Biochimica et Biophysica Acta, 568 (1979) 454—467

BBA 68749

PURIFICATION OF *THIOBACILLUS DENITRIFICANS* SIROHEME SULFITE REDUCTASE AND INVESTIGATION OF SOME MOLECULAR AND CATALYTIC PROPERTIES

M. SCHEDEL * and H.G. TRÜPER

Institut für Mikrobiologie der Universität Bonn, Meckenheimer Allee 168, D-5300 Bonn 1 (F.R.G.)

(Received November 6th, 1978)

Key words: Siroheme sulfite reductase; (Purification, properties, Thiobacillus denitrificans)

Summary

A siroheme-containing sulfite reductase was isolated from *Thiobacillus denitrificans*, purified to an electrophoretically homogenous state, and investigated with regard to some of its molecular and catalytic properties. The enzyme was a tetramer with a molecular weight of 160 000, consisting of two types of subunits arranged to an $\alpha_2\beta_2$-structure. The molecular weight of the α-subunit was 38 000, that of the β-subunit 43 000. As prosthetic groups siroheme and Fe/S groupings could be detected. The absorption spectrum showed maxima at 273 nm, 393 nm, and 594 nm; the molar extinction coefficients at these wavelengths were 280, 181, and $60 \cdot 10^3$ $cm^2 \cdot mmol^{-1}$, respectively. With reduced viologen dyes the enzyme reduced sulfite to sulfide, thiosulfate and trithionate. In many properties *T. denitrificans* sulfite reductase closely resembled desulfoviridin, the dissimilatory sulfite reductase of *Desulfovibrio* species. It is proposed that the physiological function of this enzyme is not to reduce but rather to form sulfite from reduced sulfur compounds in the course of dissimilatory sulfur oxidation in *T. denitrificans*.

Introduction

Aerobic thiobacilli have been shown to contain an oxygenase which oxidizes elemental sulfur to sulfite [1,2]. The facultative anaerobe *Thiobacillus denitrificans* which conducts sulfur oxidation in the absence of atmospheric oxygen

* Present address: Bayer AG, PH-Forschungszentrum, Institut für Biochemie, D-5600 Wuppertal, F.R.G.
Abbreviation: SDS, sodium dodecyl sulfate.

should possess a different enzyme to catalyze this reaction.

In a general study on the anaerobic oxidation of reduced sulfur compounds in this organism we were able to demonstrate the presence of a siroheme-containing sulfite reductase which, under appropriate reaction conditions, reduced sulfite to sulfide [3]. Two reasons led us to believe that the physiological function of this enzyme was not to reduce but rather to form sulfite and, therefore, should be regarded as an oxygen-independent anaerobically active sulfide and/or elemental sulfur-oxidizing enzyme corresponding to the oxygenase present in aerobic thiobacilli: (1) Wagner et al. [4] calculated the average redox potential of the six electrons transferred in the sulfite reductase reaction as −120 mV; this means that a reversed sulfite reductase reaction, in which sulfite is formed from either sulfide or elemental sulfur, can proceed under physiological conditions if an appropriate electron acceptor is present. (2) Growth of *T. denitrificans* is obligately dependent on the oxidation of reduced sulfur compounds; the organism would have no need for an assimilatory sulfite reductase activity.

We report here the purification of the enzyme to an electrophoretically homogeneous state; some molecular and catalytic (tested as sulfite reductase) parameters are also described.

Materials and Methods

Growth and harvest of T. denitrificans cells. T. denitrificans strain 'RT' was grown in a medium (pH 7.0) which contained in 1 l: 12.5 g $Na_2S_2O_3 \cdot 5\ H_2O$, 6.0 g KNO_3, 0.5 g $MgCl_2 \cdot 6\ H_2O$, 2 ml 10-fold concentrated trace element solution (SL4 of Pfennig and Lippert [5]), 2.7 g KH_2PO_4 and 3.9 g $NaHCO_3$. The last two components were autoclaved separately from the rest, and the three components combined after cooling. Cells were cultivated anaerobically at 30°C, harvested after 36 h at room temperature and stored at −20°C.

Sulfite reductase assay. Sulfite reductase activity was measured manometrically in Warburg flasks under H_2 at 30°C, following basically the method described by Yoshimoto and Sato [24]. The main compartment contained in a total volume of 2.5 ml: 1 ml *Desulfovibrio gigas* hydrogenase which was free from sulfite and thiosulfate reductase activities, 0.1 ml 75 mM methylviologen, 0.1 ml 1 M potassium phosphate (pH 7.0) and 0.1—1.3 ml enzyme solution. The center well contained 0.2 ml 5 N NaOH. The reaction was started by adding 0.3 ml 50 mM Na_2SO_3 from the side arm. One enzyme unit (U) was defined as that amount of protein which consumed 1 μmol H_2/min.

Preparation and assay of hydrogenase from D. gigas. D. gigas DSM 496 was grown in a medium (pH 7.0) which contained in 1 l: 12 ml 45% sodium lactate solution, 2 g NH_4Cl, 2 g $MgSO_4 \cdot 7\ H_2O$, 4.4 g Na_2SO_4, 1 g yeast extract, 2 ml 10-fold concentrated trace element solution (SL4 of Pfennig and Lippert [5]) and 20 ml 1 M potassium phosphate. Potassium phosphate was autoclaved separately and added after cooling. Cells were cultivated anaerobically at 30°C, harvested after 2 days at room temperature and stored under H_2 at −18°C. For preparation of hydrogenase the cell paste was thawed, mixed with 1 volume of H_2-saturated 30 mM potassium phosphate (pH 7.0) at 0°C and stirred in an ice bath under H_2 for 5 min. The suspension was centrifuged and the dark-red

supernatant collected. The washing procedure was repeated 4 times with the cell pellet. To the combined supernatants alumina Cγ-gel was added which had been suspended in H_2-saturated 30 mM potassium phosphate (pH 7.0). After centrifugation the hydrogenase-containing yellow supernatant was concentrated under H_2 in a Diaflo cell (membrane: PM 10) and filtered through a Sephadex G-75 column which has previously been equilibrated with H_2-saturated 30 mM potassium phosphate (pH 7.0). Hydrogenase-containing fractions of the eluate were combined, concentrated under H_2 in a Diaflo cell and stored at −18°C.

Hydrogenase activity was measured manometrically in Warburg flasks under H_2 at 30°C [25]. The main compartment contained 1.55 ml enzyme solution and 0.15 ml 1 M potassium phosphate (pH 7.0). The reaction was started by adding 0.3 ml 4% methyl viologen from the side arm. Hydrogenase activity was calculated from the initial rate of H_2 consumption. One enzyme unit (U) was defined as that amount of protein which consumed 1 μmol H_2/min.

Analytical determinations. Sulfide was determined titrimetrically or colorimetrically with the method given by Trüper and Schlegel [6]. Thiosulfate, trithionate and tetrathionate were assayed following basically the cyanolytic method of Kelly et al. [7]. Sulfite was determined titrimetrically or colorimetrically according to Grant [8]. Acid-labile sulfur was determined according to King and Morris [9], and iron according to Sandell [10]. Protein determinations were made according to Lowry et al. [11]. The molar extinction coefficients used for calculations were: S^{2-}: $21 \cdot 10^3$; $S_2O_3^{2-}$, $S_3O_6^{2-}$ and $S_4O_4^{2-}$: $8.6 \cdot 10^3$; SO_3^{2-}: $32 \cdot 10^3$ and Fe^{2+}: $11 \cdot 10^3$ $cm^2 \cdot mmol^{-1}$.

Preparative methods. Trithionate was prepared according to Stamm and Goehring [12] and dithionate according to Pfanstiel [13]. Alumina Cγ-gel was prepared following the method of Willstätter and Kraut [14].

Enzymological methods. Analytical polyacrylamide gel electrophoresis was carried out in cylindrical gels (7.5% acrylamide, 8 cm × 0.5 cm) at 4°C for 4—6 h at 5 mA/gel. Two buffer systems were used: Tris/glycine, pH 8.3 (upper reservoir negative) and alanine/acetate, pH 4.0 (upper reservoir positive). 30—80 μg protein was applied to each gel. Bromophenol blue (pH 8.3) or methyleneblue (pH 4.0) were used as marker. The gels were stained with Coomassie blue. SDS-polyacrylamide gel electrophoresis was carried out according to Weber et al. [15] in 8 cm × 0.5 cm gels (7.5% acrylamide, 0.1% SDS) at room temperature and 8 mA/gel. 120-μl samples containing 2 μg sulfite reductase or, in addition, 2 μg each of marker proteins, 2.5 μg Bromophenol blue and 5 μl mercaptoethanol were applied to each tube. The gels were stained with Coomassie blue. The protein samples were prepared as follows: 0.1 ml protein solution (40 μg sulfite reductase or 20 μg marker protein) was mixed with 1 ml 10 mM potassium phosphate (pH 7.0), 1% (w/v) SDS, 1% (v/v) mercaptoethanol, incubated at 100°C for 3 min and used after cooling.

Chemicals. Chemicals were of reagent grade. Ecteola-cellulose, methyl and benzyl viologen were purchased from Serva, Heidelberg.

Results

Purification of sulfite reductase

Frozen cell paste was thawed and suspended in 50 mM potassium phosphate

(pH 7.0). The cells were broken by passing the suspension two or three times through a French pressure cell at about 138 MPa. The crude extract was brought to pH 4.5 by adding 1 vol. 300 mM ammonium acetate (pH 4.1). The precipitated material was centrifuged down and discarded. This fraction contained the dissimilatory *T. denitrificans* nitrite reductase (cytochrome *cd*) which, like the siroheme sulfite reductase, reduced sulfite with viologen dyes as electron donors. It contributed to about 50% to the total sulfite reductase activity (14 mU/mg protein) found in crude extracts of *T. denitrificans*. The supernatant was adjusted to pH 5.5 with 1 M K_2HPO_4 and then fractioned with $(NH_4)_2SO_4$. The protein precipitating between 45 and 70% saturation was collected by centrifugation, dissolved in 50 mM potassium phosphate (pH 7.0) and applied to Ecteola-cellulose previously equilibrated with the same buffer. The protein which had adsorbed to the gel was eluted with a linear gradient between 50 and 350 mM potassium phosphate (pH 7.0). Fractions with sulfite reductase activity (intensely green coloured, eluted at about 220 mM) were combined, dialysed against 50 vols. 50 mM potassium phosphate (pH 7.0) and then stirred into an appropriate volume of alumina Cγ-gel slurry suspended in water. The gel to which sulfite reductase adsorbed was centrifuged, washed once with 50 mM potassium phosphate (pH 7.0) and then eluted with stepwise increasing concentrations of potassium phosphate (pH 7.0; 80 mM, 110 mM, 140 mM, 170 mM and 210 mM). The eluates at 110 mM, 140 mM and 170 mM, which contained sulfite reductase, were combined and brought to 80% saturation with solid $(NH_4)_2SO_4$. The precipitated protein was collected by centrifugation, dissolved in 50 mM potassium phosphate (pH 7.0) and applied to a Sephadex G-200 column, which had previously been equilibrated with the same buffer. Green fractions of the eluate were combined and frozen at −20°C. A typical purification is summarized in Table I.

Control of purity

Fig. 1 shows the elution diagram after Sephadex G-200 gel filtration. Only one symmetrical protein peak was obtained, which coincided with the sulfite reductase peak located by measuring the enzyme activity or simply the absorp-

TABLE I

PURIFICATION OF *T. DENITRIFICANS* SULFITE REDUCTASE

Fraction	Protein (mg)	Sulfite reductase activity	
		Total (U)	Spec. act. (mU/mg protein)
Crude extract	18 400	258	14
Ammonium acetate fraction	5 280	121	31
Ammonium sulfate fraction	3 540	71	20
Ecteola eluate	386	113	293
Alumina Cγ eluate	312	106	339
Sephadex G-200 filtrate	254	84	331

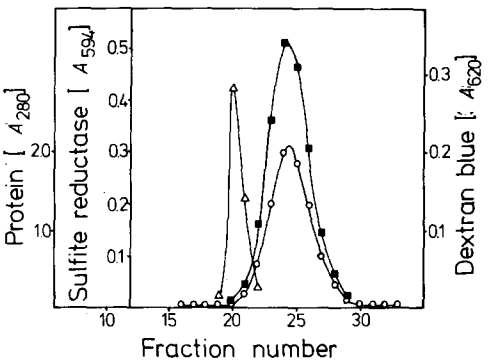

Fig. 1. Filtration of a purified preparation of *T. denitrificans* sulfite reductase through Sephadex G-200. 312 mg protein in a volume of 19 ml 50 mM potassium phosphate (pH 7.0) were filtered through a 3 cm × 100 cm column. The gel had been equilibrated with 50 mM potassium phosphate (pH 7.0) and was eluted with the same buffer. The void volume of the column was determined with Dextran blue. Protein (○) was measured as absorbance at 280 nm, sulfite reductase (■) at 594 nm and Dextran blue (△) at 620 nm.

tion at 594 nm. The absorbance ratio $A_{594} : A_{280}$ was almost constant throughout the peak; it varied between 0.26 (fraction 20 in Fig. 1) and 0.24 (fraction 28). Analytical electrophoresis of the Sephadex G-200 fraction in polyacrylamide gels at two different pH values (pH 8.3 and pH 4.0) and staining with Coomassie blue gave one strong (R_F values: 0.35 at pH 8.3 and 0.07 at pH 4.0) and several diffuse minor bands. The result of SDS gel electrophoresis (see below), which showed only two bands indicating no more than two different polypeptides, showed that the minor protein bands were obviously due to different forms of sulfite reductase.

Spectral properties

Fig. 2A gives the spectra of oxidized and dithionite-reduced sulfite reductase. No significant spectral change occurred upon reduction. The oxidized form had maxima at 274 nm, 393 nm and 594 nm. The molar extinction coefficients at these wavelengths were $280 \cdot 10^3$, $181 \cdot 10^3$ and $60 \cdot 10^3$ $cm^2 \cdot mmol^{-1}$, respectively.

Identification of heme prosthetic groups as siroheme

The heme prosthetic group of *T. denitrificans* sulfite reductase was extracted by mixing 1 vol. enzyme solution in 50 mM potassium phosphate (pH 7.0) with 9 vols. 50 mM HCl in acetone at 0°C. Fig. 2B gives the spectrum of the supernatant obtained after centrifugation of the denatured protein material. The absorption maxima were at 370 nm and 594 nm. The spectrum of *T. denitrificans* sulfite reductase heme prosthetic group was similar to siroheme preparations obtained from *Escherichia coli* sulfite reductase and desulfoviridin.

To identify the *T. denitrificans* sulfite reductase heme prosthetic group as siroheme, metal-free methyl esters of the chromophore released by acetone/HCl treatment were prepared following the method described by Murphy and

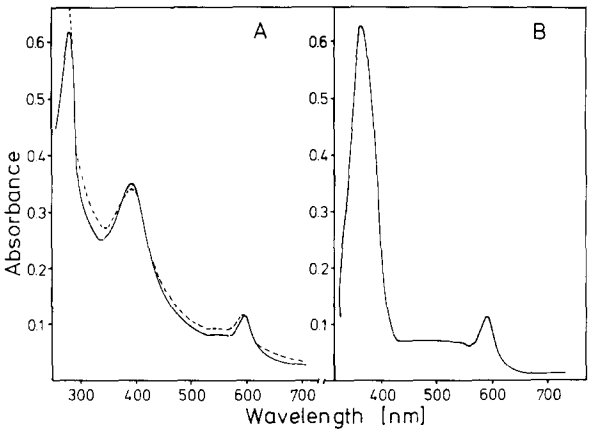

Fig. 2. (A) Absorption spectrum of *T. denitrificans* sulfite reductase. The spectrum of a 2 μM sulfite reductase solution in 50 mM potassium phosphate (pH 7.0) was recorded in a 1 cm cell against buffer at room temperature. ———, sulfite reductase oxidized; - - - - -, sulfite reductase reduced with dithionite. (B) Absorption spectrum of siroheme isolated from *T. denitrificans* sulfite reductase with acetone/HCl. 0.5 ml of a 40 μM sulfite reductase solution in 50 mM potassium phosphate (pH 7.0; A_{393}, 7.3; A_{594}, 2.6) was mixed with 4.5 ml 50 mM HCl in acetone. After centrifuging down the denatured protein the spectrum of the supernatant was determined in a 1 cm cell at 0°C against acetone/HCl.

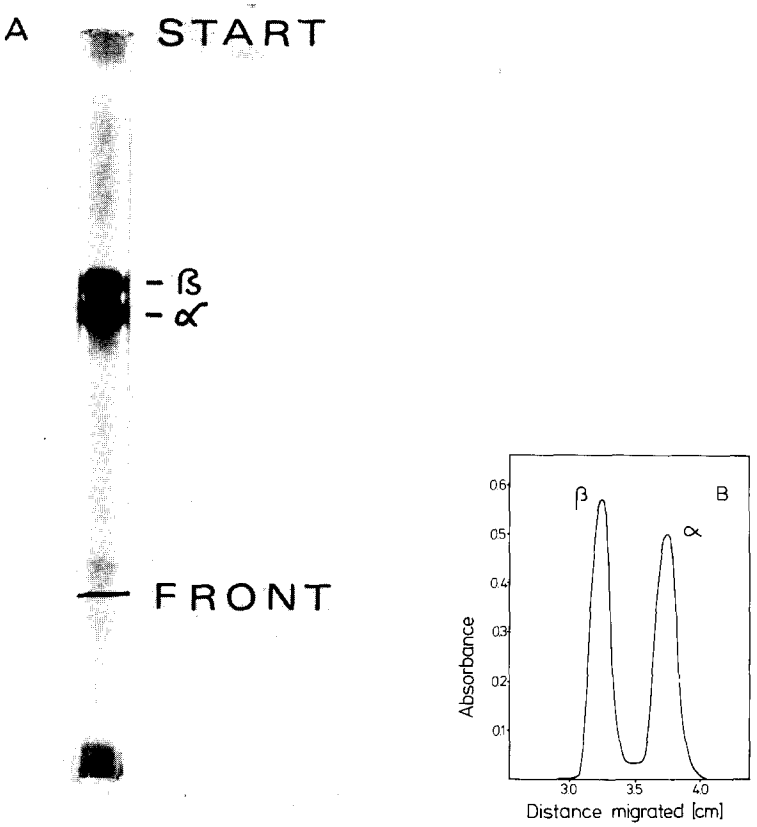

Fig. 3. Electrophoresis of *T. denitrificans* sulfite reductase in 7.5% acrylamide gels containing 0.1% sodium dodecyl sulfate. For details of sample treatment, electrophoresis, staining and destaining see Materials and Methods. (A) Photograph of gel stained with Coomassie blue. (B) Scan of adsorbance associated with each band with a Gilford Spectrophotometer at 620 nm.

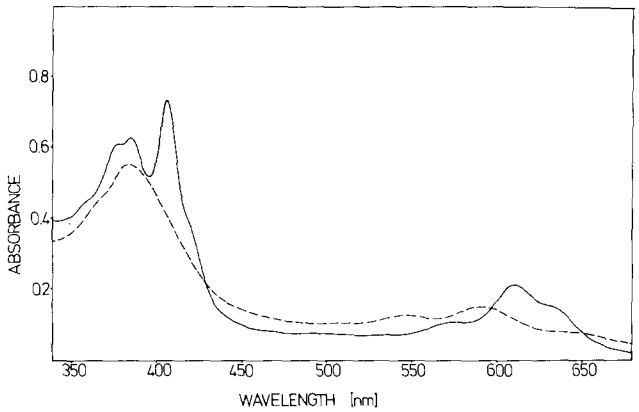

Fig. 4. Absorption spectra of demetallized esterified heme chromophore released from *T. denitrificans* sulfite reductase. Demetallization and esterification was carried out according to Murphy and Siegel [16]. Concentration of chromophore solution: 64 μg/ml. ———, methanol containing 1/20 vol. of H_2SO_4; - - - - - -, piperidine.

Siegel [16]. Fig. 4 gives the absorption spectra of the methyl esters of metal-free *T. denitrificans* sulfite reductase chromophore in piperidine and methanol/H_2SO_4. The corresponding fluorescence excitation and emission spectra are shown in Fig. 5. All spectra were almost identical to the ones reported for metal-free porphin methyl esters of siroheme isolated from *E. coli* sulfite reductase and desulfoviridin [16,23].

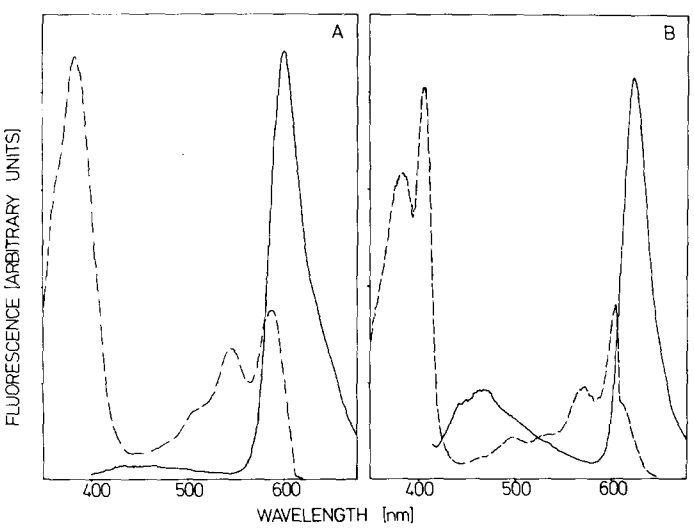

Fig. 5. Fluorescence excitation and emission spectra of demetallized esterified heme chromophore released from *T. denitrificans* sulfite reductase. Concentration of chromophore solution 64 μg/ml. - - - - - -, excitation; ———, emission. (A) Piperidine. The excitation spectrum was recorded by measuring the fluorescence at 597 nm, the emission spectrum was determined with exciting light at 384 nm. (B) Methanol containing 1/20 vol. H_2SO_4. The excitation spectrum was recorded by measuring the fluorescence at 622 nm, the emission spectrum was determined with an exciting light at 400 nm.

Molecular weight of native enzyme and subunits

The molecular weight of native *T. denitrificans* sulfite reductase was estimated by comparing its elution volume after gel filtration through Sephadex G-200 with that of several marker proteins with known molecular weight. Sulfite reductase eluted at $V_e/V_0 = 1.21$ corresponding to a molecular weight of 160 000. To determine the number and size of subunits *T. denitrificans* sulfite reductase was subjected to SDS-polyacrylamide gel electrophoresis. After staining with Coomassie blue two bands were obtained (Fig. 3A), a faster migrating one termed α-band (R_F value: 0.52) and a slower migrating one termed β-band (R_F value: 0.45). A scan of the absorbance (Fig. 3B) associated with each band indicated that *T. denitrificans* sulfite reductase contained as many α-subunits as it did β-subunits. The same pattern was obtained when the gels were stained with amido black. When sulfite reductase was coelectrophorized with several marker polypeptides, the molecular weight of the α-subunit could be determined to 38 000 and that of the β-subunit to 43 000. With 160 000 as molecular weight of the native enzyme and equal amounts of α- and β-subunits the enzyme structure can be expressed as $\alpha_2\beta_2$.

Content of iron and acid-labile sulfur

Iron and acid-labile sulfur were determined as described in Materials and Methods. The iron content was 24 Fe/enzyme molecule (average of four determinations), that of acid-labile sulfur 20 'S'/enzyme molecule (average of six determinations).

General catalytic properties

T. denitrificans sulfite reductase reduced sulfite but not thiosulfate, di-, tri- or tetrathionate. Enzymatic activity was obtained with methyl or benzyl viologen but not with $NADH_2$ or $NADPH_2$. The pH optimum was in the slightly acid region at about 6.0. The turnover number of the electrophoretically homogeneous enzyme preparation was 331 nmol H_2/min per mg protein. This corresponds to 53 H_2/min per enzyme molecule.

Stoichiometry of sulfite reduction

In order to determine the products of sulfite reduction catalyzed by *T. denitrificans* sulfite reductase a known amount of sulfite was enzymatically reduced in the manometric assay system and the products were colorimetrically determined after completion of the reaction (when no more hydrogen was consumed). The result of a typical experiment is shown in Table II. Only about 40% of the sulfite sulfur appeared as sulfide, the remainder as trithionate (36%) and thiosulfate (20%). 3.8 μmol H_2 were consumed for the reduction of 2.74 μmol SO_3^{2-}. This corresponded well with the calculated value of 4.0 μmol necessary to reduce 2.74 μmol sulfite to the products given in Table II.

Attempts to demonstrate sulfite formation from sulfide with T. denitrificans sulfite reductase

Sulfite reduction liberates electrons of a mean redox potential to $E'_0 = -120$ mV. Under appropriate reaction conditions it should, therefore, be possible to form sulfite from reduced sulfur compounds with *T. denitrificans* sulfite

TABLE II

STOICHIOMETRY OF SULFITE REDUCTION CATALYZED BY *T. DENITRIFICANS* SULFITE REDUCTASE

Sulfite was reduced in the reaction mixture described in Materials and Methods in a Warburg vessel with two side arms. 0.4 mg of sulfite reductase was added. The reaction was started with sulfite added from the first arm. When hydrogen was no longer consumed 0.2 ml 3 M ammonium acetate (pH 5.0) was added from the second arm. After 20 min sulfide was determined in the content of the center well, trithionate and thiosulfate in that of the main compartment.

		μmol/vessel		% 'S'
SO_3^{2-}	added	2.74		100
H_2	consumed	3.80		
$S_3O_6^{2-}$		0.32		36
$S_2O_3^{2-}$		0.28		20
S^{2-}		1.07		39
			found:	94

reductase. Sulfide and also reduced (R-S_x-H, e.g. Glu-S_2-H) not, however, oxidized (R-S_x-R, e.g. Glu-S_3-Glu or polythionates) polysulfane sulfur-containing compounds could be shown to reduce *T. denitrificans* sulfite reductase and donate electrons to the enzyme. As compounds of the type R-S_x-H are in aqueous solution in equilibrium with sulfide and the corresponding oxidized compound (2 R-S_x-H ↔ H_2S + R-S_{2x-1}-R) sulfide was the only true in vitro substrate available for a reversed sulfite reductase reaction. From ten artificial electron acceptors with $E'_0 \geqslant -120$ mV which were assayed for the ability to accept electrons from sulfide-reduced *T. denitrificans* reductase seven were reduced directly by sulfide (concentration: 0.2 mM). The remaining three (triphenyltetrazolium, menadion, methylene blue) could not be reduced by sulfide when

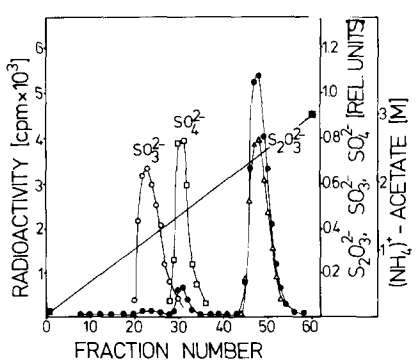

Fig. 6. Products of sulfide oxidation with substrate amounts of *T. denitrificans* sulfite reductase. The experiment was carried out as described in the text. 70 ml low molecular weight fraction obtained after Sephadex G-25 filtration was mixed with cold $S_2O_3^{2-}$, SO_3^{2-} and SO_4^{2-} (1 mmol each), adsorbed to Dowex resin (Dowex Cl^- form, 1 × 2, 200—400 mesh; 0.7 cm × 6 cm column) and eluted with a linear ammonium acetate (pH 5.0) gradient. 5-ml fractions were collected. $S_2O_3^{2-}$, SO_3^{2-} and SO_4^{2-} were determined as described in Materials and Methods. 0.5 ml of each fraction was mixed with 5 ml scintillation cocktail and the radioactivity determined by liquid scintillation counting.

T. denitrificans sulfite reductase was added to a concentration of 1 μM. As a consequence no continuous assay system for a reversed sulfite reductase could be worked out. To demonstrate qualitatively the ability of *T. denitrificans* sulfite reductase to oxidize sulfide to sulfur of the oxidation state of sulfite, substrate amounts of oxidized enzyme (1.6 μmol) were incubated in a total volume of 3.2 ml 50 mM potassium phosphate (pH 7.0) under N_2 at 30°C in a Warburg vessel with a small excess of [^{35}S]sulfide (6.7 μmol with 0.33 Ci/mol added from the side arm). After 10 min incubation time 0.2 ml 3 M ammonium acetate (pH 5.0) was added from a second side arm and the remaining sulfide adsorbed in 2.0 ml 5 N NaOH in the center well. 0.97 μmol of sulfide (average to three experiments) had been oxidized as compared to a control without enzyme. The reaction mixture was removed from the main compartment and filtered through Sephadex G-25. About 1/3 of the radioactivity remained enzyme bound, 2/3 appeared in the low molecular weight fraction. This fraction was mixed with cold $S_2O_3^{2-}$, SO_3^{2-} and SO_4^{2-} (0.1 mmol of each), adsorbed to Dowex resin and eluted with a linear gradient of ammonium acetate (pH 5.0). As shown in Fig. 6 90% of the radioactivity of the non-enzyme-bound oxidation products appeared in the $S_2O_3^{2-}$, the remainder in the sulfate fraction.

Discussion

The molecular and catalytic properties of *T. denitrificans* sulfite reductase reported in this paper are summarized in Table III and compared with those of the well characterized *Desulfovibrio vulgaris* sulfite reductase (desulfoviridin) which represents the dissimilatory type sulfite reductase involved in respiratory

TABLE III

COMPARISON OF MOLECULAR AND CATALYTIC PROPERTIES OF *T. DENITRIFICANS* SULFITE REDUCTASE WITH THOSE OF *D. VULGARIS* SULFITE REDUCTASE

Data of *D. vulgaris* sulfite reductase were taken from Lee et al. [17] and from Kobayashi et al. [18,19]. n.d., not determined.

	T. denitrificans	*D. vulgaris*
Absorption maxima and molar extinction coefficients ($cm^2 \cdot mmol^{-1} \times 10^3$)	274 nm; 280 393 nm; 181 594 nm; 60	279 nm 390 nm 408 nm; 150 580 nm 628 nm; 50
Molecular weight of native enzyme	160 000	226 000
Number of molecular weight of subunits	2 α (38 000) 2 β (43 000)	2 α (42 000) 2 β (50 000)
Molecular structure	$\alpha_2\beta_2$	$\alpha_2\beta_2$
Iron content (Fe/enzyme molecule)	24	n.d.
Acid-labile sulfur ('S'/enzyme molecule)	20	n.d.
μmol H_2 consumed/μmol SO_3^{2-} reduced	1.4	0.8
Products of sulfite reduction (% of sulfur found)		
$S_3O_6^{2-}$	36	66
$S_2O_3^{2-}$	20	16
S^{2-}	39	15
pH optimum	6.0	5.5—6.0
Turnover number (2 e^-/enzyme molecule · min^{-1})	53	58

sulfate reduction in bacteria of the genus *Desulfovibrio*. Table III shows that the two enzymes are very similar in their properties: both have an $\alpha_2\beta_2$-structure, similar molecular weights and comparable specific extinction coefficients. They do not reduce sulfite completely to sulfide but form trithionate and thiosulfate as additional products. Both enzymes are only active with reduced viologen dyes but not with reduced pyridine nucleotides. The pH optimum lies in the slightly acid region indicating bisulfite (HSO_3^-) as actual enzyme substrate. The turnover number of about 50 H_2/enzyme molecule · min^{-1} is extremely low. In all these properties *T. denitrificans* sulfite reductase is fundamentally different from assimilatory type sulfite reductases that have been isolated and characterized from a variety of organisms. The *E. coli* enzyme [20], e.g. is a macromolecule with 12 subunits arranged to an $\alpha_4\beta_8$-structure which carries siroheme, FMN, FAD and Fe/S clusters as prosthetic groups and which possesses a complex intramolecular electron transport chain through which electrons are transferred from $NADPH_2$ to sulfite. The *E. coli* enzyme like other assimilatory sulfite reductases reduces sulfite quantitatively to sulfide, shows a pH optimum in the slightly alkaline region (the actual enzyme substrate then is SO_3^{2-}) and has a significantly higher turnover number (3900 H_2/enzyme molecule · min^{-1} with reduced methyl viologen).

T. denitrificans sulfite reductase has been manometrically tested as sulfite-reducing enzyme with reduced methyl viologen as electron donor. We have not been able to work out an assay system for a reversed sulfite reductase. We could, however, demonstrate that sulfur in the oxidation state of sulfite (sulfone sulfur of thiosulfate) is formed as one of the oxidation products of sulfide oxidation when substrate amounts of enzyme are incubated with a small excess of sulfide. As it is not known how many of the six electrons liberated during oxidation of sulfide to sulfite can be stored within the enzyme molecule the conclusions which may be drawn from this experiment are limited.

The ultimate experimental proof for the assumption that the physiological function of *T. denitrificans* sulfite reductase is not to reduce but rather to form sulfite from either sulfide or elemental sulfur in the course of dissimilatory oxidation of reduced sulfur compounds has still to be furnished. The following considerations, however, support this idea:

(1) Unpublished experiments carried out in this laboratory have shown that *T. denitrificans* does not perform assimilatory sulfite reduction: neither sulfite nor sulfate were used by whole cells as a sulfur source for biosynthetic processes; during growth on thiosulfate cell sulfur was exclusively obtained from the outer (oxidation level of elemental sulfur) and not from the inner sulfur atom (oxidation level of sulfite) of the molecule. An assimilatory function of *T. denitrificans* sulfite reductase as sulfite-reducing enzyme can, therefore, be excluded.

(2) To prepare an electrophoretically homogeneous enzyme preparation a 50-fold enrichment over the specific activity in crude extracts was sufficient. One has to bear in mind that only about 50% of the specific sulfite reductase activity in crude extracts of *T. denitrificans* is contributed by siroheme sulfite reductase, the remainder by nitrite reductase. The high cellular content of *T. denitrificans* sulfite reductase which can be estimated as about 2% of the total protein would indicate a dissimilatory rather than an assimilatory function of the enzyme.

(3) As already mentioned the redox potential of the half reaction S^{2-} + 3 $H_2O \rightleftharpoons SO_3^{2-}$ + 6 H^+ at pH 7 is $E'_0 = -120$ mV [4]. This means that under physiological conditions the sulfite reductase reaction can easily be reversed in the presence of an appropriate electron acceptor of $E'_0 = -120$ mV.

(4) Table III shows that *T. denitrificans* sulfite reductase is very similar in most of its properties to dissimilatory type sulfite reductase, so for instance to desulfoviridin isolated from *D. vulgaris*. Adenylylsulfate reductase is another enzyme of sulfur metabolism which occurs in both thiobacilli and sulfate reducers for which similar catalytic and molecular parameters have been found when purified enzyme preparations from either of the two groups or organisms were investigated [21]. The adenulylsulfate (APS) reductase reaction AMP + $SO_3^{2-} \rightleftharpoons APS^{2-}$ + 2 e^- also shows a midway redox potential which at pH 7 is $E'_0 = -60$ mV [20]. In analogy to adenylylsulfate reductase which in sulfate reducers has been shown to reduce in thiobacilli to form adenylylsulfate, sulfite reductase should be envisaged as an enzyme which in sulfate reducers takes part in sulfite reduction, in *T. denitrificans*, however, in sulfite formation.

The finding that the facultative anaerobe *T. denitrificans* contains a sulfite-forming enzyme which shows close similarity to desulfoviridin, the sulfite reductase of dissimilatory sulfate-reducing bacteria, is in accordance with an early proposal put forward by Peck [22] who discussed the possibility that the oxidation of reduced sulfur compounds and the reduction of sulfate are enzymologically identical, however, differently directed processes; so far only APS reductase has been shown to occur both in sulfur-oxidizing and in sulfate-reducing bacteria. Siroheme sulfite reductase is now the second enzyme which has been demonstrated in dissimilatory sulfate reducers and in at least one sulfur oxidizer.

It is of great interest to investigate other anaerobic sulfur-oxidizing bacteria for the presence of siroheme sulfite reductase to answer the question whether this enzyme is in general responsible for anaerobic sulfite formation and, therefore, corresponds to elemental sulfur oxygenase active in aerobic thiobacilli. Experiments with phototrophic sulfur bacteria are presently carried out in this laboratory.

Acknowledgments

We thank Miss Maria Vanselow for skillful technical assistance and Dr. Bayer for recording the absorption and fluorescence excitation and emission spectra of demetallized and esterified heme chromophore. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

References

1 Suzuki, I. (1965) Biochim. Biophys. Acta 110, 97
2 Suzuki, I. (1974) Annu. Rev. Microbiol. 28, 85
3 Schedel, M., Le Gall, J. and Baldensperger, J. (1975) Arch. Microbiol. 105, 339
4 Wagner, G.C., Kassner, R.J. and Kamen, M.D. (1974) Proc. Natl. Acad. Sci. U.S. 71, 253
5 Pfennig, N. and Lippert, K.D. (1966) Arch. Microbiol. 55, 245
6 Trüper, H.G. and Schlegel, H.G. (1964) Antonie van Leeuwenhoek 30, 225
7 Kelly, D.P., Chambers, L.A. and Trudinger, P.A. (1969) Anal. Chem. 41, 898
8 Grant, W.M. (1947) Ind. Eng. Chem. Anal. Ed. 19, 345

9 King, T.E. and Morris, R.O. (1967) Methods Enzymol. 10, 634
10 Sandell, E.B. (1944) Colorimetric Determinations of Traces of Metals, Interscience Publishers, New York
11 Lowry, O.H., Rosebrough, H.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265
12 Stamm, H. and Goehring, M. (1942) Z. Anorg. Allg. Chem. 250, 226
13 Pfanstiel, R. (1946) Inorg. Synth. 2, 167
14 Willstätter, R. and Kraut, H. (1923) Chem. Ber. 56, 1117
15 Weber, K., Pringle, J.R. and Orborn, M. (1972) Methods Enzymol. 26, 3
16 Murphy, M.J. and Siegel, L.M. (1973) J. Biol. Chem. 248, 6911
17 Lee, J.P., Le Gall, J. and Peck, H.D. Jr. (1973) J. Bacteriol. 115, 529
18 Kobayashi, K., Takahashi, E. and Ishimoto, M. (1972) J. Biochem. 72, 879
19 Kobayashi, K., Seki, Y. and Ishimoto, M. (1974) J. Biochem. 75, 519
20 Siegel, L.M. (1975) in Metabolic Pathways (D.N. Greenberg, ed.), 3rd edn., Vol. 7, p. 217, Academic Press, New York
21 Kirchhoff, J. and Trüpper, H.G. (1974) Arch. Microbiol. 100, 115
22 Peck, H.D., Jr. (1962) Bacteriol. Rev. 26, 67
23 Murphy, M.J., Siegel, L.M., Tove, S.R. and Kamin, H. (1974) Proc. Natl. Acad. Sci. U.S. 71, 612
24 Yoshimoto, A. and Sato, R. (1968) Biochim. Biophys. Acta 153, 555
25 Peck, H.D., Jr. and Gest, H. (1956) J. Bacteriol. 71, 70