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Purification and properties of the sulfur oxygenase/reductase from the acidothermophilic archaeon, *Acidianus* strain S5

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Abstract The sulfur oxygenase/reductase (SOR) of *Acidianus* strain S5 was purified and characterized after expressing the *SOR* gene in a recombinant strain of *Escherichia coli*. The N-terminal sequence of the purified SOR protein was the same as the deduced amino acid sequence from previously cloned *SOR* genes. Enzymatic studies indicated that the SOR catalyzed the conversion of elemental sulfur (S^o) to sulfite, thiosulfate, and sulfide. The optimal pH and temperature were 5.0 and 70 °C, respectively. Comparison of this SOR and that of *A. ambivalens* revealed several differences between these two SORs. The most striking difference is that the SOR of *Acidianus* S5 had maximal activity at acidic pH. By application of anti-SOR serum and the Western blot technique, it was found that SOR proteins existed in *A. brierleyi* and in *Acidianus* S5 cells cultivated with thiosulfate as the sole energy source, indicating that SOR may also play a role in thiosulfate metabolism.

Keywords Sulfur oxygenase/reductase · *Acidianus* · Archaeon · Sulfur metabolism

Introduction

Biological conversions of elemental sulfur (S^o) to sulfate, sulfide, or other forms of sulfur compounds are important aspects of sulfur metabolism of many chemolithotrophic bacteria and archaea. Through these reactions the bacteria or archaea obtain the energy for growth and physiological activities. Friedrich (1998) and Kappler and Dahl (2001) summarized the up-to-date understanding of the physiology and enzymology of sulfur-oxidizing bacteria and archaea. Applications of these sulfur metabolizers in biomining and in biological metal processing have also been reviewed (Norris et al. 2000). The genus *Acidianus* contains examples of extremely thermophilic, chemolithotrophic sulfur-metabolizing archaea, and it comprises three species: *A. ambivalens, A. infernus*, and *A. brierleyi*. These species are physiologically characterized by their capability of obtaining energy through oxidizing S^o to sulfate and simultaneously reducing sulfur to sulfide in the absence of molecular hydrogen (H_2) (Segerer et al. 1986). This property is attributed to the existence of sulfur oxygenase/reductase (SOR) in their cells.

The SOR was first described in *A. ambivalens* by Kletzin (1989). Under aerobic conditions, SOR can oxidize and reduce S^o simultaneously, with sulfite plus thiosulfate and hydrogen sulfide as the products. However, our knowledge of the SOR is very limited. The SOR from *A. ambivalens* is the only characterized member of this category of enzymes. Better understanding of the properties of SOR needs more studies on SOR from different *Acidianus* species. In this paper, we describe the purification and characterization of the SOR of *Acidianus* strain S5, which was isolated from an acidic thermal vent in Tengchong (a county of Yunnan province located in southwestern China), by exploitation of a recombinant strain of *Escherichia coli* that actively expresses the *SOR* gene of *Acidianus* S5.

Materials and methods

Bacterial strains, media, and cultural conditions

Acidothermophilic, sulfur-oxidizing archaeon strain S5 was briefly described previously (He et al. 2000) and has been deposited in the China General Microbiological Culture Collection center (CGMCC, Beijing, China). *Acidianus brierleyi* (DSM1651) was obtained from the Japan Collection of Microorganisms (JCM, Riken, Japan). Cultivations of these archaeal strains were carried out in modified Allen medium with 2% S^o (Allen 1959; Brock et al.1972).

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Escherichia coli HB101 harboring pBV220SOR was constructed in our laboratory and was cultivated in LB broth containing 100 mg/l of ampicillin, with rotary shake of 300 rpm at 30 °C. SOR expression was induced by a temperature shift from 30 °C to 42 °C. Construction of pBV220SOR and expression of SOR in *E. coli* HB101 were described previously (He et al. 2000).

Preparation of cellular lysates

Cells were collected by centrifugation at 5,000 rpm for 30 min at room temperature (GL-20G-II centrifuge, Shanghai Anting Corp., China). Cell pellets were resuspended in Tris-acetate buffer (20 mM, pH 8.0) and were broken by sonification (150 cycles of 5 s each at 400 W; JY92-II sonificator, Scientz, China) 3 times, with cooling on ice. Debris was removed by centrifugation (12,000 rpm, 10 min, 4 °C, Biofuge Fresco, Heraeus, Germany). The supernatant was treated at 75 °C for 10 min. Crude enzyme solution was obtained by removal of denatured proteins from the heated supernatant through centrifugation in the same conditions as described above.

DEAE-52 and Sephadex G-200 chromatography

All steps were conducted at room temperature. Fifty milliliters of crude enzyme solution was mixed with 10 g DEAE-52 (Whatman) that were equilibrated with buffer A (70 mM Tris-HCl, pH 7.5). After 30 min the resin was collected and the supernatant was discarded. The SOR absorbed on the resin was eluted with 150 ml of buffer B (70 mM Tris-HCl + 0.5 M NaCl, pH 7.5). As indicated by the results obtained in this study, this DEAE step did not help much for the purification, and our later purification did not include this step. The eluent from DEAE treatment was concentrated to 4 ml by ultrafiltration (Centriplus YM-10, Millipore, Mass., USA). This concentrate was loaded onto Sephadex G-200 (Pharmacia, N.J., USA) column (10×80 cm, 75 ml), which was equilibrated with buffer C (70 mM Tris-HAc, pH 7.4). The column was fractionated with a total of 225 ml of buffer C at a flowing rate of 0.9 ml min^{-1}. After the first 100 ml of eluent, 0.5 ml was collected each for SOR activity analysis, for determination of protein concentration, and for SDS-PAGE. Fractions with high SOR activity that exhibited only one band on SDS-PAGE were collected and were pooled for further study.

Protein determination, electrophoresis, and Western blotting

Protein concentrations were determined according to Bradford (1976). Proteins in the samples were separated by SDS-electrophoresis (at constant voltage 150 V) on 12% polyacrylamide gels that were prepared according to Laemmli (1970). Proteins on the gel were visualized with silver staining (Sambrook et al. 1989).

To perform Western blots, proteins on the gel were transferred to a PVDF membrane by electro-blotting, with transition buffer under constant voltage (30 V) for 90 min (Liu et al. 1998). For detection of the SOR, the immunological reactions were exploited using an anti-SOR serum and an anti-rabbit IgG/HRP (Jackson Immmnol. Res. Lab, Penn., USA). The anti-SOR serum was raised in the rabbit by subcutaneous injection of purified SOR proteins.

N-terminal sequence determination

N-terminal sequence of the purified SOR was determined by the method of Edmann degradation with a PE/ABI-491 instrument (England).

Enzymatic activity analysis of SOR

The SOR activities were determined as described by Kletzin (1989). One unit (U) is defined as the amount of enzyme required for formation of 1 μmol of sulfite plus thiosulfate per minute.

Chemical analysis

SO_3^{2-} was determined by application of a test kit (Merck, Darmstadt, Germany). S^{2-} was determined according to the method described by King and Morris (1967).

Results

Purification and N-terminal determination of SOR from *Escherichia coli* HB101 (pBV220SOR)

Heat treatment, DEAE-52 anion exchange, and Sephadex G-200 chromatography were applied to obtain pure SOR proteins. Heat treatment was very effective in removing most of the contaminating proteins from the cellular lysate of *Escherichia coli*, as most *E. coli* proteins are not heat tolerant. We also found that the total activities and the specific activities of SOR increased significantly after heat treatment (Table 1). Since the SOR is a thermophilic archaeal protein that was synthesized in mesophilic *E. coli* cells, it is possible that high temperature (heat treatment) facilitated correct folding of SOR peptides. After heat treatment, SOR proteins were further purified with DEAE-52 at pH 7.5. After elution of SOR from the DEAE-52 and concentration of the eluent, samples were further fractionated with Sephadex G-200 chromatography (Fig. 1). During Sephadex G-200 chromatography, the SOR proteins eluted at 150 ml of eluent. Fractions from 150 ml to 151.5 ml (corresponding to fractions 10–12) were collected. By adoption of all these procedures, the final yield was 11.7% (Table 1), and SDS-PAGE revealed only one band at 35 kD, as visualized by silver staining (Fig. 2).

The N-terminal sequence of the purified SOR was determined. The first nine amino acids of N-terminal (P-K-P-Y-I-A-I-N-M) are identical to the amino acids deduced from the cloned *SOR* gene (He et al. 2000),

Table 1 Purification of SOR from recombinant *Escherichia coli* HB101harboring pBV220SOR

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)
Crude extract	75.62	1051.1	13.9	100
Heat treatment	16.98	1847.4	108.8	175.8
DEAE-52	3.56	200.4	56.3	19.1
Sephadex G-200	0.66	123.2	186.7	11.7

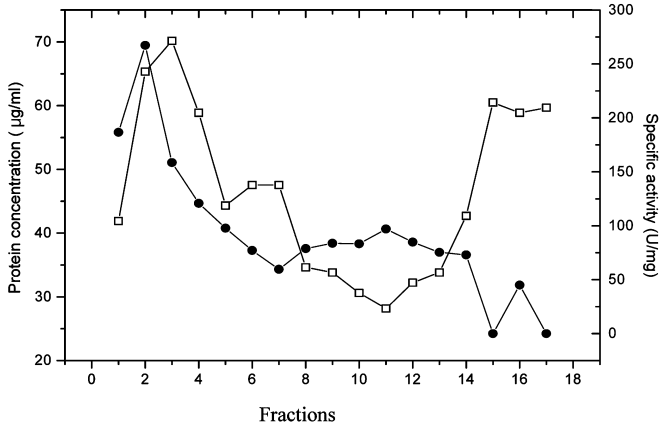

Fig. 1 Elution profile of Sephadex-200 chromatography. *Square* Protein concentration, *solid circle* SOR-specific activity

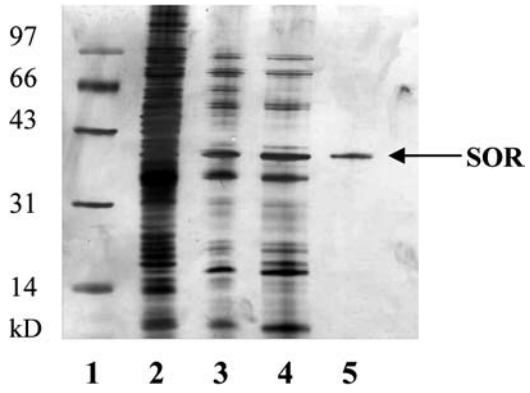

Fig. 2 Purification of SOR from recombinant *Escherichia coli* HB101 harboring pBV220SOR. Proteins were visualized with silver staining. *Lane 1* Marker, *lane 2* cellular lysate of *E. coli* HB101 (pBV220SOR), *lane 3* after heat treatment, *lane 4* after DEAE-52, *lane 5* after Sephadex G-200

indicating the identity of this purified recombinant SOR to the SOR of *Acidianus* S5.

Catalytic properties of the SOR

The purified SOR catalyzed the biological conversion of S^o to sulfite, thiosulfite, and sulfide. The general reaction is showed in the following equation:

$$5S + O_2 + 4H_2O \xrightarrow{\textbf{SOR}} H_2SO_3 + H_2S_2O_3 + 2\,H_2S$$

The effects of pH (3.0–9.0) and temperature on the SOR activities were studied and the results are shown in Fig. 3. As seen from Fig. 3A, maximal SOR activity occurred at pH 5.0. This was confirmed by application of three other different buffers (phosphoric acid/sodium citrate, citric acid/sodium citrate, and acetic acid/sodium acetate).

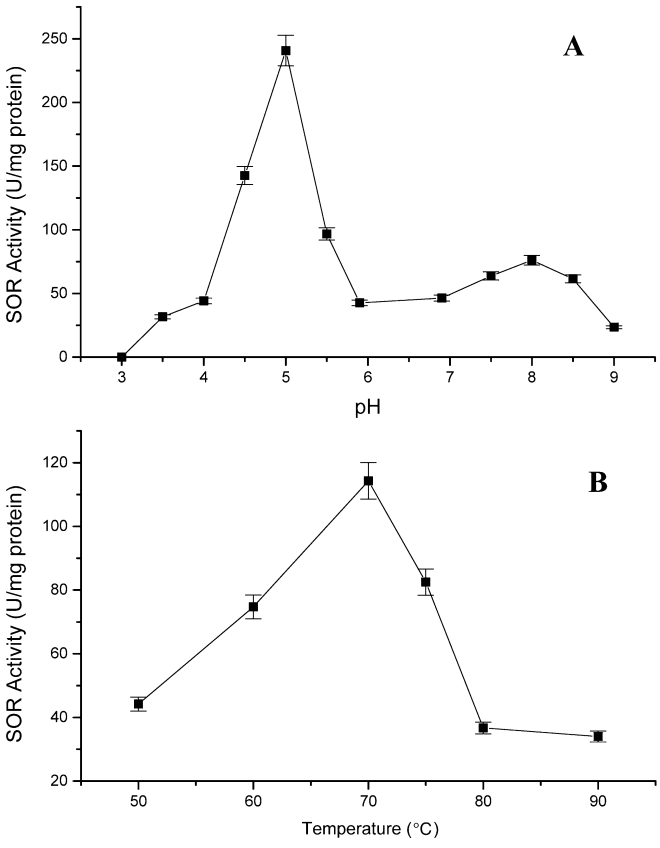

Fig. 3 Effect of pH (**A**) and temperature (**B**) on SOR activity. The reaction was carried out in 70 mM Tris-acetate buffer at pH values and temperatures as indicated within the figures

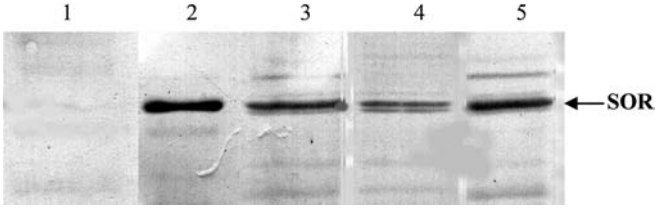

Fig. 4 Identification of SOR proteins in *Acidianus brierleyi* and in cells of *Acidianus* S5 cultivated with thiosulfate as the sole energy sources. *Lane 1 E. coli*/ pBV220, *lane 2 E. coli*/ pBV220SOR, *lane 3 Acidianus* S5 cultivated with sulfur, *lane 4 Acidianus* S5 cultivated with thiosulfate as the sole energy source, *lane 5A. brierleyi* cultivated with sulfur

The optimal temperature of SOR was determined to be 70 °C (Fig. 3B), which corresponds to the optimal growth temperature (70 °C) of *Acidianus* S5.

Identification of SORs in other species of *Acidianus*

From the purified SOR proteins, anti-SOR serum was prepared by immunizing a rabbit. The specificity of the anti-SOR serum to the SOR protein was confirmed (Fig. 4, lanes 1–3). By application of the obtained anti-SOR serum, SOR proteins were detected in S^o-oxidizing

cells of *A. brierleyi* (Fig. 4, lane 5). SOR was also detected in cells of *Acidianus* S5 that were cultivated with thiosulfate as the sole energy source (Fig. 4, lane 4).

Discussion

SOR was previously purified and characterized from *Acidianus ambivalens* (Kletzin 1989), and the *SOR* genes have been cloned and sequenced from both *A. ambivalens* and *Acidianus* S5 (Kletzin 1992; He et al. 2000). Analysis of amino acid sequence indicated 80% similarity, indicating the conservative nature of SOR. Western blot results of this study extended this to other species of *Acidianus*. By applying anti-SOR serum, SOR proteins were detected in *A. brierleyi*. We noticed that a sulfur oxygenase from *A. brierleyi* similar to the SOR of *A. ambivalens* was reported previously (Emmel et al. 1986). The detection of SOR proteins in *Acidianus* S5 cells that were cultivated with thiosulfate (without sulfur) was surprising, as the SOR was believed to be an enzyme specific to the conversion of S^o. The expression of SOR in *Acidianus* S5 with thiosulfate as the sole energy source indicated that SOR might also be involved in the metabolism of thiosulfate, thus reopening the question of the physiological function of SOR (Friedrich 1998).

Although the SORs from *Acidianus* S5 and *A. ambivalens* catalyze the same reaction – i.e., conversion of S^o to sulfite, thiosulfate, and sulfide – significant differences between the two SORs were observed. The optimal pH and temperature of SOR from *Acidianus* S5 are 5.0 and 70 °C, respectively, while those of the SOR from *A. ambivalens* are 7.5 and 85 °C, respectively (Kletzin 1989). These differences might be related to the differences in their primary amino acids and might also be related to the adaptation of the organisms to their habitats. It was found that substitutions of valine residues with isoleucine residues in the SORs of *Acidianus* S5 and of *A. ambivalens* occurred (He et al. 2000).

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