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Purification and characterization of three proteins from a halophilic sulfate-reducing bacterium, *Desulfovibrio salexigens*

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SUMMARY

Hydrogenase, desulfoviridin and molybdenum proteins have been isolated from a halophilic sulfate-reducing bacteria, *Desulfovibrio salexigens* strain British Guiana. At least 50% of the hydrogenase was found to be located in the periplasm. The hydrogenase has a typical absorption spectrum, a 400/280 nm ratio of 0.28, a molecular weight by sedimentation equilibrium of 81 000 and is composed of two subunits. It has one nickel, one selenium and 12 iron atoms per molecule. The sulfite reductase has a typical desulfoviridin absorption spectrum, a molecular weight of 191 000 and iron and zinc associated with it. The molybdenum-iron protein is gray-green in color and exhibits an absorbtion spectrum with peaks around 612, 410, 275 nm and a shoulder at 319 nm. It is composed of subunits of approximately 13 250 and has an approximate molecular weight of 110 000. Three molybdenum and 20 iron atoms are found associated with it.

An extensive study of these three proteins will allow a better understanding of the function of these enzymes and also of their possible role in microbially caused corrosion.

INTRODUCTION

Sulfate-reducing bacteria have been implicated in the phenomenon of microbially caused corrosion in neutral anaerobic environments [13]. Hydrogen consumption and sulfate reduction contribute to this corrosion process. Hydrogen is consumed by the hydrogenase enzyme from either the metal surface [58] or from the iron sulfide film on the metal [28]. Hydrogen sulfide is produced by the sulfate reduction system and acts as an anodic reactant [59], a cathodic reactant [12] or in the formation of a reactive phosphorous compound [26].

Desulfovibrio (D.) salexigens strain British Guiana is the only well known halophilic strain in the genus *Desulfovibrio* and only studies on its cytochrome C_3 [16] flavodoxin and rubredoxin [41] have been published.

Hydrogenases have been purified to apparent homogeneity from many bacterial species, including anaerobic microorganisms such as sulfate reducers [46] and methanogenic bacteria [5,18,27].

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They are involved either in the hydrogen consumption in which hydrogen is used as a reductant for CO_2 fixation or for energy generation via electron transport or in hydrogen production which enables bacteria to dispose of excess electrons [1,50].

An important enzyme in sulfate reduction is dissimilatory sulfite reductase or bisulfite reductase [47]. This enzyme has been purified from the cytoplasm of many sulfate-reducing bacteria [46] and is believed to be involved in ATP production in these micro-organisms [44]. Reduction of sulfite either involves a cyclic scheme, utilizing intermediates trithionate and thiosulfate to sulfide [3] or a direct six electron reduction to sulfide [46].

A molybdenum protein has been found in some species of sulfate reducers from the genus *Desulfovibrio (D.) gigas* [40], *D. africanus* [21], *D. desulfuricans* strains Berre Eau [7] and Berre Sol (our unpublished results). It is characterized by not only containing molybdenum but also iron and labile sulfide. Its function is still unknown.

A purification scheme and partial characterization of hydrogenase, desulfoviridin (bisulfite reductase) and molybdenum protein from *D. salexigens* is reported. A study of these proteins may help elucidate the phenomenon of microbially caused corrosion.

MATERIALS AND METHODS

Growth of cells. Desulfovibrio salexigens strain British Guiana (NCIB 8403) was grown at 37°C on lactate/sulfate medium [54] with 3.0% NaCl. Cells for localization studies were harvested by centrifugation and immediately used. Cells (250 g) for enzyme purification were harvested, resuspended in 10 mM Tris-HCl (pH 7.6) lysed with a French press and then frozen at −80°C until used.

Assay and metal determinations. Hydrogenase activity was measured at 32°C either by the hydrogen evolution assay from dithionite-reduced methyl viologen [45] using an Aerograph A-90 P3 gas chromatograph or by hydrogen consumption with benzyl viologen as electron acceptor using Warburg respirometry [8]. One unit of hydrogenase activity is defined as the amount of enzyme which catalyses the evolution or the consumption of 1 μmol H_2/min. Dissimilatory sulfite reductase activity was measured by a manometric assay at 32°C [31] using pure hydrogenase from *D. gigas* to reduce methyl viologen under H_2. The initial rate of hydrogen utilization is proportional to the amount of sulfite reductase. Protein was determined by a modification of the Lowry method as proposed by Markwell et al. [38]. Iron and nickel were determined by plasma emission spectroscopy using the Jarrel-Ash Model 75 atomcomp.

Optical spectra. Ultraviolet and visible absorption spectra were recorded on a Beckman DU 7 spectrophotometer.

Electrophoresis and molecular weight determination. Purity of the enzymes was established by polyacrylamide disc electrophoresis [11] and by comparison of published absorption ratios for similar type pure proteins. Subunit structure was determined by SDS-polyacrylamide gel electrophoresis [17]. Molecular weight was determined by SDS electrophoresis, gel filtration using a TSK 3000 SW analytical column (high-pressure liquid chromatography) or sedimentation equilibrium [49].

RESULTS

Location of the hydrogenase

Lysing the cells with the French press and centrifuging a 120 000 × *g* for 70 min released about 80% of the hydrogenase as soluble protein. The cells were washed in a 1:1 w/v buffer solution (pH 8.0) of 50 mM Tris-HCl/10 mM EDTA/500 mM glucose/3% NaCl, incubated in the same buffer at a 1:50 w/v ratio with 8 mg of lysozyme/ml and then centrifuged. This results in 70–80% of the hydrogenase being found in the supernatant. A visible spectrum showed 30% of the desulfoviridin was also found in the supernatant. Further experiments using $MgCl_2 6H_2O$ instead of NaCl found almost 50% of the hydrogenase in the supernatant and less than 5% of desulfoviridin. This seems to indicate a periplasmic origin for at least 50% of the hydrogenase enzyme. It is important to add NaCl or

Table 1

Purification of hydrogenase from *D. salexigens*

Fraction	Protein (mg)	Total activity* (μmol H_2/min)	Specific activity* (μmol H_2/min per mg)
Crude extract	10750	47290	4.4
DEAE Bio-Gel A column	562	24000	42.7
Hydroxylapatite column	28	15552	561
Gel exclusion by HPLC	3.9	7170	1830

* in H_2 evolution.

$MgCl_26H_2O$ to the washing or incubating buffers or the cells clump together. When Na_2SO_4 was substituted at the same concentration, the cells clumped together and there was some cell lysis.

Purification of hydrogenase, desulfoviridin and molybdenum-iron sulfur protein

All purification procedures were carried out in air at 4°C and the pH of the buffers, Tris-HCl and phosphate (KPB), were 7.6 (measured at 5°C).

The lysed cells were slowly defrosted and centrifuged for 1.5 h at 20 000 × *g*. The supernatant was centrifuged at 120 000 × *g* for 2 h, dialized against 10 vol. of 10 mM Tris-HCl for 24 h and then centrifuged at 120 000 × *g* for 1 h. The centrifuged extract was loaded onto a DEAE Bio-Gel A column (5 × 25 cm) equilibrated with 10 mM Tris-HCl and the column washed with 500 ml of the same buffer. A gradient of 1500 ml 10 mM Tris-HCl and 1500 ml 400 mM Tris-HCl was set up. A molybdenum-iron protein, gray in color, came off at 100–125 mM Tris-HCl. The hydrogenase and desulfoviridin eluted off together at about 200–250 mM Tris-HCl. The hydrogenase activity recovered in the H_2 evolution was 51%.

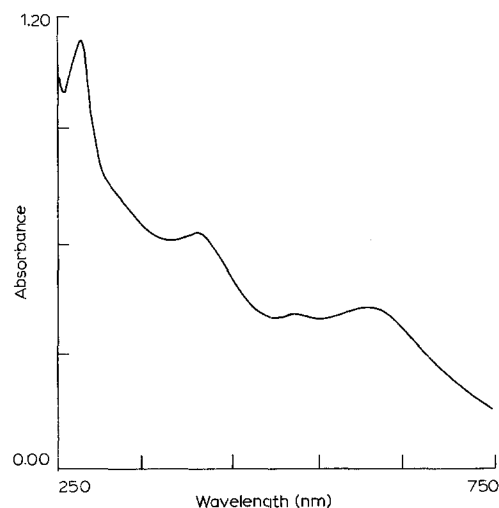

Fig. 1. Electronic absorption spectrum of oxidized molybdenum iron protein from *Desulfovibrio salexigens* recorded at 25°C.

This last fraction was loaded onto a Bio-Rad hydroxylapatite column (HTP) (4.3 × 26 cm) equilibrated with 250 mM Tris-HCl. The column was washed with 250 mM Tris-HCl and a reverse gradient of 400 ml 250 mM Tris-HCl and 400 ml 10 mM Tris-HCl was set up. The column was then washed with 200 ml of 10 mM KPB. A gradient of 1500 ml 10 mM KPB and 1500 ml 500 mM KPB was set up. The desulfoviridin eluted off at about 200 mM to 250 mM KPB and had a 409/630 nm ratio of 2.80 and a 279/630 nm ratio of 4.7. The hydrogenase band (brown) began to migrate at about 450 mM KPB and was collected at 500 mM KPB. The activity was 65% of that from the DEAE Bio-Gel column. The sample was concentrated in a diaflow apparatus using a YM 30 membrane. It was loaded onto a high-pressure liquid chromatography gel exclusion column which was equilibrated with 500 mM KPB (pH 7.5). Different fractions were analyzed spectroscopically and those with the highest 400/280 nm ratios were combined. Gel electrophoresis revealed one major band for the hydrogenase and the protein was estimated to be 95%

pure. The final yield of hydrogenase was 15.2%. A summary of the hydrogenase purification is found in Table 1.

The desulfoviridin appeared to be pure by the absorbancy ratios which were identical to that obtained for pure desulfoviridin from *D. gigas* [30] and *D. vulgaris* Hildenborough [31]. Before staining the electrophoresis gel there was one green band about 25% down the gel and a red band which migrated near the bromophenol blue. After staining, one major band was found in the location of the green band. The location of the red band stained but slowly dissipated after 24 h. This band is believed to be a siroheme which is part of the desulfoviridin [51]. The enzyme was judged to be about 95% pure.

The gray protein from the DEAE Bio-Gel column was loaded onto a HTP column (5 × 24 cm) equilibrated with 150 mM Tris-HCl. The column was developed with a KPB gradient up to 1 M and very little of the gray protein came off. The column was then washed with 2 M KPB and the majority of the protein eluted off the column. The 278/612 nm ratio is 2.92. The absorbance spectrum of this protein (Fig. 1) appears to be very similar to the molybdenum-iron-sulfur protein spectrum of *D. africanus* [21] and has the same 278.5:615 nm ratio.

Characterization of hydrogenase

The hydrogenase of *D. salexigens* is brown in color and its native form exhibits a typical hydrogenase UV/visible absorption spectrum with a broad shoulder around 400 nm (Fig. 2). SDS gel electrophoresis showed that it is composed of two different subunits of molecular weight 62 000 ± 5000 and 35 000 ± 3000. The total molecular weight by addition of the subunits is 97 000 but the molecular weight found by sedimentation equilibrium is 81 000. The ratio of 400/280 nm is 0.28 and the extinction coefficient at 400 nm using a molecular weight of 81 000 is 46 $mM^{-1} \cdot cm^{-1}$.

A metal content of 12.3 iron atoms, 0.8 nickel atoms and 0.86 selenium atoms per molecule of hydrogenase was determined. The hydrogenase does not exhibit an activation phase before maximum H_2 evolution activity. The specific activity in the H_2

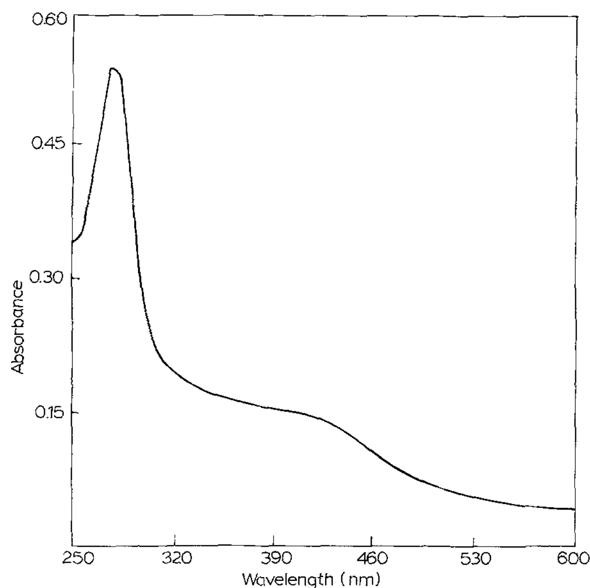

Fig. 2. Electronic absorption spectrum of oxidized *Desulfovibrio salexigens* hydrogenase at 25°C.

evolution is 1830 μmol/min per mg protein and is 1300 μmol/min per mg protein in H_2 consumption.

Characterization of desulfoviridin

The optical spectrum of desulfoviridin shows absorption bands at the following wavelengths (nm): 630 (0.595), 584 (0.374), 409 (1.668), 391.5 (1.544) and 279 (2.79); relative intensities are indicated in parenthesis. The molecular weight, determined by sedimentation equilibrium, is 191 000. The metal content is 31 iron atoms and 1.4 zinc atoms per molecule. The specific activity is 136 nmol H_2 consumed/min per mg protein. The activity was found to be greater at pH 6.0 than at pH 7.6 which indicates that bisulfite is the substrate [42]. An end product of the reaction was sulfide, appearing as a yellow precipitate of cadmium sulfide, which was formed in the center well of the reaction vial.

Characterization of molybdenum protein

The molybdenum-iron protein is gray-green in color and exhibits an absorbance spectrum with peaks around 612, 410, 275 nm and a shoulder at 319 nm (Fig. 1). Denaturing gel electrophoresis

Table 2

Properties of assimilatory (A) and dissimilatory (D) sulfite reductases from anaerobic bacteria

Sulfite reductase	Mr ($\times$ 10^{-3})	Activity[a]	Active center	Reference
Desulfuromonas acetoxidans (A)	23.5	906	Sir + Fe-S(α)	[35]
D. vulgaris (A) Hildenborough	27	900	Sir + Fe-S(α)	[25]
M. barkeri (DSM800) P 590 (A)	23	2790	Sir + Fe-S(α)	[42]
Desulfatomaculum nigrificans P 582 (D)	145	65*	Sir + Fe-S	[4]
D. gigas desulfoviridin (D)	200	632	Sir + Fe-S($\alpha_2\beta_2$)	[30]
D. vulgaris Hildenborough desulfoviridin (D)	226	260	Sir + Fe-S($\alpha_2\beta_2$)	[31]
D. desulfuricans Norway 4 desulforubidin (D)	225	410	Sir + Fe-S($\alpha_2\beta_2$)	[32]
D. baculatus 9974 desulforubidin (D)	n.d.	198	Sir + Fe-S($\alpha_2\beta_2$)	Fauque (unpublished)
Thermodesulfobacterium commune desulfofuscidin (D)	167	2000	Sir + Fe-S($\alpha_2\beta_2$)	[22]

[a] Activity expressed in nmol H_2 consumed/min per mg protein at pH 6 at 30°C except for *Thermodesulfobacterium commune* at 65°C.
* partially purified P 582.
Sir, siroheme; Fe-S: iron sulfur center.
s.d., not determined.

yielded bands of molecular weights of approximately 13 250, 26 000, 42 500 and 64 300. These results suggest a protein of several subunits of molecular weight of 13 250. The conditions were probably not sufficient to completely dissociate the protein. The molecular weight as determined by high-presure liquid chromatography analytical gel exclusion column is approxiately 110 000. The enzyme has three molybdenum atoms and 20 iron atoms for 110 000 molecular weight. The amount of molybdenum protein in the bacterium was large. The final amount is about 50 mg per 250 g of cells.

DISCUSSION

The location of a hydrogenase in the periplasmic space of *D. salexigens* is not unusual. Periplasmic origin of hydrogenase is common in sulfate-reducing bacteria of the genus *Desulfovibrio: D. Vulgaris* strains Hildenborough [57], Marburg [6], Miyazaki [2], *D. gigas* [9], *D. desulfuricans* (NRC 49001) [39], and *D. baculatus* strain 9974 [55].

The existence of more than one hydrogenase in the same species of the genus *Desulfovibrio* has been postulated [43].

The need for chloride at high concentration (> 2%) in order to prevent the clumping of cells from *D. salexigens* may be related to some function in the outer membrane or in response to the use of a high concentration of EDTA. It has been found that *D. salexigens* has an absolute requirement for chloride [37] and the cells in our buffer conditions exhibited an abnormal response to less than 2% of it.

It has been proposed that a periplasmic location

of hydrogenase in anaerobic bacteria is a specific adaptation important for utilization of low level of H_2 and for interspecies H_2 transfer [9]. The periplasmic location of hydrogenase would be important in hydrogen utilization from metal surfaces or iron sulfide films. However, attempts to show direct correlation between hydrogenase activity and bacterial corrosion have shown mixed results. [19]. Recently, a marine strain of a sulfate-reducing bacterium has been found to utilize cathodically produced hydrogen from a metal surface [20]. Conclusions concerning 'hydrogenase-less' sulfate-reducing bacteria will have to be revised because many of these strains are now known to have hydrogenase activity, e.g., *Desulfotomaculum orientis* [34] and *Desulfovibrio desulfuricans,* El Agheila Z (our own unpublished results).

Desulfovibrio salexigens hydrogenase is similar to the recently purified Fe-Ni-Se hydrogenases found in *D. desulfuricans* Norway 4 [48] and *D. baculatus* strain 9974 [55]. The only known selenium-containing hydrogenase without nickel is from *Methanococcus vannielii* [60]. The function of selenium in this enzyme is not known. Other nickel-containing hydrogenases are present in sulfate-reducing bacteria of the genus *Desulfovibrio,* i.e., *D. gigas* [33], *D. desulfuricans* ATCC 27774 [29] and *D. multispirans* [15]. The *D. salexigens* hydrogenase does not exhibit an activation phase as found in two nickel-containing hydrogenases from *Desulfovibrio* [10,15,36]. There is no indication in the published results of an activation phase in the nickel-selenium-containing hydrogenases. The specific activity in hydrogen evolution of *D. salexigens* hydrogenase is the highest presently known value for nickel or nickel-selenium hydrogenases from *Desulfovibrio.* This specific activity is higher than previously reported with the hydrogenase isolated following a different purification procedure [14]. The native, partially reduced and fully reduced states, using hydrogen or dithionite as electron donors, of the *D. salexigens* hydrogenase have been analyzed by electron paramagnetic resonance spectroscopy [56].

The dissimilatory sulfite reductases of *Desulfovibrio* are complex structures ($\alpha_2\beta_2$ subunit structures) with molecular weights of about 200 000, Fe-S clusters and sirohemes (Table 2). The absorption spectrum and molecular weights of *D. salexigens* desulfoviridin are similar to that found in *D. gigas* [30] and *D. vulgaris* Hildenborough [31]. The specific activity of *D. salexigens* desulfoviridin is lower than other dissimilatory type sulfite reductases from *Desulfovibrio* and of assimilatory type sulfite reductases from sulfate or sulfur reducers and methanogenic bacteria (Table 2). Comparison of sulfite reductase activity from lysed cell extracts of different *Desulfovibrio* showed that *D. salexigens* had also the lowest activity (our unpublished results). Dissimilatory sulfite reductases may under different assay conditions form trithionate and thiosulfate in addition to sulfide. Desulfoviridin from this bacterium was able to qualitatively form sulfide in our assay conditions.

The optical absorbance spectrum and molybdenum/iron content of the *D. salexigens* molybdenum/iron protein are very similar to the one found in *D. africanus* [21]. Similarity between cytochrome c_3 of *D. africanus* Benghazi and *D. salexigens* British Guiana have also been found [52]. However, antiserum to *D. africanus* Benghazi cells reacts weakly to *D. salexigens* British Guiana cells [53].

The function of the molybdenum protein in *Desulfovibrio* is unknown. Molybdenum in bacteria is found associated with the nitrogenase, formate, dehydrogenase and nitrate reductase enzymes [24]. The amount of molybdenum protein in this bacterium is large as is found in *D. aficanus* (30 mg/250 g wet weight cells) and implies an important function. In *D. salexigens* the amount of cytochromes is low and perhaps the molybdenum protein may function as an electron transport protein. The high molecular weight and the subunit structure may also imply a metal storage capacity for this protein. Further studies on the physiological function of this protein are in progress.

Metal corrosion in sea water is an important problem for the off-shore oil industry. *Desulfovibrio salexigens* is an obligative halophilic sulfate-reducing bacterium and may be used as an reference organism. Hydrogenase, sulfite reductase, and molybdenum protein (metal storage protein) which can be key enzymes in anaerobic bacterial corrosion

deserve to be well defined in this bacterium. A study of these important enzymes will allow a better understanding of how these enzymes function, their role in microbially caused corrosion, and a more rational development of biocides to control these bacteria.

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