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Identification of two inactive forms of the central sulfur cycle protein SoxYZ of *Paracoccus pantotrophus*

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Abstract **The central protein of the sulfur-oxidizing enzyme system of *Paracoccus pantotrophus*, SoxYZ, reacts with three different Sox proteins. Its active site Cys110^{Y} is on the carboxy-terminus of the SoxY subunit. SoxYZ "as isolated" consisted mainly of the catalytically inactive SoxY-Y(Z)$_2$ heterotetramer linked by a Cys110^{Y}-Cys110^{Y} interprotein disulfide. Sulfide activated SoxYZ "as isolated" 456-fold, reduced the disulfide, and yielded an active SoxYZ heterodimer. The reductant tris(2-carboxyethyl)phosphine (TCEP) inactivated SoxYZ. This form was not re-activated by sulfide, which identified it as a different inactive form. In analytical gel filtration, the elution of "TCEP-treated" SoxYZ was retarded compared to active SoxYZ, indicating a conformational change. The possible enzymes involved in the re-activation of each inactive form of SoxYZ are discussed.**

Keywords: Inactivation; Interprotein disulfide; Protein conformation; SoxYZ protein; Sulfur oxidation

1. Introduction

The oxidation of inorganic sulfur compounds to sulfuric acid is mediated by aerobic chemotrophic eubacteria and archaea and by anaerobic phototrophic eubacteria [1,2]. The periplasmic sulfur-oxidizing (Sox) enzyme system of the facultatively chemotrophic alpha-proteobacterium *Paracoccus pantotrophus* is composed of four proteins, SoxYZ, SoxXA, SoxB and Sox(CD)$_2$. Together these proteins oxidize sulfide, sulfur, thiosulfate and sulfite in vitro. The heme enzyme complex SoxXA oxidatively links the sulfur substrate to the sulfhydryl of the single cysteine-110-residue at the carboxy-terminus of SoxY (112 aa; 10977 kDa). SoxY forms with SoxZ (108 aa; 11719 kDa) the SoxYZ complex. This active site cysteine is on a peptide swinging arm [3,4]. The sulfane–sulfur atom (–S^{-}) of the Cys110^{Y}-persulfide is oxidized by the sulfane dehydrogenase Sox(CD)$_2$ to sulfone (–SO$_3^{-}$) which is released as sulfate by the sulfate thiohydrolase SoxB (Fig. 1) (reviewed in [5]).

SoxYZ "as isolated" appears in a major fraction with associated subunits and a minor fraction of subunits covalently linked via interprotein disulfides with Cys110^{Y}-Cys110^{Y} being the most stable one [6]. Activation of SoxYZ by disodium sulfide results in a conformational change of the complex as evident from FTIR spectroscopy. Moreover, the SoxYZ heterodimer forms a SoxY-Y(Z)$_2$ heterotetramer with the SoxY-subunits linked by the Cys110^{Y}-Cys110^{Y} interprotein disulfide [6]. It was unresolved if the tetramer was accidental or an intermediate of the Sox reaction cycle. Therefore, it was necessary to identify different secondary, tertiary and quaternary structures of SoxYZ and assign these to the catalytic activity.

We here report the separation of different forms of SoxYZ by analytical gel filtration and that its tetramer was catalytically inactive. SoxYZ changed its conformation upon reduction as identified by different chromatographic techniques. These findings point to different enzymes to re-activate SoxYZ.

2. Materials and methods

2.1. Strain, cultivation and extract preparation

P. pantotrophus GB17 [7] was cultivated aerobic chemotrophically with thiosulfate at 30 °C. Cells were harvested at about 100 mg dry cell weight per litre, collected by centrifugation, washed twice and stored at −20 °C. Cell free extracts were prepared by a French press as described [6].

2.2. Sox protein purification

The proteins SoxYZ, SoxXA, SoxB and Sox(CD)$_2$ were purified from the 200000 × *g* supernatant to homogeneity involving ammonium sulfate precipitation and different chromatographic steps for each Sox protein as detailed previously [6]. SoxYZ "sulfide-treated" was prepared from SoxYZ "as isolated" (100 µl; 35 µM) treated with 11 µl of 100 mM Na_2S for 20 min at 30 °C. Sulfide was removed by using ultrafiltration spin columns with a cut-off of 5 kDa (Vivaspin, Sartorius, Göttingen, Germany). SoxYZ "as isolated" was treated with the non-sulfur reductant tris(2-carboxyethyl)phosphine (TCEP): 100 µl of 35 µM SoxYZ were incubated with 2 µl 100 mM TCEP for 20 min at 30 °C and designated SoxYZ "TCEP-treated".

2.3. Analytical procedures

Analytical gel filtration. Multimeric forms of SoxYZ were separated by analytical gel filtration with a Superdex-75 column (Ø 10 mm, length 300 mm) equilibrated with 20 mM Bis-Tris-buffer, pH 6.5, with 1 mM $MgSO_4$. SoxYZ (200 µM, 80 µl) was eluted at a rate of 0.5 ml/min and fractions of 0.50 ml were collected. Protein was monitored at 280 nm. The activity of fractions was assayed from 100 µl to reconstitute the Sox enzyme system.

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Abbreviations: AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; Sox, sulfur oxidation; TCEP, tris(2-carboxyethyl)phosphine

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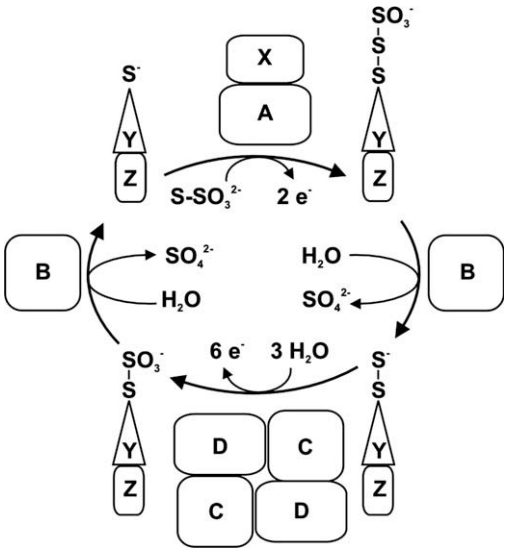

Fig. 1. Model of the reaction cycle of thiosulfate oxidation by the Sox enzyme system of *P. pantotrophus* ([5], altered, with permission). XA, heterodimeric cytochrome *c* complex SoxXA; B, dimanganese sulfate-thioesterase SoxB; YZ, sulfur-binding complex SoxCD; CD, $\alpha_2\beta_2$-heterotetrameric SoxCD complex of the molybdoprotein SoxC and the cytochrome *c* SoxD.

Electrophoresis. The molecular masses of denatured proteins were determined by SDS–PAGE [8]. Adduct formation of SoxYZ was examined by SDS–PAGE without 2-mercaptoethanol. The size of native proteins was determined by density gradient gel electrophoresis (DGGE) [9]. Protein was determined according to Bradford [10].

2.4. Sox enzyme assay

The thiosulfate-dependent oxygen uptake rate of cells was determined with an oxygen electrode (Rank Brothers) [11] and expressed as μmol O_2 consumed min^{-1} (mg of protein)$^{-1}$. The assay (0.70 ml) for thiosulfate-dependent cytochrome *c* reduction contained 50 mM of Bis-Tris-buffer, pH 6.0, 70 μM of horse heart cytochrome *c*, 0.5 μM of SoxXA, SoxYZ, SoxB, and SoxCD if not otherwise stated. The reaction was started by the addition of 7 μl of 10 mM thiosulfate. One unit (U) of activity of the Sox enzyme system is defined as 1 μmol horse cytochrome *c* reduced per min. The specific activity (U/mg of protein) relates to the total Sox proteins present in the assay.

3. Results

3.1. Catalytic activities of differently treated SoxYZ

The thiosulfate-dependent oxygen uptake rate as determined from *P. pantotrophus* cells cultivated chemoautotrophically and harvested in the early stationary phase was about 1 μmol O_2/min^{-1} (mg of protein)$^{-1}$. This activity was lower than that of exponentially grown cells. From these cells the Sox proteins were purified to homogeneity [12]. In the thiosulfate-dependent cytochrome *c* reduction assay SoxYZ yielded a uniform almost inactive preparation (Table 1) while the other homogeneous proteins SoxXA, SoxB and SoxCD were highly active when reconstituted with activated SoxYZ (data not shown; [6]). Thus, SoxYZ was not degraded but appeared to be inactive as growth ceased.

The specific activity of 0.55 mU mg protein^{-1} of the reconstituted Sox system with SoxYZ "as isolated" was marginal (Table 1). The almost inactive SoxYZ "as isolated" was highly activated (456-fold) in the "sulfide-treated" form (Table 1).

Table 1
Specific thiosulfate-dependent cytochrome *c* reduction rates of the reconstituted Sox enzyme system with differently treated SoxYZ preparations

Treatment of SoxYZ "as isolated"	Spec. cytochrome *c* reduction rate (mU/mg of protein)
None	0.55
10 mM Na_2S	251.16
2 mM TCEP	11.51
Na_2S; TCEP	11.41
TCEP; Na_2S	10.38
Na_2S; TCEP; Na_2S	14.69

35 μM SoxYZ in 25 mM $KNaPO_4$-buffer, pH 6.5, with 1 mM $MgSO_4$ was treated with 10 mM Na_2S or 2 mM TCEP for 20 min at 30 °C. Sulfide was removed from the sample by triple spin filtration (see Section 2).

Surprisingly, SoxYZ "as isolated" was activated 21-fold when treated with the TCEP (Table 1) while intermediate active SoxYZ is inactivated by TCEP [6]. However, when the active "sulfide-treated" SoxYZ was treated with TCEP the specific activity decreased by 95.5% to 10.38% mU/mg of protein. SoxYZ "TCEP-treated" was not activated by sulfide (Table 1). This important result demonstrated that TCEP rendered SoxYZ to a conformation which was not reversed by sulfide.

3.2. Separation of active and inactive SoxYZ-forms

The impact of the sulfide- and TCEP-treatments on the tertiary structure was examined by SDS–PAGE without reductant. A 25 kDa band was most pronounced from SoxYZ "as isolated" and represented the SoxY-Y dimer (Fig. 2) as previously identified by its mass and N-terminal sequencing [13]. DGGE of SoxYZ "as isolated" showed a major band of 45 kDa (data not shown) of the SoxY-$Y(Z)_2$ heterotetramer as identified previously [6,12,13]. The 25 kDa band of SoxYZ "TCEP-treated" was less intense than that observed from SoxYZ "sulfide-treated". Moreover, SoxY-Y of SoxYZ "TCEP-treated" appeared at 25.5 kDa (Fig. 2, lanes 3, 4). The slight increase in size of SoxY-Y may have indicated a conformational switch of the SoxY subunit which led to the difference in migration of the dimer but not of the monomer.

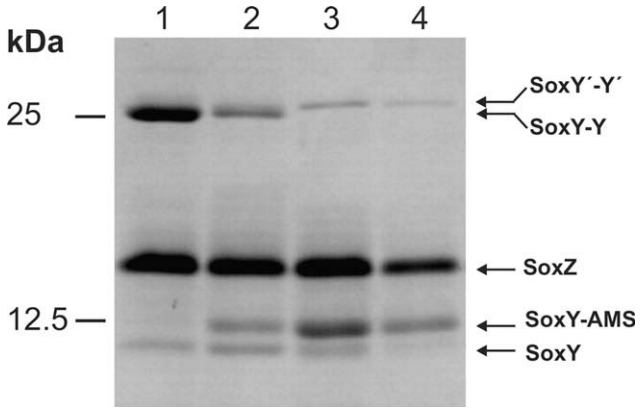

Fig. 2. SDS–PAGE of SoxYZ without reductant. To each well 5.5 μg of SoxYZ were applied. Lane 1, SoxYZ "as isolated"; lane 2, SoxYZ "sulfide-treated"; lane 3, SoxYZ "TCEP-treated"; lane 4, SoxYZ "sulfide/TCEP-treated". The samples were separately incubated with AMS for 2 h to block free sulfhydryls.

SoxYZ requires the free thiol of $Cys110^{Y}$ for activity. To examine this the thiols were blocked by 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS). Addition of AMS increased the size by about 0.5 kDa. No free thiols were observed from SoxY of SoxYZ "as isolated" (Fig. 2, lane 1) which correlated with its marginal activity. SoxY of SoxYZ "sulfide-treated" was shifted as expected from the high catalytic activity. $Cys110^{Y}$ of SoxYZ "TCEP-treated" was reduced and SoxY was shifted by AMS (Fig. 2, lane 3) but SoxYZ was almost inactive (Table 1).

To examine the catalytic activity the differently treated samples were subjected to analytical gel filtration. SoxYZ "as isolated" eluted from the column in three peaks. The first major peak (75% of total protein) eluted at 41.7 kDa as evident from the linear calibration curve ($R^2 = 0.9995$; data not shown) and represented the SoxY-$Y(Z)_2$ tetramer (theoretical mass 45 388.6 kDa). The second peak (20% of total protein) appeared at 27.9 kDa and represented the SoxYZ heterodimer (22 695.3 kDa). The dimer was followed by a third small peak being about 5% of the total protein eluted around 17 kDa (Fig. 3A) similar to the size of the monomers. The low thiosulfate-oxidizing activity eluted between the heterodimers and monomers and may have been due to altered Stoke's radii or re-aggregation of the monomers.

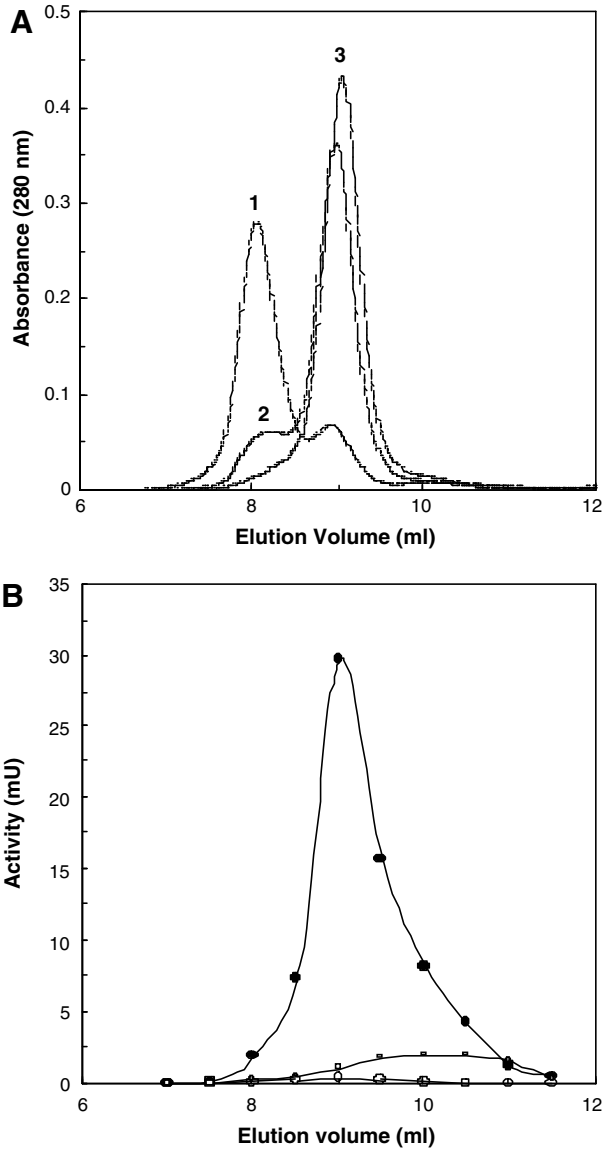

Fig. 3. Analytical gel filtration of SoxYZ. (A) Absorbance at 280 nm; (B) thiosulfate-dependent cytochrome *c* reduction rate. The activity was determined from 100 µl of each fraction (500 µl); 0.36 mg of homogeneous SoxYZ were applied to the column; SoxYZ "as isolated" (1,○), SoxYZ "sulfide-treated" (2,●); SoxYZ "TCEP-treated" (3,□).

Table 2
Recovery of the catalytic activities of SoxYZ from analytical gel filtration

SoxYZ preparation	Total thiosulfate-oxidizing activity (mU)[a]		
	Applied	Recovered	Yield (%)
As isolated	9.1	5.3	58.0
Sulfide-treated	923.4	345.0	37.4
TCEP-treated	80.4	47.2	58.7

[a]A total of 16 nmol SoxYZ (0.363 mg) was applied to each column.

The elution of the SoxYZ heterodimers and the SoxY-$Y(Z)_2$ heterotetramer deviated from their masses which was attributed to differences in Stoke's radii. The length to diameter ratio of SoxYZ of *P. pantotrophus* is 1.54 [4]. Also, the subunits of SoxYZ do not migrate according to their molecular masses (Fig. 2).

SoxYZ "sulfide-treated" eluted from analytical gel filtration mainly as heterodimer (81.5% of total protein) together with the SoxYZ-activity. A minor peak (14% of total protein) represented an inactive tetramer SoxY-$Y(Z)_2$ (Fig. 3B). The thiosulfate-oxidizing activity of the heterodimer tailed slightly (Fig. 3B) possibly as a result of other SoxYZ conformations.

SoxYZ "TCEP-treated" eluted in one major peak as heterodimer with a shoulder towards lower masses. The protein eluted slightly retarded as compared to the heterodimer of the sulfide-activated sample (Fig. 3A). The low thiosulfate-oxidizing activity of SoxYZ "TCEP-treated" was not correlated with the major protein peak but shifted between the heterodimer and lower masses (Fig. 3B). Therefore, this heterodimeric SoxYZ was completely inactive.

3.3. TCEP-treated SoxYZ

The almost inactive SoxYZ "as isolated" was moderately activated by TCEP (Table 1). The ratio of activities of SoxYZ "as isolated" and "TCEP-treated" applied to the column were 8.8 and that recovered was 9.2 and represented the degree of activation by TCEP (Table 2). Previously, SoxYZ preparations of various intermediate activities were inactivated by TCEP by 20–90% [3,6]. These seemingly conflicting observations result from differences in the structural properties of the almost inactive and intermediate active SoxYZ "as isolated" preparations. Consequently, and in accordance with the previous reports TCEP-inactivated the highly active SoxYZ "sulfide-treated" by 95.5% (Table 1).

4. Discussion

We have identified two different inactive forms of SoxYZ, the central protein of chemotrophic sulfur oxidation. First, the inactive SoxY-$Y(Z)_2$ tetramer was linked by a

$Cys110^Y$-$Cys110^Y$ interprotein disulfide and highly activated upon reduction by disodium sulfide. Second, highly active SoxYZ was almost completely inactivated by the non-sulfur reductant TCEP, and this form was not re-activated by sulfide. Third, evidence was obtained that SoxYZ-inactivation by TCEP affected the conformation of SoxY.

The active SoxYZ of *P. pantotrophus* facilitates the accidental formation of SoxY-$Y(Z)_2$ heterotetramers. A SoxY-Y disulfide was described from the crystal structure of SoxY alone from the green sulfur bacterium *Chlorobaculum thiosulfatophilum* produced in *Escherichia coli* [14]. Inactivation of SoxYZ of *P. pantotrophus* by blocking the active site $Cys110^Y$ creates the need for either its re-activation or de novo synthesis to maintain the sulfur-oxidizing reaction cycle. SoxYZ is activated by sulfide in vitro and reduction by dithiothreitol keeps the Sox enzyme system active in vivo [11,15]. Sulfide not only reduces the $Cys110^Y$-$Cys110^Y$ interprotein disulfide but also decreases the β-sheet character of SoxYZ by 4% [6].

The periplasmic thiol-disulfide oxidoreductase SoxS is the prime candidate to reduce the interprotein disulfide-of SoxY-$Y(Z)_2$. SoxS is essential for chemotrophic growth of *P. pantotrophus*, is crucial for the function of the Sox enzyme system in vivo but is not required for the in vitro reaction [11]. Without SoxS the SoxYZ protein is rapidly inactivated but not degraded in vivo which suggests SoxYZ re-activation rather than de novo synthesis. Moreover, SoxY is the target of SoxS as evident from trapping by mixed disulfides of $Cys16Ala^S$ [15]. Therefore, it is proposed that the thiol-disulfide oxidoreductase SoxS reduces the interprotein disulfide of one mol of the inactive SoxY-$Y(Z)_2$ heterotetramer to yield two moles of SoxYZ. The structure of SoxS indicates a triple thioredoxin-glutaredoxin-DsbC functionality [Y. Carius, unpublished data] which makes it unlikely that SoxS exerts only a single function to re-establish the active SoxYZ.

The inactivation of SoxYZ by TCEP appears to be very different from the oxidation of the active site as (i) the active site thiol of $Cys110^Y$ stays reduced, (ii) SoxY undergoes a conformational change and (iii) the catalytic activity is not re-established by sulfide-treatment. Very likely, re-activation of TCEP-inactivated SoxYZ requires a different reaction than that of a thioredoxin-like protein.

The basis for the search of an enzyme activity which may reverse an inactivation as similarly exerted by TCEP is the expression of a phenotype not related to the proteins of the Sox enzyme system. The only phenotype other than for SoxS was discovered for the flavoprotein SoxF. SoxF is beneficial but not essential for chemotrophic thiosulfate oxidation of *P. pantotrophus* in vivo [16]. Homogeneous SoxF has a sulfide dehydrogenase activity in vitro [17]. This activity, however, is not considered to be of relevance in vivo since *P. pantotrophus* harbors three different sulfide-oxidizing activities [18] and SoxF affects the thiosulfate metabolism [16]. Moreover, SoxF does not function in sulfur oxidation in vitro in the Sox enzyme system as reconstituted from homogeneous active Sox proteins. As SoxYZ is the only protein of the Sox enzyme system susceptible to inactivation SoxF may be suited to re-activate the second inactive form of SoxYZ.

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