) (*Pd*), *Chlorobium limicola* (Klarskov *et al.*, 1998) (*Cl*), *Chlorobium tepidum* (The Institute for Genomic Research. URL <http://www.tigr.org>) (*Ct*), *Aquifex aeolicus* (Deckert *et al.*, 1998) (*Aa*) and *Rhodospseudomonas palustris* (DOE Joint Genome Institute. URL http://www.jgi.doe.gov/JGI_microbial/rhodo_homepage.html) (*Rp*). The alignments were generated with the program ClustalW using default parameters (see <http://www.ebi.ac.uk/clustalw/>). Secondary structural elements (β -sheet shown by blue arrows and α -helices by red bars) are indicated above the *R.sulfidophilum* sequence. The four residues indicated by Pitts (1998) to be diagnostic for formation of each cytochrome *c* fold are highlighted in green, and the four conserved helices of the folds of the two domains ($\alpha 1$ – $\alpha 4$ and $\alpha 1'$ – $\alpha 4'$) are labelled. CXXCH haem-binding motifs and second axial ligands are indicated in yellow. Polypeptides contributing to the interface of the SoxAX complex and forming part of interface region A (see text) are boxed in blue; those forming part of interface region B are boxed in red. A consensus sequence showing identical residues is also given.

domain are >10 Å apart, a distance greater than the maximum for a disulfide bridge (Sowdhamini *et al.*, 1989). The question then naturally arises as to whether, never-

theless, the A_N domain of the *R.palustris* and *Chlorobium* sp. SoxA proteins can adopt the cytochrome *c* fold but substitute a disulfide bridge for the *c* haem to form a

Table I. Data collection and refinement statistics

Data collection	MAD data collection space group $P2_1$ $a = 81.2 \text{ \AA}$ $b = 102.9 \text{ \AA}$ $c = 114.4 \text{ \AA}$ $\beta = 110.5^\circ$	Oxidized, space group $P2_12_12$ $a = 66.4 \text{ \AA}$ $b = 87.3 \text{ \AA}$ $c = 71.7 \text{ \AA}$		Reduced, space group $P2_12_12$ $a = 65.8 \text{ \AA}$ $b = 87.2 \text{ \AA}$ $c = 71.5 \text{ \AA}$	
	$\lambda 1 = 1.729 \text{ \AA}$	$\lambda 2 = 1.741 \text{ \AA}$	$\lambda 3 = 1.000 \text{ \AA}$	$\lambda = 0.933 \text{ \AA}$	$\lambda = 0.98 \text{ \AA}$
Resolution ($\text{\AA}$)	2.5	2.5	2.55	1.75	1.5
Completeness (%)	95.0 (92.2)	94.8 (89.6)	98.2 (88.7)	93.4 (91.4)	99.3 (98.4)
R_{sym}^a (%)	4.9 (9.1)	5.1 (10.4)	3.1 (5.0)	7.0 (16.8)	4.1 (28.4)
R_{anom}^b (%)	4.4 (7.1)	3.5 (8.7)	1.8 (5.4)	—	—
$\langle I \rangle / \langle \sigma(I) \rangle > 3$ (%)	92.9 (82.4)	90.6 (73.9)	96.6 (89.2)	78.7 (44.2)	78.6 (48.4)
Independent reflections	61 072	61 950	58 706	42 647	66 141
Total reflections	903 590	901 411	101 074	262 534	670 264
Overall temperature factor ($\text{\AA}^2$)	34.7	36.7	36.7	17.8	13.2
SOLVE mean FOM ^c	0.74				
Refinement statistics	$P2_1$	$P2_12_12$ oxidized	$P2_12_12$ reduced		
Wavelength ($\text{\AA}$)	1.000	0.933	0.98		
Resolution range ($\text{\AA}$)	20–2.55	20–1.75	25–1.5		
Refined structure ^d	SoxA 1–261, SoxX 1–138, + 691 water	SoxA 1–261, SoxX 1–51, 55–137, + 353 water	SoxA 1–261, SoxX 1–51, 55–137, + 667 water, + 2 ethylene glycol		
R_{cryst}^e	21.0 (29.6)	17.7 (21.3)	19.9 (23.5)		
R_{free}^f	26.4 (36.8)	21.4 (23.0)	23.0 (27.4)		
R.m.s.ds					
Bonds ($\text{\AA}$)					
C–N	0.005	0.009	0.005		
N–C $_{\alpha}$	0.009	0.014	0.008		
C $_{\alpha}$ –C	0.012	0.016	0.009		
Angles ($^\circ$)					
N–C $_{\alpha}$ –C	0.88	1.08	1.36		
C–N–C $_{\alpha}$	1.30	1.77	2.16		
Mean temperature factors ($\text{\AA}^2$)					
Main chain atoms	18.5	16.5	14.4		
Side chain atoms	19.1	21.9	17.3		

Figures in parentheses refer to data in the highest resolution bin.

^a $R_{\text{sym}} = \sum \sum |I_i| - \langle I \rangle / \langle I \rangle$, where $\langle I \rangle$ is the average of symmetry equivalent reflections and the summation extends over all observations for all unique reflections.

^b $R_{\text{anom}} = \sum |I_i| - \langle I \rangle / \sum |I_i| + \langle I \rangle$.

^cAverage figure of merit (FOM) calculated by SOLVE (Terwilliger and Berendzen, 1999).

^dThe final structural model for the $P2_1$ crystal form contained four copies of the indicated residues of SoxA and SoxX in the asymmetric unit.

^e $R_{\text{cryst}} = \sum ||F| - |F_c|| / \sum |F|$.

^fFor R_{free} , the summations extends over a subset (5%) of reflections excluded from all stages of refinement.

different superfamily. An important observation in this respect is that the haem-less SoxA domains preserve the cluster of hydrophobic residues at positions A61 (6), A65 (10), A134 (94) and A137 (97) (tuna cytochrome *c* residue numbering in parentheses; see Figure 2A) in the N- and C-terminal helices of the fold. These four residues are not involved in haem binding and are conserved in otherwise highly divergent cytochromes *c*. This, together with the absence of an apparent functional role for these residues, has led to the suggestion that they are involved in a common folding nucleus of all subfamilies of *c*-type cytochromes (Ptitsyn, 1998). It therefore appears reasonable to conclude that the haem-less SoxA_N domain in these proteins may adopt a modified cytochrome *c* fold.

The nature of the subunit interface

The SoxAX complex is purified readily from the periplasm of *R.sulfidophilum*, suggesting a subunit interaction that is

both specific and of relatively high affinity (Appia-Ayme *et al.*, 2001). At present, the complex of yeast cytochrome *bc*₁ with cytochrome *c* (Lange and Hunte, 2002) is the sole example of a crystal structure of a productive electron transfer complex involving two cytochrome *c* proteins. Furthermore, there are few examples of stable, structurally characterized homo-oligomeric proteins with the cytochrome *c* fold. Under these circumstances, the nature of the subunit interface in the SoxAX complex is of some interest. In an attempt to determine whether the SoxA_C:SoxX packing is structurally unique, the PQS server (Henrick and Thornton, 1998) was used to generate an independent set of stable cytochrome *c* dimer packings. Adding to these the domain packings observed in the two-domain cytochromes *c*, a total of 11 independent cytochrome *c*:cytochrome *c* domain packings were found. Comparison reveals that the SoxA_C:SoxX domain packing bears a passing resemblance to that observed

between monomers of the cytochrome c_6 from the green alga *Chlamydomonas reinhardtii* although the relative orientation of the haems is significantly different. It is appropriate to note that there is some evidence that the dimerization of the latter has functional significance (Kerfeld *et al.*, 1995). Notably, there is no similarity between the packing of cytochrome c domains at the SoxA_C:SoxX interface and that observed in the yeast cytochrome bc_1 :cytochrome c complex (Lange and Hunte, 2002). Thus, the packings of the c -type cytochrome domains both within SoxA and at the subunit interface are essentially unique. Cytochrome c domains are limited in their packing arrangements if the haem groups are to fall within suitable minimum edge–edge distances for electron transfer without the requirement for other cofactors. This requirement is clearly illustrated in the packing of domains within the SoxA subunit.

Analysis of the high resolution structure reveals a subunit interface that is well packed. This is evidenced by the shape complementarity statistic (Sc) (Lawrence and Colman, 1993), which has a value of 0.74 and is consistent with previously compiled statistics on permanent heterodimeric complexes (Jones and Thornton, 1996). Despite being well packed, the interface is also apparently fairly plastic. The largest r.m.s.d. between pairs of high and low resolution structures of the complex is ~ 0.6 Å (based on all α -carbon atoms), and this difference can be accounted for in the most part by a rigid body screw domain displacement of SoxX relative to SoxA_C of 4° and a translation of 0.2 Å. As is often the case, the root cause of this difference in the SoxAX structures may be attributable to crystal lattice packing contacts. However, whilst the haem edge–edge distances and interplanar angles are not affected greatly by the excursions of the SoxX subunit relative to SoxA (Table II), the observed plasticity at least suggests that conformational changes may occur on binding of SoxYZ. The increased mobility of SoxX is also reflected in the average atomic temperature factor for atoms in this subunit (25.5 Å²) being greater than that for the SoxA subunit (15.1 Å²).

The interface of the complex is predominantly hydrophobic in nature, in keeping with that observed in other stable electron transfer complexes (Mathews and White, 1993). Approximately 2500 Å² is buried on complex formation (6.8% of the total accessible surface area), of which apolar and aromatic residues contribute 42%. This buried area is consistent with that observed in other heterodimeric complexes (Jones and Thornton, 1996). The interface is composed of two distinct, non-overlapping contact regions which we label A and B. Four short polypeptide stretches in SoxA form the majority of the interaction surface with SoxX (Figure 2A). Those contributing to region A are 183–184 and 187–191, whilst 168–175 and 224–232 contribute to region B. In SoxX, a number of short polypeptides are involved in forming the interface: residues 37–44 and 55–60 (region A), and residues 89–93 (region B) (Figure 2B). These three polypeptides contribute $\sim 50\%$ of the total buried surface area of the SoxX subunit in the complex. The remainder comes from residues 101–110 (region B) which form part of a loop extending from the core domain structure to form an intimate association with residues of SoxA. Notably, this interface loop (residues 99–118) constitutes a major

insertion in SoxX relative to other members of the cytochrome c superfamily. Interestingly, this loop is also missing in the SoxX proteins from *Chlorobium tepidum*, *Aquifex aeolicus* and *R. palustris* (Figure 2B). In addition, the overall sequence homology of the other contact peptides in SoxX relative to the corresponding regions in the loop-less proteins appears to be low (Figure 2B) and this may have important consequences in terms of the stability of their complexes. It should be noted that the absence of a permanent SoxAX complex in these organisms may imply generation of a means of stabilizing one-electron-oxidized intermediates.

A single midpoint potential using NADH as reductant has been observed for *R. sulfidophilum* SoxAX with a value of +180 mV that has been assigned to two redox-linked haems (Little, 2000). The packing of SoxA_C to SoxX at the subunit interface provides a haem–haem distance of closest approach of 10.7 Å. This distance is marginally shorter than that seen in the di-haem cytochrome c peroxidase (14.5 Å) and the cytochrome c_4 protein (11.2 Å) and involves transversing a region of the interface predicted to be solvent accessible. However, electron transfer should be rapid and essentially independent of the environment between the haems for a separation of 14 Å or less (Page *et al.*, 1999). We therefore assume that haems 2 and 3 constitute the redox-linked pair. The haem groups arranged closest by the SoxA_C:SoxX packing are the C-pyrrole rings. Several lines of evidence have implicated the exposed edge of the haem pyrrole ring C in electron transfer in c -type cytochromes (Pelletier and Kraut, 1992).

Post-translational modification to catalytic haem ligand

Fourier maps calculated using a fully refined structural model based on the predicted amino acid sequence of SoxA reveal regions of unexplained difference electron density adjacent to the cysteine axial haem ligand to haem 2 in the vicinity of its terminal sulfur atom. The $|F_o - F_c|$ difference electron density peaks are at $>5.2\sigma$ and 11.5σ from the mean electron density calculated over the volume of the molecule (where σ is the standard deviation of the distribution), indicating the presence of at least one additional atom (Figure 4A). The refined cysteine sulfur to ferric haem iron distance is 2.57 Å in this model. Given the likely geometry of the modified amino acid as indicated by the electron density, cysteine persulfide and cysteine sulfenic acid are the most probable derivatives. There is precedent for both modifications in other protein crystal structures (Claiborne *et al.*, 1999; Bordo *et al.*, 2000). We attempted to refine both possibilities against our X-ray data with the result that both the cysteine persulfide sulfane sulfur to haem iron distance and the alternative cysteine sulfenic acid terminal oxygen to haem iron distance refined to 2.41 Å. However, whilst the cysteine persulfide form showed only minor residual difference electron density after the modification, the cysteine sulfenic acid residue showed a $+7.0\sigma$ difference electron density peak in the vicinity of the sulfenic oxygen atom. This strongly suggests that cysteine persulfide is the post-translational modification observed for CysA222. Unfortunately, anomalous difference Fourier maps calculated using the iron K-edge wavelength data set ($\lambda 1$ in Table I), which could positively identify a terminal sulfane

sulfur atom on this residue, were inconclusive due to the proximity of the additional atom to the iron centre. So, in the absence of an unambiguous sulfur anomalous scattering signal, further supportive evidence was sought for this interpretation.

While the additional density associated with the haem 2 cysteine ligand does not give an unambiguous sulfur anomalous scattering signal, additional spectroscopic and biochemical evidence strongly supports the assignment of

a sulfur atom to this density. A combined EPR and MCD analysis of oxidized SoxAX indicates that haems 1 and 2 both have thiolate haem iron ligands (Cheesman *et al.*, 2001). Haem 2, therefore, should be directly co-ordinated by a sulfur atom. To confirm the assignment of this atom as sulfur, we undertook a chemical analysis of the purified SoxAX protein and determined that SoxAX contains 1.0 mol of labile sulfur per three haems. Thus, there is chemical evidence for one atom of labile sulfur in each SoxAX molecule. Parallel quantitation of the low spin ferrocyanochrome EPR signals indicates that there is 1.0 mol labile sulfur per pair of thiolate-ligated haems (data not shown). The modification to CysA222 was therefore taken to be the cysteine persulfide, and a view of the final electron density map is shown in Figure 4B.

Persulfide groups are generated in the catalytic cycles of various enzymes, for example, rhodanese (thiosulfate sulfurtransferase) (Gliubich *et al.*, 1996; Bordo *et al.*,

2000) and the biosynthetic enzyme, ThiI (Palenchar *et al.*, 2000). In all cases, the persulfide groups are stabilized by the influence of their protein environment. This is certainly also the case in the SoxAX complex where co-ordination of the cysteine persulfide sulfane sulfur to haem ferric iron will stabilize the anionic form of the modified residue.

The enzyme active site

Amongst other indicators, the unprecedented post-translational modification to the CysA222 ligand to haem 2 and its accessibility to substrates suggest this haem to be the most likely site of thiosulfate oxidation (Figure 4C). A number of conserved arginine residues (A190, A206 and A218) plus other less well-conserved basic residues (LysA208 and ArgA224) can be found in the vicinity. This cluster of residues provides a highly basic electrostatic potential at the molecular surface in this region lining a channel which provides access to the active site for anionic substrates (Figure 3C). Electrostatic field calculations carried out with SPOCK (Christopher, 1997) show that in the vicinity of the presumed thiosulfate-binding site adjacent to the cysteine persulfide ligand to haem 2, the field rises to a value of ~ 20 kT/e (ionic strength 0.15 M; relative dielectric constants of protein and solvent taken to be 4 and 80, respectively). The guanidyl groups of arginines at positions A218 and A206 are found in close proximity (the distance of closest approach is 3.4 Å) and that of ArgA218 approaches to within 3.5 Å of the haem 2 plane. This suggests a low pK_a for one (or both) of these residues and neutral charge at the pH of the crystal. The guanidyl group of ArgA218 also lies within 3.8 Å of the persulfide sulfane sulfur of the side chain of CysA222 and the side chain populates a minor conformer of arginine (χ_1 ,

χ_2) space (Kleywegt and Jones, 1998). It is certainly the case that ArgA218 is found in an unusual environment, and circumstantial evidence from the final Fourier maps also suggests that this residue may adopt alternative conformation(s) (Figure 4A). Our inference is that the side chain of this residue may be conformationally flexible, although the side chain average atomic B -value for this residue is not unusual, having a value of 16.4 Å^2 compared with a molecule average of $\sim 20 \text{ Å}^2$. Also evident from inspection of a molecular surface representation of the active site region is a groove running across the face of the complex, which may serve as part of the docking site for the SoxYZ co-substrate (Figure 3C). Preliminary docking studies using a Gly-Gly-Cys-Gly-Gly pentapeptide as a model for the flexible C-terminal region of SoxY demonstrate that there is sufficient space to locate the acceptor cysteine sulfur adjacent to haem 2 but insufficient for it to approach closely enough to act as an alternative haem iron axial ligand (data not shown).

The structure of dithionite-reduced SoxAX

Upon reduction of the SoxAX complex with dithionite, a change in the ligands of haem 2 is detectable spectroscopically (Cheesman *et al.*, 2001). The suggested interpretation is that the thiolate ligand has become either protonated or replaced by an unidentified ligand. In the latter case, replacement of the thiolate haem ligand with one provided by the protein is envisaged. Redox-linked switches of amino acid ligands have been observed in other haemoproteins (Williams *et al.*, 1997; Lanzilotta *et al.*, 2000). In the absence of a major conformational change in SoxAX, ArgA218 is the only residue of the active site that could provide an alternative ligand for

Fig. 3. The overall fold of the SoxAX complex. (A) The architecture of the complex. α -helices are shown in blue, and β -strands in yellow for SoxA, whilst for SoxX they are shown in red and green, respectively. The SoxA subunit is composed of two cytochrome *c* domains related by a pseudo 2-fold axis and contains two *c*-type haems with histidine–cysteine axial ligands. SoxX consists of a single cytochrome *c* fold with a histidine–methionine co-ordinated haem. (B) A stereo view of the C_α trace of the SoxAX molecule with every twentieth residue labelled. The SoxX subunit is coloured yellow, whilst for SoxA, the N-terminal 50 amino acids are coloured grey and the SoxA_N and SoxA_C cytochrome *c* domains are coloured blue and red, respectively. An insertion in the SoxX sequence from *R.sulfidophilum* relative to other cytochromes *c* (shown in purple) forms a major component of the subunit interface. (C) A close-up view of the electrostatic potential at the solvent-accessible molecular surface showing the substrate access channel and the binding groove for the C-terminal peptide of SoxYZ. The positions of arginine, lysine and histidine residues contributing to a basic substrate access funnel are shown. (D) Comparison of SoxA (left) with the two-domain cytochrome *c* fold of cytochrome *c*₄ from *Pseudomonas stutzeri* (right). The C-terminal domains of SoxA and cytochrome *c*₄ (yellow haems) are presented in the same orientation as determined by DALI (Holm and Sander, 1993). The four conserved helices (α_1 – α_4) of the cytochrome *c* fold are shown for each domain in red. The N-terminal 50 amino acids of SoxA (shown in grey) pack against the face of the SoxA_C domain, which in the previously characterized two-domain cytochromes *c* interacts with the N-terminal domains.

Table II. Haem geometric parameters and solvent accessibility

	Haems 1–2	Haems 2–3	
Distance (Fe–Fe) (Å)	31.4 (31.0)	19.0 (18.8)	
Distance (closest approach) (Å)	23.9 (23.4)	10.7 (10.6)	
Interplanar angle (°)	56.1 (55.1)	80.95 (78.3)	
	Haem 1	Haem 2	Haem 3
Ligand distances (Å)			
Fe–S	2.45 (2.37)	2.41 (2.35)	2.34 (2.43)
Fe–N	2.08 (2.11)	2.08 (2.08)	2.05 (2.07)
Haem solvent accessibility (Å ²)	179.2 (179.7)	56.7 (53.3)	80.8 (82.9)

The figures in parentheses refer to the average calculated over the four independent molecules of the $P2_1$ cell.

haem 2. To gain insight into this phenomenon, we wished to compare the structures of the enzyme in the oxidized and reduced states. Crystals of the enzyme were therefore treated with dithionite and the enzyme verified to be in the haem reduced state by single crystal microspectrophotometry. The reduced enzyme structure was solved by molecular replacement and refined to convergence at 1.5 Å resolution. The refined oxidized and reduced structures are very similar, with an r.m.s.d. of 0.17 Å over all 394 C $_{\alpha}$ atoms, rising to 0.35 Å when calculated over all atoms (including the haem groups). When the superposition was performed over all atoms within a 12 Å radius of the terminal sulfane sulfur of A222, the r.m.s.d. drops to 0.13 Å. Thus, the active site is essentially unchanged. In particular, the ArgA218 side chain does not move on reduction, being almost superimposable on that of the oxidized protein. The average B -value of its side chain atoms (10.5 Å² compared with a molecule average of ~16 Å²) drops slightly from that of the oxidized state, but no other systematic change in this residue was detected. The peak in the $F_o - F_c$ Fourier map observed for the oxidized enzyme adjacent to the side chain C γ atom is still present, albeit diminished, having a maximum value of 3.5 σ (the corresponding value in the oxidized enzyme map was ~5 σ). Thus, at least in the crystalline state, ligand switching is not observed in the reduced SoxAX heterodimer and, by elimination, protonation of the thiolate ligand becomes the likely explanation for the observed spectroscopic changes. However, the physiological relevance of this observation made on a chemically reduced enzyme in the absence of substrate remains unclear.

A model for the mechanism of SoxAX

Friedrich *et al.* (2001) have ascribed the role of the SoxAX complex to the two-electron oxidation of substrates such as thiosulfate and sulfite during transfer to SoxY, yet the mechanism for enzymatic oxidation of reduced sulfur compounds is not well characterized. (Note that SoxY forms part of a complex with SoxZ, and subsequent reference to it in the text should be taken to imply involvement of the SoxYZ complex.) What insights then can the crystal structure of the enzyme provide to help explain the molecular basis for thiosulfate oxidation? A useful paradigm is found in the sulfurtransferases, such as rhodanese, which catalyse the transfer of a sulfane sulfur atom from an anionic donor substrate (such as thiosulfate) to a thiophilic acceptor via a sulfur-substituted enzyme

covalent intermediate (Westley, 1973). The intermediate form (Figure 5Ab) is characterized by formation of a persulfide linkage to a catalytic cysteine residue, readily cyanolysed to restore the cysteinate (Figure 5Ac). Crystal structures are available for both forms of the enzyme from mammalian (Gliubich *et al.*, 1996) and bacterial (Bordo *et al.*, 2000) sources. These reveal an active site which is characterized by an unusually strong positive electrostatic field. This is generated by a combination of the unique alignment of peptide dipoles from an active site hexapeptide loop and from five basic residues which lie within 7 Å of the catalytic cysteine. One of these, a mobile arginine residue (Arg235 in the *Azotobacter vinelandii* enzyme), lies within 3.3 Å of the catalytic cysteine persulfide sulfane sulfur. The result of this field is to produce a highly stabilized persulfide form of the catalytic cysteine residue and also to provide a strong anion-binding site suitable for the substrate, thiosulfate. The electrostatic field in this binding site calculated for the bacterial enzyme (18 kT/e) is similar in magnitude to that calculated for the corresponding site in SoxAX.

The SoxAX and rhodanese structures have different folds and use different methods to stabilize the anionic form of a catalytic cysteine residue, yet arguably may share common features in their mechanisms. We have drawn on these parallels to suggest two possible catalytic mechanisms for reduced sulfur compound oxidation by SoxAX (Figure 5B). Whilst rhodanese transfers a sulfur atom from donor to acceptor groups without loss of electrons (consistent with the absence of a redox active group on the enzyme), SoxAX must perform the two-electron oxidative transfer of thiosulfate to the carrier protein SoxY. This is achieved by means of haems 2 and 3, which accommodate the two electrons liberated by the reaction. The enzyme must also provide a means for stabilization of the anionic forms of the catalytic cysteine residue and its persulfide derivative, and this is achieved by using this residue as axial ligand to an oxidized ferric c -type haem. Note that although cysteine persulfide is the crystallographically observed form for this residue in SoxAX, minor structural adjustments in this region would allow the alternative cysteinate form to bind to the haem 2 iron. Thus, as with rhodanese, it appears that both forms of the enzyme can be stabilized. The array of basic residues in the vicinity of the active site provides a strong electropositive potential which may also contribute to stabilization of the cysteinate form. The proximal arginine,

Fig. 4. The active site of SoxAX. (A and B) Post-translational modification of the haem 2 axial ligand. (A) The $(F_o - F_c)$ Fourier map contoured at the 3.5σ level (shown in red), where σ represents the r.m.s. electron density for the unit cell. This map has been calculated using structure factor amplitudes and phases calculated from a refined model with a cysteine at residue A222. Note the small region of difference electron density adjacent to the side chain of ArgA218. This feature is also found in $(F_o - F_c)$ maps calculated using the final refined structural model containing a cysteine persulfide haem ligand. The atoms and chemical bonds of haem 2 are coloured grey, except the haem iron which is magenta. (B) Stereo view of the $(2F_o - F_c)$ electron density map around haem 2 calculated at 1.75 Å resolution and contoured at the 1.2σ level. This map has been calculated from a refined structural model with a cysteine persulfide at residue A222. (C) Orthogonal views of the active site region. CysA222 and HisA181 provide the axial ligands to the iron atom of haem 2. The basic residues A218, A206 and A208 form a possible proton-transfer relay from the active site.

Fig. 5. Insights into the mechanism of thiosulfate oxidation. (A) The mechanism of the sulfurtransferase, rhodanese. (B) The proposed mechanisms for thiosulfate oxidation by SoxAX. Note that the point at which reoxidation of haems 2 and 3 occurs is not known.

A218, in the SoxAX structure shares features in common with Arg235 of rhodanese, lying immediately adjacent to the catalytic cysteine. We suggest a similar primary role for ArgA218, that is to provide an anionic-binding site suitable for the substrate sulfonyl group, orienting the substrate sulfane sulfur relative to the terminal catalytic cysteinyl sulfur (Figure 5Ba). Transfer of the thiosulfate moiety to CysA222 now takes place, generating a two-electron-oxidized SoxA–thiocysteine-S-sulfate intermediate. The electrophilic character of ArgA218 polarizes the terminal sulfur–sulfur bond leaving the outer sulfane sulfur as the preferred axial ligand to ferrous haem iron. We presume that this results in a conformational change in the side chain of this residue leading to rearrangement of the ferrous haem iron axial ligand (Figure 5Bb).

One of two possibilities now occurs. In the first case, docking of SoxY results in formation of the SoxY–thiocysteine-S-sulfate product (Figure 5Bc). Diffusion of modified SoxY from the active site and reoxidation of haems 2 and 3 will restore the active resting state of SoxAX (Figure 5Bd). However, in this case, the crystallographically observed cysteine persulfide is not represented. In a second scenario, binding of SoxY results in generation of the alternative SoxY–cysteine-S-sulfate derivative through transfer of sulfite. The cysteine

persulfide-modified enzyme (Figure 5Be) now represents a direct intermediate in the oxidative pathway. Importantly, it should also be possible to transfer the terminal enzyme-bound sulfane sulfur from this enzyme form to SoxY. In this case, SoxAX is acting as a sulfurtransferase, i.e. as a rhodanese-like enzyme. However, it may be possible that in the absence of SoxY and cytochrome *c*₂, the intermediate (Figure 5Bb) can break down by a two-electron reduction process to give the crystallographically observed CysA222 persulfide with release of sulfite (not shown). We note that this is likely to occur during enzyme purification since thiosulfate is present in the cell lysate while the acceptor forms of SoxY and cytochrome *c*₂ can no longer be regenerated. Note also that sulfite is predicted to be a free intermediate in this scheme as is observed during oxidation of thiosulfate by *R.sulfidophilum* (Appia-Ayme *et al.*, 2001). Without further experimentation, it is difficult to conclude which of these pathways is more likely to be operative *in vivo*.

Conclusions

The crystal structure of the haemoprotein SoxAX complex presented here gives the first insights into the molecular basis for oxidation of reduced sulfur compounds by enzymes of the sulfur cycle. The analysis reveals a unique

cysteine persulfide–histidine ligand pair to the presumed catalytic *c*-haem. Structural analysis of the dithionite-reduced enzyme reveals no significant differences compared with the crystal structure of the oxidized enzyme. Similarities between the active sites of the SoxAX complex and the sulfurtransferase, rhodanese, have allowed us to propose plausible mechanistic schemes that can now be tested by a combination of biochemical, structural and genetic approaches.

Materials and methods

Crystallization and crystal characterization

The SoxAX complex from *R.sulfidophilum* DSM1374^T was purified as described previously (Appia-Ayme *et al.*, 2001) from cultures grown photolithotrophically in the presence of thiosulfate. Crystals of typical dimensions $200 \times 50 \times 5 \mu\text{m}^3$ grew from vapour diffusion experiments using 15% (w/v) PEG 8K and 0.2 M magnesium acetate in 100 mM sodium MES buffer at pH 6.5. The crystals could be cryoprotected by transferring them to stabilization solution containing 25% (v/v) ethylene glycol. Initial X-ray data collection indicated that the crystals belong to space group $P2_1$ with cell parameters of $a = 81.2 \text{ \AA}$, $b = 102.9 \text{ \AA}$ and $c = 114.4 \text{ \AA}$, with $\beta = 110.5^\circ$ at 100 K, and contain four copies of SoxAX per asymmetric unit. Subsequent data collection indicated the presence of a second crystal form growing under the same condition. These crystals belong to space group $P2_12_12$ with cell parameters of $a = 66.4 \text{ \AA}$, $b = 87.3 \text{ \AA}$ and $c = 71.7 \text{ \AA}$, and contain a single copy of SoxAX per asymmetric unit.

MAD phasing and structure solution of the $P2_1$ crystal form

Data for the MAD phasing were collected from a single crystal at three different wavelengths ($\lambda 1$ – $\lambda 3$) around the iron K-edge on station BM14 at ESRF, Grenoble, and processed using DENZO (Otwinowski and Minor, 1997) to 2.55 Å resolution. Determination of heavy atom sites and initial phase estimates were obtained using the program SOLVE (Terwilliger and Berendzen, 1999) using $\lambda 1$ as the reference data set, and improved by solvent flattening using DM (Cowtan, 1994) at a nominal solvent content of 49%. The mean figure of merit for acentric reflections to 2.55 Å resolution was 0.814 after solvent flattening, and Fourier maps calculated with these phases showed clear and contiguous electron density of sufficient quality to trace residues 4–261 of SoxA and residues 1–51 and 55–137 of SoxX. Model building was performed using the program O (Jones *et al.*, 1991), and all refinement procedures performed with CNS (Brünger *et al.*, 1998). Application of the non-crystallographic symmetry (NCS) operators followed by rigid body refinement for this model gave an R_{cryst} of 36.9% and R_{free} (Brünger, 1992) of 36.5%. Manual rebuilding and simulated annealing refinement resulted in a model consisting of residues 1–261 of SoxA and residues 1–51 and 55–137 of SoxX, with an R_{cryst} of 28.6% and R_{free} of 30.1%. Incorporation of cysteine persulfide and residues 52–54 of SoxX followed by a single round of conjugate gradient refinement with an NCS restraint of 30 kcal/mol/Å² resulted in an R_{cryst} of 26.4% and an R_{free} of 29.4%. After addition of 691 water molecules into significant residual electron density, the structure refined to give a final R_{cryst} of 21.0% and an R_{free} of 26.4%. When analysed for stereochemical quality using PROCHECK (Laskowski *et al.*, 1993), the final structure (1H31) has 99.1% of residues in the most favoured regions of the Ramachandran plot, and only three residues from each copy of SoxAX fall in generously allowed regions.

Structure solution of the $P2_12_12$ crystal form

High resolution data were collected from a single crystal on station ID14.2 at the ESRF, Grenoble, and processed using DENZO to 1.75 Å resolution. Molecular replacement using MOLREP of the CCP4 program suite (CCP4, 1994) was performed to transform the preliminary MAD-phased model into the crystallographic cell of the 1.75 Å high resolution data. Simulated annealing refinement resulted in a model with an R_{cryst} of 25.3% and R_{free} of 27.3%. After incorporation of the cysteine persulfide, the three N-terminal residues of SoxA and 353 water molecules, and temperature factor refinement, the model converged to give a final R_{cryst} of 17.7% and R_{free} of 21.4%. The final structure (1H33) has 99.4% of residues in the most favoured (91.1%) and additionally allowed (8.3%) regions of the Ramachandran plot, and only two residues fall in generously allowed regions. These two residues, ArgA190 and AlaX107, both lie on the extremities of β -turns which line the periphery

of the active site cleft. Searches for structural homology were performed using DALI (Holm and Sander, 1993). DynDom (Haywood and Berendsen, 1998) was used for analysis of domain motions. Analysis of the interface was carried out using programs from the CCP4 program suite (CCP4, 1994) and Sc (Lawrence and Colman, 1993). Calculations of molecular electrostatics were performed with SPOCK (Christopher, 1997). Figures were produced using a combination of the programs MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merriett, 1997).

Structure solution of reduced SoxAX

Aerobically grown single crystals of SoxAX were transferred to a reducing solution containing 15% (w/v) PEG 8K, 0.2 M magnesium acetate, 20 mM sodium dithionite, 25% (v/v) ethylene glycol and 100 mM sodium MES buffer at pH 6.5, and equilibrated for 30 min at 20°C before transfer to liquid nitrogen. High resolution data were collected from a single crystal on station 14.2 at the SRS, Daresbury Laboratory, and processed using DENZO to 1.5 Å resolution. The crystal was essentially isomorphous to that used for high resolution data collection from the oxidized form, being of space group $P2_12_12$ with cell parameters $a = 66.4 \text{ \AA}$, $b = 87.3 \text{ \AA}$ and $c = 71.7 \text{ \AA}$ at 100 K. Structure solution and refinement proceeded as for the high resolution oxidized crystal form. After incorporation of the 261 residues of SoxA, including a cysteine persulfide, and 667 water molecules, and temperature factor refinement, the model converged to give a final R_{cryst} of 19.9% and R_{free} of 23.0%. The final structure (1H32) has 99.4% of residues in the most favoured (91.1%) and additionally allowed (8.3%) regions of the Ramachandran plot. As with the high resolution oxidized structure, only residues ArgA190 and AlaX107 fall in generously allowed regions. Absorbance spectra measured at 100 K from this crystal before and following X-ray data collection using a 4DX Systems microspectrophotometer verified that crystalline SoxAX was reduced during the X-ray data collection experiment.

Sulfur analysis

The labile sulfur content of SoxAX was determined as the sulfide released following denaturation of the protein by guanidine hydrochloride in the presence of dithiothreitol (Beinert, 1983). Haem was quantified by the alkaline pyridine ferrohaemochrome method (Bartsch, 1971). The low-spin ferric EPR signals were acquired and quantified as described (Cheesman *et al.*, 2001), with the total content of thiolate-ligated haems being taken as the sum of the LS_{1a} , LS_{1b} and LS_2 signals.

Acknowledgements

We thank Judith Mayne, David Clarke and Jason Southgate for technical assistance. We acknowledge access to the UEA Centre for Metalloprotein Spectroscopy and Biology. This work was funded by the BBSRC through grant 83/B15211 and by the Wellcome Trust through project grant 059531/Z/99/Z.

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Received December 11, 2001; revised August 26, 2002;
accepted September 4, 2002