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NOTES

Characterization of the Chlorate Reductase from *Pseudomonas chloritidismutans*

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A chlorate reductase has been purified from the chlorate-reducing strain *Pseudomonas chloritidismutans*. Comparison with the periplasmic (per)chlorate reductase of strain GR-1 showed that the cytoplasmic chlorate reductase of *P. chloritidismutans* reduced only chlorate and bromate. Differences were also found in N-terminal sequences, molecular weight, and subunit composition. Metal analysis and electron paramagnetic resonance measurements showed the presence of iron and molybdenum, which are also found in other dissimilatory oxyanion reductases.

Pseudomonas chloritidismutans (strain AW-1) is a recently isolated facultative anaerobic chlorate-reducing bacterium (22). In chlorate-reducing bacteria, chlorate is reduced to chlorite by a chlorate reductase, and in a second reaction, chlorite is disproportionated to chloride and oxygen by a chlorite dismutase. Two closely related strains of *P. chloritidismutans, Pseudomonas stutzeri* DSM 50227 and DSM 5190^T (with 16S ribosomal DNA similarities of 100% and 98.6%, respectively), were not able to grow by dissimilatory chlorate reduction. Accordingly, both *P. stutzeri* strains lack chlorite dismutase activity. However, in addition to nitrate reductase activity, these strains also showed chlorate reductase activity, which has been observed before for other denitrifying bacteria (6, 15). *P. chloritidismutans* differed from other (per)chlorate-reducing bacteria in that it was only able to use chlorate as a terminal electron acceptor. Other chlorate-reducing bacteria can also couple the reduction of perchlorate or nitrate to growth (1, 13). Although cell extracts also showed bromate reductase activity, *P. chloritidismutans* could not grow on bromate (22).

Up to now, two enzymes that can reduce chlorate and/or perchlorate have been purified and characterized. A chlorate reductase C has been purified from the denitrifying strain *Proteus mirabilis*, as well as two nitrate reductases (15). The only known substrate of chlorate reductase C is chlorate, which was reduced to chlorite. It was not demonstrated that *Proteus mirabilis* was able to couple the reduction of chlorate to growth. A second (per)chlorate reductase has been purified from strain GR-1 (10). Experiments showed that one enzyme is responsible for both chlorate and perchlorate reduction activity. Besides (per)chlorate, nitrate, iodate, and bromate were also reduced by the (per)chlorate reductase of strain GR-1. Perchlorate-grown cells were unable to oxidize nitrate or nitrite, indicating that another nitrate reductase may be involved in nitrate-grown cells (17). The purified chlorate reductase of *P. chloritidismutans* reported here is the first chlorate reductase derived from a chlorate-reducing bacterium that is capable of only dissimilatory chlorate reduction.

P. chloritidismutans (DSM 13592) was grown under strictly anaerobic conditions at 30°C, as described before (1). Strain GR-1 (DSM 11199) was grown as described previously (10, 17). Chlorate (10 mM) was used as the electron acceptor, while acetate (10 mM) was used as the electron donor and carbon source. Cell extracts of *P. chloritidismutans* lost chlorate reduction activity when stored under air. Therefore, the preparation of cell extracts and all purification steps were done in an anaerobic glove box containing an atmosphere of 96% N_2 and 4% H_2, and purified enzyme fractions were kept on ice (4°C). Cells were collected by centrifugation at 9,000 rpm for 10 min at 4°C. The cell pellet was suspended (1:2 [wt/vol]) in 15 mM potassium-sodium phosphate buffer, pH 7.2 (buffer A), and cells were disrupted by ultrasonic disintegration (Sonics & Materials Inc., Danbury, Conn.). Cell debris and whole cells were removed by centrifugation at 13,000 rpm for 10 min at 4°C. The supernatant fraction was subjected to an ultracentrifugation step, 110,000 × *g* for 1 h at 4°C. This resulted in a red supernatant containing the cytoplasmic and periplasmic fraction. The pellet (membrane fraction) was suspended in buffer A.

Enzyme purification was started by loading the red supernatant fraction onto a Q-Sepharose column (2 by 30 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5. The chlorate reductase eluted at the start of a linear gradient of 0 to 1 M potassium chloride in 50 mM Tris-HCl buffer, pH 7.5. Active fractions were pooled and desalted by ultrafiltration (150-ml stirred cell; filter pore size, 10 kDa; Filtron Technology). The

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concentrated enzyme fraction was diluted with 10 mM Tris-HCl, pH 7.2. This fraction was loaded on a hydroxyapatite column equilibrated with 10 mM Tris-HCl, pH 7.2. A linear gradient was established from 10 mM Tris-HCl, pH 7.2, to 450 mM potassium phosphate, pH 7.2. Active fractions were combined and loaded onto a Mono-Q column which was equilibrated with 50 mM Tris-HCl buffer, pH 7.5. A linear gradient was established from 0 to 1 M potassium chloride in 50 mM Tris-HCl buffer, pH 7.5.

A 400-μl aliquot of the active fraction was subsequently loaded onto a Superdex 200 column (1.6 by 70.5 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 100 mM NaCl. Chlorate reductase activity was measured anaerobically in stoppered glass cuvettes by monitoring the oxidation of reduced methyl viologen at 578 nm and 30°C in a Hitachi spectrophotometer (U-2010) as described earlier (6). The following electron acceptors (all sodium salts) were tested: ClO_4^-, ClO_3^-, ClO_2^-, NO_3^-, NO_2^-, BrO_3^-, SO_4^{2-}, SeO_4^{2-}, IO_3^-, and IO_4^-. The enzyme activity was measured at different pHs (5.5 to 10) and temperatures (30 to 90°C). Kinetic parameters were obtained by a computer-aided direct fit of the Michaelis-Menten curve. The chlorate concentration was varied between 10 μm and 4 mM.

Chlorite dismutase and catalase activities were determined by measuring oxygen production with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, Ohio) as described earlier (22). One unit of activity is defined as the amount of enzyme required to convert 1 μmol of substrate per minute. The protein content of the enzyme fractions was determined by the method of Bradford, with bovine serum albumin as the standard (4).

For localization of the chlorate reductase, a culture of *P. chloritidismutans* or strain GR-1 was centrifuged for 10 min at 9,000 rpm and 4°C. The cell pellet was suspended (1:2 [wt/vol]) in EDTA buffer (50 mM Tris, 50 mM EDTA, 170 mM Na_2CO_3, pH 9) (20). This cell suspension was incubated for 30 min at room temperature, followed by a centrifugation step (13,000 rpm, 10 min at 4°C). The red supernatant (periplasmic fraction) was separated from the pellet. The pellet was suspended in buffer A (1:2 [wt/vol]), disrupted by ultrasonic disintegration, and further processed as described above.

A gentler method to disrupt the cells is a freeze-thaw procedure (23). A cell pellet was suspended in buffer A. After the suspension was frozen in liquid N_2, it was thawed by immersing it in running water (40°). This procedure was repeated four times. A small amount of DNase I (Sigma) was added to reduce the viscosity due to the released DNA. The solution was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant (cell extract) was centrifuged at 110,000 × *g* for 1 h at 4°C. The supernatant (cytoplasmic and periplasmic fraction) was separated from the pellet. The pellet (membrane fraction) was suspended in buffer A.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (12) with 12% acrylamide gels. To calculate the molecular mass of the subunits and the native enzyme, the SDS-PAGE and Superdex 200 column were calibrated with standard proteins. For determination of the N terminus of the subunits, the subunits were blotted onto a Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad) following the manufacturer's instructions. The N-terminal sequences were determined as described earlier (18).

TABLE 1. Summary of the purification of chlorate reductase from *P. chloritidismutans*

Step	Vol (ml)	Protein concn (mg/ml)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Soluble fraction	13	27.5	726	2	100	1
Q-Sepharose	20	2.8	335	6	46.1	3
Hydroxyapatite	5	3.5	155	8.7	21.4	4.4
Mono-Q	2	3.3	95	14.4	13.1	7.2
Superdex 200	10	0.18	75	42	10.3	21

Electron paramagnetic resonance spectra were recorded on a Bruker ER-200D spectrometer with peripheral equipment and data handling as described previously (16). The modulation frequency was 100 kHz. The presence of metals was measured by inductively coupled plasma mass spectrometry (Elan 6000; Perkin-Elmer).

During the first purification step, the chlorate reductase was separated from a red fraction which eluted just before the chlorate reductase and contained chlorite dismutase activity. On the other columns used, the chlorate reductase eluted at the start of the linear gradient. These fractions had a brown-reddish color. The purification scheme is shown in Table 1. The active fraction, from the Superdex 200 column, was run on SDS-PAGE, and three bands were obtained, with molecular masses of 97, 38, and 34 kDa. The molecular mass for the native enzyme was about 167 kDa, as determined by gel filtration with the Superdex 200 column. These results suggest that all subunits are present in a 1:1:1 stoichiometry and that the chlorate reductase is a heterotrimer ($\alpha_1\beta_1\gamma_1$). The N-terminal sequences of this enzyme can be retrieved through the Swiss-Prot sequence database, European Bioinformatics Institute, under accession numbers P83448, P83449, and P83450. The same stoichiometry has been found for the chlorate reductase C from *Proteus mirabilis* (Table 2), although the sizes of the subunits were different. Chlorate reductase of strain GR-1 showed a different stoichiometry, resulting in a different molecular mass of the native enzyme (420 kDa). This enzyme was found to consist of an α and a β subunit, but a third (γ) subunit was anticipated (Table 2).

For chlorate, K_m and V_{max} values of 159 μM and 51 U/mg were found, respectively. Besides chlorate, only bromate served as an electron acceptor. Under standard conditions, specific activities of 50 and 26 U/mg were found for chlorate and bromate, respectively. Because bromate could not be used as an electron acceptor for growth (1), the chlorate reductase of *P. chloritidismutans* is apparently not involved in another oxyanion dissimilatory reduction pathway. The enzyme from strain GR-1 showed activity with chlorate, perchlorate, bromate, iodate, and nitrate (6).

The optimum pH for the chlorate reductase was pH 7.5. At pH 5.5 and 10, the specific activity was approximately 50% of the optimal value at pH 7.5. The purified enzyme had a temperature optimum of 75°C. This is remarkable because *P. chloritidismutans* is a mesophilic bacterium (1), which does not grow above 42°C. The optimal temperature of the enzyme from strain GR-1 was only 45°C. However, the trimethylamine *N*-oxide (TMAO) reductase of *Salmonella enterica* serovar Typhimurium also functions at

TABLE 2. Summary of enzyme characteristics

Enzyme	Localization	Electron acceptor(s)	Subunits (kDa)	Native enzyme composition	EPR of Mo(V) (*g* values)	ClO_3^-	
						V_{max} (U/mg)	K_m (μM)
P. mirabilis chlorate reductase C	Membrane	Chlorate[a]	75, 63, 56	Heterotrimer ($\alpha_1\beta_1\gamma_1$)			
GR-1 (per)chlorate reductase	Periplasm	Perchlorate, chlorate, nitrate, iodate, bromate	95, 40	Trimers of heterodimers ($\alpha_3\beta_3$)	2.002, 2.017	13.2	<5
P. chloritidismutans chlorate reductase	Cytoplasm	Chlorate, bromate[b]	97, 38, 34	Heterotrimer ($\alpha_1\beta_1\gamma_1$)	2.002, 2.024, 2.076	51.3	159

[a] Electron acceptors other than chlorate and nitrate were not tested.
[b] ClO_4^-, ClO_2^-, NO_3^-, NO_2^-, IO_4^-, IO_3^-, SO_4^{2-}, and SeO_4^{2-} were tested but did not show reductase activity.

higher temperatures (below 75°C) than the optimal growth temperature, which was 37°C (11). Remarkably, besides the reduction of TMAO, this enzyme also showed chlorate and bromate but not nitrate reduction activity (11, 21).

Analysis of the N-terminal sequences of the three subunits via a BLAST search in the databases did not result in any similar N-terminal sequence for the α (LNMLEPVGETLA SEYPYXK; Swiss-Prot P83448) and γ (EXSEQNPNILEIKPG DTVKVXT; Swiss-Prot P83450) subunits. However, a significant similarity was found for the N-terminal sequence of the β subunit (TVKXQLSMVLDLNKEIGGQTXTAA; Swiss-Prot P83449) when this sequence was compared to the β subunit of the dimethylsulfide dehydrogenase of *Rhodovulum sulfidophilum* (73%) (14), the β subunit of the nitrate reductase of *Haloarcula marismortui* (61%) (24), the anaerobic ethylbenzene dehydrogenase subunit B of *Azoarcus* sp. strain EB1 (60%) (9), and the β subunit of the selenate reductase of *Thauera selenatis* (55%) (19). The similarity to the β subunit of the (per)chlorate reductase of strain GR-1 was 27%. A common characteristic of these enzymes is the presence of molybdenum.

Chlorate reductase activity was found mainly in the cytoplasmic fraction (Table 3). In contrast to the chlorate reductase, chlorite dismutase activity was found predominantly in the periplasmic fraction. The absence of catalase activity in the periplasmic fraction confirmed the efficiency of the localization method used. It is known that nitrate reductases are located almost exclusively in the cytoplasmic membrane (5). By disrupting cells of *H. marismortui* with an ultrasonic oscillating device, it was shown that nitrate reducing activity was easily released into the soluble fraction (10). With a gentler method (freeze-thaw procedure), cells of *H. marismortui* were disrupted, and the nitrate reductase was extracted from the resulting membrane fraction. Therefore, the enzyme was considered an extrinsic membrane protein that binds to the surface of the cytoplasmic membrane by hydrophobic interaction. This freeze-thaw procedure was also used for cells of *P. chloritidismutans* and resulted in the highest chlorate reductase activity in the cytoplasmic/periplasmic fraction (95.7 U) compared to the membrane fraction (8.2 U). Therefore, it is suggested that the chlorate reductase of *P. chloritidismutans* is cytoplasmic.

TABLE 3. Localization of chlorate reductase, chlorite dismutase, and catalase activity in cell fractions of strain AW-1

Fraction	ClO_3^- reductase (U/mg)	ClO_3^- reductase (U total)	ClO_2^- dismutase (U/mg)	ClO_2^- dismutase (U total)	Catalase (U/mg)	Catalase (U total)
Periplasmic	6.2	7.5	236	284	NA[a]	NA
Cytoplasmic	29.6	47.2	20.6	32.7	15.7	24.9
Membrane	3.8	2	9.3	4.8	3.3	1.7

[a] NA, no activity.

It is not yet clear how the respiratory pathway is arranged in chlorate-reducing bacteria. The (per)chlorate reductase of strain GR-1 is located in the periplasm, whereas the chlorate reductase described here is located in the cytoplasm. This means that the ways in which energy is conserved in these bacteria must also be different. The opposite locations of the chlorate reductase and chlorite dismutase (Table 3) imply that transport systems are required to transport chlorate from the periplasm to the cytoplasm and to transport chlorite back to the periplasm. Because chlorite is highly oxidative, it might be favorable to translocate chlorite back to the periplasm. We do not have any insight into the transport of chlorate and chlorite. However, a chlorate-chlorite antiport system might be most appropriate to avoid accumulation of toxic chlorite in the cell. A similar situation exists in *Azospirillum brasilense* Sp7 (5) and *Paracoccus denitrificans* (2, 3), both denitrifying bacteria that possess cytoplasmic nitrate reductases and periplasmic nitrite reductases. It was proposed that nitrate uptake might proceed via an energy-independent nitrate-nitrite antiport system. This process prevents intracellular accumulation of toxic levels of nitrite and allows further detoxification in the periplasm through the action of nitrite reductase.

Electron paramagnetic resonance analysis of the anaerobically purified enzyme revealed an unusual signal with all *g* values greater than the free-electron value, $g_e = 2.002$, namely, $g_z = 2.076$ and $g_{xy} = 2.024$ (Fig. 1, trace A). Double integration versus an external copper standard gave a spin count of 0.2 $S = 1/2$ per 167-kDa $\alpha_1\beta_1\gamma_1$ heterotrimer. The signal was very similar in shape and integrated intensity to that of the $\alpha_3\beta_3$ heterohexameric (per)chlorate reductase from strain GR-1 (6). Previously, this signal was suggested to come from Mo(V) with Se coordination (6). In line with this suggestion, we find that the intensity of the present signal was reduced to 0.13 spins upon incubation with sodium dithionite; it diminished further to 0.05 spins upon light reduction with the deazaflavin-EDTA system.

The present enzyme did contain molybdenum, but selenium was not measured. The molar ratio of [Fe] to [Mo] of ≈16 was different from the molar ratio of [Fe] to [Mo] of ≈11 found for strain GR-1 (6). Moreover, in the GR-1 enzyme, a complex $2[4Fe\text{-}4S]^{1+}$ spectrum developed upon dithionite reduction with an intensity of approximately 2 spins, and oxidation with potassium ferricyanide afforded a $[3Fe\text{-}4S]^{1+}$ signal equivalent

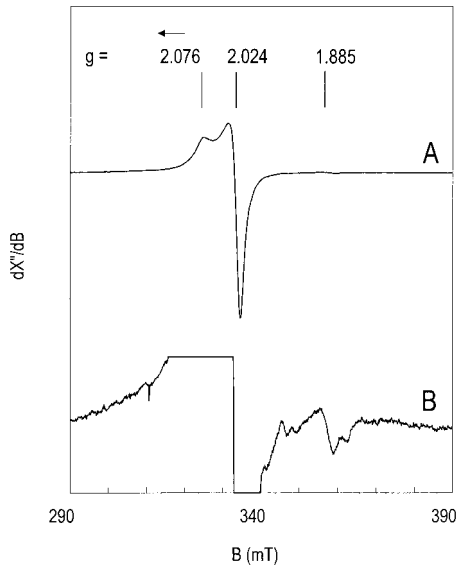

FIG. 1. Electron paramagnetic resonance spectra of *P. chloritidismutans* chlorate reductase. The enzyme concentration was 2.3 mg/ml or 14 μM. Trace A is from the enzyme isolated anaerobically; trace B is from dithionite-reduced enzyme. The amplification for trace B is 25 times that for trace A. Electron paramagnetic resonance conditions: microwave frequency, 9.43 GHz; microwave power, 126 mW; modulation frequency, 100 kHz; modulation amplitude, 0.63 mT; temperature, 16 K.

to 1 spin (6). In contrast to this, we found only very weak Fe/S signals for the *P. chloritidismutans* enzyme. The Fe/S spectrum integrated to approximately 1% of the protein concentration. Addition of dithionite caused an increase to some 0.02 spin (Fig. 1, trace B). The amplitude did not increase further upon light reduction. The lack of Fe/S electron paramagnetic resonance signals is puzzling; however, a few examples of [4Fe-4S] cluster-containing enzymes in which (part of) the clusters are electron paramagnetic resonance silent are known (7). Nevertheless, based on these results, we suggest that the Fe in the protein is nonheme iron.

The purification of both the (per)chlorate reductase of strain GR-1 and the chlorate reductase of *P. chloritidismutans* showed that molybdenum enzymes are involved in (per)chlorate reduction. In an overview by Hille (8) about mononuclear molybdenum enzymes, the dimethyl sulfoxide reductase family is described. It is remarkable that in this dimethyl sulfoxide reductase family, two other enzymes are present, the TMAO reductase and the dissimilatory nitrate reductases, both of which show chlorate reductase activity. The results shown in this paper suggest that the chlorate reductase of *P. chloritidismutans* is different from the nitrate reductases. Sequencing of the chlorate reductase gene coupled with crystallographic information and detailed spectroscopic characterization by electron paramagnetic resonance can give more insight into the mechanism of action of the chlorate reductase in comparison to dimethyl sulfoxide reductases.

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