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Purification and Characterization of (Per)Chlorate Reductase from the Chlorate-Respiring Strain GR-1

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Strain GR-1 is one of several recently isolated bacterial species that are able to respire by using chlorate or perchlorate as the terminal electron acceptor. The organism performs a complete reduction of chlorate or perchlorate to chloride and oxygen, with the intermediate formation of chlorite. This study describes the purification and characterization of the key enzyme of the reductive pathway, the chlorate and perchlorate reductase. A single enzyme was found to catalyze both the chlorate- and perchlorate-reducing activity. The oxygen-sensitive enzyme was located in the periplasm and had an apparent molecular mass of 420 kDa, with subunits of 95 and 40 kDa in an $\alpha_3\beta_3$ composition. Metal analysis showed the presence of 11 mol of iron, 1 mol of molybdenum, and 1 mol of selenium per mol of heterodimer. In accordance, quantitative electron paramagnetic resonance spectroscopy showed the presence of one [3Fe-4S] cluster and two [4Fe-4S] clusters. Furthermore, two different signals were ascribed to Mo(V). The K_m values for perchlorate and chlorate were 27 and <5 μM, respectively. Besides perchlorate and chlorate, nitrate, iodate, and bromate were also reduced at considerable rates. The resemblance of the enzyme to nitrate reductases, formate dehydrogenases, and selenate reductase is discussed.

The oxyanions chlorate (ClO_3^-) and perchlorate (ClO_4^-) [(per)chlorate] are used extensively for a variety of purposes. Chlorate is used as a herbicide or defoliant, and it is released when chlorine dioxide (ClO_2) is used as a bleaching agent in the paper and pulp industry. Perchlorate has been manufactured in large quantities as an energetic compound in solid rocket fuel. Mishandling of these compounds and the fact that they are chemically stable in water have led to harmful concentrations in surface waters and groundwaters (14). Conventional chemical and physical water treatment technologies are not effective in the removal of (per)chlorate. In contrast, biological removal of these anions can be viewed as very promising (22, 30). Various microorganisms are known to be capable of reducing (per)chlorate, either to chlorite (ClO_2^-) or completely to chloride. The former reaction has been known for a long time and is performed by denitrifying bacteria with the enzyme nitrate reductase or chlorate reductase (8, 23, 26). These organisms are probably not able to respire with (per)chlorate. However, the alternative complete reduction of (per)chlorate to chloride has recently been shown to be coupled to growth in several new isolates (2, 21, 25, 30). These (per)chlorate-respiring microorganisms have been reviewed by Herman and Frankenberger (14) and by Logan (20). Although anaerobic respiration by using (per)chlorate as the terminal electron acceptor has now been demonstrated for several microorganisms, knowledge about the biochemistry of the reductive pathway is still limited. In all cases, perchlorate reducers also reduced chlorate, suggesting that an identical pathway is involved.

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The best-studied (per)chlorate respirer is strain GR-1, a gram-negative facultative anaerobe that uses various fatty acids and dicarboxylic acids as electron donors and that can use oxygen, nitrate, and Mn(IV) as terminal electron acceptors in addition to (per)chlorate (25). The pathway for (per)chlorate reduction has been proposed to be as follows: $ClO_4^- \rightarrow ClO_3^- \rightarrow ClO_2^- \rightarrow Cl^- + O_2$. It has been shown that the organism disproportionates the harmful chlorite into chloride and oxygen by using a chlorite dismutase. This enzyme has recently been purified and characterized as a novel type of heme-iron enzyme, distinct from catalases and peroxidases (29). The complete reduction pathway also involves the (per)chlorate reductase. In the present paper we describe the purification and characterization of the (per)chlorate reductase from strain GR-1. This report represents the first description of a (per)chlorate reductase from a (per)chlorate-respiring organism.

MATERIALS AND METHODS

Growth of organism. Strain GR-1 (DSM 11199) was grown at 30°C in a mineral medium supplemented with 0.02 g of yeast extract/liter, as described before (25), except that the amount of sodium selenite was lowered from 10 to 1 μM. Acetate (24 mM) was used as the electron donor, and chlorate (18.8 mM) or perchlorate (16.4 mM) was used as the primary electron acceptor. For routine culturing, stoppered serum bottles (300 ml), which contained 200 ml of medium and which were flushed with N_2 before inoculation, were used. For enzyme purification large bottles containing 10 liters of medium were used. Resazurin (0.5 mg/liter) was present as an indicator of the redox potential. During growth the bottles were pink due to the metabolic formation of oxygen in the chlorite dismutase reaction. When all the (per)chlorate was consumed, the pink color disappeared because the oxygen concentration was sufficiently lowered by the organisms. At that moment cells were harvested by continuous centrifugation and used for extract preparation.

Enzyme assays. Chlorate reductase and perchlorate reductase levels were measured anaerobically in stoppered quartz cuvettes, by monitoring the oxidation of reduced methyl viologen (MV) at 578 nm and 30°C. The assay mixture (1 ml) consisted of 50 mM Tris-Cl^- buffer (pH 7.5), 0.5 mM MV, and an appropriate amount of enzyme. The assay mixture was prereduced by a small amount of a dithionite solution (0.2 M) until an absorbance of 1.5 was reached, and then the reaction was started by the addition of 10 μl of chlorate (1 M) or perchlorate

(1 M). Specific activities were calculated from the linear decrease in absorbance, with an extinction coefficient of 9.7 mM^{-1} for MV. However, one should realize that the reduction of (per)chlorate leads to chlorite, which is further disproportionated to chloride and oxygen by a chlorite dismutase. Crude extracts have been shown to contain a high level of activity of this enzyme (145 $U \cdot mg^{-1}$) (29). Thus, upon addition of perchlorate, MV is oxidized by perchlorate itself but also by chlorate and oxygen. This means that 8 or 6 mol of MV is oxidized per mol of perchlorate or chlorate, respectively. When the chlorite dismutase is completely removed from (per)chlorate reductase preparations (final purification step), 4 or 2 mol of MV is oxidized, respectively. For the calculation of the activities, these different stoichiometries were taken into account. One unit of activity is defined as the amount of enzyme required to oxidize 2 μmol of reduced MV per min.

Chlorite dismutase activity was determined as described before by measuring the production of oxygen with a Clark-type oxygen electrode (29).

Alternative electron acceptors were tested in the same assay system, except that (per)chlorate was replaced by NO_3^-, NO_2^-, IO_3^-, BrO_3^-, SO_4^{2-}, SO_3^{2-}, SeO_3^{2-}, or $HAsO_4^{2-}$ at a concentration of 2 mM.

Preparation of extracts and localization of (per)chlorate reductase. Cell extract was prepared by suspending wet cells (1:1 [wt/vol]) in 50 mM potassium phosphate buffer (pH 6.0; buffer A) containing 0.1 mg of DNase I $liter^{-1}$. The cell suspension (37 ml; whole-cell fraction) was treated in a French pressure cell at 110 MPa and subsequently centrifuged at 5,000 × *g* for 15 min. The pellet fraction was washed once with buffer A, and the centrifugation step was repeated. The pellet fraction was resuspended in buffer A and adjusted to 50 ml (cell debris fraction). The supernatant fractions (crude extract fraction) were combined and subjected to ultracentrifugation at 110,000 × *g* for 1 h (4°C), yielding 40 ml of a red supernatant (soluble fraction) and 10 ml of membrane fraction. To the soluble fraction, which contains both cytoplasmic and periplasmic proteins, 4 ml of glycerol was added.

To determine the localization of the chlorate reductase, the various fractions that were obtained during the preparation of the cell extract were analyzed for chlorate reductase activity. Additionally, another whole-cell fraction was treated with lysozyme. Therefore, an anaerobically prepared cell suspension in buffer A (1.3 mg [dry weight] $\cdot ml^{-1}$), containing 0.3 M sucrose, 5 mM sodium-EDTA, and lysozyme (2 $mg \cdot ml^{-1}$), was incubated for 1 h at 37°C. Afterwards, magnesium sulfate was added to give 50 mM, and after a few minutes sucrose was added to give a final concentration of 0.6 M. Microscopic examination confirmed the formation of spheroplasts and deformed rods. The spheroplasts were separated from the periplasm fraction by centrifugation (5 min; 11,350 × *g*), and the pellet was resuspended in buffer A to the initial volume. The pellet fraction was sonicated for 30 s, and cell debris was removed by centrifugation (5 min; 11,350 × *g*). All handlings of cell suspensions and cell extracts were performed in an anaerobic glove box. The chlorate reductase activities of the periplasmic and spheroplast fractions were determined. The level of malate dehydrogenase, a cytoplasmic marker enzyme, was determined as described by Bergmeyer (4).

Protein was determined with Coomassie brilliant blue G250 as described by Bradford (5), with bovine serum albumin as a standard.

Purification of (per)chlorate reductase. Since the (per)chlorate reductase was slightly oxygen sensitive, the various enzyme fractions were kept under a nitrogen atmosphere and all purification steps were performed in an anaerobic glove box containing an atmosphere of 96% N_2 and 4% H_2. All buffers contained 10% glycerol. The soluble fraction was used for purification. This fraction was applied to a column of S-Sepharose (3.2 by 13 cm) equilibrated in buffer A containing 10% glycerol. The active enzyme, perchlorate as well as chlorate reductase, eluted from the column with the chlorite dismutase at the start of a linear gradient (300 ml) of 0 to 1 M potassium chloride in buffer A. Active fractions were combined and loaded on a hydroxyapatite column (Bio-Scale CHT5-I), equilibrated in 10 mM potassium phosphate buffer, pH 7.2, containing 10% glycerol. (Per)chlorate reductase eluted from the column at the end of a linear gradient of 10 to 450 mM potassium phosphate, pH 7.2 (300 ml). Active fractions were pooled and concentrated approximately sevenfold in Microsep (30K) concentrators (Filtron). Aliquots of 0.45 ml were subsequently subjected to gel filtration on a Superdex 200 column (1.6 by 70.5 cm) equilibrated in 50 mM potassium phosphate buffer, pH 7.0, containing 10% glycerol and 100 mM NaCl. (Per)chlorate reductase-containing fractions were pooled and stored under N_2 gas until used.

Determination of molecular mass. The elution volume of the (per)chlorate reductase on a Superose-6 HR 10/30 column was used to estimate the molecular mass of the native enzyme. The column was calibrated with the following standard proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the procedure of Laemmli with a 15% acrylamide gel (19). The gel was stained with Coomassie brilliant blue R250.

Kinetic analysis. Kinetic parameters were determined for chlorate and perchlorate by using the standard assay system at 30°C. The concentrations of chlorate and perchlorate were varied between 10 μM and 10 mM. Kinetic parameters were obtained by a computer-aided direct fit of the Michaelis-Menten curve.

TABLE 1. Chlorate reductase activity in various cell fractions[a]

Fraction	Sp act (U/mg)	Total activity (U)	Recovery (%)
Whole cell	0.66	443	100
Crude extract	0.39	247	56
Cell debris	0.65	241	54
Soluble	0.51	232	52
Membrane	0.27	19	4
Periplasmic	1.43 (5.0)	0.96 (3.4)	97 (15)
Spheroplast	0.02 (14.5)	0.03 (18.6)	3 (85)

[a] The values in parentheses represent the data found for malate dehydrogenase.

Metal analysis. The metal content of the purified enzyme was analyzed by inductively coupled plasma mass spectrometry (17). Protein samples were introduced by electrothermal evaporation.

Spectroscopy. Electron paramagnetic resonance (EPR) spectra were recorded on a Bruker ER-200 D spectrometer with peripheral equipment and data handling as described previously (24). The modulation frequency was 100 kHz. UV-visible spectra were recorded on a Beckman DU-7500 diode array spectrophotometer.

N-terminal amino acid sequence analysis. The N-terminal sequence of the purified chlorate reductase was determined according to the Edman degradation method and was performed by the sequencing facility of the Institute for Organic Chemistry and Biochemistry of the University of Freiburg (Freiburg, Germany). Both subunits were electroblotted from an SDS-polyacrylamide gel on a polyvinylidene difluoride membrane prior to analysis.

Materials. S-Sepharose, Superdex 200, Superose-6, and protein standards for gel filtration were purchased from Pharmacia (Woerden, The Netherlands). Hydroxyapatite (Bio-Scale CHT5-I) and SDS-PAGE standards were obtained from Bio-Rad (Veenendaal, The Netherlands). Lysozyme and DNase I were from Boehringer GmbH (Mannheim, Germany). MV was from Sigma Chemie (Bornem, Belgium). All other chemicals were of analytical grade.

RESULTS

Activity in crude extracts and localization. (Per)chlorate reductase activity could be easily demonstrated by using reduced MV as the artificial electron donor. The activity was proportional to the amount of extract added (up to 0.5 mg of protein), but the activity towards ClO_3^- was approximately threefold higher than the activity towards ClO_4^- (not shown). The same ratio was observed in cell extracts that were derived from either ClO_3^- or ClO_4^--grown cells, suggesting that a single enzyme is responsible for both activities.

The (per)chlorate reductase activity was oxygen sensitive. Aerobically stored extracts showed a half-life of inactivation of approximately 2 to 3 days (data not shown). Reactivation in the presence of reducing compounds (50 mM dithiothreitol or β-mercaptoethanol) and Fe^{2+} [20 mM $(NH_4)_2Fe(SO_4)_2$] was not successful. Anaerobically stored extracts also showed some inactivation, but this could be diminished by the addition of 10% glycerol.

Fractionation of the cells enabled localization of the (per) chlorate reductase (Table 1). Activity in the whole-cell fraction was detectable. Since MV is not able to pass the membrane (18), this suggested a localization of the reductase outside the cytoplasmic membrane. After French pressure treatment the activity was found to be about equally distributed over the crude extract and the cell debris fractions. Further fractionation of the crude extract by ultracentrifugation showed that the enzyme is soluble and not intrinsically bound to the membrane. The periplasmic localization was confirmed by the production of spheroplasts. After lysozyme treatment of whole cells, 97% of the total chlorate reductase activity was released from the cells (Table 1). The major part of the cytoplasmic marker enzyme malate dehydrogenase (85%) remained present in the spheroplast fraction.

TABLE 2. Purification of perchlorate and chlorate reductase from strain GR-1

Step	Volume (ml)	Protein concn (mg/ml)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Perchlorate reductase						
Soluble fraction	44	11.5	75.2	0.15	100	1
S-Sepharose	92	1.05	68	0.71	90	4.8
Hydroxyapatite	51	0.64	50	1.53	66	10.4
Superdex 200	17	0.18	11.5	3.65	15.3	24.3
Chlorate reductase						
Crude extract	44	11.5	247	0.49	100	1
S-Sepharose	92	1.05	207	2.15	84	4.4
Hydroxyapatite	51	0.64	120	3.69	48	7.6
Superdex 200	17	0.18	41	13.2	16.6	26.9

Purification of (per)chlorate and chlorate reductase. Table 2 shows the results of the purification of the perchlorate and chlorate reductase. Both the chlorate and perchlorate reductase activities did not bind to anion exchange columns such as the Q-Sepharose and Mono-Q columns. Even at an increased pH value (pH 9.0), the enzyme(s) did not bind, suggesting a high isoelectric point (pI) of the enzyme(s). Accordingly, binding was achieved on a cation exchange column (S-Sepharose) at pH 6.0. The chlorate and perchlorate reductase and also the chlorite dismutase eluted at the same point at the start of the salt gradient, separated from a major part of the contaminating proteins in the flowthrough fraction. On the second column (hydroxyapatite), the chlorate and perchlorate reductase eluted again together at the end of the phosphate gradient, separated now from the majority of the chlorite dismutase. In the final gel filtration step both reductase activities again coeluted from the column in one single brown peak. Analysis by SDS-PAGE resulted in two bands of 95 and 40 kDa (Fig. 1). From the 25-fold purification, it follows that the reductase constitutes approximately 4% of the total cell protein.

Gel filtration on Superose-6 indicated an apparent molecular mass of the native enzyme of 420 kDa. Together with the SDS-PAGE results, this suggested that the native enzyme is composed of a trimer of heterodimers ($\alpha_3\beta_3$).

UV-visible spectroscopy. The UV-visible spectrum of the (per)chlorate reductase (N_2 atmosphere) as isolated showed no specific characteristics (not shown). Only a shoulder at 320 nm and a faint shoulder at 400 nm were visible. This pointed to the presence of iron-sulfur centers, which typically cause a shoulder at 320 nm in the reduced state and a peak at 390 to 400 nm in the oxidized state. There were no indications of the presence of heme-like chromophores.

Catalytic properties. From the identical chromatographic behavior during the various purification steps it was concluded that both reductase activities reside on a single enzyme. The stoichiometry of the assay procedure (see also Materials and Methods) was investigated by using the purified enzyme (Table 3). The results show that, for each mole of perchlorate or chlorate, 4 and 2 mol of MV are oxidized, respectively, and that in the presence of the chlorite dismutase almost 4 mol of MV is also oxidized.

Analysis of the temperature dependence showed an identical optimum for both activities of 45°C (data not shown). Kinetic parameters for both electron acceptors were determined. For perchlorate, K_m and V_{max} values of 27 ± 7 μM and 3.8 ± 0.4 U · mg^{-1}, respectively, were found. The K_m for chlorate was difficult to determine because of its extremely low value. No decrease in the initial rate of activity was found down to 10 μM chlorate, indicating a K_m value of less than 5 μM. The apparent V_{max} amounted to 13.2 U · mg^{-1}.

Various other electron acceptors were tested in the assay. Significant activity was found with NO_3^- (6.2 U · mg^{-1}), IO_3^- (5.3 U · mg^{-1}), and BrO_3^- (10.1 U · mg^{-1}) compared to ClO_3^- (11.3 U · mg^{-1}). Minor activity was found with NO_2^- (0.3 U · mg^{-1}), IO_4^- (0.15 U · mg^{-1}), SO_4^{2-} (0.3 U · mg^{-1}), and SO_3^{2-} (0.6 U · mg^{-1}). No activity was found with SeO_3^{2-} or $HAsO_4^{2-}$.

Metal analysis. The analysis of the purified enzyme for metals revealed the presence of 30.6 mol of iron, 2.9 mol of molybdenum, and 2.9 mol of selenium, based on an apparent molecular mass of 420 kDa. These results are in line with the proposed subunit composition; i.e., each heterodimer contains 1 Mo, 1 Se, and approximately 10 Fe.

N-terminal sequencing of the subunits. The α-subunit (95 kDa) did not give a sequence, possibly because the N terminus was blocked. The β-subunit (40 kDa) gave a sequence which was for the most part unambiguous. A BLAST search in the databases did not result in any similar sequence. However, comparison with the β-subunit of the recently described selenate reductase of *Thauera selenatis* (27) did reveal several identical amino acids, as shown in Table 4.

EPR of (per)chlorate reductase. Low-temperature EPR spectra of the oxidized and the reduced (per)chlorate reductase are characteristic of several iron-sulfur clusters and are presented in Fig. 2. When the enzyme is anaerobically oxidized

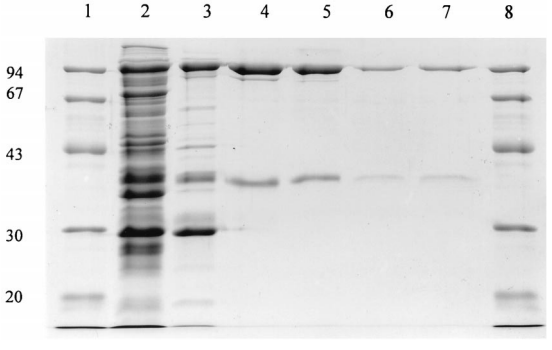

FIG. 1. SDS-PAGE of the (per)chlorate reductase from strain GR-1. Lanes 1 and 8, set of marker proteins with their molecular masses indicated; lane 2, soluble fraction (115 μg of protein); lane 3, S-Sepharose pool (10.5 μg of protein); lanes 4 and 5, hydroxyapatite pool (9.6 μg of protein); lanes 6 and 7, Superdex 200 pool (3.6 μg of protein). Proteins were stained with Coomassie brilliant blue R250.

TABLE 3. Stoichiometry of the MV-dependent reduction of perchlorate and chlorate[a]

Enzyme	Perchlorate added (μM)	Chlorate added (μM)	MV oxidized (μM)	MV/(per)chlorate ratio	
				Measured	Predicted
(Per)chlorate reductase	9.8		41.2	4.2	4
(Per)chlorate reductase		9.8	22.6	2.3	2
(Per)chlorate reductase + chlorite dismutase	9.8		67.0	6.83	8
(Per)chlorate reductase + chlorite dismutase		9.8	51.2	5.22	6

[a] Reactions were performed by the standard assay using purified (per)chlorate reductase alone (0.01 U) or together with an excess of purified chlorite dismutase (19 U) as indicated.

with potassium ferricyanide, a sharp spectrum with minor *g* anisotropy ($g_z = 2.017$) around the free-electron value ($g_e = 2.002$) is found. This spectrum is the fingerprint of the $[3Fe\text{-}4S]^{1+}$ cluster. Double integration versus an external copper standard affords a spin count of 0.96 $S = 1/2$ per αβ heterodimer of 135 kDa.

Reduction with sodium dithionite gives the complex spectrum in the lower trace of Fig. 2. The *g* value range is indicative of $[4Fe\text{-}4S]^{1+}$ or $[2Fe\text{-}2S]^{1+}$; the former is indicated by severe broadening of the signals upon raising the temperature above 40 K (data not shown). The doubly integrated intensity corresponds to 1.5 $S = 1/2$ spins.

When the microwave power is increased, with the reduced enzyme another set of signals is detected at lower magnetic field values (Fig. 3). The signals at effective *g* values 5.93, 4.51, and 3.40 are readily assigned to the two intradoublet transitions of an $S = 3/2$ system of intermediate rhombicity (11). Quantitation of the two signals as effective $S' = 1/2$ spectra (1) gives a spin count of approximately 0.5 $S = 3/2$ spins per αβ dimer.

The enzyme as isolated is in an intermediate redox state (not necessarily equilibrated) because it exhibits signals of substoichiometric intensity both from the 3Fe and 4Fe clusters (data not shown). When the temperature is raised to 40 K, these signals are largely broadened away, and we observe the spectrum given in Fig. 4. Simulation indicates that the spectrum is dominated by two different signals, one with all *g* values greater than g_e, the other with all *g* values less than g_e. The latter signal has weak hyperfine satellite lines whose multiplicity and relative intensity are consistent with molybdenum (25.2%; nuclear spin $I = 5/2$). Both signals are tentatively ascribed to Mo(V). Their sum intensity corresponds to approximately 0.2 $S = 1/2$ spins per αβ dimer.

DISCUSSION

The pathway for perchlorate reduction to chloride involves chlorate and chlorite as intermediates (25). Here, we report on the purification and characterization of the key enzyme of the reduction pathway, the (per)chlorate reductase. The fact that there is an identical purification scheme for both the perchlorate and chlorate reductase and that the purified enzyme catalyzed both activities in the same ratio observed in crude extracts demonstrated that one enzyme was responsible for both activities. The analysis of the substrate specificity showed that the enzyme also exhibits significant bromate, iodate, and nitrate reductase activity. The last property is not unusual; many nitrate reductases also have chlorate reductase activity (16). Whether GR-1 contains a separate nitrate reductase when grown on nitrate is not known. Perchlorate-grown cells were unable to convert nitrate or nitrite, suggesting that another nitrate reductase may be involved in nitrate-grown cells (25). The kinetic data anyhow show that the reductase is very efficient in the reduction of perchlorate ($K_m = 27$ μM) and chlorate ($K_m < 5$ μM). Compared to K_m values found for nitrate (0.1 to 1.3 mM [16]), the value for chlorate is remarkably low.

The (per)chlorate reductase could easily be isolated from cells without alkaline heat treatment or the aid of detergents, suggesting that it is a soluble protein. The high reductase activity in whole cells and the release of almost all the activity

TABLE 4. Alignment of the N-terminal amino acid sequence of the β-subunit of the (per)chlorate reductase from strain GR-1 and the β-subunit of the selenate reductase from *T. selenatis*

Reductase source	Sequence
Strain GR-1	A N V M K A P R R Q L T Y V T D X N
T. selenatis	S Q R Q L A Y V F D L N

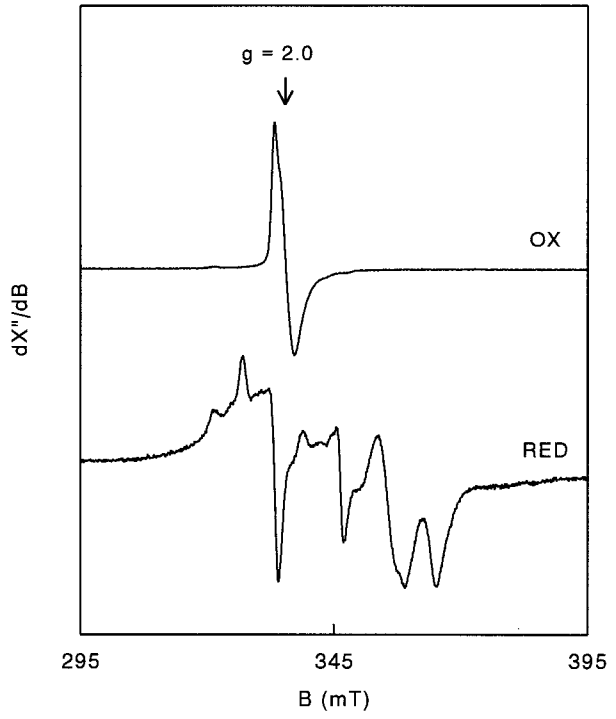

FIG. 2. $S = 1/2$ EPR spectra of [3Fe-4S] and [4Fe-4S] clusters in chlorate reductase. The enzyme concentration was 5.3 mg/ml (39 μM αβ dimer) in 50 mM potassium phosphate buffer (pH 7.0)–10% (vol/vol) glycerol. The upper trace was obtained after anaerobic incubation with 0.4 mM potassium ferricyanide for 4 min at ambient temperature. Similarly, the lower trace was obtained after 5 min of incubation with 5 mM sodium dithionite. EPR conditions: microwave frequency, 9,414 MHz; microwave power, 5 mW; modulation amplitude, 0.5 mT; temperature, 14 K. OX, oxidized; RED, reduced; X″, the imaginary part of the magnetic susceptibility; B, magnetic field.

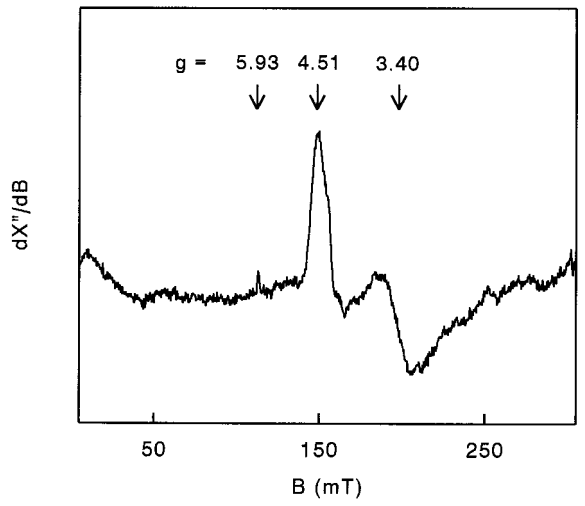

FIG. 3. Low-field $S = 3/2$ signals from [4Fe-4S] in dithionite-reduced chlorate reductase. The sample was the same as that for Fig. 2, trace RED. The EPR conditions were also the same, except for a higher electronic gain and a microwave power of 126 mW. X″ and B are as defined in the legend to Fig. 2.

by a lysozyme treatment indicated a periplasmic location of the enzyme. This would mean that the previously described soluble chlorite dismutase, catalyzing the disproportionation of chlorite, is also a periplasmic enzyme. Although the (per)chlorate reductase is soluble, it is expected to be part of an electron transport chain, because (per)chlorate reduction by GR-1 is coupled to growth (25). It is, however, not yet clear how the reductase is coupled to the membrane in a way that energy can be conserved. A comparison with analogous enzyme systems, such as nitrate reductases, could give some indications in this respect. However, most respiratory nitrate reductases reside on the inner aspect of the membrane (16). Some nitrate reductases are also soluble and are located in the periplasm, but their physiological role is not entirely clear. For example, *Alcaligenes eutrophus*, *Rhodobacter sphaeroides* (chlorate reducing), and *Thiosphaera pantotropha* contain a periplasmic nitrate reductase in addition to a typical respiratory-membrane-bound nitrate reductase (3, 6, 28).

According to the results of the SDS-PAGE and the native molecular mass of 420 kDa, it is concluded that the (per)chlorate reductase is composed of a trimer of heterodimers of 95 and 40 kDa ($\alpha_3\beta_3$). This is consistent with the results of the metal analysis; i.e., next to 30.6 mol of iron, approximately 3 mol of molybdenum and 3 mol of selenium are present per multimeric enzyme. These results again point to a resemblance to nitrate reductase, an enzyme that is often purified as a dimer ($\alpha\beta$) with subunits of approximately 104 to 150 (α) and 52 to 63 kDa (β) (16). The α-subunit contains iron-sulfur centers and molybdenum and constitutes the active site for nitrate reduction. The complete nitrate reductase also contains a γ-subunit of approximately 20 kDa, which is the nitrate reductase-specific cytochrome b_{556}. The γ-subunit is assumed to be essential for assembly of the α- and β-subunits into the membrane but is not required for activity, as determined with viologen dyes. Another interesting analogy is found in selenate reductase, which was recently purified from the selenate-respiring *T. selenatis* (27). The periplasmic selenate reductase is a heterotrimer whose components have molecular masses of 96, 40, and 23 kDa and which contains an average of 12.9 iron atoms, 1 molybdenum atom, and 1 heme *b* molecule per trimer. The enzyme is, however, not active with chlorate or nitrate. Nevertheless, the N-terminal amino acid sequence of the β-subunit of the selenate reductase shows significant similarity with the sequence of the β-subunit of the (per)chlorate reductase.

The presence of selenium in the enzyme is rather unusual. None of the chlorate or nitrate reductases described to date contain selenium. However, several formate dehydrogenases from enterobacteria or clostridia are known to contain selenium as selenocysteine in addition to molybdenum and iron-sulfur clusters (13). For example, the formate dehydrogenase isoenzyme FDH_N from *Escherichia coli* contains selenium in its 110-kDa α-subunit. In addition to the α-subunit, a 30-kDa β-subunit and a 20-kDa γ-subunit, which are a ferredoxin-like protein and a membrane-internal cytochrome b_{556}, respectively, are present (9). Selenium is presumably also present as selenocysteine in the (per)chlorate reductase, although this has to be confirmed by chemical and/or genetic analysis. Taken together, these comparisons suggest that, for the (per)chlorate reductase from GR-1 also, a third cytochrome-type subunit, which accomplishes the connection to the membrane and which is apparently lost during the isolation and purification of the enzyme, is probably involved.

The nature of the metal centers in the (per)chlorate reductase could be probed in the EPR experiments. When the active enzyme is oxidized with ferricyanide under anaerobic conditions (to avoid possible oxidative breakdown of cubane clusters) a $[3Fe\text{-}4S]^{1+}$ signal whose integrated intensity is stoichiometric with the $\alpha\beta$ dimer is found. The dithionite-reduced enzyme exhibits a complex spectrum with integrated intensity of 1.5 $S = 1/2$ and 0.5 $S = 3/2$ spins. It is quite common for

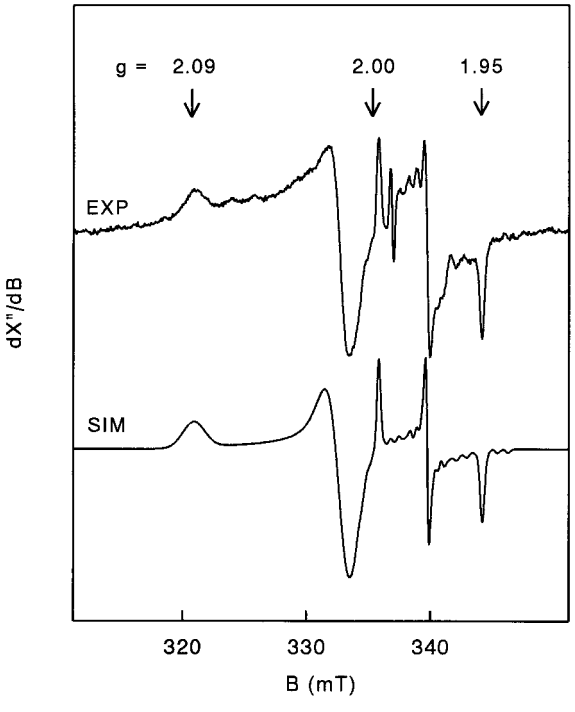

FIG. 4. Putative Mo(V) EPR signals from chlorate reductase as isolated (6 mg/ml). The spectrum was simulated as a sum of two signals. Parameters for one signal: $g = 2.091$, 2.016, and 2.016; isotropic line width, 1.5 mT; parameters for the other signal: $g = 1.999$, 1.976, and 1.950; molybdenum hyperfine splitting, 0.65, 0.5, and 0.83 mT; line width, 0.25, 0.2, and 0.3 mT. EPR conditions: microwave frequency, 9,396 MHz; microwave power, 0.5 mW; modulation amplitude, 0.32 mT; temperature, 40 K. EXP, experimental; SIM, simulated. X″ and B are as defined in the legend to Fig. 2.

$[4Fe\text{-}4S]^{1+}$ clusters to occur in noninterchangeable mixtures of ground state $S = 1/2$ and $S = 3/2$ (10). Therefore, we ascribe the sum EPR intensity of 1.5 plus 0.5 spins to [4Fe-4S] clusters. We have thus detected one [3Fe-4S] cluster and two [4Fe-4S] clusters in the αβ dimer of chlorate reductase, which gives a total of 11 iron atoms per dimer.

Molybdenum enzymes typically exhibit a variety of Mo(V) signals depending on the history of the samples (15). It is thus not surprising to find multiple signals in an enzyme as isolated. The sharp signal with $g < g_e$ in Fig. 3 is readily assigned to Mo(V). The g values are consistent with a d^1 configuration, and the relaxation is much slower than that of the Fe-S clusters. The signal exhibits weak satellite lines. When the two Mo isotopes with nuclear spin $I = 5/2$ are included in the stimulation of Fig. 4, the relative intensities of the resulting satellite lines approximately fit the experimental spectrum. Assignment of the second signal, i.e., with $g > g_e$, is less straightforward. The g_z value of 2.091 is unusually high for molybdenum enzymes, although such a high value ($g_z = 2.10$) has been found in the tungsten and selenium enzyme formate dehydrogenase from *Clostridium thermoaceticum* (7). Similar values have also been reported for Mo ($g_z = 2.07$) and W ($g_z = 2.09$) selenolate model compounds (12). A second argument to assign the signal to Mo(V) is the lack of a reasonable alternative interpretation. However, further work, for example, with isotope-enriched samples, is required to determine both the electronic and catalytic natures of the centers that give rise to these signals.

In conclusion, the (per)chlorate reductase shows a resemblance to nitrate reductases with respect to its substrate use and structural composition, but on the other hand clear differences concerning the presence of selenium and the N-terminal sequence of the β-subunit exist. Moreover, energy-conserving nitrate reductases reside on the inner aspect of the membrane, indicating that the mechanism by which the periplasmic (per) chlorate reductase is coupled to the membrane in a way that energy can be conserved must also be different.

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