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Structural Identification of the Interactions of SoxA and SoxX During the Oxidation of Sulfur Anions via the Novel Global Sulfur Oxidizing (Sox) Operon*

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Abstract: In this study, we have tried to elucidate the structural basis of the involvements of SoxA and SoxX in the binding and in electron transport during oxidation of sulfur anions via the novel global sulfur oxidation cycle. SoxA is known to be a di-heme cytochrome c protein, which helps in binding sulfur anions with SoxY. SoxX, a mono-heme cytochrome c protein, binds to SoxA forming a complex, which helps in efficient transport of electrons during oxidation of sulfur anions. We employed homology modeling to construct the three-dimensional structures of the SoxA and SoxX from *Pseudaminobacter salicylatoxidans* and established the geometry of the cytochrome c region and that of the active site of SoxAX complex. The docking studies with SoxA and SoxX allowed us to identify the details of SoxA-SoxX interactions. The structural basis of the formation of the hetero-dimeric complex of SoxA-SoxX were also demonstrated to predict the biochemical pathway of sulfur anion binding and transport of electrons via these proteins in the novel global sulfur cycle as SoxAX complex is the initiator of the sulfur anion oxidation pathway. Since there have been no previous reports regarding the structural biology of these proteins, results from this study will be important for the understanding of the three dimensional structures of SoxA and SoxX as well as to elucidate the structural basis of their mode of actions.

Key words: Sulfur oxidation, cytochrome c, homology modeling, molecular dynamics, protein-protein interaction

INTRODUCTION

Microbial redox reactions of sulfur are mainly responsible for cycling of this element in the environment to maintain environmental sulfur balance. Sulfur has a unique range of oxidation states that varies from +6 to -2 and as a result several important biological processes involving transformations of sulfur from one form to other have been evolved. Sulfur based chemo- or photolithotrophy is one of such processes in which electron transfer from reduced sulfur compounds is used by phylogenetically diverse prokaryotes (Friedrich, 1998; Fowler and Crundwell, 1999; Okabe *et al*., 1999). Various sulfur anions such as, sulfide, polysulfide, thiosulfate, polythionates, sulfites as well as elemental sulfur are the different forms of sulfur in the environment

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(Le Faou *et al*., 1990), which serve as the electron donors in the process of respiration or photosynthesis of the sulfur oxidizing prokaryotes. Only little is understood about the molecular mechanism of this oldest known process.

Recent studies with both chemo- and photolithotrophic α-Proteobacteria, such as *Paracoccus pantotrophus* (Para), *Pseudaminobacter salicylatoxidans* (KCT), revealed that multiple-gene cluster, shxVW (soxVW) and soxXYZABCDEFGH, is associated with metabolism sulfur anions (Deb *et al*., 2004; Mukhopadhyaya *et al*., 2000). SoxXA, SoxYZ, SoxB and SoxCD are required for sulfur-dependent cytochrome c reduction. The eight-electron oxidation of a molecule of thiosulfate is governed by cytochrome c complex multienzyme system (TOMES) encoded by soxXYZABCD (Rother *et al*., 2001; Friedrich *et al*., 2001).

It is to be noted that *Pseudaminobacter salicylatoxidans* is also involved in the oxidative hydrolysis of highly toxic halo-alkanes SoxA and SoxX of KCT are proteins with 286 and 154 amino acid residues respectively. SoxA and SoxX are a di-hem (Mukhopadhyaya *et al*., 2000) and mono-heme cytochrome c proteins respectively. Sequence analysis of the SoxX protein from some other organisms like *Paracoccus pantotrophus* reveal that it contains a mono-heme subunit (Rother *et al*., 2001; Friedrich *et al*., 2001). During the sulfur oxidation process SoxA and SoxX combine with each other to form a complex (SoxAX complex). The complex accepts electrons from sulfur anions and thereby facilitates the binding of sulfur anions to a conserved cysteine residue of SoxY to form SoxY-thiocysteine-S-sulfate, the first covalently bonded sulfur adduct during sulfur anion oxidation by *sox* operon (Friedrich *et al*., 2001; Mukhopadhyaya *et al*., 2000). However to date there are very few reports regarding the structural aspect of the involvements of these proteins in the global sulfur cycle. In the present study our aim is to understand the structural basis of the interaction of SoxA and SoxX from KCT to investigate the molecular mechanism of sulfur anion oxidation via *sox* operon. We describe the three-dimensional structures of these proteins obtained by homology modeling. We have docked the two proteins in order to investigate their favorable binding modes. Molecular dynamics simulations have been performed on the protein complex in order to properly find out the amino acid residues involved in the protein-protein interaction. Binding interactions between SoxA and SoxX have been analyzed to predict the possible molecular mechanism of electron transport during sulfur anion oxidation. These studies provide detailed structural information regarding the molecular biochemistry of the binding of SoxA with SoxX from KCT. As this is the first report regarding the structural aspects of the interaction of SoxA and SoxX from KCT in the process of oxidation of sulfur anions via sox operon, our studies are expected to contribute towards the understanding of the molecular details of the biochemical pathway of sulfur anion oxidation by these ecologically important microorganisms.

MATERIALS AND METHODS

Sequence Analysis and Homology Modeling of Monomeric SoxA and SoxX

The amino acid sequences of SoxA and SoxX of KCT were obtained from Entrez database (Accession No. CAB94219 and CAH59732 respectively). These amino acid sequences were used separately to search Brookhaven Protein Data Bank (PDB) (Berman *et al*., 2000) using the software BLAST (Altschul *et al*., 1990) for finding suitable template for homology modeling. The BLAST search result of SoxA and SoxX showed 54 and 50% sequence similarities respectively with the X-ray crystal structure of SoxAX complex from *Rhodovulum sulfidophilum* (pdb code: 1H33). In the complex 1H33 A and B chains referred to the crystal structures of SoxA and SoxX respectively. The models of SoxA and SoxX were built using the A and B chains respectively of the crystal 1H33 as templates.

Homology modeling was performed using the program Homology of Insight II (Accelrys, San Diego, USA) on a Silicon Graphics Indigo II workstation.

Modeled structures were then superimposed separately on each of the crystal templates without altering the coordinate system of atomic positions in the respective templates (A and B chains of 1H33 for SoxA and SoxX, respectively). The mean r.m.s deviations for the superimpositions were 0.4Å and 0.3Å for SoxA and SoxX respectively on their corresponding crystal templates (Fig. 1 and 2, respectively). Short contacts and bad regions were rectified manually by Insight II.

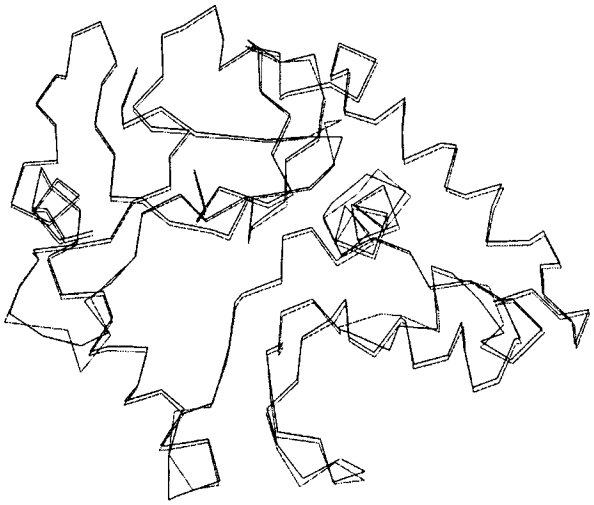

Fig. 1: Superimposition of the α-carbon backbones of SoxA (Black) on the A chain of 1H33 (Red)

Fig. 2: Superimposition of the α-carbon backbones of SoxX (Black) on the B chain of 1H33 (Red)

The models were then energy minimized fixing the backbones to ensure proper interactions. Conjugate Gradient (CG) method was employed for minimization with the Consistent Valence Force Field (CVFF) (Dauber-Osguthorpe *et al*., 1988) using the program DISCOVER3 of Insight II until all the structures reached the final derivative of 0.001 Kcal/mol and were validated using VERIFY3D (Eisenberg *et al*., 1997). PROCHECK (Laskowski *et al*., 1993) analysis was performed in order to assess the stereo-chemical qualities of the three dimensional models and Ramachandran plots (Ramachandran and Sashisekharan, 1968) were drawn. No residues were found to be present in the disallowed regions of the Ramachandran plot.

The accessible surface areas of the predicted cytochrome c motifs of the final structures of SoxA and SoxX were calculated with the program NACCESS (Hubberd, 1992).

Docking of Iron-prophyrine Complex with SoxA and SoxX

To study the interactions involved in the binding of the Iron-Prophyrine complex with SoxA and SoxX, the cytochrome c motifs of the proteins were superimposed on the corresponding cytochrome c motifs of SoxA and SoxX of 1H33 in presence of the Iron-Prophyrine complex. The r.m.s deviations of superimpositions were 0.3 Å for the two cytochrome c motifs of SoxA and 0.4 Å for the cytochrome c motif of SoxX on the corresponding regions in the crystal templates. The complex was subsequently merged with the model to mimic a model- Iron-Prophyrine complex.

Docking of SoxA and SoxX

To study the interactions involved in the binding of SoxA and SoxX, the modeled proteins were superimposed separately on the A and B chains of the crystal structure of 1H33. The models were subsequently merged with the crystal templates and the crystals were removed to produce a modeled SoxAX complex. The docked structure of the SoxAX complex, thus obtained, was subjected to energy minimization using the program DISCOVER3 of Insight II initially by Steepest Descent (SD) and then by CG with CVFF keeping the backbone atoms fixed, until model reached the final derivative of 0.001 kcal/mol.

Molecular Dynamics Calculations

Molecular Dynamics (MD) simulations were performed on the docked structure to predict the favourable binding interactions. The docked structure was solvated with an average of 2000 simple point charges (Berendsen *et al*., 1981) water molecules. The system was minimized initially keeping the water and the backbones of the proteins fixed. In the next step of minimization, the protein complex was kept fixed and the water molecules were allowed to move. The first few rounds of minimizations were performed by steepest descent (SD) method and then CG method was employed. The minimized system was equilibrated for a period of 10 ps with positional restraints. Then a 120 ps MD run was performed without restraints. Weak coupling of the protein to a solvent bath of constant temperature (300 K) and constant pressure (1 bar) was maintained with a coupling of 1.0 ps. For all energy minimizations and MD simulations GROMACS molecular simulation package (Lindahl *et al*., 2001) was used. All the structures were finally analyzed by PROCHECK.

RESULTS AND DISCUSSION

Description of the Structure of SoxA

The modeled structure is a 286 amino acid residue long protein. The predicted structure is similar to X-ray crystal structure of SoxA protein from *Rhodovulum sulfidophilum* (Bamford *et al*., 2002). The first 27 residues show the characteristic feature of a signal peptide. This was predicted by PrediSi

(Hiller *et al*., 2004). The protein contains two domains. The first domain (amino acid residues 1 to 143) starts with a helix (amino acid residues 9 to 15) followed by a three-stranded parallel β-sheet (amino acid residues 33 to 35, 38-40, 43 to 44). The first cytochrome c motif consists of a helix (amino acid residues 100 to 104) followed by a short bend, which ends in another helix (amino acid residues 107 to 110). The central core of the protein is made up of helices interspersed with β-sheets connected among themselves by short turns and bends. The second cytochrome *c* motif (amino acid residues 202 to 206) is also made up of a helix, which is connected to a β-sheet by short turns and bends. The rest part of the protein is made up of helices and sheets connected among themselves by loops and turns. The structure is presented in Fig. 3.

Description of the Structure of SoxX

The model consists of 154 amino acid residues. The predicted structure is similar to the SoxX protein from *Rhodovulum sulfidophilum*. The first 21 amino acids show the presence of a typical signal peptide. The protein is made up of helices and sheets connected by loops and bends. The cytochrome c motif consists of a bent helix (amino acid residues 61 to 67) followed by loops and bends and ends in another helix (amino acid residues 87 to 90). The rest part of the protein is made up of helices (amino acid residues 93 to 102 and 139 to 147) and sheets (amino acid residues 126 to 127 and 132 to 133) connected by loops and bends. Figure 4 represents the structure of the modeled protein.

Comparison with Other Cytochrome C Proteins

SoxX has strong structural similarities with other known cytochrome *c* proteins viz., *Bacillus pasterii* (1C75) (Benini *et al*., 2000), *Thermus thermophilus* (1C52) (Than *et al*., 1997), *Desulfovibrio vulgaris* (1C53) (Nakagawa *et al*., 1990), *Monoraphidium braunii* (1CTJ) (Frazao *et al*., 1995). Superimpositions of the cytochrome c motif of SoxX with the corresponding motifs in 1C75, 1C52, 1C53 and 1CTJ give r.m.s deviations of 0.8, 0.9, 0.8 and 0.7 Å, respectively showing that the overall structure of SoxX is similar to these well-known cytochrome c family proteins (Fig. 5).

Residue-wise accessibility of the cytochrome c motifs of both the proteins were determined and shown in Table 1. The Table 1 shows that out of a total of 10 residues 3 residues are above 30% solvent exposed. This predicts that the cytochrome c motifs are buried in the protein core. Similar results were also shown for other proteins having cytochrome c motif (Hasegawa *et al*., 2000).

Table 1: Residue-wise absolute and % accessibilities of the cytochrome c motifs of SoxA and SoxX of KCT

	C	A	S	C	H
SoxA					
Sequence: CASCH (100-104)					
	29.4	26.2	61.7	90.1	53.7
	(19.9)	(17.8)	(25.9)	(60.6)	(33.2)
Sequence: CASCH (202-206)					
	20.6	9.00	20.8	40.6	36.0
	(13.2)	(10.2)	(13.5)	(38.7)	(23.0)
	C	L	A	C	H
SoxX					
Sequence: CASCH (62-66)					
	21.7	6.8	24.8	27.9	23.6
	(17.4)	(4.6)	(14.8)	(27.3)	(15.2)

*Accessibilities are in square angstrom, *Percent accessibilities are shown in parenthesis

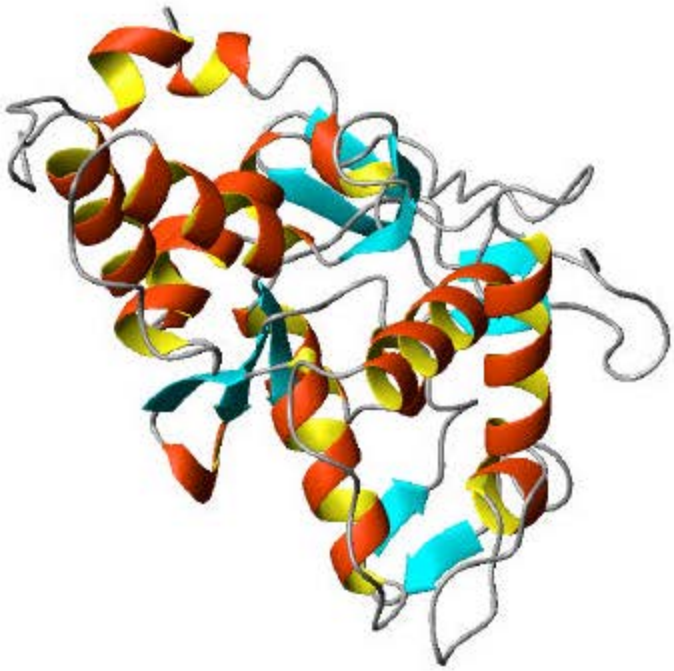

Fig. 3: Ribbon representation of modeled SoxA. -helices (Red and Yellow) and -sheets (Cyan) are shown as helices and ribbons, respectively. The rest are shown as loops. The figure was prepared by MOLSCRIPT (Kraulis, 1991)

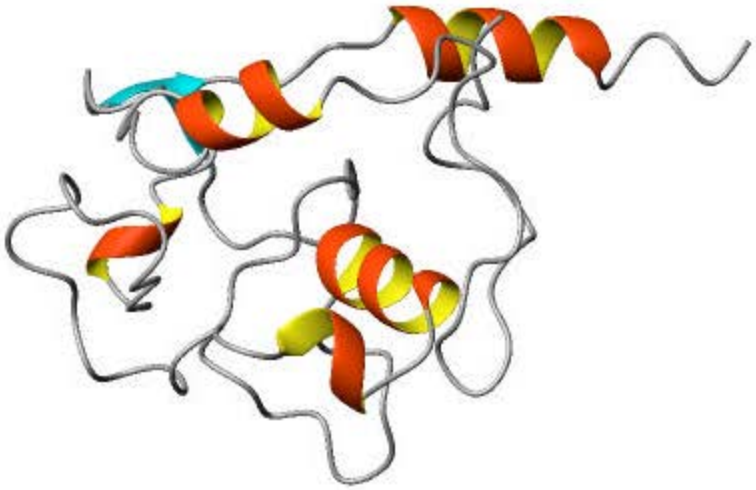

Fig. 4: Ribbon representation of modeled SoxX. -helices (Red and Yellow) and -sheets (Cyan) are shown as helices and ribbons respectively. The rest are shown as loops. The figure was prepared by MOLSCRIPT (Kraulis, 1991)

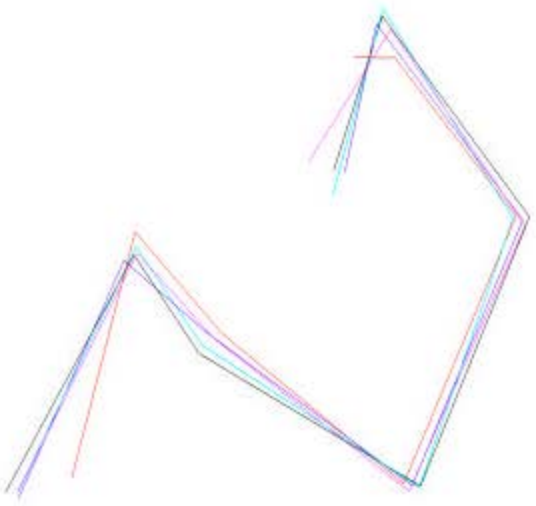

Fig. 5: Superimposition of the -carbon backbones of the cytochrome c motif of SoxX (red) on the similar motifs of 1C75 (violet), 1C52 (cyan), 1C53 (black) and CTJ (mauve). The mode of superimposition is similar for the cytochrome c motifs of SoxA; therefore only one is shown for clarity

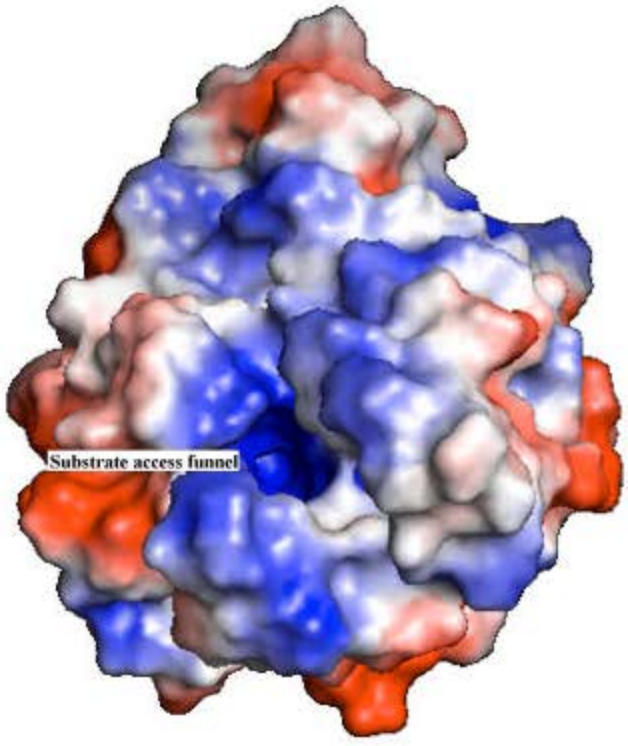

Fig. 6: Electrostatic potential map of the surface of the SoxAX complex showing the positively charged substrate active funnel

SoxA- Iron Porphyrine Interaction

In the crystal structure of 1H33 (A chain) the first heme group is covalently bound to the protein matrix through thioether bonds, between CysA76 and CysA114 Sγ atoms and the heme Cα positions 2 and 4, respectively (Bamford *et al*., 2002). Superimposition of the first cytochrome c motif of SoxA on the same motif of 1H33 produced an r.m.s deviation of 0.3 Å. The distance of the Sγ atoms of

Table 2: Residues of SoxA and SoxX involved in ionic interaction in the SoxAX complex

SoxA:	Arg194, Gln197, Asn199, His206, Asp208, Lys212, Arg215, Asp217, His218, Asn242, Arg243, Arg249, Asp250, Arg252, Glu254
SoxX:	Asn56, Arg57, Asn61, His66, Glu74, Gln75, His77, Lys104, Glu110, Asn124, Arg126, Lys127, Asp128, Lys132

Cys100 and Cys138 of SoxA (similar to the CysA76 and CysA114, respectively of 1H33) and the heme Cα atoms at positions 2 and 4, respectively are found to be 1.47 and 1.76 Å. This implies these groups are close enough to form covalent bonds with the protein matrix as observed in case of 1H33.

The crystal structure of 1H33 (A chain) also shows that the Fe atom of the heme group of the iron-porphyrine complex is coordinated to the Nε atom of HisA80 (Bamford *et al*., 2002). In SoxA the distance between the Nε atom of His104 and the Fe atom of the iron-porphyrine complex is 2.34, which is close enough to form a coordinate covalent bond with the Fe atom.

Similarly, for the second cytochrome c motif of SoxA, Cys202 and His206 are involved in the interactions with the iron-porphyrine complex analogus to the CysA177 and HisA181 in 1H33.

SoxX- Iron Porphyrine Interaction

In the crystal structure of 1H33 (B chain) the heme group is covalently bound to the protein matrix through thioether bonds, between CysB42 Sγ atom and MetB92 Sδ atom and the heme Cα positions 2 and 4, respectively (Bamford *et al*., 2002). The Fe atom of the Iron porphyrine complex is coordinated to the Nε atom of HisB46. Superimposition of the cytochrome c motif of SoxX on the similar motif of 1H33 produced an r.m.s deviation of 0.4 Å. In the SoxX-Iron porphyrine complex, Cys62, Met113 and His46 are found to be present at the identical positions.

Interaction of SoxA and SoxX

SoxA and SoxX are found to interact strongly with each other. The protein-protein interface is found to contain mainly the polar amino acid residues. The interior of the complex is made up of hydrophobic amino acids. There are extensive H-bonding interactions involving both the main and the side chains of the two protein molecules. The side chains of the polar amino acid residues of SoxA and SoxX are involved in H-bonding among themselves. The main chains of Gly196, Tyr213 and Ala253 of SoxA are found to form H-bond with the main chain of Ile123, Val80 and Ala64 of SoxX, respectively. The SoxAX complex is also stabilized by ionic interactions. The residues of the proteins involved in the formation of ion-pair are presented in Table 2. Out of the two cytochrome c motifs of SoxA only the His206 of the second cytochrome c motif of SoxA and Ala64 and His66 of the cytochrome c motif of SoxX are involved in the interaction. The main chain of Ala64 of the cytochrome c motif of SoxX forms H-bond with the side chain of Arg215 of SoxA. The side chain of His206 of SoxA is involved in ionic interaction with that of His66.

Probable Mode of Action of the SoxAX Complex in Oxidation of Sulfur Anions

According to the mechanism of sulfur anion oxidation, the SoxAX complex acquires electrons from the sulfur anions to facilitate the binding of sulfur anions to the SoxY protein of the SoxYZ complex. From the structural arrangement of SoxAX complex, it is easily notable that there is a highly positively charged cluster of basic amino acid residues lining a channel, which provides access to the anionic sulfur

substrates. As observed in other proteins with well-conserved cytochrome c motif (Bamford *et al*., 2002), SoxA too has an abundance of positively charged amino residues on the surface of the SoxAX complex. The catalytic channel is surrounded by the side chains of Arg215, Arg231, Asn233, Arg243 and Arg249 as observed in other well-characterized cytochrome c proteins (Bamford *et al*., 2002). From Table 1 it is evident that, the residues of the second cytochrome c motif of SoxA as well as the cytochrome c motif of SoxX are buried inside the SoxAX protein complex whereas the first cytochrome c motif of SoxA is more accessible. Formation of SoxAX complex would help to bring the two cytochrome c motifs (the second cytochrome c motif of SoxA and the cytochrome c motif of SoxX) in close proximity (14 Å). This closeness helps in the transport of electrons efficiently from one part of the protein to the other part. According to the mechanism of sulfur anion oxidation (Friedrich *et al*., 2001), the conserved cysteine residue at the N-terminal region of SoxY protein binds the sulfur anion to form SoxY-thiocysteine-S-sulfate, the first covalently bound sulfur adduct in the novel global sulfur anion oxidation cycle. When the model of SoxY protein (pdb code: 2CVN) was docked to SoxAX complex, it has been found that the N-terminal region of SoxY interacts strongly with the positively charged amino acids residues (viz., Arg215, Arg231, Asn233, Arg243 and Arg249) lining the catalytic channel. Therefore, it may be concluded that during the bio-geochemical sulfur oxidation reaction cycle, SoxAX complex first binds the sulfur anions via the positively charged substrate-binding channel on the surface of the SoxAX complex via polar interactions as found in case of 1H33 (Bamford *et al*., 2002). Then SoxY interacts with the SoxAX complex and removes the sulfur anion by making a covalent bond between the Sγ atom of the N-terminal conserved cysteine residue of itself and the sulfur atom of the sulfur anion. Since, the covalent bond strength is much more than polar interactions (Lee, 1991), SoxY would easily remove the sulfur anion from SoxAX complex to perform other steps of the reaction cycle.

CONCLUSIONS

In this study, we have tried to elucidate the structural basis of the involvements of SoxA and SoxX in the binding and in electron transport during oxidation of sulfur anions via the novel global sulfur oxidation cycle. We have described the three dimensional structures of the SoxA and SoxX proteins and established the geometry of the cytochrome c region and that of the active site of SoxAX complex. The docking studies with SoxA and SoxX have allowed us to identify the details of SoxA-SoxX interactions. The structural basis of the formation of the hetero-dimeric complex of SoxA-SoxX has also been demonstrated to predict the biochemical pathway of sulfur anion binding and transport of electrons via these proteins in the novel global sulfur cycle as SoxAX complex is the initiator of the sulfur anion oxidation pathway. Since there have been no previous reports regarding the structural biology of these proteins, results from this study will be important for the understanding of the three dimensional structures of SoxA and SoxX as well as to elucidate the structural basis of their mode of actions. Our model provides a rational framework for designing experiments aimed at determining the contribution of various amino acid residues in these proteins to predict the molecular basis of the interactions both among themselves as well as with various sulfur anions in these biologically as well as ecologically important micro-organisms.

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