Purification and characterization of dissimilatory nitrate reductase from a denitrifying halophilic archaeon, *Haloarcula marismortui*

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Abstract Dissimilatory nitrate reductase was purified from a denitrifying halophilic archaeon, *Haloarcula marismortui*, to an electrophoretically homogeneous state. The purified enzyme was inferred to be a homotetramer composed of a 63 kDa polypeptide. The electron paramagnetic resonance spectrum of the purified enzyme revealed typical rhombic signals which were ascribed to Mo(V) in the Mo–molybdopterin complex. Like the bacterial membrane-bound (Nar-) enzyme, the purified enzyme supported the catalysis of chlorate. The enzyme was activated in extreme saline conditions and the values of k_{cat} and K_m toward nitrate were 145 s^{-1} and 79 μM, respectively, in the presence of 2.0 M NaCl.
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Key words: Nitrate reductase; Denitrification; Halophilic archaeon; Molybdenum; *Haloarcula marismortui*

1. Introduction

Excessive accumulated ammonia in an environment is aerobically oxidized to nitrate through a bacterial nitrification process, then the resulting nitrate is converted to N_2 gas by denitrifying microorganisms and released into the atmosphere. Denitrification can be understood as one kind of anaerobic respiration in which nitrate is employed as a terminal electron acceptor and N_2 is formed as a final product. The biochemical system of denitrification has been well investigated in bacterial denitrifiers [4,30]. In the bacterial denitrification, nitrate is successively reduced to N_2 through nitrite, NO and N_2O and each reduction step is catalyzed by the respective redox enzymes.

Denitrifying microorganisms are distributed not only in the bacteria, but also in the eukaryotic microorganisms and the archaea [30]. Denitrifying activity in fungi was found by Shoun and Tanimoto [23]. Although the biochemistry of fungal denitrification resembles that of bacteria, there are two significant differences: (i) the NO reductase of fungi is a novel cytochrome P-450-type enzyme and (ii) denitrifying fungi release N_2O as the final product of denitrification due to the lack of N_2O reductase [19,23].

In archaea, some halophiles and hyperthermophiles have been reported to have denitrifying activity [26,28]. Nitrate reductases have been purified from three denitrifying halophilic archaea in genus *Haloferax* to date [1,6,15]. Subunit constructions of the enzymes from *Haloferax denitrificans* and *Haloferax volcanii* are reported to be similar to that of the bacterial membrane-bound enzyme, while their prosthetic cofactors have not been characterized at all [6,15]. Copper-containing nitrite reductase has been reported only from *H. denitrificans* [16]. Further, there has been no information about the presence of NO and N_2O reductases in any denitrifying archaea to date.

To elucidate the total biological mechanism of denitrification in archaea, we have advanced the characterization of each denitrifying enzyme in a denitrifying halophilic archaeon, *Haloarcula marismortui*. In this paper, we describe the purification of dissimilatory nitrate reductase from the microorganism. Structural and functional properties of the purified enzyme are characterized and compared with those of the bacterial and the other archaeal counterparts. The effect of salt concentration on the catalytic property of the purified enzyme is discussed.

2. Materials and methods

2.1. Cultivation of the organism

H. marismortui ATCC43049 was cultivated anaerobically in the presence of nitrate. The growth medium contained 1.0 g peptone (Difco, Detroit, MI, USA), 1.0 g yeast extract (Difco), 5.0 g K_2SO_4, 5.1 g $NaNO_3$, 125 g NaCl, 160 g $MgCl_2 \cdot 6H_2O$, 0.13 g $CaCl_2$, 1.0 mg $MnSO_4$, 0.83 mg $Fe_2(SO_4)_3$, 10 μg $CuSO_4 \cdot 5H_2O$, 10 μg $(NH_4)_6$-$Mo_7O_{24} \cdot 4H_2O$ per liter. The pH of the medium was adjusted to 7.0. After it was autoclaved, 40 mg/l of Na-ascorbate was added to the medium to remove dissolved oxygen. Cultivation was performed at 40°C for about 3 days. Cells at the late-exponential growth stage were harvested by centrifugation and stored at −20°C until experimental use.

2.2. Physical measurement

PAGE was performed according to Davis [10]. SDS–PAGE was carried out by the method of Schègger and von Jagow [22]. Protein concentration was determined by the modified Lowry method [11] or by the method of Bradford [7] using bovine serum albumin as the standard. The molecular weight of the purified enzyme in a mature state was determined using a HPLC system (Jasco, Tokyo, Japan) equipped with a G3000SW$_{XL}$ pre-packed column (Tosoh, Tokyo, Japan) that was equilibrated with 10 mM Mops buffer (pH 7.0) containing 0.2 M NaCl. Metal concentrations were determined with an inductively coupled plasma (ICP) atomic emission spectrometer SPS 1500 VR (Seiko Instruments, Tokyo, Japan) after the samples had been dialyzed against 10 mM Tris–HCl buffer, pH 8.0, containing 1 mM EDTA for 12 h. Acid-labile sulfur was analyzed as described by Brumby et al. [8]. Spectroscopic measurements in the UV/visible regions were performed using a 220A spectrophotometer (Hitachi, Tokyo, Japan) with a 1 cm lightpath cuvette. A fluorescent pterin derivative was extracted and prepared by the method of Johnson and Rajagopalan [17] and analyzed using an F-3000 fluorescence spectrophotometer (Hitachi). Electron paramagnetic resonance (EPR) spectra were measured with a JEOL RE-1X X-band spectrometer

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(JEOL, Tokyo, Japan) at 77 K. The magnetic field was calibrated with diphenylpicrylhydrazine and MnO. D_2O of 99.8% minimum purity (Nacalai Tesque, Kyoto, Japan) was used for EPR measurement.

2.3. Assay conditions

The nitrate reducing activity was measured according to the method of MacGregor et al. [18] using a couple of dithionite and methyl viologen as the electron donor. After an appropriate reaction time, the nitrite thus generated was assayed by a diazo-coupling method [20]. Activity staining of the enzyme in a polyacrylamide gel was performed as follows: after electrophoresis of the enzyme preparation, the gels were soaked in the staining solution containing 0.1 M Na-phosphate buffer (pH 7.0), 0.2 mM methyl viologen, 2.3 mM dithionite, 1.0 M NaCl and 10 mM $NaNO_3$ or 10 mM $NaClO_3$. The gels were totally stained with the blue color of reduced methyl viologen except for a colorless band based on the nitrate- or chlorate-reducing activities of the enzyme. Electron donating abilities of several substrates to the enzyme were tested under the same assay conditions but using 1.0 mM duroquinol (Sigma, St. Louis, MO, USA), 1.0 mM NADH (Wako Pure Chemicals, Osaka, Japan) or 3.5 μM H. marismortui ferredoxin instead of methyl viologen. Duroquinone was reduced using $NaBH_4$ as the reductant. H. marismortui ferredoxin was purified from the cultured cells according to the method of Werber and Mevarech [29] with some modifications.

2.4. Purification of nitrate reductase

Cultured archaeal cells (30 g wet weight) were suspended in 180 ml of 10 mM Na-phosphate buffer (pH 7.0) containing 2.0 M NaCl. Cells were disrupted by a freeze–thaw procedure. After the suspension was frozen using liquid N_2, it was thawed immediately by immersing it in running water. The freezing and thawing treatment was repeated five times, then a small amount of DNase (Sigma) was added to the resulting solution to remove the viscosity that was due to the released DNA. After being stirred gently for 12 h, the suspension was centrifuged at $10\,000\times g$ for 30 min to precipitate intact cells. The cell-free extract thus obtained was centrifuged at $70\,000\times g$ for 60 min. The precipitant was re-suspended in 10 mM Na-phosphate buffer (pH 7.0) containing 2.0 M NaCl and centrifuged again at $70\,000\times g$ for 60 min. The precipitant thus obtained was used as the membrane fraction for the purification of dissimilatory nitrate reductase, while the supernatant was the starting material for the purification of ferredoxin as described above.

The membrane fraction was suspended in 10 mM Tris–HCl buffer (pH 8.0) containing 2.0 M NaCl at a protein concentration of 5 mg/ml. Then, a stock solution (20% w/v) of Triton X-100 (Sigma) was added to the suspension to reduce it to 1% for extraction of the enzyme. The resulting suspension was gently stirred for 12 h and then centrifuged at $70\,000\times g$ for 60 min. The supernatant obtained was dialyzed against 10 mM Tris–HCl buffer (pH 8.0) for 12 h. The resulting solution was subjected to anion-exchange chromatography on a DEAE-Toyopearl 650M (Tosoh) column (2.0×10 cm) that had been equilibrated with 10 mM Tris–HCl buffer (pH 8.0). After the column had been washed with 200 ml of 10 mM Tris–HCl buffer (pH 8.0) containing 200 mM NaCl, the enzyme that was adsorbed on the column was eluted with a linear gradient generated from 100 ml each of 10 mM Tris–HCl buffer (pH 8.0) containing 200 mM NaCl and the buffer containing 500 mM NaCl. The eluate that showed nitrate reducing activity was collected, then dialyzed against 10 mM Tris–HCl buffer (pH 8.0). The enzyme preparation was concentrated using a small size DEAE-Toyopearl column (0.5×2.0 cm), then subjected to a Sephacryl S-300 (Pharmacia, Uppsala, Sweden) gel-filtration column (2.0×100 cm) equilibrated with 10 mM Tris–HCl buffer (pH 8.0) containing 200 mM NaCl. The eluate that contained the enzymatic activity was collected and used as the purified enzyme preparation. All the purification procedures were performed at 4°C.

3. Results and discussion

Dissimilatory nitrate reductase was purified from H. marismortui that had been cultivated anaerobically in the presence of nitrate. By disrupting the cultivated cells with an ultrasonic oscillating device, nitrate reducing activity was easily released into the soluble fraction. Therefore, the enzyme is considered
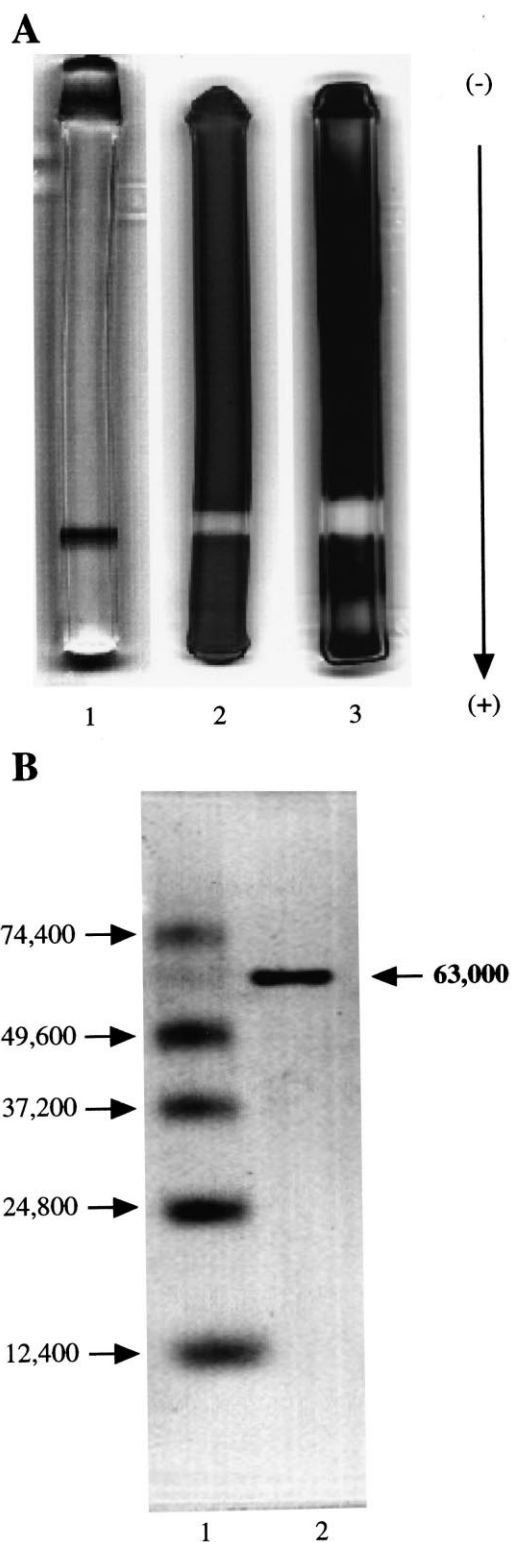

Fig. 1. PAGE analysis of the purified dissimilatory nitrate reductase from H. marismortui in the absence (A) or in the presence (B) of SDS. A: A single Coomassie Brilliant Blue-stained band appeared on the 7.0% polyacrylamide gel at lane 1 after loading 7.8 μg of the purified enzyme. The enzyme was also stained by nitrate-reducing activity staining reagent (lane 2) and by chlorate-reducing activity staining reagent (lane 3). B: Purified enzyme (2.6 μg) was treated with 2% SDS and loaded onto the gel in lane 2. Standard proteins, with their molecular masses shown beside the gel, were in lane 1.
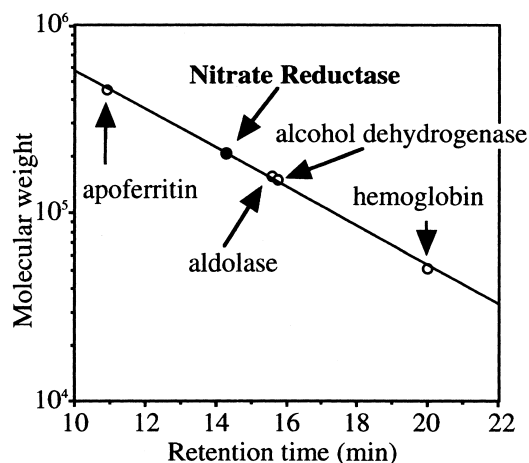
Fig. 2. Estimation of the molecular weight of *H. marismortui* nitrate reductase by size-exclusion HPLC. Experimental conditions are described in Section 2. Hemoglobin (Mw = 67 000), yeast alcohol dehydrogenase (150 000), aldolase (158 000) and apoferritin (450 000) were used as the molecular weight standards.

to be a membrane-extrinsic protein that combines with the surface of the cytoplasmic membrane by hydrophobic interactions. The membrane fraction was prepared from cells that had been disrupted by the freezing–thawing method and then the enzyme was extracted from the resulting membrane fraction by treating with a non-ionic detergent Triton X-100. Because the enzyme was stable even in the absence of NaCl, ion-exchange chromatography could be used to purify the enzyme. The purified enzyme preparation was obtained with 45% final recovery through two chromatographic steps as summarized in Table 1.

Electrophoresis of the purified enzyme preparation revealed one Coomassie Brilliant Blue-positive band on the polyacrylamide gel as shown in Fig. 1A (lane 1). Activity staining bands, due to the nitrate-reducing activity (lane 2) and chlorate-reducing activity (lane 3), were also observed in the same position on the gels. The enzyme was composed of only one kind of polypeptide, which had an estimated molecular weight of 63 000 from SDS–PAGE (Fig. 1B). The molecular weight of the purified enzyme in a mature state, based on the size-exclusion HPLC experiment as shown in Fig. 2, was estimated to be 208 000, suggesting that the purified nitrate reductase was a tetramer of the 63 kDa polypeptide.

In the bacterial denitrifiers, two types of dissimilatory nitrate reductases, membrane-bound nitrate reductase (Nar) and periplasmic enzyme (Nap) are known [4,30]. Nar-enzyme contains three subunits of which the molecular masses are 150, 60 and 20 kDa with a 1:1:1 stoichiometry [30]. The largest, the α-subunit, is the catalytic core of the enzyme, while the β- and γ-subunits mediate an intermolecular electron transport from

quinol to the catalytic center of the enzyme [5]. Another enzyme, Nap, is a heterodimeric complex composed of 90 and 16 kDa subunits [4]. The archaeal dissimilatory nitrate reductase purified in this study is, therefore, differentiated from the bacterial counterparts by the subunit construction.

The purified enzyme catalyzed the two-electron reduction of nitrate to nitrite using a couple of dithionite and methyl viologen as the electron donating system. The purified enzyme did not react with duroquinol or NADH, which are potent electron donors to the Nar-enzyme and the assimilatory nitrate reductase, respectively. Further, as previously reported by Werber and Mevarech, archaeal [2Fe–2S]-type ferredoxin did not react with the enzyme [28]. When the Nar-enzyme was isolated from the membrane particles of *Escherichia coli* by the heat-extraction method, a two-subunit enzyme was obtained by loss of the γ-subunit [18]. The resulting two-subunit enzyme retains the ability to react with methyl viologen, while it lacks the quinol-oxidizing activity [30]. Therefore, the possibility should be considered that the purified enzyme has lost some of the components that mediate the electron transport from the physiological reductant to the catalytic 63 kDa polypeptide during the purification process. Further, it should be noted that the enzyme reduced chlorate, which has a structure homologous to nitrate. It is already known that the Nar-enzyme and another Mo-containing DMSO reductase catalyze the reduction of chlorate, while the Nap-enzyme does not [2,27].

The nitrate-reducing enzymes, including the Nar- and Nap-enzymes from several bacteria, are known to contain a Mo–molybdopterin complex as the catalytic center [30]. The present archaeal enzyme was also shown to contain a Mo–molybdopterin complex by the EPR measurement. The spectrum of the purified enzyme in the air-oxidized state is presented in Fig. 3A. The spectrum was typical of a rhombic Mo(V) having g_1, g_2 and g_3 = 2.004, 1.985 and 1.959, respectively, and each signal component was split into a doublet by the super-hyperfine splitting, A_1, A_2 and A_3 = 1.66, 1.25 and 1.32 mT, respectively. The measurement in D_2O produced an unsplit signal, as shown in Fig. 3B, indicating that an exchangeable proton from bulk water interacts with the ligand of Mo.

The EPR signals of the purified enzyme were reminiscent of the 'low pH' signals of *E. coli* Nar-enzyme [25] and the 'high-*g* split' signal of DMSO reductase [9]. On the other hand, the anisotropy factor ($g_1 - g_3 = 0.045$) in the EPR signals of the purified enzyme was quite large compared with that of the Nap-enzyme from denitrifying *Paracoccus denitrificans* [3]. These EPR-spectroscopic characteristics suggest a structural similarity in the ligand geometry of the Mo atom between the *H. marismortui* nitrate reductase and the Nar-enzyme that reflects the chlorate-reducing activities of both enzymes.

The purified nitrate reductase was greenish brown in color.

Table 1
Purification of dissimilatory nitrate reductase from denitrifying cells of *H. marismortui*

Purification step	Volume (ml)	Total protein[a] (mg)	Specific activity (nmol/s/mg)	Total activity (μmol/s)	Purification (x-fold)	Yield (%)
Membrane fraction	38.0	176	41.1	7.23	1	100
Solubilization	39.0	164	43.8	7.18	1.07	99.3
Anion-exchange chromatography	19.6	16.6	368	6.11	8.95	84.5
Gel-filtration	13.0	2.30	1410	3.24	34.3	44.8

[a]Protein concentrations were quantified using a modified Lowry method [11].
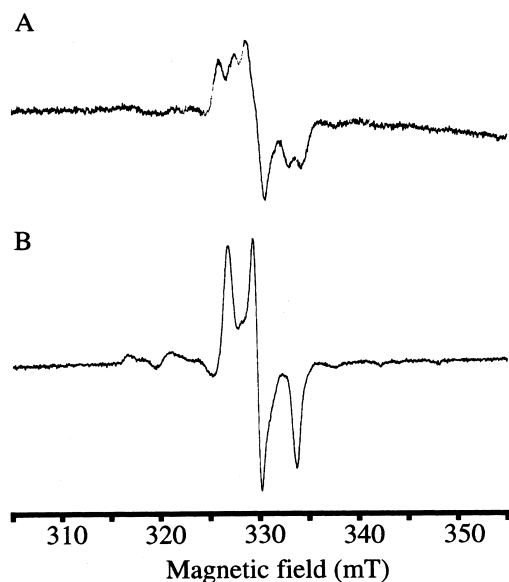
A

B

Fig. 3. EPR signals of *H. marismortui* nitrate reductase in H_2O (A) and in D_2O (B). A: Enzyme (5.2 mg protein/ml) dissolved in 10 mM Tris–HCl buffer (pH 7.8) containing 2 M NaCl. B: Enzyme (6.3 mg protein/ml) dissolved in D_2O (pH 7.8). Conditions of EPR run were: microwave frequency, 9.21 GHz; microwave power, 6.25 mW; modulation amplitude, 1.0 mT; sweep time, 8 min; time constant, 0.1 s.

The air-oxidized enzyme produced a broad peak around 400 nm in the absorption spectrum as shown in Fig. 4. The spectrum of the enzyme in the visible region was diminished by the addition of a reducing reagent. Reduction of the purified enzyme was very slow: it required more than 15 min for sufficient reduction after an addition of dithionite. These spectroscopic properties revealed the presence of iron–sulfur centers, presumably [4Fe–4S]- or [3Fe–4S]-type clusters [24]. Neither cytochrome nor flavin moiety was found in the purified preparation as judged from the absorption spectra.

Mo, Fe and acid-labile sulfur were quantified in the purified enzyme. The concentration of Mo in the enzyme was 0.33 ± 0.03 (mol per mol of the 63 kDa polypeptide) as the mean value of six samples purified individually, and those of

Fe and acid-labile sulfur were 4.49 ± 0.17 and 4.38 ± 0.14 (mol per mol of the 63 kDa polypeptide), respectively, from three individual samples. No tungsten was detected in the enzyme (data not shown). A fluorescent pterin derivative was extracted from the purified enzyme, an emission spectrum of the extracted material showed peaks at 292, 304 and 380 nm and an excitation spectrum showed a peak at 469 nm. The spectra were almost identical with those of the form B pterin derivative that was reported by Johnson and Rajagopalan [17]. Further, multiple [4Fe–4S]-type clusters were identified in the purified enzyme preparation based on the EPR spectroscopic measurement at a liquid He temperature (Yoshimatsu et al., unpublished results).

These results indicate that the Mo–molybdopterin complex and iron–sulfur centers are present as prosthetic cofactors of the enzyme, but the concentrations of the cofactors in the enzyme seem to be very low and are inconsistent with the predicted stoichiometries of each element against the enzyme molecule. Although both modified Lowry and Bradford methods were applied to the protein quantification of the purified preparation, only a small difference was observed between the protein concentration values that were measured by each method. Therefore, it is possible that both methods are unsuitable for the protein quantification of the present enzyme, or that partial dissociation of cofactors might occur during the purification.

Although the purified enzyme was stable even in the absence of NaCl, salt-dependent enhancement of the enzymatic activity was observed, as shown in Fig. 5. The value of the catalytic velocity constant (k_{cat}) was determined to be 71 (mol of NO_2^- per mol of 63 kDa polypeptide per s) in the absence of NaCl, while it was about doubled to 145 s^{-1} in 2.0 M NaCl. In assay mixtures containing NaCl higher than 2 M, catalytic activity of the enzyme was inhibited. Instead, the apparent affinity constant (K_m) for nitrate decreased continuously with an increase of NaCl in the assay solution. The value of K_m was estimated as 79 μM in the assay mixture containing 2.0 M NaCl. The catalytic parameters of the enzyme in 2 M of NaCl are comparable with those of Nar- and Nap-enzymes [30].

Generally, proteins and enzymes from extremely halophilic
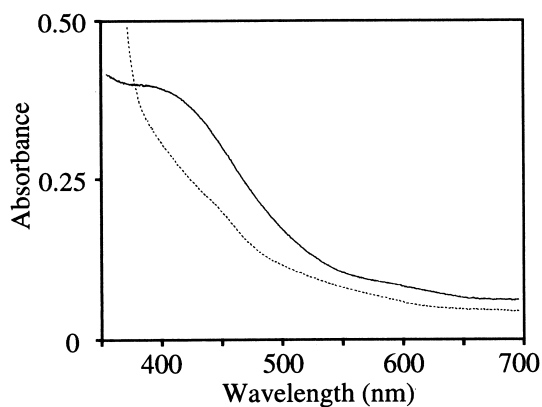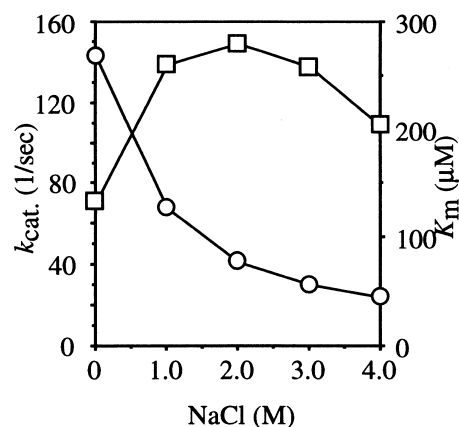

Fig. 4. Absorption spectra of *H. marismortui* nitrate reductase. Purified enzyme (0.89 mg protein/ml) was dissolved in 0.1 M Na-phosphate buffer (pH 7.0) containing 2.0 M NaCl. The spectra of the purified enzyme in the air-oxidized state and in the dithionite-reduced state are shown by solid and dotted lines, respectively.

Fig. 5. Effect of NaCl concentration on the enzymatic parameters of *H. marismortui* nitrate reductase. Values of catalytic velocity constant k_{cat} (□) and apparent affinity constant $K_{m(app)}$ (○) of the enzyme toward nitrate were determined in several NaCl concentrations. Assay solutions were buffered with 0.1 M Na-phosphate buffer to pH 7.0.

archaea are known to require high concentrations of salts for their structural stability and/or enzymatic activities [13,14]. Recent crystallographic analysis of halobacterial enzymes has shown the presence of clusters of negatively charged residues on the surface of the protein molecules [12,21]. The 'halophilic adaptation' phenomenon of the halobacterial enzymes is, therefore, explained by the presence of the negative-charge clusters that lead to instability of the protein molecule from the electrostatic repulsion among the clusters in a condition of low ionic strength. In the present enzyme, the enzymatic activity was found to be enhanced in a hypersaline condition, while a high concentration of salts was not required for the structural stability, as previously observed in a moderate halophilic dihydrofolate reductase [21]. On the other hand, it has been reported that the other archaeal nitrate reductases from *Haloferax* species did not require high salt concentration for either stability or activity [1,6,15].

In this study, we purified the dissimilatory nitrate reductase from a denitrifying halophilic archaeon, *H. marismortui*. The purified enzyme was a homotetramer of a 63 kDa polypeptide. Reports of purification of archaeal dissimilatory nitrate reductases have also been published from three *Haloferax* species; heterodimeric (116 and 60 kDa) or heterotrimeric (100, 60 and 31 kDa) structures have been reported as the subunit constructions of *Haloferax denitrificans* [15] and *Haloferax volcanii* enzymes [6], respectively. Like the presence of Nar- and Nap-enzymes in the denitrifying bacteria, a significant difference between the subunit constructions of the *Haloarcula* enzyme and *Haloferax* enzymes indicates the diversity in the structures of archaeal dissimilatory nitrate reductases.

The purified enzyme was found to contain a Mo–molybdopterin complex and iron–sulfur centers, presumably [4Fe–4S]-type clusters, while their stoichiometries in the enzyme molecule still remain unclear. Although EPR analysis and the enzymatic properties suggested an analogous ligand geometry in the Mo atom between the archaeal nitrate reductase and the bacterial Nar-enzyme, the archaeal enzyme was discriminated from the bacterial Nar-enzyme based on the significant differences in the molecular weight and subunit construction. To elucidate the correlation between the structure and function in the enzyme, further physicochemical and genetic investigations are required in the future.

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