

Jan M. Visser · Govardus A. H. de Jong
Lesley A. Robertson · J. Gijs Kuenen

A novel membrane-bound flavocytochrome *c* sulfide dehydrogenase from the colourless sulfur bacterium *Thiobacillus* sp. W5

Received: 15 October 1996 / Accepted: 7 January 1997

Abstract A novel membrane-bound sulfide-oxidizing enzyme was purified 102-fold from the neutrophilic, obligately chemolithoautotrophic *Thiobacillus* sp. W5 by means of a six-step procedure. Spectral analysis revealed that the enzyme contains haem *c* and flavin. SDS-PAGE showed the presence of two types of subunit with molecular masses of 40 and 11 kDa. The smaller subunit contains covalently bound haem *c*, as was shown by haem staining. A combination of spectral analysis and the pyridine haemochrome test indicated that the sulfide-oxidizing heterodimer contains one molecule of haem *c* and one molecule of flavin. It appeared that the sulfide-oxidizing enzyme is a member of a small class of redox proteins, the flavocytochromes *c*, and is structurally most related to the flavocytochrome *c* sulfide dehydrogenase of the green sulfur bacterium *Chlorobium limicola*. The pH optimum of the enzyme is 8.6. At pH 9, the V_{\max} was $2.1 \pm 0.1 \mu\text{mol cytochrome } c \text{ (mg protein)}^{-1} \text{ min}^{-1}$, and the K_m values for sulfide and cytochrome *c* were $1.7 \pm 0.4 \mu\text{M}$ and $3.8 \pm 0.8 \mu\text{M}$, respectively. Cyanide inhibited the enzyme by the formation of an N-5 adduct with the flavin moiety of the protein. On the basis of electron transfer stoichiometry, it seems likely that sulfur is the oxidation product.

Key words *Thiobacillus* sp. W5 · Sulfide oxidation · Sulfur formation · Flavocytochrome *c* · *Chlorobium limicola* · *Chromatium vinosum* · Thiobacilli

Abbreviations *FSDH* Flavocytochrome *c* sulfide dehydrogenase · *TCA* Trichloroacetic acid · *SQR* Sulfide quinone reductase

Introduction

Oxidation of inorganic sulfur compounds by bacteria has been studied for almost a century (Winogradsky 1887; Nathansohn 1902), and particular interest has been shown with respect to the chemolithotrophic organisms that play an important role in the global sulfur cycle (Ehrlich 1990). Most of the autotrophic sulfur oxidizers are placed within the genus *Thiobacillus*. The bacteria in this genus possess very diverse physiological characteristics, and they are linked by the fact that they are all gram-negative and rod-shaped and obtain energy from the oxidation of reduced sulfur compounds (Kuenen et al. 1992). Within this genus, diversity is also reflected by 16S rRNA analysis, showing that the thiobacilli are scattered throughout the beta subdivision of the Proteobacteria (Lane et al. 1992). Biochemical and physiological research on the oxidation of reduced sulfur compounds by thiobacilli has shown that different metabolic pathways operate in different organisms (Kelly 1989). The metabolic routes involving the oxidation of thiosulfate, tetrathionate and sulfite have received much attention and have led to the isolation of several enzymes (Lyric and Suzuki 1970; Schedel and Trüper 1979; Toghrol and Sutherland 1983; Lu and Kelly 1988; Meulenberg et al. 1993; Tano et al. 1996) and enzyme complexes (Oh and Suzuki 1977; Lu and Kelly 1983) responsible for the conversion of these compounds.

Despite the fact that sulfide is an important substrate in natural systems, few studies have been devoted to the metabolic pathways involving sulfide oxidation. Sulfide reacts spontaneously with oxygen. Thiosulfate, elemental sulfur and sulfite have been described as products of this chemical oxidation (Chen and Morris 1972; Janssen et al. 1995). However, due to their high affinity for both sulfide and oxygen, the sulfide-oxidizing bacteria can successfully compete with the spontaneous oxidation reaction (Kuenen and Bos 1989). At concentrations above 150 μM , sulfide may even be toxic to the thiobacilli (Hirayama and Vetter 1989). All thiobacilli thus far investigated are able to oxidize sulfide completely to sulfate. In

J. M. Visser · G. A. H. de Jong · L. A. Robertson (✉)
J. G. Kuenen
Department of Microbiology and Enzymology,
Delft University of Technology, Julianalaan 67, 2628 BC Delft,
The Netherlands
Tel. +31-15-2782421; Fax +31-15-2782355
e-mail l.a.robertson@stm.tudelft.nl

the facultatively chemolithoautotrophic *Thiobacillus versutus* (now *Paraccocus versutus*; Katayama et al. 1995), it is proposed that sulfide is oxidized by an enzyme B without any intermediate being detectable (Schneider and Friedrich 1994).

In most obligate and facultative sulfide oxidizers, intermediate sulfur appears as a major or minor product during the oxidation of sulfide (Hazeu et al. 1988; Chan and Suzuki 1993; Van den Ende and Van Gernerden 1993; Steffes et al. 1996). This suggests that zero-valent sulfur may be an intermediate in the sulfide-oxidation pathway. This suggestion is supported by the observation that membrane fractions of *Thiobacillus concretivorus* (now *Thiobacillus thiooxidans*; Kuenen et al. 1992) shows cytochrome-*c*-dependent sulfide oxidation to some form of membrane-bound polysulfur (Moriarty and Nicholas 1970). However, a bottleneck in the study of the oxidation pathway of sulfide has been that, until now, sulfide-oxidizing enzymes have not been isolated from obligately chemolithoautotrophic *Thiobacillus* species.

This paper reports the isolation of a novel membrane-bound, sulfide-oxidizing enzyme from the obligately chemolithoautotrophic *Thiobacillus* sp. W5 (LMD 94.73). This bacterium has been shown to be the dominant organism in a sulfur-producing reactor and appears to be a new species closely related to *Thiobacillus neapolitanus* (J. M. Visser, L. A. Robertson, and J. G. Kuenen, unpublished results). Characterization of the enzyme revealed that it belongs to a small class of redox proteins, the flavocytochromes *c*, and is structurally related to the soluble flavocytochrome *c* sulfide dehydrogenase from the anoxygenic green sulfur bacterium *Chlorobium limicola* (Yamanaka and Fukumori 1980).

Materials and methods

Organism and cultivation

Thiobacillus sp. W5 (LMD 94.73) was obtained from the Delft Culture Collection. Sulfide-limited (50 mM) chemostat cultures were grown on mineral salt medium at a dilution rate of 0.10 h⁻¹ and a dissolved oxygen tension of 50% (Visser et al. 1996).

Oxygen uptake experiments

The maximum sulfide-dependent oxygen uptake rate by whole cells taken directly from the chemostat cultures was measured in a biological oxygen monitor. Oxygen consumption was measured polarographically at 25°C using a Clark-type oxygen electrode mounted in a thermostatically controlled cell (Yellow Springs Instruments, Yellow Springs, Ohio, USA). Sulfide (25 µM) was added to obtain maximum oxidation rates. At this concentration, the spontaneous oxidation of sulfide was sufficiently low so as not to disturb the accurate measurement of biological conversion.

Enzyme purification

Cells (75 g wet weight) collected at 4°C from a sulfide-limited chemostat were centrifuged and resuspended in 200 ml 25 mM Tris-HCl (pH 7.5). Subsequently, the cell suspension was passed three times through a French pressure cell (American Instrument,

Silver Spring, Md., USA) at 110 MPa. The broken cells were treated with DNase, after which whole cells and cell debris were removed by centrifuging at 10,000 × *g* for 30 min. The membrane fraction was obtained by centrifuging at 200,000 × *g* for 4 h. The membranes were then washed and resuspended in 50 mM Tris-HCl (pH 7.5). The membrane proteins were solubilized for 1 h at 4°C by the addition of 1% β-D-lauryl maltoside (Anatrace, Maumee, Ohio, USA). After centrifuging at 200,000 × *g* for 6 h, the supernatant was loaded on a DEAE-Sepharose column (5 × 40 cm; Pharmacia, Roosendaal, The Netherlands) previously equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.05% lauryl maltoside (buffer A). Proteins were eluted with a linear gradient (1,000 ml) of 0–0.5 M KCl in buffer A at 6 ml min⁻¹. Active fractions were concentrated (Centricon 10; Amicon, Capelle a/d IJssel, The Netherlands) and applied to a prepacked Superdex 200 HR 10/30 gel filtration column (Pharmacia) previously equilibrated with 0.2 M KCl in buffer A. Pooled active fractions were again concentrated (Centricon 10; Amicon), desalted (PD 10; Pharmacia), and further purified by loading on a prepacked Mono Q HR 10/10 column (Pharmacia) pre-equilibrated with buffer A. Proteins were eluted with a linear gradient (60 ml) of 0–0.5 M KCl in buffer A at 1 ml min⁻¹. The final purification step was performed by a second gel filtration step (Superdex 200 HR 10/30). During the procedures, active fractions were stored at –80°C.

Enzyme assay

The standard reaction mixture (1 ml) for measuring sulfide-oxidizing activity contained 50 mM Tris-HCl (pH 9.0), 25 µM horse-heart cytochrome *c* (Sigma, St. Louis, Mo., USA), 10 µM Na₂S and enzyme. The measurements were performed at 25°C and were started by the addition of sulfide. Reduction of horse-heart cytochrome *c* was measured at 550 nm in an HP 8524A diode array spectrophotometer using an extinction coefficient of 19.6 mM⁻¹ cm⁻¹. One unit of activity (U) is defined as 1 µmol of horse-heart cytochrome *c* reduced per minute.

Substrate specificity and electron acceptors

The substrate specificity of the enzyme was investigated using the enzyme assay described above. Sodium thiosulfate (1 mM), potassium tetrathionate (1 mM), potassium trithionate (1 mM), potassium pentathionate (1 mM), sodium sulfite (1 mM), sulfur (10 mM) and polysulfide (10–25 µM) were tested as substrates. A concentrated polysulfide solution was prepared by heating a solution containing 10 mM Na₂S and 100 mM sulfur for 10 min at 110°C. Trithionate and pentathionate were kind gifts from Prof. Ralph Steudel (Technische Universität Berlin, Germany).

Inhibitors

The effect of potential inhibitors was studied using the enzyme assay described above. The assay mixture containing the enzyme was preincubated with the inhibitors for 5 min at 25°C. The following inhibitors (concentration) were used: sodium azide (1 mM), EDTA (1 mM), *N*-ethylmaleimide (1 mM), potassium cyanide (0.025–1 mM), sodium sulfite (0.05–2 mM) and sodium thiosulfate (0.05–2 mM).

Protein electrophoresis

SDS-PAGE electrophoresis was performed at room temperature on vertical 18% polyacrylamide slab gels according to the method of Laemmli (1970) using Mini Protean equipment (Biorad, Veenendaal, The Netherlands). Gels were stained for protein with silver staining. A low-molecular-mass calibration kit (Pharmacia) was used to derive the molecular masses. Haem staining was done with 3,3',5,5'-tetramethylbenzidine (Thomas et al. 1976). Pre-

Table 1 Purification of a membrane-bound sulfide-oxidizing enzyme from *Thiobacillus* sp. W5

Purification step	Total protein (mg)	Total activity (U)	Recovery (%)	Specific activity (mU/mg protein)	Purification (-fold)
Crude extracts	2,068	40.3	100	19.5	1.0
Membranes	973	37.0	91.8	39.0	2.0
Detergent extraction	589	33.0	81.9	56.6	2.9
DEAE-Sepharose	78	22.0	54.6	283	14.5
Gel filtration	34	14.8	36.7	435	22.3
Mono Q	5.2	7.4	18.4	1,420	72.8
Gel filtration	1.2	2.4	5.9	1,989	102

stained markers (Biorad) were used when gels were stained for haem.

UV/visible spectroscopy

UV/visible spectra of purified enzyme in 50 mM Tris-HCl (pH 7.5) were recorded on an SLM Aminco DW-2000 spectrophotometer. The spectral band width was 1 nm, while the scanning speed was 2 nm s⁻¹. Reduced spectra were obtained by adding sulfide to a final concentration of 10 µM. The pyridine haemochrome method (Berry and Trumpower 1987) was used to determine the haem *c* content.

Analytical procedures

The chain length of polysulfide was determined by measuring the amount of sulfur after acidifying the polysulfide solution. Sulfuric acid (9 ml, 1 M) was added to 1 ml of the concentrated polysulfide solution. The acidified solution was bubbled with air for 15 min to remove the hydrogen sulfide and was subsequently centrifuged for 10 min at 13,000 × *g*. The sulfur pellet was extracted overnight with 10 ml acetone. The sulfur concentration was determined by cyanolysis (Bartlett and Skoog 1954). Protein concentrations were determined with the BCA Protein Assay Reagent Kit (Pierce, Rockford, Ill., USA).

Results and discussion

Purification of the sulfide-oxidizing enzyme

With horse-heart cytochrome *c* as electron acceptor, 92% of the sulfide-oxidizing enzyme activity was found in the membrane fraction of disrupted *Thiobacillus* sp. W5 cells. Solubilization of the membrane proteins using the non-ionic detergent β-D-lauryl maltoside resulted in selective extraction because 54% of the protein and 89% of the activity were recovered in the solubilized fraction. The rest remained in the membranes. Solubilized membrane protein was applied to an anionic exchange column. Active fractions were further subjected to gel filtration, anionic exchange and further gel filtration, resulting in a 102-fold purification with a final recovery of 5.9% (Table 1).

Characterization of the sulfide-oxidizing enzyme

UV/visible absorption spectroscopy of the sulfide-reduced enzyme showed characteristic maxima at 410 (γ band), 524 (β band) and 552 nm (α band) (Fig. 1), revealing the presence of *c*₅₅₂ haem. Dithionite did not increase

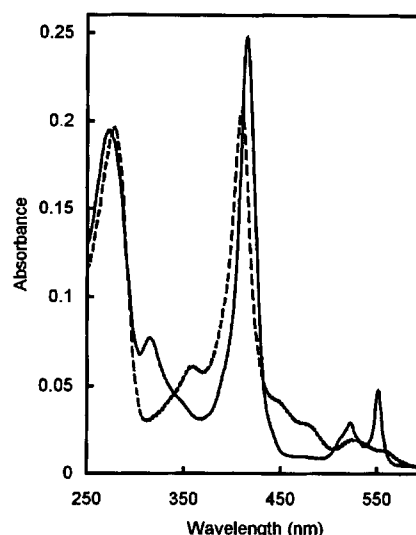


Fig. 1 UV/visible absorption spectrum of purified sulfide-oxidizing enzyme (1.8 µM). The absorption spectrum is shown from 250 to 600 nm. It was recorded with an Aminco DW2000 spectrophotometer at room temperature. The air-oxidized spectrum is represented by the dotted line. The reduced spectrum was obtained by the addition of sulfide (10 µM) and is represented by the solid line

the level of reduction of the enzyme, indicating that sulfide is capable of totally reducing it. The absorption spectrum of the air-oxidized enzyme showed maxima at 450 and 480 nm, which is characteristic for the presence of flavin.

The spectral characteristics show that this enzyme is related to a small family of redox proteins, the flavocytochromes *c*. Only two types of flavocytochromes *c* have been reported to possess sulfide-oxidizing activity. Both types were originally isolated as soluble haem proteins with unknown functions, the first from the phototrophic purple sulfur bacterium *Chromatium vinosum* (Bartsch and Kamen 1960) and the second from the phototrophic green sulfur bacterium *Chlorobium thiosulfatophilum* (Meyer et al. 1968). It took, however, until 1973 to realize that these flavocytochromes *c* were sulfide-oxidizing enzymes (Kusai and Yamanaka 1973). This group of enzymes was given the name flavocytochrome *c* sulfide dehydrogenase (FSDH) because of their function as sulfide dehydrogenases (Cusanovich et al. 1991). Detailed structural analysis has shown that the *Chromatium* FSDH dif-

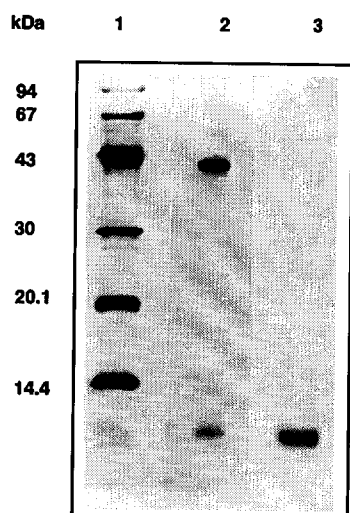


Fig. 2 Silver- and haem-stained SDS-polyacrylamide gel (18%) after electrophoresis of the sulfide-oxidizing enzyme from *Thiobacillus* sp. W5. Lane 1 Silver-stained low-molecular-mass markers; lane 2 silver staining (3 µg purified protein), lane 3 haem staining (5 µg purified protein)

fers fundamentally from flavocytochrome b_2 and flavocytochrome- c - p -cresol methylhydroxylase (Chen et al. 1994).

SDS-PAGE of the purified enzyme showed two protein bands with molecular masses of 40.2 ± 0.8 and 10.7 ± 0.5 kDa, respectively (Fig. 2). Haem staining revealed the exclusive presence of covalently bound haem c in the smaller subunit (Fig. 2). The purified protein resisted native gel electrophoresis. However, comparison of the data on the subunits and the UV/visible absorption spectrum of the *Thiobacillus* sp. W5 enzyme with those of the phototrophic organisms (Cusanovich et al. 1991) indicated that the new enzyme is a heterodimer with a molecular mass of 51 kDa. The absorption maximum of the pyridine haemochrome complex was used to determine the haem concentration. It was calculated that 1 mol of sulfide-oxidizing enzyme contains 1.09 ± 0.04 mol of haem c , indicating a molar haem- c -enzyme ratio of 1:1. Absorption

ratios are characteristic of the two types of flavocytochrome c . Once the ratio is known for a particular enzyme, the apparent $A_{280\text{ox}}/A_{410\text{ox}}$ ratio is often used as a measure of the purity of an enzyme preparation. The $A_{280\text{ox}}/A_{410\text{ox}}$ ratio of the pure *Thiobacillus* sp. W5 enzyme was found to be 0.95. Furthermore, an $A_{475\text{ox}}/A_{525\text{ox}}$ ratio of 1.5 was observed, which is indicative for a full complement of unmodified flavin (Cusanovich et al. 1991). The millimolar extinction coefficient (ϵ^{mM}) of 16 at 480 nm, calculated from the absorption spectrum, indicated the presence of 1 mol of flavin/mol of *Thiobacillus* enzyme. Trichloroacetic acid (TCA) precipitation of the enzyme did not result in the detachment of the flavin moiety from the protein, indicating a covalent attachment to the protein subunit. Attempts to assign the flavin to one of the two subunits failed because the native enzyme could not be separated into its subunits by differential extraction after TCA precipitation (Yamanaka et al. 1979) or by denaturing gel filtration in 6 M urea. However, on the basis of its structural similarities to the other FSDH (Table 2), it is assumed that the flavin resides in the larger subunit. Further biochemical studies are necessary to confirm that the flavin is in the larger subunit and to determine the exact nature of the flavin (FMN, FAD).

Spectral features and structural properties of the flavocytochromes c from *Thiobacillus* sp. W5 and the two phototrophic bacteria are listed in Table 2. Comparison of the sulfide-oxidizing enzyme from *Thiobacillus* sp. W5 with the FSDH from the phototrophic sulfur bacteria (Table 2) suggests that the new enzyme belongs to the FSDH family of proteins. However, this requires confirmation. *Thiobacillus* sp. W5 FSDH seems structurally most closely related to the FSDH of the green sulfur bacterium *Chlorobium limicola* since they both apparently possess a mono-haem cytochrome subunit. A major difference, however, is the fact that the *Thiobacillus* sp. W5 enzyme is membrane-bound while the *Chlorobium* enzyme is a soluble protein. A membrane-bound location has also been shown for the sulfide-oxidizing activity in *Thiobacillus concretivorus* cell-free extracts (Moriarty and Nicholas 1970).

Table 2 Spectral and structural properties of flavocytochrome c sulfide dehydrogenase from *Thiobacillus* sp. W5, *Chlorobium limicola* and *Chromatium vinosum*

	<i>Thiobacillus</i> sp. W5	<i>Chl. limicola</i>	<i>Chr. vinosum</i>
Absorption maxima (oxidized, nm)	410, 450, 480	410, 450, 480	410, 450, 480
Absorption maxima (reduced, nm)	416, 523, 552	417, 523, 553	416, 523, 552
$A_{280\text{ox}}/A_{410\text{ox}}$	0.95	0.85	0.54
$A_{475\text{ox}}/A_{525\text{ox}}$	1.5	1.5	1.5
$A_{280\text{ox}}/A_{552\text{red}}$	4.11	3.90	3.73
Molecular weights			
Native flavocytochrome c	51,000	58,000	57,000
Flavoprotein subunit	40,000	47,000	46,000
Cytochrome subunit	11,000	11,000	21,000
Localisation	Membrane-bound	Soluble	Soluble
Haem c (mol/mol)	1	1	2
Flavin (mol/mol)	1	1 (FAD)	1 (FAD)

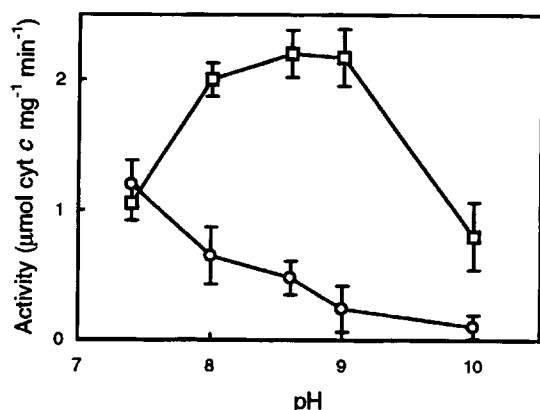


Fig. 3 pH Dependence of the chemical (○) and enzymatic (□) reduction of horse-heart cytochrome *c*. The standard assay contained 2 μg of enzyme. The pH of the buffer was adjusted by the addition of 0.1 M HCl or 0.1 M KOH and was measured before and after the determination of enzyme activity

pH Dependence and kinetic properties of the flavocytochrome *c* sulfide dehydrogenase

The pH optimum for the enzyme was 8.6. The activity of the FSDH at pH 7.4 and 10.0 was 2- and 2.8-fold lower, respectively. It was also noticed that the spontaneous chemical reduction of horse-heart cytochrome *c* increased markedly with decreasing pH, being fivefold higher at pH 7.4 than at pH 9.0 (Fig. 3). These results show that it is most efficient to measure the enzyme activity at pH 9.0, since the difference between the chemical and the enzymatic reduction of horse-heart cytochrome *c* is largest at this pH.

The kinetic properties of the FSDH were determined at pH 9.0 and 25°C by the addition of various amounts of substrate. It was observed that sulfide oxidation and cytochrome *c* reduction followed Michaelis-Menten kinetics. From the Lineweaver-Burk plot (Fig. 4), an apparent V_{\max} value of $2.1 \pm 0.1 \mu\text{mol cyt } c \text{ (mg protein)}^{-1} \text{ min}^{-1}$ was calculated. The apparent K_m values for sulfide and horse-heart cytochrome *c* were $1.7 \pm 0.4 \mu\text{M}$ and $3.8 \pm 0.8 \mu\text{M}$, respectively.

The V_{\max} value was equivalent to 107 mol cyt *c* (mol of flavocytochrome *c*)⁻¹ min⁻¹. This catalytic activity falls between the levels of activity observed for the *Chlorobium* and *Chromatium* FSDH [304 and 7 mol cyt *c* (mol of flavocytochrome *c*)⁻¹ min⁻¹, respectively] (Yamanaka and Fukumori 1980). An apparent sulfide turnover rate (k_{cat}) of 1.8 s⁻¹ was calculated. The V_{\max} value reflects only approximately 1% of the maximum *in vivo* activity as calculated from oxygen uptake experiments using sulfide as a substrate [$2.0 \pm 0.2 \mu\text{mol O}_2 \text{ (mg protein)}^{-1} \text{ min}^{-1}$]. The low V_{\max} value and sulfide turnover rate clearly do not reflect *in vivo* rates, which is probably due to the use of an artificial electron acceptor in the *in vitro* assay. Indeed, preliminary results with purified soluble cytochrome *c* from *Thiobacillus* sp. W5 showed tenfold higher activities.

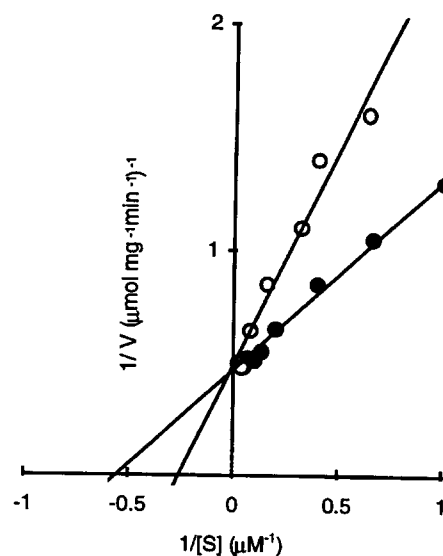


Fig. 4 Substrate concentration dependence of the purified flavocytochrome *c* sulfide dehydrogenase. Data are shown in a Lineweaver-Burk plot. ● Horse-heart cytochrome *c* dependence; sulfide was maintained constant at 20 μM. ○ Sulfide dependence; horse-heart cytochrome *c* was maintained at 50 μM

Substrate specificity

Reduction of horse-heart cytochrome *c* was not observed when thiosulfate, tetrathionate, trithionate, pentathionate, sulfite or sulfur replaced sulfide as the substrate in the FSDH enzyme assays. The addition of polysulfide ($S^{2-3.5 \pm 1}$) resulted in an initial reduction rate of half that found with sulfide. However, it was observed that the oxidation of 1 mol of polysulfide resulted in the reduction of only 0.04 ± 0.02 mol of cytochrome *c*. The possibility that this cytochrome *c* reduction was due to trace amounts of sulfide in the polysulfide solution cannot be excluded. Aerobic cultures of *Thiobacillus* sp. W5 are capable of rapidly oxidizing polysulfide to sulfate. The inability of the enzyme to oxidize polysulfide to equivalent amounts of sulfur indicates that accessory proteins are required in addition to the FSDH, or that a totally different enzyme is responsible for the oxidation of polysulfides. Sulfide, thus, appears to be the sole substrate for the enzyme.

Effect of inhibitors on the flavocytochrome *c* sulfide dehydrogenase activity

The enzyme was not inhibited by sodium azide, the sulfhydryl binding reagent N-ethylmaleimide and the chelating agent EDTA. Heating the enzyme to 80°C for 2 min resulted in total loss of activity.

The addition of 25, 100 or 500 μM cyanide to an assay containing approximately 2 μg enzyme resulted in 5, 75 or 95% inhibition, respectively. Cyanide has been reported to inhibit the enzymes of the phototrophic sulfur bacteria, when it became virtually irreversibly bound to

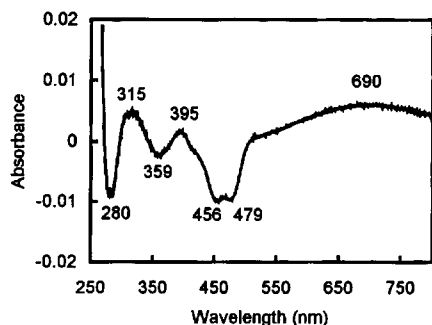


Fig. 5 Difference absorption spectrum of air-oxidized + cyanide minus air-oxidized flavocytochrome *c* sulfide dehydrogenase from *Thiobacillus* sp. W5. The cuvette (200 μ l) contained approximately 14 μ g enzyme and 100 μ M cyanide

the flavin (Yamanaka and Kusai 1976). UV/visible spectroscopy revealed that the addition of cyanide to air-oxidized *Thiobacillus* sp. W5 FSDH resulted in spectral changes that were also observed with the FSDH of the phototrophic bacteria. The air-oxidized + CN^- minus air-oxidized difference spectrum (Fig. 5) showed troughs at 280, 359, 456 and 479 nm that were due to the loss of absorption of oxidized flavin. The peaks at 315 and 395 nm can be attributed to an N-5 adduct formed between the cyanide and the flavin. The broad increase between 600 and 800 nm is probably a consequence of charge transfer from the adduct to a protein component (Meyer and Bartsch 1976). The addition of sulfite and thiosulfate (both 2 mM), which also have been shown to form flavin ligands with flavocytochromes *c*, decreased enzyme activity by 50 and 30%, respectively.

Electron transfer stoichiometry

The electron transfer stoichiometry was determined with the standard enzyme assay under anoxic conditions in order to minimize the effect of the chemical oxidation of sulfide. On the basis of an extinction coefficient for horse-heart cytochrome *c* of 19.6 $\text{mM}^{-1} \text{cm}^{-1}$ at 550 nm, it was calculated that the oxidation of 1 mol of sulfide resulted in the reduction of 1.95 ± 0.09 mol of horse-heart cytochrome *c*. This shows that approximately two electrons per molecule of sulfide are transferred by the enzyme to the electron acceptor. Thus, it is most likely that zero-valent sulfur is the product ($\text{HS}^- \rightarrow \text{S}^0 + \text{H}^+ + 2\text{e}^-$). Similar reaction stoichiometry has been observed for the FSDH of the phototrophic sulfur bacteria, which are known to produce elemental sulfur intracellularly or extracellularly. In 1974, Suzuki suggested that zero-valent sulfur is an intermediate in the oxidation of sulfide by most thiobacilli. Isolation of an apparently sulfur-producing enzyme from *Thiobacillus* sp. W5, as reported here, supports this hypothesis.

Sulfide quinone reductase activity in *Thiobacillus* sp. W5

Recently, sulfide quinone reductase (SQR) activity has been observed in crude extracts of *Thiobacillus* sp. W5 with ubiquinone-2 as an acceptor (J. M. Visser, unpublished results). FSDH of *Thiobacillus* sp. W5 did not show activity with the quinone acceptor. *Chlorobium* has also been shown to have both SQR and FSDH activities (Shahak et al. 1992). It has been proposed that SQR plays a major role in sulfide-dependent, anoxygenic photosynthesis (Arieli et al. 1994), but this is obviously not the case in the obligate chemolithoautotrophic *Thiobacillus* sp. W5. The physiological significance of the possession of two enzymes with apparently the same function in *Thiobacillus* sp. W5 is currently being studied.

Acknowledgements This work (Project no. IMB 91208) was financially supported by the Innovation-oriented Research Programme on Environmental Biotechnology, which is financed by the Ministry of Economic Affairs and by the Ministry of Housing, Physical Planning, and the Environment, The Netherlands.

References

- Arieli B, Shaka Y, Taglicht D, Hauska G, Padan E (1994) Purification and characterization of sulfide-quinone reductase, a novel enzyme driving anoxygenic photosynthesis in *Oscillatoria limnetica*. *J Biol Chem* 269:5705–5711
- Bartlett JK, Skoog DA (1954) Colorimetric determination of elemental sulphur in hydrocarbons. *Anal Chem* 26:1008–1011
- Bartsch RG, Kamen MD (1960) Isolation and properties of two soluble heme proteins in extracts of the photoanaerobe *Chromatium*. *J Biol Chem* 235:825–831
- Berry EA, Trumppower BL (1987) Simultaneous determination of hemes a, b and c from pyridine hemochrome spectra. *Anal Biochem* 161:1–15
- Chan CW, Suzuki I (1993) Quantitative extraction and determination of elemental sulfur and stoichiometric oxidation of sulfide to elemental sulfur by *Thiobacillus thiooxidans*. *Can J Microbiol* 39:1166–1168
- Chen KY, Morris JC (1972) Kinetics of oxidation of aqueous sulfide by O_2 . *Environ Sci Technol* 6:529–537
- Chen Z-W, Koh M, Van Driessche G, Van Beeumen JJ, Bartsch RG, Meyer TE, Cusanovich MA, Mathews FS (1994) The structure of flavocytochrome *c* sulfide dehydrogenase from a purple phototrophic bacterium. *Science* 266:430–432
- Cusanovich MA, Meyer TE, Bartsch RG (1991) Flavocytochrome *c*. In: Muller F (ed) *Chemistry and biochemistry of flavoenzymes*, vol 2. CRC Press, Boca Raton, pp 377–399
- Ehrlich HL (1990) *Geomicrobiology*. Dekker, New York, pp 449–498
- Hazeu W, Batenburg-Van der Vegte WH, Bos P, Van der Pas RK, Kuenen JG (1988) The production and utilization of intermediary sulfur during the oxidation of reduced sulfur compounds by *Thiobacillus ferrooxidans*. *Arch Microbiol* 150:574–579
- Hirayama A, Vetter RD (1989) Kinetics of sulphide and thiosulphate oxidation by the hydrothermal vent bacterium *Thiomicrospira crunogena* and comparison with *Thiobacillus neapolitanus* (abstract I43). Proceedings of the annual meeting, American Society for Microbiology, Washington, DC
- Janssen AJH, Sleyster R, Van der Kaa C, Jochemsen A, Bontsema J, Lettinga G (1995) Biological sulphide oxidation in a fed-batch reactor. *Biotechnol Bioeng* 47:327–333
- Katayama Y, Hiraishi A, Kuraishi H (1995) *Paracoccus thio-cyanatus* sp. nov., a new species of thiocyanate-utilizing facultative chemolithotroph, and transfer of *Thiobacillus versutus* to the genus *Paracoccus* as *P. versutus* comb. nov. with emendation of the genus. *Microbiology* 141:1469–1477

- Kelly DP (1989) Physiology and biochemistry of unicellular sulfur bacteria. In: Schlegel HG, Bowien B (eds) *Autotrophic bacteria*. Science Tech Publishers, Madison, Wis., pp 93–218
- Kuenen JG, Bos P (1989) Habitats and ecological niches of chemolitho(auto)trophic bacteria. In: Schlegel HG, Bowien B (eds) *Autotrophic bacteria*. Science Tech Publishers, Madison, Wis., pp 53–80
- Kuenen JG, Robertson LA, Tuovinen OH (1992) The genera *Thiobacillus*, *Thiomicrospira* and *Thiosphaera*. In: Balows A, Trüper HG, Dworkin M, Harder H, Schleifer KH (eds) *The prokaryotes*, vol. 3. Springer, Berlin Heidelberg New York, pp 2636–2657
- Kusai A, Yamanaka T (1973) Cytochrome *c* (553, *Chlorobium thiosulfatophilum*) is a sulphide-cytochrome *c* reductase. *FEBS Lett* 34:235–237
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 227:680–685
- Lane DJ, Harrison AP, Stahl D, Pace B, Giovannoni SJ, Olsen GJ, Pace R (1992) Evolutionary relationships among sulfur- and iron-oxidizing eubacteria. *J Bacteriol* 174:269–278
- Lu W-P, Kelly DP (1983) Partial purification of two principal enzymes of the thiosulphate-oxidizing multi-enzyme system from *Thiobacillus* A2. *J Gen Microbiol* 129:3549–3564
- Lu W-P, Kelly DP (1988) Cellular location and partial purification of the „thiosulphate-oxidizing enzyme“ and „trithionate hydrolyase“ from *Thiobacillus tepidarius*. *J Gen Microbiol* 134:877–885
- Lyric M, Suzuki I (1970) Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thio-parus* III. Properties of thiosulfate-oxidizing enzyme and proposed pathway of thiosulfate oxidation. *Can J Biochem* 48:344
- Meulenbergh R, Pronk JT, Hazeu W, Van Dijken JP, Frank J, Bos P, Kuenen JG (1993) Purification and partial characterization of thiosulphate dehydrogenase from *Thiobacillus acidophilus*. *J Gen Microbiol* 139:2033–2039
- Meyer TE, Bartsch RG (1976) The reaction of flavocytochromes *c* of the phototrophic sulfur bacteria with thiosulfate, sulfite, cyanide and mercaptans. In: Singer TP (ed) *Flavins and flavoproteins*. Elsevier, Amsterdam, pp 312–317
- Meyer TE, Bartsch RG, Cusanovich MA, Mathewson JH (1968) The cytochromes of *Chlorobium thiosulfatophilum*. *Biochim Biophys Acta* 153:854–861
- Moriarty DJW, Nicholas DJD (1970) Products of sulphide oxidation in extracts of *Thiobacillus concretivorus*. *Biochim Biophys Acta* 197:143–151
- Nathansohn A (1902) Über eine neue Gruppe von Schwefelbakterien und ihren Stoffwechsel. *Mitt Zool Stn Neapel* 15:655–680
- Oh JK, Suzuki I (1977) Resolution of a membrane-associated thiosulphate-oxidizing complex of *Thiobacillus novellus*. *J Gen Microbiol* 99:413–423
- Shahak Y, Arieli B, Padan E, Hauska G (1992) Sulfide quinone reductase (SQR) activity in *Chlorobium*. *FEBS Lett* 299:127–130
- Schedel M, Trüper HG (1979) Purification of *Thiobacillus denitrificans* siroheme sulfite reductase and investigation of some molecular and catalytic properties. *Biochim Biophys Acta* 568:454
- Schneider A, Friedrich C (1994) Sulfide dehydrogenase is identical with the SoxB protein of the thiosulfate-oxidizing enzyme system of *Paracoccus denitrificans* GB17. *FEBS Lett* 350:61–65
- Steffens GC, Torremans RAM, De Schrijver R, Robertson LA, Kuenen JG (1996) Quantitative measurement of sulphur formation by steady-state and transient-state continuous cultures of autotrophic *Thiobacillus* species. *Appl Microbiol Biotechnol* 45:169–175
- Suzuki I (1974) Mechanisms of inorganic oxidation and energy coupling. *Annu Rev Microbiol* 28:85–105
- Tano T, Kitaguchi H, Harada M, Nagasawa T, Sugio T (1996) Purification and some properties of a tetrathionate decomposing enzyme from *Thiobacillus thiooxidans*. *Biosci Biotechnol Biochem* 60:224–227
- Thomas PE, Ryan D, Levin W (1976) An improved staining procedure for the detection of the peroxidase activity of cytochrome P450 on sodium dodecyl sulfate polyacrylamide gels. *Anal Biochem* 75:168–176
- Toghrol F, Southerland WM (1983) Purification of *Thiobacillus novellus* sulfite oxidase. *J Biol Chem* 258:6762–6766
- Van den Ende FP, Van Gemerden H (1993) Sulfide oxidation under oxygen limitation by a *Thiobacillus thio-parus* isolated from a marine microbial mat. *FEMS Microbiol Ecol* 13:69–78
- Visser JM, De Jong GAH, Robertson LA, Kuenen JG (1996) Purification and characterization of a periplasmic thiosulfate dehydrogenase from the obligately autotrophic *Thiobacillus* sp. W5. *Arch Microbiol* 166:372–378
- Winogradsky S (1887) Über Schwefelbakterien. *Botan Ztg* 45: 489–507, 513–523
- Yamanaka T, Fukumori Y (1980) A biochemical comparison between *Chlorobium* and *Chromatium* flavocytochromes *c*. In: Yagi K, Yamano T (eds) *Flavins and flavoproteins*. Japan Scientific Societies Press, Tokyo, pp 631–639
- Yamanaka T, Kusai A (1976) The function and some molecular features of cytochrome *c*-553 derived from *Chlorobium thiosulfatophilum*. In: Singer TP (ed) *Flavins and flavoproteins*. Elsevier, Amsterdam, pp 292–301
- Yamanaka T, Fukumori Y, Okunuki K (1979) Preparation of subunits of flavocytochromes *c* derived from *Chlorobium limicola* f. *thiosulfatophilum* and *Chromatium vinosum*. *Anal Biochem* 95:209–213