Sulfur oxidation of *Paracoccus pantotrophus*: the sulfur-binding protein SoxYZ is the target of the periplasmic thiol–disulfide oxidoreductase SoxS

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The periplasmic thiol–disulfide oxidoreductase SoxS is essential for chemotrophic growth of *Paracoccus pantotrophus* with thiosulfate. To trap its periplasmic partner, the cysteine residues of the CysXaaXaaCys motif of SoxS (11 kDa) were changed to alanine by site-directed mutagenesis. The disrupted *soxS* gene of the homogenote mutant GB Ω S was complemented with plasmids carrying the mutated *soxS*[C13A] or *soxS*[C16A] gene. Strain GB Ω S(pRD179.6[C16A]^S) displayed a marginal thiosulfate-oxidizing activity, suggesting that Cys13^S binds the target protein. Evidence is presented that SoxS specifically binds SoxY. (i) Immunoblot analysis using non-reducing SDS gel electrophoresis and anti-SoxS and anti-SoxYZ antibodies identified the respective antigens of strain GB Ω S(pRD179.6[C16A]^S) at the 25 kDa position, suggesting an adduct of about 14 kDa, close to the value expected for SoxY migration. (ii) A mutant unable to produce SoxYZ, such as strain GB Ω X(pRD187.7[C16A]^S), did not form a SoxS(C16A) adduct, while addition of homogeneous SoxYZ resulted in the 25 kDa adduct. (iii) The SoxY and SoxZ subunits were distinguished by site-directed mutagenesis of the cysteine residue in SoxZ. SoxYZ(C53S) formed the 25 kDa adduct with SoxS(C16A). These results demonstrate that the target of SoxS is the sulfur-binding protein SoxY of the SoxYZ complex. As SoxYZ is reversibly inactivated, SoxS may activate SoxYZ as a crucial function for chemotrophy of *P. pantotrophus*.

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INTRODUCTION

The proteins that reconstitute the sulfur-oxidizing (Sox) enzyme system *in vitro* from the Gram-negative, neutrophilic, facultatively aerobic and facultatively chemolithoautotrophic thiosulfate-oxidizing alpha-proteobacteria *Paracoccus versutus* and *Paracoccus pantotrophus* have been characterized (reviewed by Kelly *et al.*, 1997; Friedrich *et al.*, 2005).

Four periplasmic proteins, SoxYZ, SoxB, SoxCD and SoxXA, reconstitute the Sox enzyme system *in vitro*, which oxidizes hydrogen sulfide, sulfur, thiosulfate and sulfite, and transfers the electrons to horse cytochrome *c*. The heterodimeric haem enzyme SoxXA binds the sulfur substrate and covalently links it to the thiol of the single cysteine110 residue of the SoxY subunit (10 977 Da) (Bamford *et al.*, 2002). With SoxZ (11 719 Da), SoxY forms the metal- and cofactorless SoxYZ complex. With sulfide as substrate, SoxY-cysteine persulfide is formed. The outer (sulfane) sulfur atom is oxidized by sulfane dehydrogenase SoxCD (formerly designated sulfur dehydrogenase) to sulfone, yielding SoxY-cysteine-S-sulfate. SoxCD is an $\alpha_2\beta_2$ tetramer of the molybdoprotein SoxC

(43 897 Da) and the dihaem cytochrome *c* SoxD (38 899 Da). Finally, SoxY-cysteine-S-sulfate is hydrolysed by the dimanganese SoxB protein (58 611 Da) to yield sulfate and to regenerate SoxY for a new reaction cycle of SoxYZ (Friedrich *et al.*, 2001, 2005).

The SoxYZ complex is redox-active, very likely via the carboxy-terminal Cys-110^Y, which is exposed and solvent-accessible, as is evident from its crystal structure (Sauvé *et al.*, 2007). Cys-110^Y accidentally forms interprotein disulfides to yield e.g. SoxY–Y(Z)₂ (Quentmeier *et al.*, 2007). This heterotetramer is catalytically inactive (A. Quentmeier, unpublished data), and the reduction of the interprotein disulfide would reactivate the SoxYZ complex.

The Sox proteins that catalyse sulfur oxidation *in vitro* are encoded by seven genes. However, the *sox* gene region of *P. pantotrophus* comprises 15 genes, *soxRSVWXYZA–H*, which are organized in three transcriptional units (Rother *et al.*, 2001). The *soxSR* genes encode the respective proteins and are transcribed divergently to *soxVWXYZA–H* with *soxR* being located downstream of *soxS*. SoxR, a repressor of the ArsR family, binds to two intergenic regions of the *soxVWXYZA–H* cluster (Rother *et al.*, 2005). The mature SoxS (99 aa, 11 077 Da) is a periplasmic thiol–disulfide oxidoreductase

Abbreviations: Sox, sulfur oxidation; ssDNA, single-stranded DNA.

and is essential for chemotrophic growth with thiosulfate, as is evident from the homogenote mutant *P. pantotrophus* GB Ω S. SoxS is not required for sulfur oxidation *in vitro*, as the Sox system of cell-free extracts of strain GB Ω S has identical activity to that of the wild-type. Genetic complementation of strain GB Ω S demonstrates that SoxS activates the Sox enzyme system *in vivo*. SoxS is not involved in the expression of the *sox* genes (Orawski *et al.*, 2007).

Thioredoxins act as antioxidants of other proteins by cysteine thiol–disulfide exchange. In the cytoplasm of *Escherichia coli*, two thiol redox systems exist: the thioredoxin and the glutaredoxin systems. Both prevent disulfide stress by reducing disulfide-bonded cytosolic proteins. The thioredoxins are reduced by respective reductases, which receive their electrons from NADPH. Glutaredoxin is reduced by glutathione oxidoreductase with glutathione as the source of electrons (reviewed by Ritz & Beckwith, 2002; Linke & Jakob, 2003). In the periplasm, thioredoxins or more specific thiol–disulfide oxidoreductases are reduced by membrane proteins, which deliver reductants via cysteine thiol–disulfide transporters. One such transporter is the DsbA/DsbB system, which plays a vital role in post-translational modifications in the folding, stability and activity of many secreted proteins (reviewed by Kadokura *et al.*, 2003; Nakamoto & Bardwell, 2004). Other systems, such as CcdA, are required for cytochrome *c* biogenesis (Thöny-Meyer, 1997; Bardischewsky & Friedrich, 2001a).

Interaction between a thiol–disulfide oxidoreductase and its redox partner has been demonstrated for the protein–disulfide-forming DsbA and its periplasmic partners by site-directed mutagenesis (Kishigami *et al.*, 1995). In the DsbA/DsbB system, DsbA oxidizes substrate cysteines to protein–disulfide, and the membrane-bound DsbB protein reoxidizes DsbA (Tapley *et al.*, 2007). One cysteine residue of the thiol–disulfide oxidoreductase CysXaaXaaCys motif interacts with a protein–disulfide and the other one reduces an intermediate state, which covalently links the thiol–disulfide oxidoreductase with its partner protein. Therefore, mutagenesis of each cysteine residue of SoxS, Cys-13^S and Cys-16^S will trap and identify the periplasmic partner of SoxS.

The cysteine–disulfide transporter SoxV of *P. pantotrophus* is a paralogue of CcdA and transfers electrons from the cytoplasm to the periplasm. SoxV is essential and specific for thiosulfate oxidation *in vivo* and is not involved in cytochrome *c* biogenesis or heavy metal resistance. Moreover, SoxV reduces the thioredoxin SoxW and very likely also SoxS. However, SoxW, in contrast to SoxS, is not essential for chemotrophic growth (Bardischewsky *et al.*, 2006; Orawski *et al.*, 2007).

The essentiality of SoxS for chemotrophic sulfur oxidation *in vivo* raises the question of the identity of its reaction partner. Here, we describe cysteine residue exchanges in the thioredoxin motif of SoxS and the SoxZ subunit of the SoxYZ complex, and their phenotypes for sulfur oxidation. The immunochemical and protein biochemical analyses identified the sulfur-binding protein SoxY as the redox partner of SoxS.

METHODS

Bacterial strains and plasmids. Strains and plasmids used and constructed in this study are listed in Table 1.

Media and growth conditions. *E. coli* were cultivated in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) at 37 °C. Strains of *P. pantotrophus* (Rainey *et al.*, 1999; Ludwig *et al.*, 1993; Robertson & Kuenen, 1983) were cultivated mixotrophically in mineral medium, pH 7.2, with 20 mM thiosulfate plus 20 mM disodium succinate at 30 °C (Bardischewsky & Friedrich, 2001b) or heterotrophically in mineral medium, pH 6.8, with 20 mM disodium succinate at 30 °C. Antibiotics were added where appropriate (for *E. coli* 100 µg ampicillin ml⁻¹, 25 µg chloramphenicol ml⁻¹, 50 µg kanamycin ml⁻¹ or 15 µg tetracycline ml⁻¹, and for *P. pantotrophus* 300 µg kanamycin ml⁻¹ and 5 µg tetracycline ml⁻¹).

DNA and gene transfer techniques. Standard DNA techniques were applied (Sambrook *et al.*, 1989). Plasmid DNA was isolated according to Kieser (1984). Restriction enzymes and T4 DNA ligase were obtained from Promega or Roche and used as recommended by the manufacturers. Single-stranded DNA (ssDNA) was produced with plasmid pRD175.2, a derivative of vector pGEM-11ZF+ (Promega) using the helper phage R408 (Promega) according to the instructions of the supplier. The amino acid exchanges in SoxS and SoxZ were performed with the GeneEditor *in vitro* site-directed mutagenesis system from Promega. For DNA sequencing (Sanger *et al.*, 1977), plasmid DNA was prepared with the Wizard Plus SV Miniprep kit (Promega). DNA was sequenced as described previously (Rother *et al.*, 2001). DNA was transformed into *E. coli* as described previously (Chung *et al.*, 1989). *E. coli* S17-1 (Simon *et al.*, 1983) was used to mobilize plasmids into *P. pantotrophus* GB17 according to Rother *et al.* (2001).

Site-directed mutagenesis. To establish the single amino acid exchanges Cys13Ala and Cys16Ala in SoxS, several cloning steps were required. (i) The hybridized oligonucleotides S50 5'-AATTCGGTACCGATATCCCCGGGG-3' and S51 5'-GATCCCCCGGGGATATCGGTACCG-3' introduced *Kpn*I and *Sma*I restriction sites into the vector pGEM-11ZF+ linearized with *Eco*RI and *Bam*HI, resulting in plasmid pRD174.2. (ii) Plasmid pJOEB9 (Rother *et al.*, 2005) harbouring *soxR'*SVWXYZABCDE' was restricted with *Kpn*I and *Sma*I, yielding two fragments of 11 081 bp and 445 bp in size. The 445 bp fragment with *soxS'* and *soxV'* was cloned into pRD174.2, yielding plasmid pRD175.2. This vector included the *soxS* region and was used for site-directed mutagenesis. Concomitantly with the selection oligonucleotide 5'-d(pCCGCGAGACCCACCCTTGAGGCTCCAGATTTATC)-3' of the GeneEditor *in vitro* site-directed mutagenesis system, the oligonucleotides S52 5'-d(pCGAACAGCCCGCGCCCTTTATTGCGCG)-3' for the Cys13Ala amino acid exchange and S53 5'-d(pCGGCTGCCTTTATGCCGCGCGCTGGGAC)-3' for the Cys16Ala amino acid exchange were hybridized with ssDNA of plasmid pRD175.2 at the same time. Mutations were verified by DNA sequencing of the relevant regions. The altered plasmids were designated pRD175.2mutS(C13A) and pRD175.2mutS(C16A). (iii) These plasmids were restricted with *Kpn*I and *Sma*I. The 445 bp mutated fragments were ligated with the *Kpn*I–*Sma*I restricted vector pJOEB9, resulting in plasmids pRD176.1(C13A) and pRD177.28(C16A). The sequence was analysed to exclude the possibility of accidental restoration of the initial situation of vector pJOEB9. (iv) pRD176.1 and pRD177.28 were digested with *Hind*III. The 8911 bp fragments with *soxR'*mutSVWXYZABCDE' were cloned in *Hind*III-restricted pVK101 (Knauf & Nester, 1982) yielding pRD178.7 and pRD179.6. Both plasmids correspond to pVKB9 (Rother *et al.*, 2001). The mutagenized *sox* genes were expressed in the homogenote strains GB Ω S (Rother *et al.*, 2005) and GB Ω X (Bardischewsky *et al.*, 2005).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype*	Reference
<i>E. coli</i> strains		
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB); F'[traD36 proAB⁺ lacI^f lacZΔM15]</i>	Yanisch-Perron <i>et al.</i> (1985)
BMH 71-18 <i>mutS</i>	<i>thi supE D(lac-proAB), [mutS: Tn10] F'[proAB lacI^f lacZ ΔM15]</i>	Kramer <i>et al.</i> (1984); Zell & Fritz (1987)
S17-1	<i>recA pro thi hsdS</i> , RP4- <i>tra</i> -functions <i>supE44</i>	Simon <i>et al.</i> (1983)
<i>P. pantotrophus</i> strains		
GB17	Sox ⁺	Rainey <i>et al.</i> (1999); Robertson & Kuenen (1983)
GBΩS	<i>soxS::ΩSox⁻</i>	Rother <i>et al.</i> (2005)
GBΩX	<i>soxX::ΩSox⁻</i>	Bardischewsky <i>et al.</i> (2005)
Plasmids		
pGEM-11Zf(+)	Cloning vector; Amp ^r , fl ori, <i>lacZ</i>	Promega
pJOEB9	<i>soxR'-soxE'</i>	Rother <i>et al.</i> (2005)
pRD139.1	pJOEB9 with a 3048 bp <i>SstI</i> deletion	This study
pRD140.2	3048 bp <i>SstI</i> <i>soxZ'ABC'</i> fragment in pUC19	This study
pRD140.2 <i>mutZ</i>	pRD140.2 with Cys53Ser in <i>soxZ'</i>	This study
pRD141.4	3048 bp <i>SstI</i> <i>soxZ' mutABC'</i> fragment in pRD139.1	This study
pRD142.1	8911 bp <i>HindIII</i> fragment <i>soxR' SVWXYZmutABCDE'</i> from pJOEB9 in pVK101	This study
pRD174.2	Hybridized oligonucleotides S50/S51 in pGEM-11Zf(+)	This study
PRD175.2	445 bp <i>KpnI-SmaI</i> fragment of pJOEB9 in RD174.2	This study
pRD175.2 <i>mutS_C13A</i>	pRD175.2 with C13A in <i>soxS'</i>	This study
pRD175.2 <i>mutS_C16A</i>	pRD175.2 with C16A in <i>soxS'</i>	This study
pRD176.1	445 bp <i>KpnI-SmaI</i> fragment of <i>soxS'</i> with C13A in pJOEB9	This study
pRD177.28	445 bp <i>KpnI-SmaI</i> fragment of <i>soxS'</i> with C16A in pJOEB9	This study
pRD178.7	8911 bp <i>HindIII</i> fragment <i>soxR'-soxE'</i> with C13A in <i>soxS</i> in pVK101	This study
pRD179.6	8911 bp <i>HindIII</i> fragment <i>soxR'-soxE'</i> with C16A in <i>soxS</i> in pVK101	This study
pRD187.7	608 bp <i>KpnI-BamHI</i> fragment of <i>soxS</i> with C16A in pRI1	This study
pRI1	Cm ^r , Sm ^r	Pfützner <i>et al.</i> (1998)
pUC19	Ap ^r , <i>lacZ</i>	Yanisch-Perron <i>et al.</i> (1985)
pVK101	Km ^r , Tc ^r , Tra ⁻ , Mob ⁺	Knauf & Nester (1982)
pVKB9	8851 bp <i>HindIII</i> fragment <i>soxR'-soxE'</i> in pVK101	Rother <i>et al.</i> (2001)
pVKB9C	8911 bp <i>HindIII</i> fragment <i>soxR' SVWXYZABCDE'</i> from pJOEB9 in pVK101, in opposite orientation	This study

*Sox, lithotrophic growth with thiosulfate; Tra, transfer of mobilizable plasmids; Mob, mobilizability.

To establish a Cys53Ser exchange in SoxZ, four cloning steps were required. (i) Plasmid pJOEB9 was restricted with *SstI*, yielding two fragments, of 8478 and 3048 bp. The 8478 bp fragment was religated after purification by elution from the gel, resulting in plasmid pRD139.1. (ii) The 3048 bp fragment carrying the *soxZ'ABC'* genes was integrated into the linearized pUC19 vector (Yanisch-Perron *et al.*, 1985), resulting in the 5734 bp plasmid pRD140.2. This plasmid was used for site-directed mutagenesis of SoxZ. Concomitantly with the selection oligonucleotide 5'-d(CCGCGAGACCCACCCTTGGA-GGCTCCAGATTTATC)-3', the oligonucleotide for the Cys53Ser exchange in SoxZ 5'-d(CACCCCGTTCAACTCCGAAGTGAAG-CGGTTGATG)-3' was hybridized with ssDNA of plasmid pRD140.2. The altered plasmid was designated pRD140.2*mutZ* and its correct sequence was verified. (iii) pRD140.2*mutZ* was restricted with *SstI*. The 3048 bp fragment carrying the mutation in *soxZ* was ligated with the *SstI*-restricted vector pRD139.1, resulting in the 11 526 bp plasmid pRD141.4. (iv) pRD141.4 was restricted with *HindIII*. The 8911 bp fragment with *soxR' SVWXYZmutABCDE'* was

cloned in *HindIII*-restricted pVK101 (Knauf & Nester, 1982), yielding pRD142.1. This plasmid corresponds to pVKB9C.

Cloning of *soxS* for expression in a *soxY*-free background. To identify possible unspecific SoxS adducts, the *soxS* gene was cloned for expression in a background lacking *soxY* expression. A 616 bp *soxS* fragment containing *soxS*(C16A) was isolated with the restriction enzymes *KpnI* and *BamHI* from plasmid pRD177.28. The resulting fragment was integrated into plasmid pRI1, resulting in plasmid pRD187.7 (Table 1). This plasmid was transformed into *E. coli* S17-1 and conjugated therefrom to strain GBΩX and for reference into strain GBΩS.

Preparation of periplasmic extracts. Periplasmic extract was prepared by osmotic shock of the cells according to the method of Crowe & Henco (1992). Cells from 200 ml cultures (about 100 mg protein) were harvested by centrifugation, washed once with 30 mM Tris/HCl, pH 8.0, and resuspended in 40 ml of the same buffer

containing 1 mM EDTA and 20 % (w/v) sucrose. Cells were stirred at room temperature for 10 min, collected by centrifugation (8000 g, 4 °C), and subjected to osmotic shock by resuspension and stirring (10 min, 0 °C) in 40 ml 5 mM MgSO₄. The cells were collected as described above. The supernatant (40 ml, 10 mg total protein) containing the osmotic shock fluid was stabilized by addition of Tris/HCl, pH 8.0, to a final concentration of 30 mM and of 1 µM PMSF. Proteins were concentrated to 30–40 mg ml⁻¹ using a cut-off of 10 kDa (Amicon Ultra-15 Centrifugal Filter Device).

Purification of SoxS antigens. Periplasmic extract (1 ml, 38 mg total protein) was subjected to Q-Sepharose chromatography (column diameter 1.6 cm, height 3 cm). Proteins were eluted in a step gradient of 0–0.40 M sodium chloride with 0.05 M intervals. Per step, 50 ml 25 mM BisTris/HCl buffer, pH 7.2, including 1 mM magnesium sulfate and 1 µM PMSF, was collected in five fractions (10 ml). SoxS antigens eluted at 0.20 M sodium chloride, which was designated the SoxS fraction. Under these conditions SoxYZ antigens eluted predominantly at 0.15 M sodium chloride.

Analytical procedures. The thiosulfate-dependent oxygen uptake rate of whole cells was determined with an oxygen electrode (Rank Brothers). The assay (3 ml) contained 50 µl cell suspension (OD₄₃₆=30) equivalent to 150 µg protein, and 100 µmol sodium/potassium phosphate buffer, pH 8.0. Reactions were started by addition of 30 µl 0.20 M sodium thiosulfate. One unit (U) of thiosulfate oxidation activity was defined as one micromole O₂ consumed per minute. The specific activity was expressed as U (mg protein)⁻¹.

Thiosulfate was quantified according to Sørbo (1957).

Proteins were denatured and separated by SDS-PAGE according to Laemmli (1970). Proteins were stained with Coomassie blue as described by Weber *et al.* (1972). Immunoblot analysis (Towbin *et al.*, 1979) was performed according to the 'semidry' procedure using the Multiphor electrophoretic system (Pharmacia) with polyclonal antibodies raised against SoxS and the SoxYZ complex.

To preserve protein disulfide bonds, samples were treated with SDS without 2-mercaptoethanol and without boiling. Proteins were separated by SDS-PAGE without 2-mercaptoethanol.

For *in vitro* SoxS adduct studies, homogeneous SoxYZ and SoxYZ(C53S) complexes were prepared and activated chemically prior to use according to Quentmeier *et al.* (2007).

RESULTS

Site-directed mutagenesis of SoxS

The absence of SoxS in strain GBΩS can be chemically complemented by reduction of the cells with DTT, which recovers the thiosulfate oxidation rate (Orawski *et al.*, 2007). Of the two reduced cysteine residues of the CysXaaXaaCys motif of thiol–disulfide oxidoreductases, one thiol binds to a disulfide of the target protein and forms an interprotein disulfide with the protein. The other thiol re-reduces this interprotein disulfide, releasing the target protein in the reduced state and the thiol–disulfide oxidoreductase in the oxidized state (Kadokura *et al.*, 2003). To identify the crucial cysteine residue that covalently links the thiol–disulfide oxidoreductase with its periplasmic partner, each of the two active site cysteine residues of SoxS was exchanged for L-alanine by two independent mutageneses. The plasmids pRD178.7[C13A]^S

and pRD179.6[C16A]^S carrying the respective mutations were conjugated into strain GBΩS. The resulting strains GBΩS(pRD178.7[C13A]^S) and GBΩS(pRD179.6[C16A]^S) were compared with GBΩS(pVKB9) carrying the intact *soxS* gene in *soxR'*SVWXYZABCDE'.

Anti-SoxS antibodies identified SoxS antigens at a size of 11 kDa in periplasmic extracts of the wild-type, the reference strain GBΩS(pVKB9), and in the two mutants with the Cys13Ala and Cys16Ala exchange in SoxS. SoxS antigens were absent in extracts of strain GBΩS (Fig. 1). The level of *soxS* expression in the wild-type GB17 was slightly less than that of strain GBΩS(pVKB9) and the mutants derived therefrom due to the plasmid copy number (Fig. 1).

Sox activity of strains with a C13A^S or C16A^S mutation

Under mixotrophic growth conditions thiosulfate-dependent oxygen uptake was started by the reference strain GBΩS(pVKB9) after the consumption of the heterotrophic substrate succinate, and this rate was maintained at a high level over time. The SoxS-negative strain GBΩS had only a low activity during transition from heterotrophic to chemotrophic metabolism, confirming previous results (Orawski *et al.*, 2007). Such low activity was also observed for strain GBΩS(pRD179.6[C16A]^S) (Fig. 2). However, strain GBΩS(pRD178.7[C13A]^S) transiently displayed about the same high activity as the reference strain GBΩS(pVKB9), declining sharply in the early stationary phase, in which *de novo* protein synthesis ceases and reductant is no longer available (Fig. 2). This phenotypic difference between the two mutants indicated a functional difference between Cys13 and Cys16 of SoxS.

Under heterotrophic growth conditions only traces of thiosulfate-dependent oxygen uptake were observed for strain GBΩSs and GBΩS(pRD179.6[C16A]^S). Again, strain GBΩS(pRD178.7[C13A]^S) had about the same activity as strain GBΩS(pVKB9) (data not shown), also suggesting a functional difference of the two cysteine residues.

Reduction of GBΩS cells with DTT restores the thiosulfate-oxidizing ability (Orawski *et al.*, 2007). As expected,

Fig. 1. Immunoblot analysis of SoxS antigens of periplasmic extracts. Lanes: 1, *P. pantotrophus* GB17; 2, GBΩS; 3, GBΩS (pRD178.7[C13A]^S); 4, GBΩS (pRD179.6[C16A]^S), 5, GBΩS (pVKB9).

Fig. 2. Thiosulfate-dependent oxygen uptake rates of SoxS mutants of *P. pantotrophus*. Strains were cultivated mixotrophically with succinate plus thiosulfate. ■, GBΩS; ◇, GBΩS (pRD178.7[C13A]^S); ○, GBΩS (pRD179.6[C16A]^S); ▲, GBΩS (pVKB9).

addition of DTT to the medium at the end of the heterotrophic growth phase restored and stabilized the thiosulfate oxidation rate of strain GBΩS-(pRD179.6[C16A]^S) to the same level as that of strain GBΩS (Fig. 3), confirming that the target of SoxS was chemically reduced.

In the stationary growth phase cells suffer from lack of reductant. Thus, these data suggested that the Sox system remained stable when reductant was available. In this sense the data were in accordance with the stably maintained activity under mixotrophic conditions, as sulfur metabolism supplies some reductant upon the onset of chemotrophic growth conditions.

SoxS adduct detection by anti-SoxS antibodies

Anti-SoxS antibodies identified SoxS antigens from periplasmic extracts of various mixotrophically cultivated *P. pantotrophus* strains. Expression of the *soxS* gene in the wild-type was low, and did not occur in strain GBΩS. In the complemented GBΩS strains, *soxS* expression was significantly higher (Fig. 4, lanes 3–5). A major band at 11 kDa and a faint band at 25 kDa were observed from the wild-type, strain GBΩS(pVKB9) and strain GBΩS(pRD178.7[C13A]^S). In contrast, in strain GBΩS-(pRD179.6[C16A]^S), a strong signal was present at 25 kDa and a minor one at 11 kDa (Fig. 4, lane 4). These results were in accordance with the physiological data and suggested that Cys13 of SoxS is the crucial residue for formation of a mixed disulfide. Since the mixed disulfide

Fig. 3. Thiosulfate-dependent oxygen uptake rates of *P. pantotrophus* GBΩS and GBΩS(pRD179.6[C16A]^S). Both strains were cultivated mixotrophically with succinate plus thiosulfate. After reaching stationary growth phase the cultures were split; one portion of each strain was untreated; □, GBΩS; ◇, GBΩS (pRD179.6[C16A]^S); the other portion was treated with DTT (1 mM final concentration); ■, GBΩS; ◆, GBΩS (pRD179.6[C16A]^S).

was most pronounced in extracts of strain GBΩS(pRD179.6[C16A]^S), this mutant was selected for further studies.

The size of the SoxS antigens (25 kDa, Fig. 5a, lane 1) did not exclude dimerization of SoxS (expected size 22 kDa). To examine accidental formation of SoxS dimers or adducts as a result of extract preparation, iodoacetamide,

Fig. 4. Immunoblot analysis of periplasmic extracts separated by SDS-PAGE without 2-mercaptoethanol. Lanes: 1, *P. pantotrophus* GB17; 2, GBΩS; 3, GBΩS (pRD178.7[C13A]^S); 4, GBΩS (pRD179.6[C16A]^S); 5, GBΩS (pVKB9).

Fig. 5. Immunoblot analysis of periplasmic extracts and partially purified SoxS[C16A]^S. Mutated SoxS[C16A]^S was purified by resource Q chromatography. Proteins (30 µg per well) were separated by SDS-PAGE without (a, c) and with 2-mercaptoethanol (b, d). SoxS was identified by anti-SoxS antibodies (a, b), and SoxYZ by anti-SoxYZ antibodies (c, d). Lanes (a, b): 1, periplasmic extract of GBΩS (pRD179.6[C16A]^S); 2, purified SoxS(C16A) (30 µg protein per well). Lanes (c, d): 1, 1.2 µg homogeneous SoxYZ 'as isolated'; 2, periplasmic extract of GBΩS (pRD179.6[C16A]^S); 3, purified SoxS(C16A).

a chemical that traps free thiols, was added to cells of strain GBΩS(pRD179.6[C16A]^S). However, no difference in size (25 kDa) or intensity was observed from immunoblots of extracts of this strain, indicating that the 25 kDa complex was not an artefact (data not shown). Moreover, treatment of SoxS with 10 mM hydrogen peroxide did not alter the size from 11 kDa (data not shown).

Chromatography of extracts of strain GBΩS(pRD179.6-[C16A]^S) on Q-Sepharose eluted SoxS antigens in the SoxS fraction at 0.20 M sodium chloride. From SDS-PAGE without reductant, anti-SoxS antibodies identified a major band at 25 kDa and minor bands at 26 and 11 kDa (Fig. 5a, lane 2). The minor signal at 11 kDa indicated that the adduct of about 14 kDa was split off to release SoxS. The minor signal at 26 kDa was not identified, and may represent an adduct whose interprotein disulfide was not solvent-accessible, or a situation in which SoxS was linked by a thioether bond that was not split upon reduction by 2-mercaptoethanol (see also Fig. 5b, lane 2).

SoxY adduct detection by anti-SoxYZ antibodies

Polyclonal anti-SoxYZ antibodies specific for SoxYZ antigens were used to identify SoxS adducts from periplasmic extracts of mixotrophically grown strain GBΩS(pRD179.6[C16A]^S). SDS-PAGE separated intense bands corresponding to SoxY (10 977 Da) and SoxZ (11 017 Da), and migrating at 12 and 16 kDa, respectively (Rother *et al.*, 2001). The 26 kDa band observed from the SoxS fraction of Q-Sepharose chromatography (Fig. 5c) was also present in purified SoxS preparations in which SoxS antigens were identified by anti-SoxS antibodies (Fig. 5a, lane 2).

From SDS-PAGE without 2-mercaptoethanol, anti-SoxYZ antibodies identified SoxYZ antigens from homogeneous

SoxYZ at 12, 16, 25, 29 and 32 kDa (Fig. 5c, lane 1). These signals represented the SoxY and SoxZ monomers and the SoxY–Y, SoxY–Z and SoxZ–Z dimers (Quentmeier *et al.*, 2003, 2007). From periplasmic extract of strain GBΩS(pRD179.6[C16A]^S), signals at 12, 16 and 25 kDa were present (Fig. 5c, lane 2). From the Q-Sepharose SoxS fraction a major band of 25 kDa and traces of the size of SoxY were observed (Fig. 5c, lane 3). The size of the SoxY–Y dimer was identical with that of the SoxS adduct. However, SoxS antigens were identified by anti-SoxS antibodies from Q-Sepharose chromatography and these reacted with specific anti-SoxYZ antibodies (Fig. 5c, lane 3). This result argued against dimerization of either SoxS or the SoxYZ subunits. Also, a dimeric SoxS would be expected to migrate at 22 kDa, while the dimers of SoxY and SoxZ migrate at 25, 29 and 32 kDa (Fig. 5c, lane 1; Quentmeier *et al.*, 2007). Thus, these experiments suggested that SoxY is the adduct of SoxS. To verify the antibody signals at 25 kDa, SoxS adduct formation was examined in strains unable to produce SoxYZ.

SoxS(C16A) adduct formation *in vivo* requires SoxYZ

Strain GBΩX is unable to express the *soxXYZA–H* genes (Bardischewsky *et al.*, 2005), while these genes are well expressed in strain GBΩS (Orawski *et al.*, 2007). Both strains were used as background to produce SoxS and to examine adduct formation. As expected, SDS-PAGE of periplasmic extracts of strain GBΩS(pRD187.7[C16A]^S) and SDS-PAGE with reductant of strain GBΩX(pRD187.7[C16A]^S) identified SoxS[C16A]^S antigens at 11 kDa (Fig. 6a). However, without reductant, formation of an adduct to SoxS with a mobility equivalent to 25 kDa was observed exclusively from strain GBΩS(pRD187.7[C16A]^S) and not from strain

Fig. 6. Immunoblot analysis of SoxS antigens in a SoxYZ-free strain. Periplasmic extracts of GBΩS(pRD187.7[C16A]^S) (lanes 1) and GBΩX(pRD187.7[C16A]^S) unable to produce SoxYZ (lanes 2) were separated by SDS-PAGE with (a) and without (b) 2-mercaptoethanol and with antibodies against SoxS (30 µg protein per well).

GBΩX(pRD187.7[C16A]^S) (Fig. 6b). This result suggested that SoxS was linked to a protein with a mobility equivalent to a size of about 14 kDa. The size of 25 kDa differed noticeably from 22 kDa, the mass of a SoxS dimer. Therefore, such dimer formation was not considered. The fact that no SoxS adduct was observed from the background of strain GBΩX restricted the candidates for adduct formation to the Sox proteins encoded by the transcriptional unit *soxX–H*. However, of these Sox proteins, none has the appropriate size or mobility, except SoxY and SoxZ (Rother *et al.*, 2001). Therefore, *in vitro* studies were performed to distinguish the two possibilities for adduct formation.

SoxS(C16A)-SoxY adduct formation *in vitro*

Periplasmic extract of strain GBΩX(pRD187.7[C16A]^S) did not contain SoxS antigens of a molecular mass of 25 kDa (Figs 6b, lane 2, and 7, lane 2). However, when this extract was mixed and incubated with homogeneous SoxYZ 'as isolated', SoxS antigens were observed at 25 kDa. Sodium sulfide (10 mM) activates SoxYZ 'as isolated' and causes a change in conformation (Quentmeier *et al.*, 2007). Therefore, SoxS adduct formation was examined with SoxYZ 'activated'. No difference in intensity of the 25 kDa adduct band was observed (Fig. 7, lanes 3 and 4).

SoxY migrates in SDS-PAGE at 12 kDa and SoxZ at 16 kDa, and both SoxY and SoxZ contain a single cysteine residue (Friedrich *et al.*, 2000). The size difference of 14 kDa for the adduct suggested SoxY but did not allow unequivocal differentiation between the two subunits as adducts. Therefore, SoxZ was subjected to site-directed mutagenesis to generate the exchange Cys53Ser. The

Fig. 7. Complementation of extracts of strain GBΩX(pRD187.7[C16A]^S) with purified SoxYZ and SoxYZ(C53S). Periplasmic extracts of GBΩX(pRD187.7[C16A]^S) were separated by SDS-PAGE without 2-mercaptoethanol. SoxS antigens were identified by immunoblot analysis with antibodies against SoxS on a native gel (30 µg protein per well). Lanes: 1, GBΩS(pRD187.7[C16A]^S); 2, GBΩX(pRD187.7[C16A]^S); 3, GBΩX(pRD187.7[C16A]^S) + 0.8 µg SoxYZ 'as isolated'; 4, GBΩX(pRD187.7[C16A]^S) + 0.8 µg activated SoxYZ; 5, GBΩX(pRD187.7[C16A]^S) + 0.8 µg SoxYZ(C53S).

cysteine residue in SoxZ is not conserved in sulfur-oxidizing bacteria (Friedrich *et al.*, 2008).

As expected, strain GBΩX(pRD140.2[C53S]^Z) grew with thiosulfate as the wild-type did. Also, in the reconstituted enzyme system homogeneous SoxYZ(C53S) had identical activity to that of SoxYZ (data not shown). Therefore, SoxYZ(C53S) was used to complement extracts of strain GBΩX(pRD187.7[C16A]^S). This complementation revealed an identical adduct formation of 25 kDa to SoxS(C16A) (Fig. 7, lane 5). These data demonstrated that SoxY was specifically linked to SoxS via an interprotein disulfide.

DISCUSSION

The thiol-disulfide oxidoreductase SoxS of *P. pantotrophus* is essential for keeping the Sox enzyme system in the active state, but SoxS does not participate in the sulfur-oxidizing reaction (Orawski *et al.*, 2007). This observation led to the search for the periplasmic target of SoxS. To trap the target we applied site-directed mutagenesis of the active site cysteine residues of SoxS. We here present cumulative evidence that the SoxY subunit of the SoxYZ complex is the specific target of SoxS.

First, site-directed mutagenesis identified C13^S as not being crucial for sulfur oxidation *in vivo* but beneficial for maintaining a high sulfur oxidation rate, while C16^S was essential. The lack of the thiol in SoxS(C16A) made the interaction abortive with respect to catalytic turnover, suggesting that C13^S forms a disulfide link with the periplasmic partner of SoxS (Fig. 8). Second, anti-SoxS

Fig. 8. Model of disulfide reduction by the thiol–disulfide oxidoreductase SoxS (a) and mixed disulfide formation of SoxS(C16A) with SoxY (b).

antibodies detected the 11 kDa SoxS at 25 kDa in periplasmic extracts. Reduction abolished the 25 kDa species, which identified the 25 kDa molecule as a mixed disulfide with a partner of about 14 kDa. Also, an intense 25 kDa signal was detected with anti-SoxYZ antibodies, which however coincided with the size of a SoxY–Y homodimer. Third, immunoblot analysis identified the 25 kDa protein as an adduct of a Sox protein to SoxS(C16A), as a molecule of this size was absent from cells unable to express the *SoxXYZA–H* genes. Of the Sox proteins, only SoxY exhibited a mobility equivalent to a size of ~14 kDa. Fourth, the 25 kDa SoxS(C16A) adduct was detected with anti-SoxS antibodies when homogeneous SoxYZ was added to extracts of a strain unable to produce SoxYZ. Finally, the 25 kDa adduct was also obtained when homogeneous SoxYZ(C53S) was mixed with extracts containing SoxS(C16A), which excluded SoxZ as adduct.

The active site of SoxYZ is Cys110, located at the carboxy terminus on a peptide swinging arm which facilitates the access of the sulfur substrate to the thiol and its oxidative link (Sauvé *et al.*, 2007; Quentmeier & Friedrich, 2001). This structural characteristic, however, also facilitates interprotein disulfide formation. As a consequence, two SoxY subunits are covalently bound via their Cys110 residues (Quentmeier *et al.*, 2007) to yield SoxY–Y, as also observed for the SoxY subunit of *Chlorobium limicola* (Stout *et al.*, 2007). Exposure of the active site thiol results in an interprotein disulfide bond between the SoxY subunits and yields the SoxY–Y(Z)₂ heterotetramer. Also, low-molecular-mass compounds that react with free thiols bind to SoxY (Quentmeier *et al.*, 2003). Consequently, oxidation of the Cys110 thiol other than by the linkage of a sulfur substrate inactivates SoxYZ and eliminates the protein from the chemotrophic sulfur-oxidizing reaction, and this results in a drastically (80 %) decreased sulfur-oxidizing activity *in vivo* as observed for strain GBΩS (Orawski *et al.*, 2007).

The thioredoxin SoxW is reduced by the cysteine disulfide transporter SoxV (Bardischewsky *et al.*, 2006). Very likely, SoxS is also reduced by SoxV. The 25 kDa SoxS(C16A) adduct was identified by anti-SoxS antibodies. This adduct was exclusively observed either when SoxYZ was formed by the cells or when SoxYZ was added to periplasmic extracts of the SoxS(C16A) mutant. Therefore, the reduction of SoxS can be regarded as a salvage reaction for reintegration of accidentally oxidized SoxY species into the reaction cycle for chemotrophic sulfur oxidation.

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