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Purification and characterization of a sulfite:cytochrome *c* oxidoreductase from *Thiobacillus acidophilus*

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Abstract

Cell-free extracts of *Thiobacillus acidophilus* prepared at neutral pH showed oxidation of sulfite to sulfate with ferricyanide as electron acceptor. Horse heart cytochrome *c* could be used as alternative electron acceptor; however, the observed activity was only 0.1% of that found for ferricyanide. The enzyme responsible for the oxidation of sulfite was purified to homogeneity. The purified enzyme was a monomer of 42 kDa and contained one haem *c* per monomer. Electron paramagnetic resonance (EPR) spectroscopical analysis of the sulfite:cytochrome *c* oxidoreductase showed the presence of molybdenum (V), only after reduction of the enzyme with sulfite. The pH optimum for the enzymatic reaction was 7.5 and the temperature optimum 40°C. Enzymatic activity was strongly reduced in the presence of the anions: chloride, phosphate and nitrate. In contrast to other enzymes involved in sulfur metabolism and previously isolated from *T. acidophilus*, sulfite:cytochrome *c* oxidoreductase activity is not stimulated by the presence of sulfate ions.

Keywords: Thiobacillus acidophilus; Sulfite; Molybdenum; Acidophilic bacteria; Sulfur metabolism

1. Introduction

Acidophilic thiobacilli are capable of oxidizing several sulfur compounds at low pH values. The knowledge of the pathway of sulfur oxidation by acidophilic thiobacilli was rather limited, until two enzymes, trithionate hydrolase [1] and thiosulfate dehydrogenase [2], were purified to homogeneity from *Thiobacillus acidophilus*. In addition to growing autotrophically on several sulfur compounds, this organism is able to grow heterotrophically on glucose or small organic acids or mixotrophically on glucose with thiosulfate or tetrathionate [3], producing large cell yields, as compared to autotrophic growth. From a third enzyme, tetrathionate hydrolase, the activity could be established [4]; however, this enzyme has been only recently purified [5]. All three enzymes isolated from *T. acidophilus* have a pH optimum of about 3, which is a strong indication for a periplasmic location [5]. The hypothetic pathway for sulfur compound degradation by *T. acidophilus* was presented by Meulenberg [6]. This pathway predicts that the oxidation of elemental sulfur, which is produced from the hydrolysis of tetrathionate, will pro-

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ceed to sulfate via sulfite. This would mean that in the absence of chemical oxidation of sulfur or sulfite, at least two enzymes are involved — one for the oxidation of sulfur to sulfite and a second enzyme which oxidizes sulfite to sulfate. At present, no enzyme catalysing the oxidation of elemental sulfur has been isolated from any of the thiobacilli strains, whether acidophilic, neutrophilic or alkaliphilic. It should be mentioned that a sulfur dioxygenase has been purified from the archeabacterium *Sulfolobus acidocalcidarius* [7] and that GSH-dependent oxidation has been found in cell-free extracts of the acidophilic *T. thiooxidans* [8]. The enzymes involved in the oxidation of sulfite have been investigated in detail. *T. novellus* and *T. thioparus* contain a sulfite:cytochrome *c* oxidoreductase with haem *c* and molybdenum as prosthetic groups, very similar to mammalian sulfite oxidases [9,10]. A partially purified sulfite:cytochrome *c* oxidoreductase from *T. ferrooxidans* did not contain haem or non-haem iron [11]. A sulfite:ferric ion oxidoreductase has been isolated from membranes of *T. ferrooxidans* [12]. The latter enzyme is the only sulfite oxidoreductase that uses Fe^{3+} as electron acceptor. This paper describes the isolation and characterization of a sulfite oxidoreductase from *T. acidophilus* which is able to use ferricyanide and cytochrome *c* as electron acceptor.

2. Experimental

2.1. Organism and growth conditions

T. acidophilus DSM 700 was maintained as described previously [13]. High cell density mixotrophic chemostat cultures of *T. acidophilus* were grown in steady state on glucose (100 mM) and thiosulfate (50 mM) at a dilution rate of 0.05 h^{-1} as reported [3]. Autotrophic chemostat cultures were grown on thiosulfate (50 mM) at a dilution rate of 0.02 h^{-1}, in a five times diluted medium compared to the mixotrophic chemostat cultures.

2.2. Enzyme assay

Sulfite:cytochrome *c* oxidoreductase activity was assayed using sodium sulfite (1 mM) as a substrate and potassium ferricyanide (1 mM) or horse heart cytochrome *c* (10 μM) as electron acceptor in 25 mM phosphate buffer pH 7.5. EDTA (5 mM) was added to prevent metal ion-catalysed chemical oxidation of sulfite. The specific activity is defined as the reduction of 1 μmol ferricyanide or cytochrome *c* mg^{-1} protein min^{-1}.

2.3. Enzyme purification procedure

Bacterial cells (32 g wet weight) were collected on ice from an mixotrophically grown chemostat culture of *T. acidophilus* and centrifuged (10 min at $12{,}000 \times g$). The biomass was washed and resuspended in 65 ml of a buffer containing 25 mM potassium phosphate pH 7.0, and disrupted in a French pressure cell at 110 MPa. The suspension was centrifuged for 20 min at $48{,}000 \times g$ to remove the debris. The supernatant was centrifuged for 120 min at $100{,}000 \times g$. Ammonium sulfate was added to the clear supernatant until the final concentration was 1.5 M. This solution was centrifuged for 20 min at $48{,}000 \times g$ and applied to a phenyl sepharose column (5×3 cm^2) equilibrated in buffer containing 25 mM potassium phosphate and 1.5 M ammonium sulfate (pH 7). A gradient from 1.5 to 0 M ammonium sulfate in 25 mM potassium phosphate (pH 7) was applied at a flow rate of 4 ml min^{-1} during 75 min. Pooled active fractions were dialysed for 16 h against a buffer containing 5 mM potassium phosphate, pH 7, and the dialysate applied to a hydroxy apatite column (5×1 cm^2) previously equilibrated with a buffer containing 5 mM potassium phosphate, pH 7. A gradient from 5 mM to 1 M potassium phosphate, pH 7, was applied at a flow rate of 0.5 ml min^{-1} over 90 min. Pooled active fractions were diluted with an equal volume of 3.0 M ammonium sulfate, pH 7, and applied to a phenyl superose column (Pharmacia, 10/10) equilibrated in

buffer containing 25 mM potassium phosphate and 1.5 M ammonium sulfate (pH 7). A gradient from 1.5 to 0 M ammonium sulfate in 25 mM potassium phosphate (pH 7) was applied at a flow rate of 1.0 ml min^{-1} during 40 min. Pooled active fractions were dialysed for 16 h against a buffer containing 25 mM potassium phosphate, pH 7, and the dialysate applied to a Mono-S column (Pharmacia 5/5) previously equilibrated with a buffer containing 25 mM potassium phosphate, pH 7. A gradient from 0 to 1 M NaCl in 25 mM potassium phosphate, pH 7, was applied at a flow rate of 0.5 ml min^{-1} over 30 min, and the eluting active fractions stored at −80°C.

2.4. Molecular mass determination

The apparent molecular mass was determined by gel filtration on a Pharmacia Superdex 75 column equilibrated with buffer containing 0.2 M NaCl in 25 mM potassium phosphate buffer, pH 7. Molecular weight standards were bovine serum albumin (67 000), albumin egg (45 000), chymotrypsin from pancreas (25 000) and cytochrome *c* from horse heart (12 300). Subunit molecular masses were determined under denaturing conditions by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE) according to Laemmli [14] using a 10% polyacrylamide gel and Mini Protean equipment (Bio-Rad). Enzyme samples were denatured by incubation for 5 min at 100°C in 2% SDS and 10% β-mercaptoethanol. 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. Determination of pH optimum, optimum temperature

The pH optimum was determined in 25 mM potassium phosphate and 25 mM Tris/HCl. The pH of the Tris/HCl buffer was varied by addition of HCl, the pH of the potassium phosphate buffer was varied by mixing 25 mM KH_2PO_4 (pH 5) with 25 mM K_2HPO_4 (pH 9.5). The temperature optimum was studied under the standard assay conditions. The temperature was varied between 15° and 50°C and the actual temperature was measured in the cuvette.

2.6. Inhibition experiments

In order to compare the sulfite:cytochrome *c* oxidoreductase with similar enzymes from *T. novellus* and *T. thioparus*, inhibition by several anions was tested. The tested anions were Cl^-, SO_4^{2-}, NO_3^-, PO_4^{3-} and CH_3COO^-. Inhibition experiments were performed with concentrations of the investigated anions varying between 0.1 to 100 mM in combination with varying concentrations SO_3^{2-}, using the standard assay in 25 mM phosphate buffer pH 7.5 and 5 mM EDTA, at 25°C.

2.7. Spectroscopy

Ultraviolet absorbtion spectra were measured on a HP 8524A diode array spectrometer at 20°C. Calculation of the specific absorption coefficient was performed using the ratio between the absorbance at 205 and 280 nm as described [15]. Electron paramagnetic resonance (EPR) spectroscopy was performed on a Varian E-9 spectrometer operating at X-band frequency and equipped with a home-made He-flow cryostat. Sulfite cytochrome *c* oxidoreductase was reduced by the addition of 1 mM sodium sulfite.

2.8. Analytical procedures

Protein concentrations were determined according to the procedure of Lowry et al. [16].

2.9. Chemicals

All chemicals were analytical grade and obtained from commercial sources.

3. Results

3.1. Purification and physical properties

A typical autotrophic cultivation of *T. acidophilus* on 50 mM thiosulfate at 30°C produces 0.5 g wet weight cell paste per liter. Mixotrophic cultivation of *T. acidophilus* on 50 mM thiosulfate and 100 mM glucose at 30°C produces 10 g cell paste per liter. Cell-free extracts prepared at pH 7 from cells grown mixotrophically as well as autotrophically, showed similar activities towards sulfite, when assayed with ferricyanide as electron acceptor at pH 7. Activity of sulfite:cytochrome *c* oxidoreductase could not be detected at pH 3. Cell-free extracts prepared at pH 3 showed no activity when assayed at pH 3 or 7, indicating that sulfite:cytochrome *c* oxidoreductase is not stable at this pH.

Purification of sulfite:cytochrome *c* oxidoreductase was performed from mixotrophically grown cells. The purification scheme for the isolation of sulfite:cytochrome *c* oxidoreductase from such cells is given in Table 1. The total activity after hydroxyapetite increased, probably caused by the removal of an inhibitory component in this purification step. The nature of this inhibitory component is not known. Active fractions after Mono-S appeared to be homogeneous, as judged by peak purity criteria on Superdex 75 as well as by SDS/PAGE. Determination of the native molecular mass by gel filtration yielded a value of 40 kDa; subunit mass determination by SDS/PAGE yielded a value of 42 kDa.

Table 1
Purification of sulfite:cytochrome *c* oxidoreductase from *T. acidophilus*

Purification step	Total protein [mg]	Specific activity [U(mg protein)$^{-1}$)]	Yield [%]	Purification factor
Cell-free extract	1045	0.17	100	1
Phenyl sepharose	110	0.23	14.1	1.4
Hydroxyapatite	5.6	7.3	23.1	43
Phenyl superose	0.7	47.3	18.8	281
Mono-S	0.18	143	14.2	851

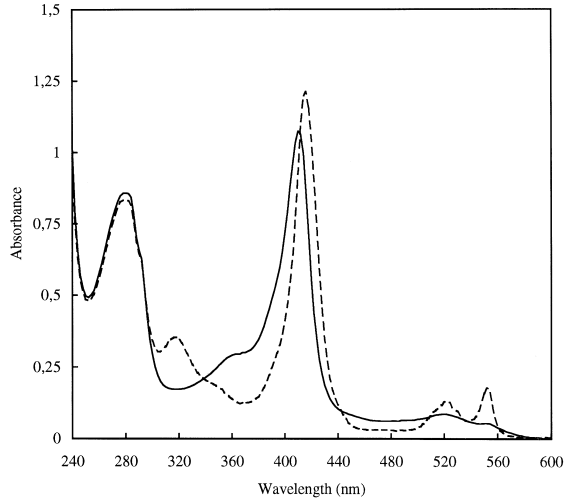

Fig. 1. Ultraviolet/visible spectra of sulfite:cytochrome *c* oxidoreductase. Sample of 11.6 μM enzyme in 25 mM potassium phosphate buffer, pH 7. (—) Air-oxidized sulfite:cytochrome *c* oxidoreductase; (---) sulfite:cytochrome *c* oxidoreductase after reduction with 1 mM sodium sulfite.

3.2. Electron acceptors

Ferricyanide used as an artificial electron acceptor gave values for the maximal activity of purified protein of 147 μmol ferricyanide mg^{-1} protein min^{-1}. Horse heart cytochrome *c* gave a value of 0.13 μmol cytochrome *c* reduced mg^{-1} protein min^{-1}, which is only 0.1% of the activity found for ferricyanide. Other artificial electron acceptors could not be used because of chemical reduction by sulfite [17]. None of the major cytochrome *c*-containing fractions of the first hydrophobic interaction column (phenyl sepharose) could be used as natural electron acceptor.

3.3. Ultraviolet / visible absorption spectra

Sulfite:cytochrome *c* oxidoreductase is isolated in a form with a spectrum characteristic for oxidized haem *c* (Fig. 1). Addition of 0.1

mM sulfite resulted in reduction of the haem *c*, with a maximal absorbance of the α band at 552 nm. Addition of dithionite did not result in further reduction of the haem *c*. Calculation of the specific absorption coefficient ($A_{280\ nm}^{0.1\%}$), using the ratio the absorbance between 205 and 280 nm [15], showed a value of 1.7.

3.4. EPR spectra

The EPR analysis of oxidized sulfite:cytochrome *c* oxidoreductase at 32 K showed no signal. After reduction with sulfite, an EPR signal for Mo (V) was observed (Fig. 2). The signal is in fact an overlap of two rhombic signals. The sharper and largest signal has $g_{zyx} = 1.985, 1.971, 1.963$ and is similar to the high pH species observed in sulfite oxidase [10]. The other signal has a split $g_z = 2.002$, whereas the g_y is probably obscured; the feature around $g = 1.952$ might be the corresponding g_x-resonance. The splitting in g_z (1.2 mT) is suggested to be due to the binding of H^+ close to the Mo (V) just like in sulfite oxidase [10].

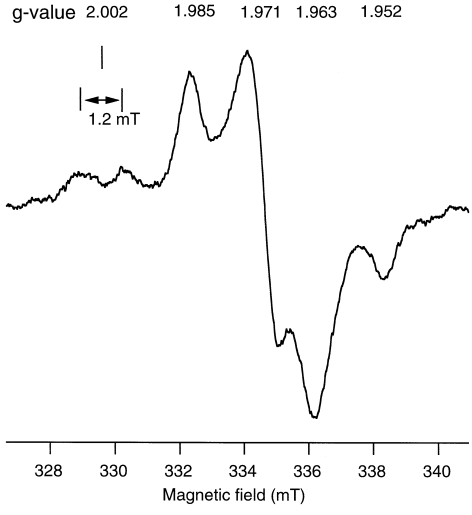

Fig. 2. The EPR spectra of sulfite:cytochrome *c* oxidoreductase after reduction with 1 mM sodium sulfite. A sample of 31 μM enzyme in 25 mM potassium phosphate buffer, pH 7, was prepared as described in Section 2. EPR conditions: microwave frequency 9.234 GHz; temperature 32 K; microwave power 2 mW; modulation amplitude 0.5 mT.

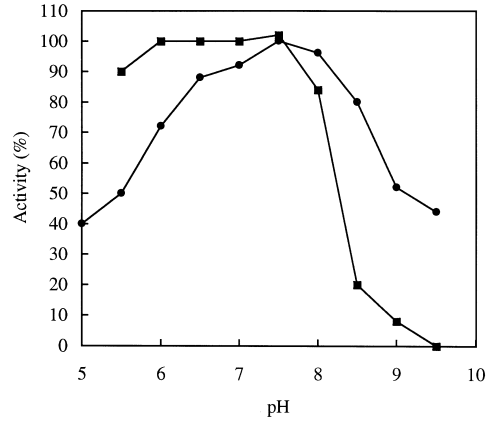

Fig. 3. Effect of pH on the oxidation of sulfite by sulfite:cytochrome *c* oxidoreductase. Activities were determined by measuring the reduction of ferricyanide in 25 mM potassium phosphate and 25 mM Tris/HCl buffer. Relative activities are given as a percentage of the maximum activity (147 μmol ferricyanide mg^{-1} protein min^{-1}). (■) Tris/HCL. (●) Potassium phosphate.

3.5. Effects of pH and temperature on enzyme activity

The effect of pH was tested to investigate the maximum rate of sulfite oxidation by sulfite:cytochrome *c* oxidoreductase with ferricyanide as electron acceptor. The maximum activity occurred at pH 7.5 (Fig. 3), irrespectively of whether Tris/HCl or potassium phosphate buffer was used. However, in contrast to potassium phosphate buffer, the Tris/HCl buffer showed a plateau in the activity between pH 5 and 7.5, as already observed for the sulfite:cytochrome *c* oxidoreductase from *T. novellus* [17].

Inhibition by phosphate at lower pH suggests that HPO_4^{2-} has a stronger inhibitory effect on the enzyme than PO_4^{3-}.

The optimum temperature of sulfite oxidation was investigated with purified sulfite:cytochrome *c* oxidoreductase and using ferricyanide as electron acceptor. The activity gradually increased from 15° to 45°C. Above 50°C, the activity decreased rapidly due to denaturation of the enzyme.

3.6. Inhibition experiments

Inhibition of sulfite:cytochrome *c* oxidoreductase was observed with Cl^-, NO_3^- and

PO_4^{3-}. Activity in the presence of these inhibitors was restored by the addition of extra substrate, showing that inhibition was competitive. The concentration of sulfite used in the activity assay was high enough to restore the inhibition, caused by using 25 mM phosphate buffer. Inhibition was studied in more detail by using NaCl at different substrate concentrations. K_i for Cl^- was 59 mM. No inhibition was observed with SO_4^{2-} ions. A strange phenomenon was observed with acetate, which showed an increase in activity up to 130% at 100 mM.

4. Discussion

Sulfite:cytochrome *c* oxidoreductase isolated from *T. acidophilus* shows remarkable similarities in properties compared to sulfite:cytochrome *c* oxidoreductases from *T. novellus* and *T. thioparus*. Both enzymes contain haem *c* and molybdenum [9,10] as found here for the *T. acidophilus* enzyme. These bacterial enzymes closely resemble mammalian sulfite oxidases, though mammalian sulfite oxidases are dimeric and contain a cytochrome *b*-type haem instead of a haem *c* observed for bacterial enzymes. The two-electron oxidation of sulfite to sulfate is probably occurring at the molybdenum centre, whereas the haem *b* functions to transfer the electrons from the molybdenum to the natural electron acceptor. Mammalian sulfite oxidases, in contrast to their bacterial counterparts, use molecular oxygen as electron acceptor in addition to ferricyanide and cytochrome *c*.

Despite the fact that *T. acidophilus* is an acidophilic bacterium with a pH optimum below 3, a close resemblance of the sulfite:cytochrome *c* oxidoreductase of this organism and similar enzymes of *T. novellus* and *T. thioparus* was found. The maximal activity of the *T. acidophilus* enzyme at neutral pH and its instability at low pH values suggest that sulfite:cytochrome *c* oxidoreductase is located in the cytoplasm of *T. acidophilus*, as has been proposed for the neutrophilic *Thiobacilli*.

Inhibition by anions, like chloride and phosphate, is also observed for the enzymes from *T. novellus* and *T. thioparus*. Although the mechanism of this inhibition is unknown, it is very characteristic for these type of enzymes. It shows that activity is strongly dependent on the concentration of the phosphate or Tris/HCl buffer. Inhibition by acetate is not observed as for sulfite:cytochrome *c* oxidoreductase from *T. novellus* [17]; in contrast, acetate showed activation up to 30% in the assay when measuring sulfite:cytochrome *c* oxidoreductase from *T. acidophilus*. At this moment, the explanation of this increase in activity is unknown.

All other enzymes previously isolated from *T. acidophilus*, involved in sulfur compound degradation, have a pH optimum around 3. Similar observations have been made for tetrathionate hydrolase of the acidophilic *T. ferrooxidans* [18]. Their low pH optimum and the fact that all enzymes are activated by sulfate indicate that the enzymes are periplasmic and thus exposed to the natural environment of these acidophiles. The environment of acidophiles normally consists of a low pH optimum and high sulfate concentration.

The resemblance between the sulfite oxidoreductases of the *Thiobacilli* suggests that the sulfite oxidation pathway in both acidophiles and neutrophiles is similar, in spite of the fact that they belong to different branches of the Proteobacteria [19]. At the same time, it is evident that the pathways of sulfur compound oxidation are different not only between acidophilic and neutrophilic thiobacilli, but also among the neutrophiles [6]. Hence, the unity in the biochemistry of sulfur compound oxidation may thus far be limited to the sulfite oxidation, although even here, a second pathway involving APS is known to exist [20].

In view of the difference found between species of thiobacilli, it is important that further investigations on the missing links in the pathway of acidophiles are focused on *T. aci-*

dophilus in which an almost complete pathway of oxidation of thiosulfate has now been demonstrated.

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