# Purification and characterization of sulfide dehydrogenase from alkaliphilic chemolithoautotrophic sulfur-oxidizing bacteria

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**Abstract Extracts of the alkaliphilic sulfur-oxidizing autotroph strain AL3 contained sulfide:cytochrome *c* oxidoreductase. This was active above pH 8, and was associated with the cell membranes. Although up to 60% of the initial activity was lost during Triton X-100 extraction, further purification resulted in an enzyme that catalyzed sulfide oxidation with horse heart cytochrome *c*. This enzyme was a 41 kDa protein containing heme $c_{554}$. The optimum pH of the membrane bound enzyme was 9.0, but after extraction this fell to 8.0. The enzyme catalyzed a single electron oxidation of $HS^-$. Hydrosulfide radical is therefore the most probable product.**



*Key words:* Sulfide dehydrogenase; Sulfur metabolism; Membrane protein; Thiosulfate; Alkaliphilic thiobacillus

## 1. Introduction

Hydrogen sulfide ($H_2S$) and the hydrosulfide ion ($HS^-$) are widely distributed reduced forms of inorganic sulfur in natural environments. They are natural substrates for sulfur-oxidizing bacteria such as those that form dense communities (sulfureta) where sulfide and oxygen flows meet. Although most of the sulfur-oxidizing bacteria thus far isolated have been shown to be able to oxidize sulfide, the mechanisms and enzymology of microbial sulfide oxidation are not fully understood. Moreover, the colorless sulfur bacteria are a heterogeneous group of only loosely related species that group over the full range of pH values and it is unlikely that the same enzymes and pathways are functional in all of them [1].

Two strains of alkaliphilic obligately autotrophic sulfur-oxidizing bacteria have recently been isolated from the Siberian soda lakes (Sorokin, D.Yu., Robertson, L.A. and Kuenen, J.G., unpublished data). These bacteria can grow autotrophically at pH values between 7.5 and 10.6 with an optimum near 9.8. They can use sulfide or thiosulfate as their energy source. One of the strains, AL3, oxidized sulfide and thiosulfate with an extremely high specific activity at pH 9–10, and was therefore chosen for research into the enzyme involved in the primary sulfide dehydrogenation by such alkaliphiles. This paper describes the detection and purification of sulfide dehydrogenase from strain AL3.

## 2. Materials and methods

### 2.1. Growth and harvesting of strain AL3

Strain AL3 was grown in a thiosulfate-limited continuous culture at pH 10 at a dilution rate of 0.15 $h^{-1}$, using a 1 l laboratory fermenter (Applicon, The Netherlands) with pH and dissolved oxygen control. The medium included the following (g/l): $Na_2CO_3$ 30, $KH_2PO_4$ 0.5, $KNO_3$ 0.5, $NaHCO_3$ up to the necessary pH. After sterilization (120°C, 20 min), 1 ml of sterile trace elements solution (Pfennig and Lippert, 1966) and sterile thiosulfate solution (final 30–80 mM) were added. Cells in the effluent were collected on ice and stored at 4°C. Cells were then concentrated in a continuous flow centrifuge and subsequently collected by centrifuging at 4°C (30 min, 10 000 × *g*) in a Sorvall GS 4 rotor, washed once in 0.1 M Tris-HCl buffer, pH 9.0 and stored at −70°C.

### 2.2. Preparation of cell extract and membrane fraction

Bacterial cells (10 g wet weight) were suspended in 20 ml 0.1 M Tris-HCl buffer, pH 9.0 and disrupted in a French pressure cell at 110 MPa. The suspension was centrifuged for 20 min at 48 000 × *g* to remove the bulk of cell debris. The supernatant was centrifuged for 120 min at 100 000 × *g*. The pelleted membranes were homogenized and suspended in 0.1 M Tris-HCl, pH 9.0.

### 2.3. Purification of sulfide dehydrogenase

The membrane fraction was treated overnight at 4°C with 1% Triton X-100 in 0.1 M Tris-HCl buffer, pH 9.0. The resulting solution was centrifuged at 50 000 × *g* for 10 min and the supernatant was applied to a DEAE-Sepharose column (15 × 2.5 cm) that had been previously equilibrated with a buffer containing 0.1 M Tris-HCl (pH 9) and 0.1% Triton X-100. A gradient from 0 to 0.4 M KCl in 0.1 M Tris-HCl (pH 9) and 0.1% Triton X-100 was used at a flow rate of 2 ml/min for 50 min. Pooled active fractions were concentrated with Centriprep-30 (Amicon) and further purified on a Pharmacia Superdex 200 column equilibrated with buffer containing 0.1 M Tris-HCl (pH 9), 0.2 M KCl and 0.1% Triton X-100, at a flow rate of 0.5 ml/min. The active fractions were diluted once with buffer containing 0.1 M Tris-HCl (pH 9) and 0.1% Triton X-100, and applied to a Mono-Q column (Pharmacia, 5/5) previously equilibrated with a buffer containing 0.1 M Tris-HCl (pH 9), 0.1 M KCl and 0.1% Triton X-100. A gradient from 0.1 to 0.4 M KCl in 0.1 M Tris-HCl (pH 9) was used at a flow rate of 0.5 ml/min over 25 min. Pooled active fractions were concentrated with Centriprep-30 (Amicon) and further purified on a Pharmacia Superdex 200 column equilibrated with buffer containing 0.1 M Tris-HCl (pH 9), 0.2 M KCl and 0.1% Triton X-100, at a flow rate of 0.5 ml/min.

### 2.4. Molecular mass determination

Subunit molecular mass was determined under denaturing conditions by SDS-PAGE according to Laemmli [2] using a 10% polyacrylamide gel and Mini Protean equipment (Bio-Rad). Purified sulfide dehydrogenase was precipitated with 10% trichloroacetic acid to remove Triton X-100. Precipitated enzyme was dissolved by incubation for 5 min at 100°C in 2% SDS and 10% β-mercaptoethanol, and subjected to SDS-PAGE. A low molecular weight calibration kit (Pharmacia) was used to derive the molecular masses. Gels were stained for protein with Coomassie brilliant blue G250.

### 2.5. Enzyme assays

Sulfide dehydrogenase activity was measured by direct comparison of the rates of sulfide-dependent horse heart cytochrome *c* reduction (550 nm) in the presence of the enzyme fraction and in a chemical control, using a double beam Hitachi spectrophotometer, at 30°C. The reaction mixture contained: 0.1 M Tris-HCl (pH 9), enzyme solution, 20 μM oxidized horse heart cytochrome *c* and 5–10 μM $HS^-$. The reaction was started by the addition of $HS^-$. It should be emphasized that the concentration of $HS^-$ was critical in this

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assay. At concentrations higher than 20 μM, the measurement became difficult, especially at pHs below 9, because of the rapid chemical reduction of horse heart cytochrome *c* by sulfide.

The affinity constants for $HS^-$ and cytochrome $c_{550}$ were calculated from the data obtained by measuring the rate of cytochrome $c_{550}$ reduction at different concentrations of $HS^-$ (1–10 μmol $HS^-$ with 20 μmol cytochrome $c_{550}$) or at 10 μmol $HS^-$ with 5–40 μmol cytochrome $c_{550}$, pH 9. The data were plotted and $K_s$ values derived as the concentration of reagent which gave a rate half that of $V_{max}$.

*2.6. Spectroscopy*

Ultraviolet/visible absorption spectra of air-oxidized, sulfide- and dithionite-reduced sulfide dehydrogenase were measured on a HP 8524A diode array spectrophotometer at 20°C.

*2.7. Measurement of substrate-dependent oxygen consumption*

The sulfide- and thiosulfate-dependent oxygen uptake rates of cell suspensions were measured polarographically at 30°C in a biological oxygen monitor with a Clark type oxygen electrode (Yellow Springs Instruments Inc., Yellow Springs, OH, USA) using a total working volume of 5 ml, pH 10 and a substrate concentration of 100 μM. Assays were performed with cell suspensions that had been taken directly from substrate-limited chemostat cultures, centrifuged (10 min at $12\,000 \times g$), washed and resuspended in mineral medium. Calculations were made on the basis of an oxygen concentration of 236 μM in air-saturated water at this temperature. The values have been corrected for endogenous respiration rates. The maximum substrate-dependent oxygen uptake rate ($V_{max}$) was calculated from a reciprocal plot, according to Hanes [3].

*2.8. Analytical procedures*

Protein concentrations of whole cells, crude extracts and membrane fractions were assayed according to Lowry et al. [4] after digestion in 1 N NaOH. In the presence of Triton X-100, protein was determined with the BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Purified sulfide dehydrogenase was first precipitated with trichloroacetic acid, and then measured according to Lowry.

## 3. Results

*3.1. Oxidation of sulfide and thiosulfate by whole cells and in cell-free extract*

Washed cells of strain AL3 that had been grown in thiosulfate- or sulfide-limited chemostat cultures at pH 10 oxidized sulfide and thiosulfate at very high rates (up to 3–3.5 μM $O_2$/mg protein/min) with a pH optimum between 9 and 10. After cell disruption, sulfide- and thiosulfate-dependent oxygen uptake disappeared completely. Moreover, the cell extract did not have any thiosulfate:ferricyanide oxidoreductase or thiosulfate:cytochrome *c* oxidoreductase activity with artificial horse heart cytochrome *c*, or with the native soluble and membrane-bound cytochrome *c*-containing fractions of strain AL3. The only activity that could be easily measured in cell extracts of strain AL3 was sulfide:horse heart cytochrome *c* oxidoreductase activity. This activity was in the range 270–320 nM/mg protein/min in 0.1 M $Na_2CO_3$ buffer, pH 9.

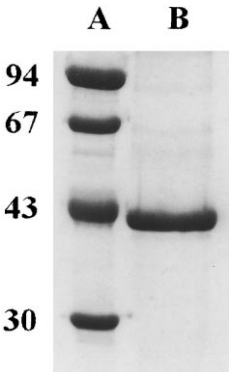


Fig. 1. SDS-PAGE of purified sulfide dehydrogenase from strain AL3. Lane A shows a low molecular weight marker, and lane B the sample.

*3.2. Location and extraction of sulfide dehydrogenase activity*

After ultracentrifuging, practically all sulfide dehydrogenase activity was recovered in the membrane fraction. The activity could be solubilized from the membranes by treatment with the non-ionic detergent Triton X-100. This treatment caused substantial loss of activity, possibly because of inhibition by the Triton X. Activity measurements were only possible after substantial dilution of Triton extracts. The addition of 1% Triton X-100 to the assay mixture also inhibited sulfide-dependent horse heart cytochrome *c* reduction. Nevertheless, further purification steps made in buffers containing 0.1% Triton X-100 allowed the successful purification of sulfide dehydrogenase.

*3.3. Purification of sulfide dehydrogenase*

Sulfide dehydrogenase activity eluted from the first anion exchange column in the main cytochrome-containing fraction at concentrations of KCl between 180 and 220 mM. The first gel filtration step showed the presence of two cytochrome-containing protein fractions, the major one being associated with sulfide dehydrogenase activity. The second anion exchange column separated the concentrated active fractions obtained after gel filtration into fractions containing the sulfide dehydrogenase activity and the contaminating cytochrome-containing protein. The second gel filtration and SDS/PAGE steps confirmed that the protein with sulfide dehydrogenase activity had been purified to homogeneity. The overall purification steps are presented in Table 1.

Table 1
Purification of sulfide dehydrogenase

| Purification step | Total protein (mg) | Specific activity[a] (U/mg) | Yield (%) | Purification factor |
|---|---|---|---|---|
| Crude extract | 2125 | 0.3 | 100 | |
| Membranes | 610 | 1.0 | 95 | |
| Membranes[b] | 405 | 0.5 | 34 | |
| DEAE-Sepharose | 33 | 1.8 | 9.3 | 6 |
| Superdex 200 | 3.2 | 15.0 | 7.5 | 28 |
| Mono-Q | 2.2 | 20 | 6.9 | 38 |
| Superdex 200 | 1.2 | 25 | 4.7 | 47 |

[a]1 unit of activity corresponds to 1 μmol of horse heart cytochrome *c* reduced per minute.
[b]After solubilization in 1% Triton X-100 for 12 h.

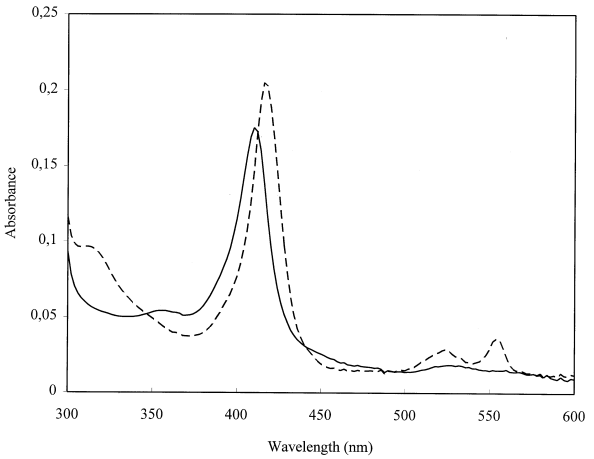


Fig. 2. Ultraviolet/visible spectra of purified sulfide dehydrogenase from strain AL3. Sulfide dehydrogenase in 0.1 M Tris-HCl (pH 9) and 0.1% Triton X-100. Solid line, air-oxidized sulfide dehydrogenase; dotted line, sulfide dehydrogenase after reduction with 1 mM sodium sulfide.

### 3.4. Properties of purified sulfide dehydrogenase

SDS-PAGE of purified sulfide dehydrogenase after trichloroacetic acid precipitation gave a single band corresponding to a molecular weight of 41 kDa (Fig. 1). The ultraviolet/visible absorption spectrum of the sulfide dehydrogenase showed the presence of oxidized heme (Fig. 2). No evidence was obtained for the presence of a flavine moiety as observed for sulfide dehydrogenases from the purple photosynthetic bacteria [5,6]. The addition of sodium sulfide to sulfide dehydrogenase resulted in reduction of the heme *c*. Adding dithionite after sulfide had no effect. The α band showed a maximum at 554 nm. Thiosulfate did not affect the oxidized sulfide dehydrogenase.

Although the pH optimum for sulfide oxidation by whole cells was about 10, sulfide dehydrogenase activity in native membranes had a pH optimum around 9. Sulfide dehydrogenase extracted from the membranes with Triton X-100 was not active in the presence of carbonates, and has a pH optimum near 8 (Table 2). Moreover it was inhibited at an ionic strength of 20 mM sodium, while whole cells remain active at sodium concentrations as high as 2 M. Purified sulfide dehydrogenase was extremely sensitive to cyanide ($K_i$ = 3 μM), and was also inhibited by sulfite and Triton X-100.

### 3.5. Kinetic constants

Purified sulfide dehydrogenase substrate constants were determined using various concentrations of sulfide and horse heart cytochrome *c*. The $K_s$ for $HS^-$ was found to be 6 μM (which is similar to that found for sulfide oxidation by whole cells). For horse heart cytochrome, the $K_s$ was 10–15 μM. When a limited amount of $HS^-$ was supplied with excess horse heart cytochrome *c*, the stoichiometry of $HS^-$ utilized to horse heart cytochrome *c* reduced was always 1:1. $HS^-$ was the only reduced sulfur compound found to be utilized as a substrate by purified sulfide dehydrogenase. Polysulfide, a possible substrate, chemically reduced horse heart cytochrome *c* so rapidly that it was not possible to test it.

The mammalian horse heart cytochrome *c* used in this assay is an artificial electron acceptor for sulfide dehydrogenase. An attempt was made to identify the natural electron acceptor among the cytochrome *c* fractions obtained during the purification of the enzyme. The only cytochrome *c* that seemed to be active with purified sulfide dehydrogenase was one from the membrane fraction that had been co-purified with the sulfide dehydrogenase protein during the first gel filtration step. However, its activity was about one order lower than for horse heart cytochrome *c*.

## 4. Discussion

Several types of enzyme involved in sulfide dehydrogenation have been characterized from various sulfur-oxidizing bacteria. In the neutrophilic colorless sulfur bacteria, *Thiobacillus versutus* (now *Paracoccus versutus* [7]) and *Paracoccus denitrificans*, sulfide dehydrogenase has been reported to be part of the thiosulfate-oxidizing system [8] previously described for *T. versutus* [9,6]. This enzyme is a manganese-containing protein (34 kDa) that oxidizes sulfide to sulfate in the presence of native cytochrome $c_{552.5}$ or external horse heart cytochrome *c*.

More is known about sulfide dehydrogenases from anaerobic phototropic bacteria. Various cytochrome *c*-containing proteins have been purified from these bacteria, but their sulfide-oxidizing activity in the presence of horse heart cytochrome *c* was only later discovered. Flavocytochromes from *Chlorobium limicola v. thiosulfatofilum* [5,10] and *Chromatium vinosum* [11,12] have been studied in detail, and Table 3 compares their properties with those of the sulfide dehydrogenase purified from strain AL3. The flavocytochrome sulfide dehydrogenases consist of two subunits containing FAD and cytochrome $c_{552}$ or cytochrome $c_{553}$. It is believed that they form stable complexes with the native electron accepting cytochrome $c_{551}$ or cytochrome $c_{555}$ [6,10].

The sulfide dehydrogenase purified from strain AL3 differs from the known enzymes with similar functions by being membrane-bound, and it could therefore not be investigated in its native form. Its effect can be seen clearly, for example, from the difference in pH dependence of native sulfide dehydrogenase activity in membranes and sulfide dehydrogenase dissolved in Triton X-100 (Table 3). The pH optimum for

Table 2
pH optima of sulfide dehydrogenase activity in whole cells, membrane fractions, and after solubilization with Triton X-100

| | pH 7 | pH 8 | pH 9 | pH 10 |
|---|---|---|---|---|
| Sulfide-dependent respiration of whole cells[a] | 25 | 70 | 100 | 80 |
| Sulfide dehydrogenase activity in native membranes[b] | ND | 35 | 100 | 40 |
| Sulfide dehydrogenase activity for purified enzyme, solubilized from membranes[c] | ND | 100 | 80 | 10 |

The values represent the percentage of the maximum activity.
[a]3.6 μM $O_2$/mg protein/min.
[b]1.0 μM cytochrome *c*/mg protein/min.
[c]25 μM cytochrome *c*/mg protein/min.
ND: not determined because of the rapid chemical reduction of cytochrome *c* by sulfide at this pH.

Table 3
Properties of sulfide dehydrogenase from the alkaliphilic, autotrophic strain AL3, as compared with flavocytochrome sulfide dehydrogenase from phototrophic sulfur bacteria

| Property | Strain AL3 | Phototrophic sulfur bacteria |
|---|---|---|
| Reaction | $HS^- \rightarrow HS^\bullet$ | $HS^- \rightarrow S^\circ$ |
| Localization | membrane bound | periplasm |
| Structure | 1 subunit, 41 kDa | 2 subunits, 20 and 47 kDa |
| Cofactors | heme $c_{554}$ | FAD and heme *c* |
| pH optimum | membrane bound: 9.0<br>solubilized: 8.0 | 8.2 |
| $K_s$ for $HS^-$ | 6 μM | 12.5 μM |
| Inhibitors | $CN^-$, $SO_3^{2-}$,<br>Triton X-100 | $CN^-$, $SO_3^{2-}$, atebrin |

sulfide oxidation by intact strain AL3 cells is close to the optimum for growth (pH 10). If the sulfide dehydrogenase is located, as might be expected, on the outer side of the cell membrane, the pH optimum for the enzyme should be in this area. Indeed, the pH optimum for sulfide dehydrogenase in isolated membranes is about 9.

The 1:1 stoichiometry of horse heart cytochrome *c* reduction by sulfide dehydrogenase isolated from strain AL3 indicated that $HS^-$ radical should be an intermediate reaction product. According to sulfide chemistry (R. Steudel, personal communication), the oxidation of sulfide to elemental polymeric sulfur most probably includes the formation of the sulfide radical. Therefore, although elemental sulfur is claimed to be a product of sulfide dehydrogenation catalyzed by purified sulfide dehydrogenase in phototropic bacteria, the pathway should include the intermediate production of sulfide radical. Without this stage, it is difficult to imagine how a polymeric sulfur molecule (either linear polysulfide or ring sulfur) is formed. In fact, the formation of polymeric sulfur bound to cell membranes during the first stage of sulfide oxidation by *Thiobacillus concretivorus* was reported more than 20 years ago [13,14]. Strain AL3 oxidizes sulfide and polysulfide very actively, but has almost no ability to oxidize elemental sulfur or sulfite. Sulfide oxidation by strain AL3 probably proceeds via sulfide radical to polysulfide, and then directly to sulfate by step to step oxidation of the terminal sulfane atom, as suggested for *P. denitrificans* and *T. versutus* [8].

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