

Purification and Characterization of the Selenate Reductase from *Thauera selenatis**

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***Thauera selenatis* is one of two isolated bacterial species that can obtain energy by respiring anaerobically with selenate as the terminal electron acceptor. The reduction of selenate to selenite is catalyzed by a selenate reductase, previously shown to be located in the periplasmic space of the cell. This study describes the purification of the enzyme from *T. selenatis* grown anaerobically with selenate. The enzyme is a trimeric $\alpha\beta\gamma$ complex with an apparent M_r of 180,000. The α , β , and γ subunits are 96 kDa, 40 kDa, and 23 kDa, respectively, in size. The selenate reductase contains molybdenum, iron, and acid-labile sulfur as prosthetic group constituents. UV-visible absorption spectroscopy also revealed the presence of one cytochrome *b* per $\alpha\beta\gamma$ complex. The K_m for selenate was determined to be 16 μM , and the V_{max} was 40 $\mu\text{mol}/\text{min}/\text{mg}$ of protein. The enzyme is specific for the reduction of selenate; nitrate, nitrite, chlorate, and sulfate were not reduced at detectable rates. These studies constitute the first description of a selenate reductase, which represents a new class of enzymes. The significance of this enzyme in relation to cell growth and energy generation is discussed.**

Selenate is an abundant oxyanion in some habitats, particularly in agricultural drainage waters from seleniferous soils (1–4). High concentrations of selenium oxyanions can have detrimental effects on wildlife such as bird species in the Kesterson Reservoir (5, 6). Unlike insoluble elemental selenium, selenate is difficult to remove from contaminated water due to its high solubility. The biological reduction of selenate to elemental selenium is therefore of interest as a potential strategy for bioremediation.

Although the reduction of selenate in sediments has been reported (2, 4, 7, 8), only three bacterial species have been isolated that can use this compound as the sole terminal electron acceptor for growth. These organisms include *Thauera selenatis* and two bacteria designated SES-1 and SES-3 (7, 9–11). Of these, SES-1 is no longer in culture. *T. selenatis* is a Gram-negative, rod-shaped bacterium of the beta subclass of the *Proteobacteria* that was isolated from selenate contaminated waste water in the San Joaquin Valley (9). It reduces selenate to selenite (9). Nitrate and oxygen can be utilized as alternative electron acceptors (10). A variety of compounds

including acetate, lactate, pyruvate, certain sugars, amino acids, fatty acids, di- and tricarboxylic acids, and benzoate can serve as electron donors and carbon sources (10). *T. selenatis* was the first characterized organism to be used to reduce selenate to selenite in a biological reactor system for selenium oxyanion bioremediation (1, 12, 13). During active denitrification in the biological reactor, selenite was further reduced to elemental selenium (13).

Little is known about the biochemistry of selenate reduction to selenite by either *T. selenatis* or SES-3. Selenate may be reduced by a specific selenate reductase or, alternatively, by an enzyme of the nitrate reduction pathway as was suggested previously (11). In *T. selenatis* the activity of selenate reductase was detected in the periplasmic space, whereas nitrate reductase activity was found in the cytoplasmic membrane (14). This suggests that the reduction of selenate and nitrate could be catalyzed by two independent enzymes.

The objective of this study was to purify and characterize the selenate reductase from *T. selenatis*. The purified enzyme consists of three heterologous subunits, designated α , β , and γ , and contains molybdenum, iron, and acid-labile sulfur atoms as well as heme *b* as cofactor constituents. This work represents the first report of the purification of a selenate reductase.

EXPERIMENTAL PROCEDURES

Growth Conditions—*T. selenatis* was grown anaerobically at 28 °C in a mineral salts medium containing yeast extract (0.4%), selenate (10 mM), and acetate (10 mM), as described previously (9). For routine cell transfers, *T. selenatis* was cultured in 10-ml anaerobic culture tubes. For enzyme purification, *T. selenatis* was grown in 5 liter batch cultures. Cultures were harvested during late log phase (after 16–18 h growth) at a final optical density (A_{600}) of 0.6–0.7. Typical cell yields were 3.0–3.6 g cells (wet weight) per 5 liters of culture.

Purification of the Selenate Reductase—Enzyme purification was performed under aerobic conditions at 4 °C unless indicated otherwise. Cells were chilled on ice (0 °C), harvested by centrifugation for 10 min at 16,000 $\times g$, and washed once with 10 mM Tris/HCl, pH 8. The periplasmic fraction was prepared by the method of Osborn and Munson (15). Throughout the procedure the bacteria were stirred gently on ice. Cells were resuspended in 30 mM Tris/HCl, pH 8, and 0.75 M sucrose at a ratio of 0.45 g wet weight cells per ml of buffer. Following incubation of the cell suspension for 5 min at 0 °C, lysozyme was added to a final concentration of 0.4 mg/ml, and incubation was continued for an additional 2 min. Two volumes of an ice cold solution of 15 mM EDTA were then added slowly over a period of 10 min. The suspension was stirred for another 10 min on ice and then placed at 37 °C for 10 min to permit the formation of spheroplasts. The spheroplasts were removed by centrifugation at 25,000 $\times g$ for 20 min. The supernatant fraction represented the periplasmic contents of the cells and contained greater than 90% of the selenate reductase activity (data not shown (14)). The proteins in the periplasmic fraction were concentrated by ammonium sulfate precipitation (50–80% saturation). The precipitated material was collected by centrifugation for 10 min at 25,000 $\times g$ and resuspended in 50 mM piperazine/HCl, pH 6, containing 2 M $(\text{NH}_4)_2\text{SO}_4$. The solution was loaded directly onto a 1-ml phenyl-Sepharose high performance hydrophobic interaction column (Pharmacia Biotech Inc.) that had been equilibrated with the piperazine-2 M $(\text{NH}_4)_2\text{SO}_4$ buffer. All chromatography procedures were performed at room temperature. Af-

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TABLE I
Summary of the purification of the selenate reductase from
T. selenatis

Purification step	Protein	Total activity	Specific activity	Purification
	mg	units ^a	units/mg	-fold
Cell extract	493	359	0.73	0
Periplasmic fraction	103	125	1.21	1.7
(NH ₄) ₂ SO ₄ precipitate	37	257	6.95	9.5
Phenyl-Sepharose	2.5	53	21.2	29.0
Superose 12	1.4	58	41.4	56.7

^a Units are defined as μmol of selenate reduced/min.

ter washing the column with 2 volumes of piperazine buffer containing 2 M (NH₄)₂SO₄, the selenate reductase was eluted from the column with a 2–0 M (NH₄)₂SO₄ gradient in piperazine buffer. During chromatography, the protein concentration was monitored by measuring the absorbance at 280 nm. Following elution, fractions containing the selenate reductase were pooled and concentrated by centrifugation using a centriprep-30 concentrator (Amicon). The resulting fraction was loaded onto a Superose 12 gel filtration column (Pharmacia) that had been equilibrated with 50 mM piperazine/HCl, pH 6. The selenate reductase was eluted in the same buffer, and the fractions containing the selenate reductase activity were concentrated and stored at -80°C .

Enzyme Assay—Selenate reductase activity was determined using an anaerobic cuvette assay by monitoring the oxidation of reduced benzyl viologen (1 mM) with selenate (10 mM) at 546 nm, as described previously (14). Reduced methyl viologen (0.3 mM) was used as an alternative electron donor where indicated. Alternate electron acceptors (10 mM) that were tested included nitrate, nitrite, sulfate, and chlorate. All enzyme activities were measured at pH 6, the pH optimum of the selenate reductase, in 100 mM piperazine/HCl (14).

Protein Determination—The protein concentration was determined with Biuret and Bradford reagents, using bovine serum albumin as the standard (16).

Determination of the Apparent Molecular Mass—The apparent molecular mass of the selenate reductase was determined using Superose 12 gel filtration (Pharmacia) chromatography. The column was developed using 20 mM piperazine/HCl, pH 6, and 100 mM NaCl at a flow rate of 0.2 ml/min. The following molecular weight standards were used: ferritin (M_r 440,000), catalase (M_r 232,000), aldolase (M_r 158,000), albumin (M_r 67,000), ovalbumin (M_r 43,000), and chymotrypsin A (M_r 25,000). Dextran blue was used to determine the void volume of the column according to the manufacturer's instructions (Pharmacia).

Absorption Spectrum—The oxidized and dithionite-reduced spectrum of the purified enzyme was recorded on a Beckman DU 640 spectrophotometer.

Gel Electrophoresis—Protein samples were prepared in 2.5% SDS, 5% β -mercaptoethanol, and 0.005% bromphenol blue as described (17). After heating for 5 min at 90°C , 1 μl of the sample was applied to a 12.5% homogenous polyacrylamide SDS gel (Phastsystem, Pharmacia). Precast polyacrylamide gels and SDS buffer strips were purchased from Pharmacia.

Metal Analysis—Non-heme iron was determined using the method of Brumby and Massey (18). Molybdenum and nickel were determined using induced coupled plasma mass spectroscopy performed by the DANR analytical laboratory, University of California, Davis. Acid-labile sulfur was determined using the method of King and Morris (19). The selenium content of the enzyme could not be accurately estimated because the organism had been grown in the presence of high levels of selenate.

N-terminal Sequence Analysis—The three subunits of the selenate reductase were blotted from a SDS-polyacrylamide gel onto a polyvinylidene fluoride membrane (Millipore). N-terminal amino acid sequence determination was carried out according to the method of Edman and Begg (20) by the microsequencing facility at the University of California at Davis (α and β subunits) and the School of Biochemistry, La Trobe University, Bundoora, Australia (γ subunit).

Chemicals—Methyl viologen, benzyl viologen, and piperazine were obtained from Sigma, and sodium selenate was obtained from Aldrich. All other chemicals were of the highest purity commercially available.

RESULTS

Purification and Subunit Composition of the Selenate Reductase—The selenate reductase from *T. selenatis* was purified

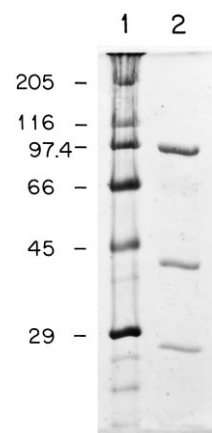


FIG. 1. SDS-polyacrylamide gel electrophoresis of the *T. selenatis* selenate reductase enzyme. Lane 1, the molecular weight standard in kDa (1.5 μg); lane 2, purified selenate reductase (2 μg).

from the periplasmic fraction of cells grown anaerobically with selenate as the terminal electron acceptor. Using phenyl-Sepharose high performance hydrophobic interaction and Superose 12 gel filtration chromatography, a 57-fold enrichment of the enzyme was achieved (Table I). After the final purification step, the enzyme was greater than 99% pure based on SDS-polyacrylamide electrophoresis (Fig. 1). The selenate reductase consists of three subunits with relative molecular weights of 96,000, 40,000, and 23,000 as determined by SDS-polyacrylamide gel electrophoresis (Fig. 1). Using gel filtration, the relative molecular weight of the native enzyme was determined to be 180,000 (data not shown). The experimentally determined size of the protein is comparable to the calculated relative molecular weight of 159,000 assuming that all subunits are present in a 1:1:1 stoichiometry. This suggests that the selenate reductase is a heterotrimer composed of an α , β , and γ subunit.

Cofactor Composition of the Selenate Reductase—The purified selenate reductase exhibited a visible absorbance spectrum characteristic of *b*-type cytochromes (Fig. 2). The absorbance maxima of the reduced enzyme were 424, 528, and 558 nm, whereas the absorbance maximum of the oxidized form was 415 nm. The cytochrome content was determined to be 1 mol of heme *b* per mol of enzyme based on the molar extinction coefficient of cytochrome *b* (21) and the calculated molecular weight of the selenate reductase complex.

The analysis of the purified selenate reductase for total iron and acid-labile sulfur revealed the presence of 12.9 and 8 mol per mol of enzyme (based on a M_r of 159,000), respectively. This suggests the presence of at least two [Fe-S] center prosthetic groups. Induced coupled plasma mass spectroscopy spectral analysis of the purified enzyme revealed 1 mol molybdenum per mol of enzyme complex. Nickel was not detected.

Activity of the Selenate Reductase—The purified selenate reductase exhibited a high specificity for its substrate, selenate. With reduced benzyl viologen as the electron donor, the K_m for selenate was determined to be 16 μM . Selenate reductase exhibits a V_{max} of 40 μmol of selenate reduced $\text{min}^{-1} \text{mg}^{-1}$ (data not shown), which corresponds to an enzyme turnover number of 387 s^{-1} . Reduced methyl viologen could also serve as an electron donor, although the specific activity of selenate reduction was 11-fold lower than the activity as measured with reduced benzyl viologen (Table II). NADH, succinate, and lactate did not act as electron donors for selenate reduction by the purified enzyme (data not shown). Other oxyanions such as nitrate, nitrite, and chlorate did not serve as electron acceptors for the selenate reductase (Table II).

¹ The abbreviation used is: M_r , relative molecular mass.

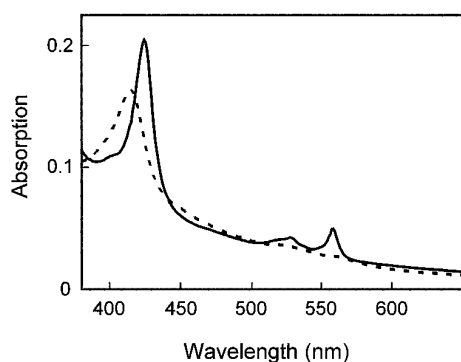


FIG. 2. Visible absorption spectrum of the *T. selenatis* selenate reductase. The purified enzyme was diluted in 50 mM piperazine/HCl, pH 6.0, to give a protein concentration of 0.11 mg/ml. The air-oxidized spectrum (dotted line) and the dithionite reduced spectrum (solid line) are shown.

TABLE II
The purified selenate reductase from *T. selenatis* is specific for selenate

Electron acceptor	Activity	
	Reduced benzyl viologen	Reduced methyl viologen
	$\mu\text{mol}/\text{min}/\text{mg protein}$	
Selenate	15.9	1.36
Nitrate	— ^a	<0.01
Sulfate	<0.01	<0.01
Chlorate	— ^a	<0.01
Nitrite	— ^a	<0.01

^a —, activities could not be determined because this compound was rapidly reduced by reduced benzyl viologen in the absence of the enzyme.

Determination of the N-terminal Amino Acid Sequence of the Selenate Reductase Subunits—The N-terminal sequence of each subunit is shown in Table III.

DISCUSSION

This report describes the first purification of a dissimilatory selenate reductase. The periplasmic enzyme from *T. selenatis* is specific for selenate reduction to selenite. This reduction is coupled to cell growth suggesting that the selenate reductase is part of an electron transport chain that generates an electrochemical gradient across the cytoplasmic membrane (9, 22). The properties of the selenate reductase are summarized in Table IV. The enzyme is a complex consisting of three heterologous subunits, and it contains molybdenum, iron, acid-labile sulfide, and heme b as cofactor constituents. The purified enzyme exhibits a high affinity for selenate. Its K_m is in the lower range of the affinities reported for *in situ* selenate reduction activities in a variety of sediments ($K_m = 7.9\text{--}720 \mu\text{M}$) (4). Nitrate, nitrite, chlorate, and sulfate do not serve as substrates. The enzyme is therefore clearly not part of another oxyanion reduction pathway. Previously reported cell yield determinations for selenate reduction with acetate as the electron donor indicated that *T. selenatis* formed ATP exclusively by electron transport phosphorylation (22). The periplasmic location of the enzyme is logical, considering the need of the organism to protect itself from the toxic effects of selenite. However, the mechanism by which selenate reduction is coupled to the cytoplasmic membrane so that energy can be conserved is not yet known. In contrast, selenite was demonstrated not to support growth of *T. selenatis* (23). Selenite is the major product of anaerobic respiration when *T. selenatis* is grown with selenate as the sole electron acceptor, whereas some minor reduction of selenite to elemental selenium may occur (9). Complete reduction of selenite takes place when both nitrate and selenate are available as electron acceptors (13) (see below).

TABLE III

N-terminal amino acid sequence of the three selenate reductase subunits

Subunit	N-terminal amino acid sequence
α	Phe-Ser-Lys-Ile-Gln-Pro-Ile-Glu-Asp-Pro-Leu-Lys-Ser-Tyr-Pro
β	Ser-Gln-Arg-Gln-Leu-Ala-Tyr-Val-Phe-Asp-Leu-Asn-Lys-Xaa-Ile-Gly-Xaa-His-Thr-Xaa-Thr-Met-Ala-Xaa-Lys-Gln-Leu-Trp-Thr-Asn
γ	Ala-Asp-Gly-Ala-Pro-Ala-Ala-Gln-Arg-Thr-Ile-Gln-Val-Leu-Ser-Val-Lys-Gly-Gly-Asp

TABLE IV

Properties of the T. selenatis selenate reductase

Subunit size	
Experimental from SDS-polyacrylamide gel electrophoresis	
α	96 kDa
β	40 kDa
γ	23 kDa
Native size	
Superose gel filtration	180 kDa
Cofactor content ^a	
Iron	12.9
Acid-labile sulfur	8
Molybdenum	1
Heme b	1
K_m^b	16 μM
V_{max}^b	40 units/mg protein

^a mol/mol of enzyme based on M_r 159,000.

^b Using reduced benzyl viologen as the electron donor.

The subunit composition and the cofactor content of the selenate reductase from *T. selenatis* bears some resemblance to the periplasmic dimethylsulfide:acceptor oxidoreductase from *Rhodobacter sulfidophilus* (24). Like the selenate reductase, the dimethylsulfide:acceptor oxidoreductase is a heterotrimer consisting of three subunits with molecular masses of 94, 38, and 32 kDa that are similar to the selenate reductase subunits (Table IV). Dimethylsulfide:acceptor oxidoreductase contains a pterin molybdenum cofactor, a cytochrome *b*, and possibly one [Fe-S] center. The enzyme may provide electrons to the photosynthetic electron transport chain when *R. sulfidophilus* is grown in light with dimethylsulfide as the electron donor. The physiological electron acceptor of dimethylsulfide:acceptor oxidoreductase that may transfer the electrons to the membrane-bound photosynthetic reaction center is not known.

Selenate reductase is dissimilar to the periplasmic nitrate reductases that have been purified from *Rhodobacter sphaeroides*, *Alcaligenes eutrophus*, and *Thiosphaera pantotropha* (3, 25–27). These enzymes consist of two subunits containing molybdopterin, possibly one [Fe-S] center and cytochrome *c* as prosthetic groups. The recently reported DNA sequence of the *napABC* operon from *R. sphaeroides* revealed the presence of an additional gene encoding NapC (27). Based on its amino acid sequence, NapC should contain a transmembrane helix and has the signatures of heme *c* binding sites. Sequence homologies to other membrane-bound, cytochrome *c*-containing subunits of electron transfer proteins led the authors to suggest a role for NapC as the mediator of electron flow between the quinone pool in the cytoplasmic membrane and the periplasmic nitrate reductase, NapAB. In contrast to all of the periplasmic nitrate reductases described thus far, the selenate reductase from *T. selenatis* does not contain cytochrome *c*. While the physiological role of the periplasmic nitrate reductases in various organisms is not clear, the selenate reductase of *T. selenatis* acts as a dissimilatory terminal reductase supporting growth when selenate is present in the environment.

T. selenatis can also grow anaerobically with nitrate as the sole terminal electron acceptor (10). Nitrate is reduced via the denitrification pathway to N_2O (10). In the absence of nitrate and/or nitrite, selenate is reduced to selenite, which is not reduced further to a significant extent (9). When both selenate and nitrate are present, *T. selenatis* reduces both substrates concomitantly (14). Interestingly, under these conditions selenite is further reduced to elemental selenium (14). This suggests a possible involvement of the denitrification pathway enzymes in the reduction of selenite to selenium. Two recent findings suggest that the nitrite reductase may catalyze the reduction of selenite to selenium (23): first, both nitrite reductase activity and the ability to reduce selenite are low in cells grown in the absence of nitrate and/or nitrite. Second, a *T. selenatis* mutant was isolated that was defective in both nitrite and selenite reduction. The physiological significance of selenite reduction in the presence of nitrate is not known.

In addition to *T. selenatis*, the anaerobic Gram-negative isolate SES-3 was described as being able to grow using selenate respiration with lactate as the electron donor (11). Apart from its ability to reduce selenate to selenite, strain SES-3 bears little resemblance to *T. selenatis*. Not only is SES-3 a strict anaerobe that is unable to use acetate as the electron donor, but it requires both peptone and yeast extract in the medium for growth (9, 11). The phylogenetic position of SES-3 has yet to be established. Like *T. selenatis*, SES-3 can utilize nitrate as an alternative electron acceptor to support growth; however, nitrate is reduced to ammonium. In strain SES-3, selenate and nitrate reduction appear to be regulated. In the presence of nitrate, the rate of selenate reduction was 2-fold lower than in cells grown in the absence of nitrate (11). Conversely, the rate of nitrate reduction was reduced 5-fold in selenate grown cells as compared with cells grown with nitrate. This differential regulation of selenate and nitrate reduction suggests that, like *T. selenatis*, SES-3 has two distinct reductases, a selenate reductase and a nitrate reductase. Therefore, in contrast to a previously stated hypothesis that selenate reduction to selenite is catalyzed by nitrate reductase (28), *T. selenatis* and probably SES-3 reduce selenate by using a selenate reductase.

Several other bacteria including *Wolinella succinogenes* (29), a *Pseudomonas stutzeri* isolate (30), and *Bacillus subtilis* (31) have been shown to reduce either selenate or selenite to elemental selenium. None of these organisms, however, can conserve energy when reducing selenate or selenite.

Biophysical studies of the selenate reductase, as well as the cloning and DNA sequence analysis of the genes for the α , β , and γ subunits are in progress and will reveal additional information regarding the mechanism of selenate reduction.

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