# Activation of the Heterodimeric Central Complex SoxYZ of Chemotrophic Sulfur Oxidation Is Linked to a Conformational Change and SoxY-Y Interprotein Disulfide Formation[†,§]

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ABSTRACT: The central protein of the four component sulfur *ox*idizing (Sox) enzyme system of *Paracoccus pantotrophus*, SoxYZ, carries at the SoxY subunit the covalently bound sulfur substrate which the other three proteins bind, oxidize, and release as sulfate. SoxYZ of different preparations resulted in different specific thiosulfate-oxidizing activities of the reconstituted Sox enzyme system. From these preparations SoxYZ was activated up to 24-fold by different reductants with disodium sulfide being the most effective and yielded a uniform specific activity of the Sox system. The activation comprised the activities with hydrogen sulfide, thiosulfate, and sulfite. Sulfide-activation decreased the predominant $\beta$-sheet character of SoxYZ by 4%, which caused a change in its conformation as determined by infrared spectroscopy. Activation of SoxYZ by sulfide exposed the thiol of the C-terminal Cys-138 of SoxY as evident from alkylation by 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid. Also, SoxYZ activation enhanced the formation of the $Sox(YZ)_2$ heterotetramer as evident from density gradient gel electrophoresis. The tetramer was formed due to an interprotein disulfide between SoxY to yield a SoxY-Y dimer as determined by combined high pressure liquid chromatography and mass spectrometry. The significance of the conformational change of SoxYZ and the interprotein disulfide between SoxY-Y is discussed.

Oxidation of reduced inorganic sulfur compounds to sulfate is a major reaction of the global sulfur cycle predominantly performed by prokaryotes. Neutrophilic chemo- and phototrophic bacteria harbor *sox* genes encoding the *s*ulfur *ox*idizing enzyme system (Sox) while acidophilic bacteria and Archaea harbor a completely different system (*1*, *2*). The periplasmic Sox enzyme system enables the Gram-negative bacterium *Paracoccus pantotrophus* to grow chemoautotrophically and to oxidize thiosulfate to sulfate (*3*). The Sox system is reconstituted *in vitro* by four proteins which together catalyze the oxidation of hydrogen sulfide, thiosulfate, polysulfide, and sulfite, and the electrons are transferred to horse cytochrome *c* as shown for the oxidation of thiosulfate [eq 1].

$$^{-}S{-}SO_3^{-} + 5\ H_2O + 8\ \text{Cyt}\ c^{3+} \leftrightarrow 2\ SO_4^{2-} + 8\ \text{Cyt}\ c^{2+} + 10\ H^{+} \quad (1)$$

The Sox enzyme system oxidizes inorganic sulfur compounds to sulfate without detectable intermediates. The sulfur substrate is covalently bound to the thiol of the single cysteine-138 residue of the SoxY protein (10 977 Da) which forms with SoxZ (11 719 Da) the metal and cofactorless heterodimeric SoxYZ complex (22 696 Da; *4*). Thiosulfate is oxidatively linked to SoxY by the heme enzyme SoxXA to yield SoxY-thiocysteine-S-sulfate [eq 2].

$$\text{SoxY-Cys-S}^{-} + {}^{-}S{-}SO_3^{-} + 2\ \text{Cyt}\ c^{3+} \leftrightarrow \text{SoxY-Cys-S-S}{-}SO_3^{-} + 2\ \text{Cyt}\ c^{2+} \quad (2)$$

SoxXA is a complex of the monoheme cytochrome *c* SoxX and the diheme cytochrome *c* SoxA, and the crystal structure of the complex has been resolved recently (*5*, *6*). SoxY-thiocysteine-S-sulfate is the substrate for the dimanganese SoxB protein (*7*), a sulfate-thioesterase which releases SoxY-cysteine persulfide and sulfate. The outer sulfur atom (sulfane) of SoxY-cysteine persulfide is then oxidized in a six electron yielding step to (sulfone) SoxY-cysteine-S-sulfate by the molybdoprotein cytochrome *c* complex sulfur dehydrogenase SoxCD (*8*). Finally, the sulfone moiety is hydrolyzed from SoxY-cysteine-S-sulfate by SoxB to regenerate the free sulfhydryl of Cys-138 of SoxY of the SoxYZ complex. The interaction of the SoxYZ protein with the various Sox proteins is summarized in Figure 1.

The non-sulfur reductant tris(2-carboxyethyl) phosphine (TCEP[1]) inhibits and inactivates at low concentrations the thiosulfate-dependent cytochrome *c* reduction rate of the reconstituted Sox enzyme system. Inhibition is evident from

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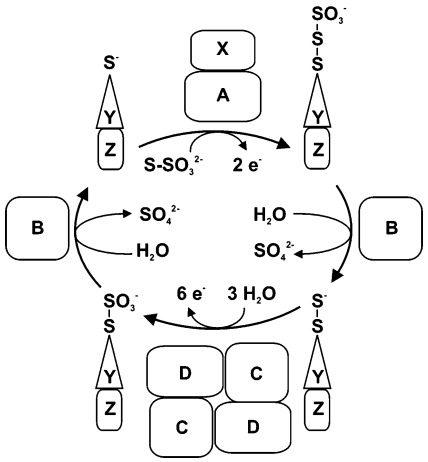


FIGURE 1: Model of the reaction cycle of thiosulfate oxidation by the Sox enzyme system of *P. pantotrophus* (*2*, altered, with permission. Copyright 2001 American Society for Microbiology). XA, heterodimeric cytochrome *c* complex SoxXA; B, dimanganese sulfate-thioesterase SoxB; YZ, sulfur-binding complex SoxYZ; CD, $\alpha_2\beta_2$-heterotetrameric SoxCD complex of the molybdoprotein SoxC and the cytochrome *c* SoxD.

the shift in thermodynamic equilibrium of the initial reaction to the left side (see eq 2). Inactivation of SoxYZ "as isolated" by TCEP is evident from the lowered constant reaction rate of the Sox enzyme system (*4*). Purification of SoxYZ resulted in various SoxYZ "as isolated" preparations which caused different specific activities of the reconstituted Sox enzyme system suggesting the presence of differently active forms of SoxYZ.

We here describe the specific activation of SoxYZ "as isolated" by disodium sulfide and other sulfur reductants. Sulfide-activation resulted in the exposure of the cysteine-thiol of SoxY and a conformational change which enabled interprotein disulfide formation to yield SoxY-Y as part of a heterotetramer Sox$(YZ)_2$. The significance of this finding is discussed.

## MATERIALS AND METHODS

*Strain, Cultivation, Extract, and Protein Preparation. P. pantotrophus* GB17 (*3*, *9*, *10*) was cultivated chemoautotrophically with thiosulfate under aerobic conditions at 30 °C. Mass cultivation was performed in a fermenter with a working volume of 220 L, and cells were harvested by continuous flow centrifugation and stored at −20 °C as described (*11*).

Cell free extracts were obtained with a French press from cells suspended in stabilizing buffer containing 2 mM thiosulfate, 1 $\mu$M phenylmethylsulfonyl fluoride, and 1 mM magnesium sulfate (*11*). The Sox proteins were purified to homogeneity after precipitation by ammonium sulfate, chromatography on Q Sepharose and phenylsepharose as described (*12*). SoxYZ was eluted from the Q Sepharose column with stabilizing buffer yielding a homogeneous preparation after concentration using ultrafiltration spin columns and chromatography on phenylsepharose as detailed previously (*12*). The "as isolated" proteins SoxYZ, SoxB, SoxXA, and SoxCD were homogeneous as judged from sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS−PAGE). The proteins were equilibrated against 25 mM sodium/potassium phosphate buffer, pH 6.5, containing 0.10 mM disodium thiosulfate and 1 mM magnesium sulfate, and stored at −20 °C.

*Sox Protein Activation.* Each of the Sox proteins was concentrated to 35 $\mu$M and was treated with a 0.10 M solution of disodium sulfide, -sulfite, -thiosulfate, dithiothreitol, 2-mercaptoethanol, or tris(2-carboxyethyl)phosphine to give a final concentration of 10 mM. Unless otherwise stated samples were incubated at 30 °C for 20 min, thereafter diluted 10-fold with 25 mM sodium/potassium phosphate buffer, pH 6.5, containing 0.10 mM disodium thiosulfate and 1 mM magnesium sulfate, and subsequently concentrated using ultrafiltration spin columns with a cutoff of 5000 Da (Vivaspin, Sartorius, Göttingen, Germany). The washing procedure was repeated three times, and 10 $\mu$L of the washed protein preparation was included to the enzyme assay (0.70 mL). The residual concentration of the chemical used for the initial treatment was less than 1.5 $\mu$M.

*Enzyme Assay.* The standard assay (0.70 mL) for inorganic sulfur-dependent cytochrome *c* reduction contained 50 mM BisTris/HCl buffer, pH 6.0, 70 $\mu$M horse cytochrome *c*, and 0.50 $\mu$M each SoxYZ, SoxXA, SoxB, and SoxCD if not otherwise stated. The reaction was started by addition of either 100 $\mu$M disodium thiosulfate (final concentration), 10 $\mu$M disodium sulfide, or 100 $\mu$M disodium sulfite (*12*). One unit (U) of activity of the Sox enzyme system is defined as 1 $\mu$mol of horse cytochrome *c* reduced per minute. The specific activity is expressed as U mg of total Sox protein$^{-1}$ with 0.50 $\mu$M of the four individual Sox proteins present in the assay.

Protein was determined according to Bradford (*13*).

*Infrared Spectrometry.* Absorbance spectra were obtained of 500 $\mu$M SoxYZ "as isolated" in 25 mM $KNaPO_4$ buffer, pH 6.5 in $D_2O$ and SoxYZ treated with 10 mM $Na_2S$ in $D_2O$, diluted, and concentrated by ultrafiltration spin columns as described above. The cuvette consisted of $CaF_2$ windows and was fixed to 8 $\mu$m path width. 10 times 256 interferograms were coadded at a resolution of 2 cm$^{-1}$ on an IFS 25 Bruker FTIR spectrometer. Second derivative spectra were calculated with the Bruker software OPUS (*14*−*17*). The relative contributions of the different secondary structure elements were obtained by multicomponent Gaussian fits, using 10 cm$^{-1}$ bandwidth. Secondary structure determination by means of infrared spectroscopy may have an error even if the fit is excellent. This occurs due to potential baseline drifts and the high number of components fitted. The respective experiments presented here and the observed shifts were fully reproducible.

*Thiol Titration.* Free thiols of SoxYZ were identified by chemical modification with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) according to Kobayashi et al. (*18*). Preparations of SoxYZ "as isolated" and SoxYZ "sulfide activated" were incubated with equal volumes of freshly prepared 50 mM Tris/HCl buffer, pH 7.5, containing 15 mM AMS, and 1% SDS. Proteins were separated by SDS−PAGE in the absence of reductants. SoxY was detected by immunoblot analysis as described (*19*).

*Protein Analytical Techniques.* The molecular masses of denatured proteins were determined by SDS−PAGE accord-

[1] Abbreviations: AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; DGGE, density gradient gel electrophoresis; DTE, dithioerythritol; DTT, dithiothreitol; ME, 2-mercaptoethanol; TCEP, tris(2-carboxyethyl)phosphine.

ing to Laemmli (*20*). The size of native proteins was determined by density gradient gel electrophoresis (DGGE) according to Andersson et al. (*21*). Proteins were stained with Coomassie blue (*22*).

*HPLC/MS Spectrometry.* HPLC/MS analyses were performed using an ion trap mass spectrometer (LCQ advantage MAX, Thermo Electron, Bremen, Germany) equipped with an electrospray ion source and a HPLC system consisting of a binary pump, autosampler, and DAD detector (Agilent 1100 series, Agilent, Waldbronn, Germany). Separations were carried out using a Jupiter C4 column (300 Å, 5 $\mu$m, 150 × 4.6 mm, Phenomenex, Aschaffenburg, Germany). Proteins were analyzed using the following conditions: the flow rate was 1 mL/min split behind the HPLC column to ca. 100 $\mu$L/min for MS and ca. 900 $\mu$L/min for UV detection; eluent A was water containing 0.1% (v/v) formic acid, eluent B acetonitrile containing 0.1% (v/v) formic acid. Gradient conditions were 0−2 min isocratic 32% B, 2−46 min 32−50% B increasing linearly. The injection volume was 10 $\mu$L per injection. The UV signal was monitored at 210 nm; electrospray mass spectra were performed in positive ion mode. For deconvolution of the spectra the BioBrowser software (Thermo Electron, Bremen, Germany) was used. The accuracy of the mass determination was 0.02%.

## RESULTS AND DISCUSSION

*Activation of SoxYZ "as Isolated".* The specific thiosulfate-oxidizing activity was determined for the Sox enzyme system as reconstituted with equimolar concentration of 0.50 $\mu$M of the homogeneous "as isolated" proteins SoxYZ, SoxXA, SoxB, and SoxCD which were kept in stock solutions of 35 $\mu$M. The specific activity of the reconstituted Sox enzyme system differed for various independent preparations of the proteins from *P. pantotrophus* up to 5-fold (data not shown). This discrepancy suggested the isolation of at least one of the Sox proteins in a less active form as the bottleneck of the overall reaction of the reconstituted Sox enzyme system. This conclusion led us to search for conditions to activate each of the Sox proteins.

Thiosulfate (2 mM) is required to stabilize the Sox proteins during purification and is present in the final preparation (0.10 mM) to stabilize the homogeneous proteins during storage (*23*). Enzyme substrates not only may stabilize but also may activate the respective enzymes. Therefore, SoxYZ "as isolated" was incubated with 10 mM of disodium thiosulfate, -sulfide, and -sulfite. These sulfur compounds not only are enzyme substrates but also represent reductants of various redox potentials (Table 1). Consequently, SoxYZ "as isolated" was also treated with 10 mM of various nonsubstrate reductants like dithiothreitol (DTT), dithioerythritol (DTE), 2-mercaptoethanol (ME), dithionite, L-cysteine, and the non-sulfur reductant TCEP and titanium(III) citrate. It should be noted that the reductants were routinely removed from the SoxYZ preparation after pretreatment.

The specific activity of the Sox enzyme system (with thiosulfate as substrate) increased 24-fold from 0.011 to 0.266 U mg of protein$^{-1}$ when reconstituted with SoxYZ pretreated with disodium sulfide. Since the increase in activity did not depend on the presence of sulfide, we concluded that SoxYZ was activated. The degree of activation depended on the reductant and decreased in the sequence sulfide > DTT > DTE > sulfite > ME > thiosulfate. Pretreatment of SoxYZ with L-cysteine, TCEP, dithionite, and titanium citrate decreased the activity (Table 1). The degree of activation/inactivation of the various chemicals differed with the activity of the SoxYZ "as isolated" preparation. However, activation of SoxYZ by sulfide led to high almost identical specific activities of the Sox enzyme system (data not shown). A slight activation of "enzyme A" of the thiosulfate-oxidizing system of *Paracoccus versutus* (formerly *Thiobacillus versutus* (*3*)) by sulfite was concluded from enzyme kinetics of thiosulfate oxidation of this strain (*24*).

Table 1: Effect of Pretreatment of SoxYZ with Chemicals with Different Redox-Potentials on the Specific Thiosulfate-Oxidizing Activity of the Sox Enzyme System[a]

| chemical for pretreatment of SoxYZ | rel act. of the Sox enzyme system | redox couple | redox potential (mV) | ref |
|---|---|---|---|---|
| none | 1.0 | | | |
| thiosulfate | 2.1 | $S_4O_6^{2-}/S_2O_3^{2-}$ | +24 | (*31*) |
| sulfide | 24.2 | $S^0/S^{2-}$ | −270 | (*31*) |
| L-cysteine | 0.4 | -S-S-/2 Cys | −250 | (*32*) |
| dithioerythrol | 5.7 | -S-S-/2[-S$^-$] | −330 | (*33*) |
| dithiothreitol | 9.8 | -S-S-/2[-S$^-$] | −330 | (*33*) |
| 2-mercaptoethanol | 2.4 | -S-S-/2[-S$^-$] | −154 | (*34*) |
| TCEP | 0.8 | $(R)_3$-P═O/$(R)_3$-P | unknown | (*35*) |
| sulfite | 3.4 | $SO_4^{2-}/SO_3^{2-}$ | −516 | (*32*) |
| dithionite | 0.5 | $SO_2^-/HSO_3^-$ | −660 | (*36*) |
| Ti(III) citrate | 0.4 | Ti(II)/Ti(III) | −700 | (*32*) |

[a] The specific activity of the reconstituted Sox enzyme system with SoxYZ "as isolated" was 0.011 U mg of protein$^{-1}$. SoxYZ "as isolated" was pretreated with the respective reductants for 20 min at 30 °C and washed three times. This preparation was used to reconstitute the Sox enzyme system with the other "as isolated" Sox proteins to give a final concentration of each Sox protein of 0.50 $\mu$M

The activation of SoxYZ "as isolated" exhibited a sigmoidal dependency on the sulfide concentration. Already 0.5 mM sulfide activated the protein notably and the maximum activation was achieved at 5 mM (Figure 2A). The degree of activation depended not only on the concentration but also on the duration of exposure, and was completed after 5 min at a concentration of 10 mM disodium sulfide (Figure 2B).

The activities of the Sox enzyme system differed when reconstituted by SoxYZ "as isolated" from different protein preparations. Batches with low activity were highly activated by sulfide while in batches with relatively high activity the degree of activation was less (Table 1, Figure 2A and 2B). The comparison of the different SoxYZ "as isolated" preparations showed that the subsequent activation with sulfide finally led to a uniformly active reconstituted Sox enzyme system.

To examine the specificity of activation of SoxYZ the Sox proteins were freshly purified to homogeneity from chemoautotrophically cultivated cells. Each of the four "as isolated" Sox proteins was separately pretreated by sulfide as the most activating reductant of SoxYZ. The pretreated Sox protein was then combined with the other untreated "as isolated" Sox proteins to reconstitute the system, and the activities with sulfide, thiosulfate, and sulfite were examined.

Using the four "as isolated" Sox proteins the specific sulfide-oxidizing activity of the reconstituted Sox enzyme system was 0.136 U mg protein$^{-1}$. The specific activities of the system with SoxYZ "sulfide-treated" and sulfide, thio-

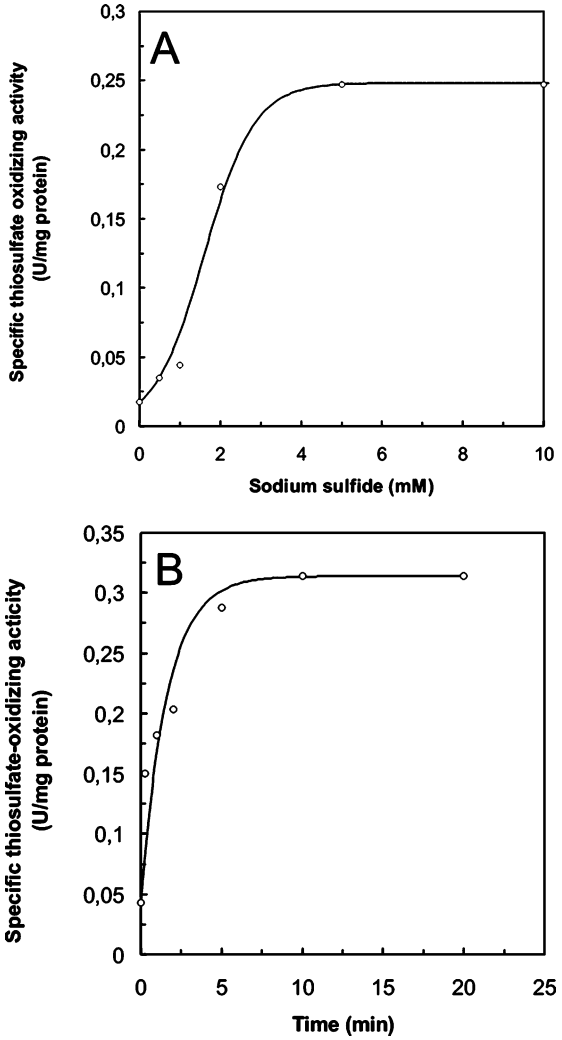


FIGURE 2: Activation of SoxYZ "as isolated" by disodium sulfide. (A) SoxYZ "as isolated" (35 $\mu$M) was incubated with different concentrations of disodium sulfide for 20 min at 30 °C. Thereafter, sulfide was removed from the enzyme as described in the Materials and Methods section. (B) For the time-course of activation of SoxYZ "as isolated" (100 $\mu$L; 35 $\mu$M) 10 $\mu$L of 100 mM disodium sulfide was added. The activation was stopped by dilution with 0.45 mL of phosphate buffer, pH 6.5, and subjected to 20-fold concentration using ultrafiltration spin columns. The dilution−concentration procedure was repeated 3 times to give a final concentration of 35 $\mu$M SoxYZ. The resulting SoxYZ preparation was reconstituted with the "as isolated" proteins SoxXA, SoxB, and SoxCD at 0.5 $\mu$M for each protein, and the thiosulfate-dependent cytochrome *c* reduction was determined as expressed as specific activity of the Sox enzyme system.

sulfate, and sulfite as enzyme substrates were increased by a factor of 4−6. Pretreatment with sulfide of SoxXA and SoxB had little effect on the activities with the three substrates. However, the activity of the system with SoxCD "sulfide-treated" with sulfide and thiosulfate was notably reduced to 51% and 69%, respectively (Table 2). It is noteworthy that the calculated specific activities and their ratios (sulfide:thiosulfate:sulfite = 0.136:0.064:0.027) were almost identical to those reported previously (*23*). Although SoxCD was susceptible to pretreatment with sulfide, the sulfite oxidation rate was not notably affected. In fact, SoxCD is not required for sulfite oxidation (*23*), and the latter observation confirms that the molybdoprotein cytochrome *c*

Table 2: Specific Sulfide-, Thiosulfate-, and Sulfite-Dependent Activities of the Sox Enzyme System Reconstituted with One Sox Protein Pretreated with Disodium Sulfide

| Sox protein pretreated with 10 mM $Na_2S$[a] | specific activity of the reconstituted sox enzyme system cytochrome *c* reduction (U mg of protein$^{-1}$) | | |
|---|---|---|---|
| | sulfide | thiosulfate | sulfite |
| none | 0.136 | 0.064 | 0.027 |
| SoxYZ | 0.518 | 0.345 | 0.160 |
| SoxXA | 0.109 | 0.058 | 0.022 |
| SoxB | 0.091 | 0.060 | 0.020 |
| SoxCD | 0.069 | 0.044 | 0.023 |

[a] The Sox proteins were pretreated as described in the Materials and Methods section and added to the other three "as isolated" Sox proteins to reconstitute the enzyme system. The final concentration of each Sox protein in the assay was 0.50 $\mu$M.

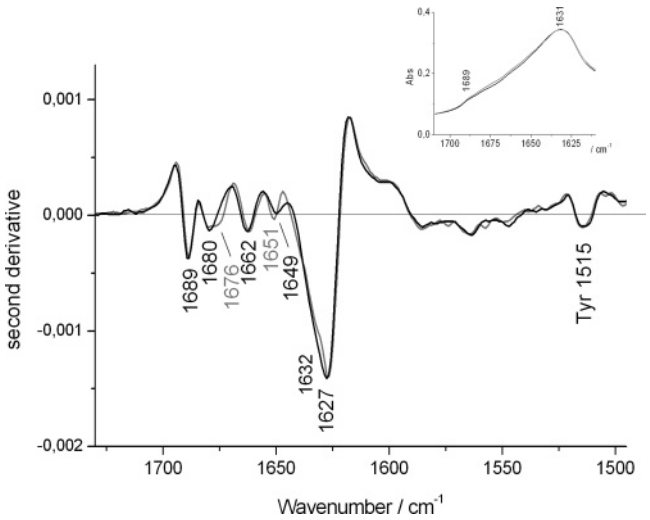


FIGURE 3: Infrared spectroscopy of SoxYZ "as isolated" in $D_2O$. The experimental conditions are detailed in the Materials and Methods section. Second derivative of the absorption spectra of SoxYZ "as isolated" (black line) and treated with 10 mM $Na_2S$ (gray line). The inset shows the absorbance spectra.

complex SoxCD is a paralogue of sulfite dehydrogenase and not involved in sulfite oxidation (*25*).

The proteins which reconstitute the Sox enzyme system are (except SoxB; P. Hellwig, unpublished data) redox active while only SoxYZ does not contain a metal or redox center (reviewed in refs *1*, *2*). SoxYZ was activated by sulfide while the other metal- and redox-centers containing Sox proteins were not activated or even slightly inactivated (Table 2). Intriguingly, the activation of SoxYZ was stably maintained after removal of sulfide or the other reductants (Table 1) and upon storage at room temperature (data not shown). These results suggested a conformational change with a high activation energy and a rearrangement of the secondary or tertiary structure of SoxYZ. Therefore, the secondary structure of SoxYZ "as isolated" and SoxYZ "sulfide-treated" was examined by infrared spectroscopy.

*Infrared Spectroscopy and Secondary Structures of SoxYZ.* The amide I signal of proteins in the mid infrared is an established tool for the determination of the secondary structure of proteins (*14*−*16*). For a clearer analysis of the broad and unspecific amide I peak, that includes the contributions of the C═O vibrational modes of the proteins backbone, we used a Gaussian multipeak fit and second derivative spectra. Figure 3 displays the second derivative spectra of the SoxYZ "as isolated" (black line) and SoxYZ

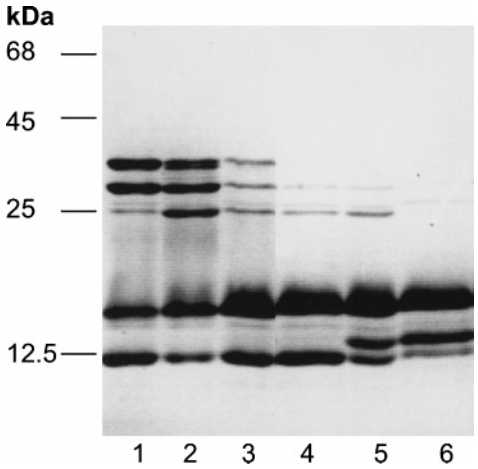


FIGURE 4: Alkylation of SoxYZ by AMS. Samples of SoxYZ were alkylated as described in the Materials and Methods section. Proteins were separated by SDS−PAGE, and 2 $\mu$g was added per well. Lane 1, SoxYZ "as isolated"; 2, "sulfide-treated" (10 mM disodium sulfide); 3, "TCEP-treated" (1 mM TCEP). Samples were chemically modified by AMS as described in the Materials and Methods section. Lane 4, SoxYZ "as isolated" plus AMS; 5, SoxYZ "sulfide-treated" plus AMS; 6, SoxYZ "TCEP-treated" plus AMS. Protein was stained with Coomassie blue.

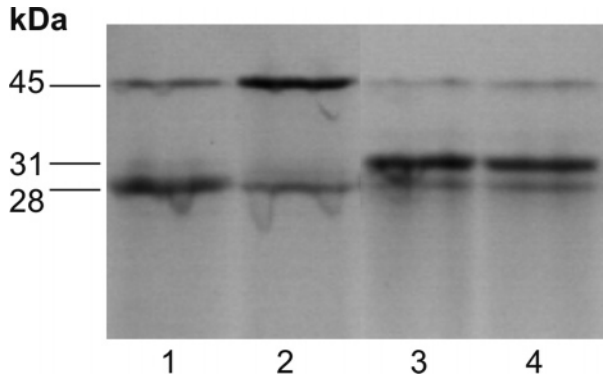


FIGURE 5: Density gradient gel electrophoresis of SoxYZ. Samples of different forms of SoxYZ (2 $\mu$g) were added per well. Lane 1, SoxYZ "as isolated"; 2, SoxYZ "sulfide-treated"; 3, SoxYZ "TCEP-treated"; 4, SoxYZ "sulfide-TCEP-treated". Protein was stained with Coomassie blue.

"sulfide-treated" (gray line) as obtained from the absorbance spectra shown in the inset. The data were normalized on the contribution of the tyrosines, at 1515 $cm^{-1}$. This signal included the ring mode of the protonated tyrosines and was found unperturbed upon pretreatment with sulfide. The overall reorganization of the protein concerns about 7% of the amide I signal.

The infrared spectra showed two dominant negative contributions at 1689 and 1680 $cm^{-1}$ specific for $\beta$-turn and antiparallel $\beta$-sheet. The signal at 1662 $cm^{-1}$ included loops, turns, and a small fraction of α-helical elements. At 1651 $cm^{-1}$ typically contributions from unordered and α-helical structural elements were involved. The broad feature at 1627−1635 $cm^{-1}$ can be attributed to $\beta$-sheet secondary structures. From SoxYZ "sulfide-treated" two clear changes occur as compared to SoxYZ "as isolated": signals arose at 1676 $cm^{-1}$, at a position specific for turns, and at 1651 $cm^{-1}$, where unordered and α-helical elements can be expected. Concomitant with the new signals, the peak at 1632 $cm^{-1}$, a position characteristic for $\beta$-sheet, decreased (Figure 3). On the basis of the Gaussian fit to the amide I band, the signals were quantified, indicating a decrease of the $\beta$-sheet character of the sample from 63 to 59% upon treatment with $Na_2S$ in the main peak at 1632 $cm^{-1}$. The contributions of unordered and α-helical elements increased from 2.3 to 3.5% and from 18.3 to 19.6% as fitted at 1646 and 1655 $cm^{-1}$, respectively. Finally the TCEP-pretreated samples were examined; however, upon exposure to TCEP no major shifts indicating conformational changes could be detected.

In summary, conformational changes upon pretreatment of SoxYZ with sulfide can be concluded, that are accompanied by some loss of the $\beta$-sheet character of the secondary structure. We suggest that these structural rearrangements play a role in the activation of the catalytic activity described above.

*Alkylation of SoxYZ by AMS.* The catalytic site of SoxY is the thiol of Cys-138. Since activation by sulfide resulted in a conformational change, the consequence for the active site was examined by alkylating free thiols with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS). Alkylation of free thiols of proteins by AMS resulted in upshifts of the molecular mass by 536 Da (*26*, *27*). SoxYZ "as isolated" and SoxYZ "sulfide-treated" were incubated with and without AMS, and the subunits were separated by SDS−PAGE without reductant to preserve disulfide bonds. As reported previously the subunits of SoxYZ "as isolated" appeared at a size of 12 kDa (SoxY) and 16 kDa (SoxZ). Also different dimers, SoxZ-Z (32 kDa), SoxY-Z (29 kDa), and traces of SoxY-Y (25 kDa) were separated (*19*). Note that besides SoxY also SoxZ has a cysteine residue which, however, is not conserved but may contribute to formation of dimers and to activation or inactivation as the case may be. In the SoxYZ "sulfide-treated" sample the SoxY-Y species was significantly enhanced at the expense of the free SoxY subunit (Figure 4, lane 2). In the SoxYZ "TCEP-treated" sample only faint bands of the dimers and a slight upshift of SoxZ were observed (Figure 4, lane 3). AMS-treated SoxYZ "as isolated" did not result in alkylation of SoxY, and no SoxZ-Z and no SoxY-Z were formed (Figure 4, lane 4). This suggested that the thiol of SoxZ was quantitatively alkylated. The missing upshift of SoxY suggested that the thiol of SoxY was not accessible to alkylation by AMS. However, in the SoxYZ "sulfide-treated" sample a strong band appeared at 12.5 kDa suggesting the SoxY−AMS adduct as the band of the SoxY subunit decreased concomitantly in intensity (Figure 4, lane 5). The data suggested that sulfide changed the conformation of SoxYZ to expose the thiol of Cys-138 of SoxY. Also, in the SoxYZ "TCEP-treated" sample a strong band appeared at 12.5 kDa suggesting the SoxY−AMS adduct (Figure 4, lane 6). Since the exposure of the thiol of Cys-138 was observed with sulfide- and with TCEP-treated SoxYZ, the activation of the protein could not be assigned to this fact. Therefore, in addition to the chemical modification and analysis of inactivated proteins by SDS−PAGE we used DGGE to analyze sulfide-activated and TCEP-inactivated SoxYZ in the native state.

*SoxYZ Analysis by DGGE.* A previous analysis of SoxYZ "as isolated" by DGGE revealed two heterodimeric forms and a heterotetrameric form (*12*). Since the significance of these forms was unknown, activated and inactivated SoxYZ was re-examined by DGGE. Surprisingly, a shift from a dimer of 28 kDa to a tetramer of 45 kDa was observed in

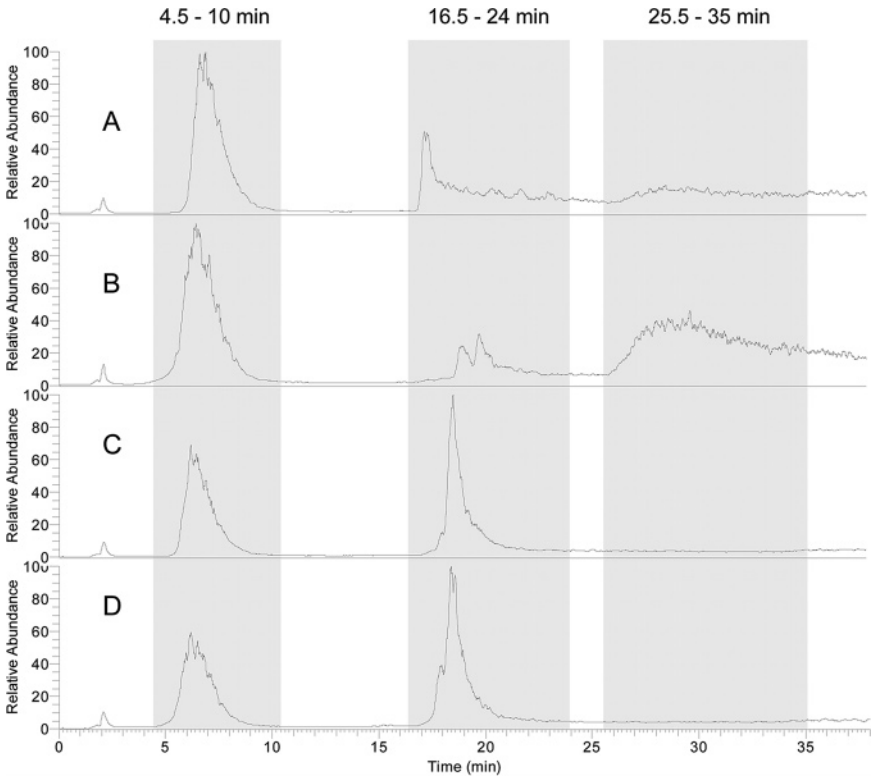


FIGURE 6: Chromatograms of different forms of SoxYZ after HPLC chromatography. The elution profile is given as total ion current. Parts A−D show the chromatograms of differently treated SoxYZ. A, SoxYZ "as isolated; B, SoxYZ "sulfide-treated; C, SoxYZ "TCEP treated"; D, SoxYZ "sulfide-treated and TCEP treated".

the SoxYZ "sulfide-treated" sample (Figure 5, lane 2). However, treatment by TCEP shifted the SoxYZ "TCEP-treated" and SoxYZ "sulfide/TCEP-treated" samples from the 28 kDa to the 31 kDa form while the tetrameric form decreased in intensity (Figure 5, lanes 3 and 4). From these data in combination with those of IR spectrometry it was concluded that sulfide-activation of SoxYZ resulted in a conformational change which exposed the C-terminus. These two changes facilitated the formation of a heterotetramer. In contrast, although inactivation by TCEP exposed the C-terminus, no conformational change was observed with IR spectroscopy. Instead, TCEP altered the dimeric form which did not allow the formation of a tetramer and which was little active although the C-terminus was exposed.

*Identification of SoxY-Y dimers by HPLC/MS.* Since the heterotetrameric form was enhanced in the sulfide-activated preparation of SoxYZ, it was of interest if the formation was crucial for activity or accidental. Samples were analyzed of SoxYZ "as isolated", SoxYZ "sulfide-treated", SoxYZ "TCEP-treated", and SoxYZ activated and subsequently inactivated, designated "sulfide-/TCEP-treated". These samples were analyzed on a high performance liquid chromatography reversed phase column which separates associated subunits but not covalently bound subunits. Eluted proteins were detected online by electrospray mass spectrometry. SoxYZ "as isolated" was eluted in three main portions. In all cases the first peak eluting between 4.5 and 10 min (Figure 6) was identified as SoxZ by its molecular mass of 11 719 Da (data not shown). The second portion eluted in peaks between 16.5 and 24 min with minor masses of 11 022 and 11 056 Da and a major mass of 11 095 Da representing the mass of SoxY (10 977 Da) +45, +79, and +118 Da. These masses were assigned as oxidized SoxY (+48 Da, either cysteate-138 or Cys-138-persulfide plus methionine sulfoxide), addition of sulfite (+80 Da), and an unknown addition. Addition of thiosulfate would be +112 Da (data not shown). The HPLC chromatogram of SoxYZ "sulfide-treated" shows a double peak between 18 and 21 min (Figure 6B). The first part was identified as SoxY with 10 976 Da (deduced mass 10 977 Da) and sodium adducts of SoxY. The second part was identified as a mixture of SoxY and SoxY with bound sulfur (11 008 Da). The HPLC chromatograms of SoxYZ "TCEP-treated" (Figure 6C) and SoxYZ "sulfide-/TCEP-treated" (Figure 6D) showed between 16.5 and 24 min just one peak at about 18 min identified as SoxY (10 977 Da; data not shown). These results confirmed a previous report (*4*).

The HPLC chromatograms show a third retention area between 25.5 and 35 min. Untreated SoxYZ had a rather weak peak in this area (Figure 6A) containing proteins with molecular masses of 21 950 Da, 21 980 Da, 22 113 Da, 22 190 Da, and 22 235 Da (Figure 7A). The first mass suggested a SoxY dimer, probably linked by a covalent interprotein disulfide (theoretical mass 21 952 Da; eq 3). The 21 980 Da protein could be assigned as a SoxY-Y-dimer + 32 Da, the mass of a sulfur atom. The SoxY-Cys-persulfide is present in SoxYZ preparations (data not shown; *4*), and this species is likely to be linked with SoxY to yield an interprotein thiodisulfide (theoretical mass 21 984 Da; eq 4) or interprotein perthiodisulfide (theoretical mass 22 016 Da; eq 5). Other masses were not identified.

$$2\ \text{SoxY-SH} \leftrightarrow \text{SoxY-S-S-Y} + 2\ [\text{H}] \quad (3)$$

$$\text{SoxY-S-SH} + \text{SoxY-SH} \leftrightarrow \text{SoxY-S-S-S-Y} + 2\ [\text{H}] \quad (4)$$

$$2\ \text{SoxY-S-SH} \leftrightarrow \text{SoxY-S-S-S-S-Y} + 2\ [\text{H}] \quad (5)$$

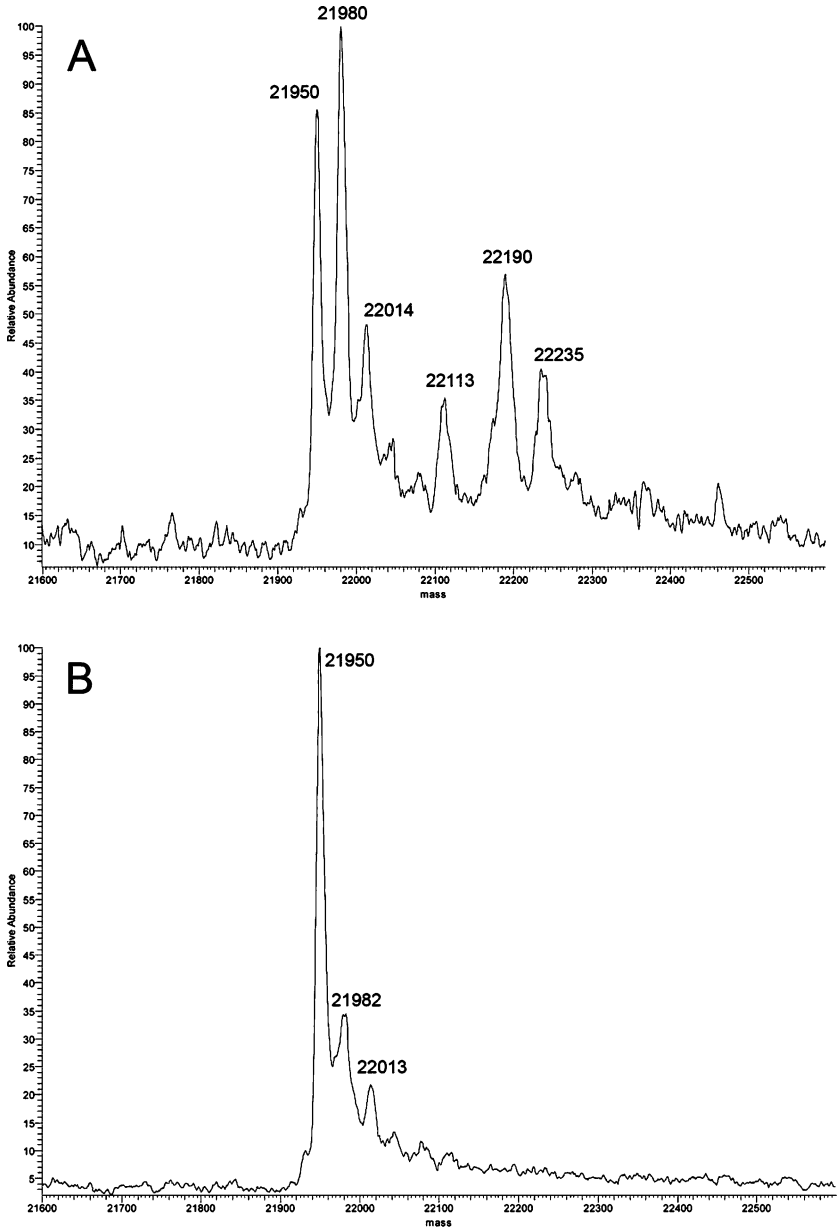


FIGURE 7: Deconvoluted mass spectrum of the HPLC eluate. The peak eluting between 25.5 and 35 min was used for mass analysis. A, SoxYZ "as isolated"; B, SoxYZ "sulfide-treated".

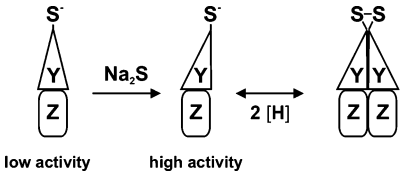


FIGURE 8: Model of the mode of action of disodium sulfide on the sulfur-binding protein SoxYZ.

The integrated peak area of SoxYZ "sulfide-treated" between 25.5 and 35 min was significantly increased as compared to SoxYZ "as isolated" (Figure 6A,B). Of this peak the molecular masses of the proteins were 21 950 Da, 21 982 Da, and 22 013 Da (Figure 7B) corresponding to covalently linked SoxY-Y homodimers ("-" stands for interprotein disulfide). The additional sulfur atoms led to an increased population of SoxY-Y homodimers possibly suggesting that the formation of the interprotein disulfide was not specific.

Incubation of SoxYZ or of SoxYZ "sulfide-treated" with TCEP resulted exclusively in elution of the monomers SoxZ and SoxY with no indication of SoxY dimers or adducts (Figure 6C,D). This demonstrated their reductive cleavage and the chemical nature of the bonds as interprotein disulfides. The sulfide treatment exposed SoxYZ to a reductive environment, and the subsequent washing removed both sulfide in the protein and the reductive environment. Therefore, to examine the impact of reduction on SoxY-Y homodimer and interprotein disulfide formation, HPLC/MS was applied to SoxYZ treated with 10 mM sulfide, however, without removal of sulfide. The elution profile was identical as shown in Figure 6B with a clear separation and major fraction of the SoxY-Y homodimer (data not shown).

## CONCLUSIONS

The substrate binding protein SoxYZ is the central and most redox-sensitive protein of the four component Sox enzyme system. SoxYZ "as isolated" was highly activated

by sulfide and significantly less activated or even inhibited by reductants of lower and very low redox-potentials. The degree of activation depended on the specific activity of SoxYZ "as isolated", and the activation was high when the specific activity of the "as isolated" protein was low. Activation by disodium sulfide led to a conformational change of the secondary structure of SoxY as evident from infrared spectroscopy. Treatment with sulfide caused a shift from the heterodimeric SoxYZ with associated subunits to a heterotetramer $Sox(YZ)_2$. Very likely, this tetramer was formed because SoxY was covalently linked by an interprotein disulfide to yield SoxY-Y and the SoxZ subunits remained associated to SoxY.

Evidence was obtained that two conditions were required for catalytic activation: (i) reduction to expose Cys-138, the active site of SoxY, as evident from thiol titration by AMS, and (ii) a distinct conformational change as evident from analysis by IR spectroscopy. As a consequence of both factors, not only the catalytic activity of SoxY was drastically increased but also the probability to undergo unspecific protein disulfide formation as evident from the occurrence of the SoxY dimers (Figure 4).

Reduction by TCEP altered the conformation of the complex (altering its mobility in DGGE). Although the C-terminus with Cys-138 of SoxY was also exposed to allow alkylation by AMS, this form exhibited a low catalytic activity and did not allow formation of the heterotetramer.

Recently, the structure of a SoxY-Y homodimer from *Chlorobium limicola*, expressed in *Escherichia coli*, has been described (*28*). In this dimer the subunits are associated with an antiparallel orientation of the C-termini which are linked by a protein disulfide. Such structure, however, is unable to enter the cleft formed by the SoxX and SoxA subunit. In this cleft, however, the active site of SoxXA is located and proposed to bind the C-terminus of SoxY (*5*, *6*).

In summary, the free thiol of SoxY-Cys-138 is suggested as the active species of SoxYZ to covalently bind the sulfur compound. The formation of the SoxY-Y dimer very likely leads to the heterotetramer $Sox(YZ)_2$ and represents an accidental process resulting from the structural change which enabled the mobility of the carboxyterminus of SoxY (Figure 8). Such dimer, however, requires rereduction to recycle the tetramer into active SoxYZ heterodimer. The flavoprotein SoxF is required for full activity of the Sox system *in vivo* (*29*), and the thioredoxin SoxS together with the thioredoxin reducing protein SoxV is essential for sulfur oxidation *in vivo* (*30*). Both are candidate systems to reduce the interprotein disulfide.

## ACKNOWLEDGMENT

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