Properties of a Dissimilatory Nitrate Reductase from the Halophilic Archaeon *Haloferax volcanii*

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Grown under anaerobic conditions in presence of nitrate, *Haloferax volcanii* shows nitrate reduction and accumulation of nitrite in the culture medium. We found a membrane-associated nitrate reductase, which could easily be solubilized by gently stirring of isolated membranes. Surprisingly, this nitrate reductase requires no NaCl for its activity. A medium pH of 7.5 and high temperatures up to 80 °C are necessary for optimum activity. Kinetic studies showed that the apparent K_M was 0.36 mmol/l for nitrate and 80 μmol/l for dithionite-reduced methyl viologen. The respiratory chain inhibitor cyanide effects nitrate reduction noncompetitively with respect to nitrate with a K_i of 0.3 mmol/l. Azide was a strong inhibitor: The concentration required for half maximal inhibition was 60 μmol/l, whereas thiocyanate and chlorate were much weaker inhibitors. The isolated enzyme was partially purified by fractionated precipitation using polyethylene glycol. SDS gel electrophoresis resulted in three putative subunits of the nitrate reductase of molecular masses of about 100, 61, and 31 kDa.

Introduction

Nitrate can be used as an electron acceptor by various microorganisms during anaerobic growth. The dissimilatory nitrate reductases seem to be closely related concerning their function and subunit composition, though nothing is known about the amino acid composition of nitrate reductases from halophilic archaea. Most of them are described to be composed of three different subunits named α, β and γ with molecular masses of about 130, 60 and 20 kDa, respectively (Carlson *et al.*, 1982; McGregor *et al.*, 1974; Craske and Ferguson, 1986; Hochstein and Lang, 1991). Nitrate reduction is inhibited by azide and cyanide which affect the reaction in a competitive and noncompetitive manner, respectively, with respect to nitrate (Craske and Ferguson, 1986; Hochstein and Lang, 1991). The halophilic archaea seem to be no exception concerning their nitrate reductases: *Haloferax denitrificans* contains a membrane-bound nitrate reductase which is composed of two subunits with molecular masses of 116 and 60 kDa (Hochstein and Lang, 1991). The surprising finding of Hochstein and Lang was, however, that the enzyme from their extremely halophilic archaeon was stable and maximally active in absence of NaCl. In contrast to the finding of Hochstein and Lang, Alvarez-Ossorio *et al.* (1992) recently reported, that *Haloferax mediterranei* contains a salt-dependent nitrate reductase. As the salt-dependent enzymes of halophilic archaea contain about 20% of acidic amino acids with proposed stabilizing function, it would be interesting to compare the different nitrate reductases on the level of their amino acid sequences. Therefore, we characterized a third nitrate reductase out of the genus *Haloferax: Haloferax volcanii*, a more moderate halophilic organism with salt reqirement of about 2 mol/l for optimum growth, shows nitrate reduction and nitrite accumulation in the medium if grown under lack of oxygen at 37 °C in presence of nitrate. To our surprise, the respective enzyme was not halophilic itself. Like the nitrate reductase from *Haloferax denitrificans*, the enzyme showed optimum activity when no NaCl was added to the medium. In the paper presented here, we describe the kinetic properties as well as the isolation and partial purification of the nitrate reductase from *Haloferax volcanii*, an archaeon which has been

Abbreviations: Hepes, N-2-hydroxyethyl piperazine-N′-ethane sulfonic acid; MV, methyl viologen; MES, morpholinoethane sulfonic acid; NR, nitrate reductase; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; Tris-buffer, tris (hydroxymethyl) aminomethane.

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isolated out of the Death Sea (Mullakhanbhai and Larsen, 1975).

Materials and Methods

Growth conditions

Haloferax volcanii WR 340 was grown in a medium containing a mixture of different salts at a final concentration of 15% (wt./vol.) (SW15), comparable to the salt concentration of the Death Sea. As a carbon source, 0.5% (wt./vol.) yeast extract and peptone were added. The pH value of the culture medium was adjusted to 7.2 using NaOH. The salt composition was: 2.1 mol/l NaCl, 0.25 mol/l $MgCl_2$, 30 mmol/l K_2SO_4 and 1 mmol/l $CaCl_2$. The usual growing temperature was 37 °C. The bacteria were usually cultured in 10 l flasks, three days aerated by a permanent air flow followed by two days of anaerobically conditions, plugging the flasks and reducing shaking to a minimum. In order to avoid foaming, a few drops of silicone oil (AR 1000, Wacker Chemie, München) were added. The cells were harvested after five days, corresponding to the optical density (520 nm) of *ca.* 1.5, according to Rodriguez-Valera *et al.* (1983).

Isolation of membranes

The cells were harvested by centrifugation, passed through a French press at 8000 psi and the membranes were isolated as described earlier (Dane *et al.*, 1992). The collected membranes were resuspended in 1.75 mol/l NaCl, 50 mmol/l Tricine pH 7.5, and 100 mmol/l $MgCl_2$. No dramatic loss in activity could be observed if the membranes were stored at −70 °C, −20 °C or +4 °C. 80% of the initial activity could be found after 7 days and 50% after one month. Storage at room temperature, however, resulted in a residual activity of 40% after 3 days.

Isolation and partial purification of nitrate reductase

To solubilize the membrane bound enzyme, isolated membranes were stirred in the above described buffer containing 1.75 mol/l NaCl at room temperature for 60 min, followed by centrifugation for 45 min at 150,000×*g*. The supernatant contained the NR activity which could be obtained by fractionated precipitation using the following polyethylene glycol (PEG) concentrations: 5, 10, 15, 20, 30% (wt./vol.). Solid polyethylene glycol (M_r 4000) was added on ice in small portions, under constant stirring, up to the indicated final concentrations. After complete solvation, the suspension was stirred for 20 min and the protein was collected by centrifugation for 20 min at 15,000×*g* and 4 °C in a Beckman centrifuge (Model J2-21). The resulting supernatant was treated in the same way using the next concentration of PEG. Every resulting sediment and supernatant was tested for nitrate reductase activity. The highest activity was found in the 15% PEG fraction. This pellet usually was resuspended in 1.75 mol/l NaCl plus 50 mmol/l Tricine pH 7.5, and stored at 4 °C.

Neither solubilization of the membranes with detergents (19 mmol/l octylglucoside plus 8 mmol/l cholic acid) nor application to the French press at 20,000 psi resulted in more effective solubilization of the NR (not shown).

The protein distribution of crude membranes and nitrate reductase fractions was studied in SDS polyacrylamide gels (15%) after silver staining. Molecular masses of polypeptides were estimated from the positions of standard proteins.

Nitrate reductase assay

Nitrate reductase activity was measured by colorimetric determination of nitrite. The assay mixture standardly contained the following reagents in a final volume of 2 ml: 1.75 mol/l NaCl, 0.05 mmol/l Tris-Cl pH 7.2, 10 mmol/l KNO_3, 0.4 mmol/l methyl viologen, 2.3 mmol/l $Na_2S_2O_4$ (freshly made and neutralized with $NaHCO_3$) and about 200 µg of membranes or 20 µg of isolated enzyme. The reaction was started by addition of the dithionite to the test tubes which were inserted into a 60 °C heated water bath. Aliqouts of 450 µl were taken out of the tube after 5, 10 and 15 min and stopped by shaking. Shaking of the mixture shifted the blue color to colorless *via* oxidation of the dithionite. After centrifugation of the samples for 4 min at 14,000×*g*, 400 µl of the supernatant was mixed with 300 µl of sulfanilamide (1% in 3 mol/l HCl) and 300 µl N-(1-naphtyl) ethylenediamine dihydrochloride (0.02%), followed by a 20 min incubation period. The yielded purple azo-

dye could be measured colorimetrically at 520 nm. The stoichiometrical relationship between the amounts of the dye and the nitrite formed was determined *via* standardization. Protein determinations were performed either according to Bensadoun and Weinstein (1976) or to Lowry *et al.* (1951).

Results

Optimum conditions for nitrate reductase activity

Induction of nitrate reductase activity in a culture of *Haloferax volcanii* was achieved by addition of 20 mmol/l $NaNO_3$ to the indicated culture medium and growth of the bacteria under anaerobic conditions. Since the preparation time could be shortened drastically if the culture methods were changed to semianaerobic conditions, after three days of aerobic culture conditions, the fully grown cultures were shifted to anaerobic conditions by stopping the aeration, plugging the flask and minimizing shaking for further two days as described by Carlson *et al.* (1982). Induction of the enzymatic activity of nitrate reductase depended on the addition of nitrate to the culture medium and switching to anaerobic conditions, as shown in Table I. The product of nitrate reduction, nitrite, was accumulated in the culture medium and not reduced to ammonium like in *E. coli* (Craske and Ferguson, 1986).

In Fig. 1 nitrate reduction by *Haloferax volcanii* membranes is plotted against the concentration of NaCl in the incubation medium. Surprisingly, optimum nitrate reductase activitiy could be found in absence of NaCl from the medium, whereas the ATPase from the same organism (Dane *et al.*, 1992) shows an activity optimum at 1.7 mol/l NaCl. *Haloferax volcanii* belongs to the moderate halophilic archaea, growing best in about 2 mol/l sodium chloride.

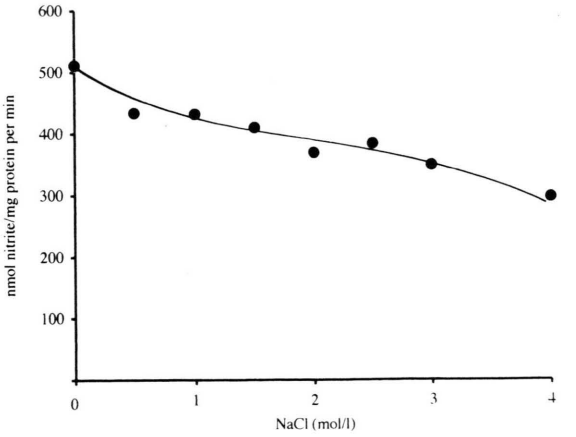

Fig. 1. NaCl concentration dependence of nitrate reduction by *Haloferax volcanii* membranes. 318 µg of protein were incubated at 60 °C in presence of 10 mmol/l KNO_3 and 0.4 mmol/l methyl viologen and the indicated NaCl concentrations. After 5 min of preincubation to reach the final temperature, start of the reaction was initiated by addition of 2.3 mmol/l sodium dithionite. Each rate was calculated from kinetical analysis.

We have found an optimum pH of 7.5 for nitrate reduction by *Haloferax volcanii*. But fairly good activity could be measured in the range of pH 6 to 8 (Fig. 2). The optimum temperature for nitrate reductase activity was 80 °C, measured at 1.75 mol/l NaCl (Fig. 3). A considerably good activity could be found with temperatures between 60 and 85 degrees. Since temperatures higher than 60 degrees tended to cause unspecific reactions, we usually tested nitrate reduction at 60 °C.

Table I. Nitrate reductase activity in different cultures of *Haloferax volcanii*. *Haloferax volcanii* was grown as described in Methods using 12.5% NaCl and 5% $MgCl_2$. The indicated cultures contained 100 mmol/l KNO_3. Measurements of the enzymatic activity were done with crude membranes after 48 h of the aerobic or anaerobic growth. The rates are calculated from kinetics of 5, 10 and 15 min. Average values of two typical experiments are shown.

Growth conditions	Activity [nmol/mg protein per min]	[%]
Anaerobiosis, with nitrate	109	100
Anaerobiosis, without nitrate	0.87	0.8
Aerobiosis, with nitrate	51	47

Nitrate reduction in presence of different cofactors, stimulating agents and inhibitors

As already shown for *Haloferax mediterranei* (Alvarez-Ossorio *et al.*, 1992), methyl viologen (MV), kept in its reduced state by dithionite, is absolutely necessary to reduce nitrate to nitrite. 0.4 mmol/l MV and 1.2 mmol/l sodium dithionite gave the best results under the here described experimental conditions (Table II). The apparent K_M for reduced methyl viologen was about 80 µmol/l

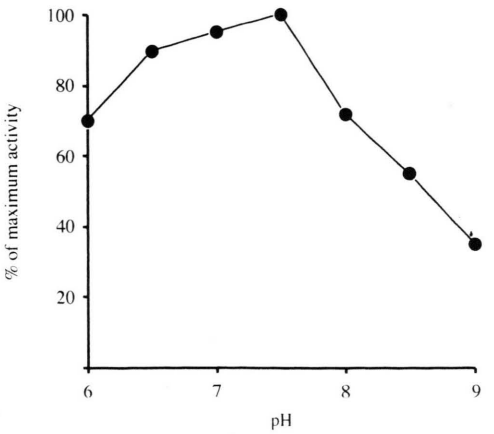

Fig. 2. Nitrate reduction by *Haloferax volcanii* membranes, dependent of the medium pH value. Nitrate reduction was performed with 300 µg of protein, other conditions as given in Fig. 1. The following buffers were used: pH 6–6.5, MES, pH 6.5–7.5 HEPES, pH 7.5–9 Tris. The rates of overlapping pH values were averaged. Control rate: 1.32 µmol nitrite·mg protein^{-1}·min^{-1}.

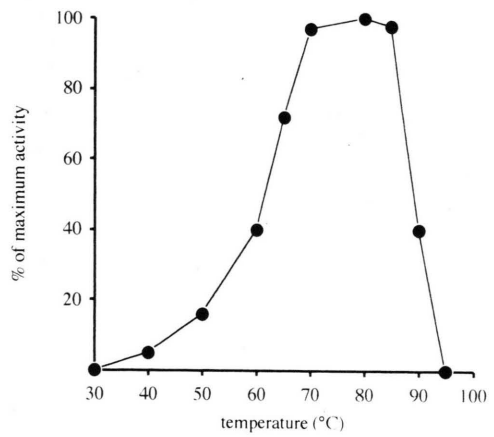

Fig. 3. Nitrate reduction by *Haloferax volcanii* membranes, as affected by different temperatures. The nitrate reductase activity of 300 µg of crude membranes was tested in the above described medium and in a waterbath which was adjusted to the indicated temperatures. Each rate was calculated by 3 point kinetical analysis. Maximum activity: 1.67 µmol nitrite·mg protein^{-1}·min^{-1}.

and that for dithionite about 50 µmol/l. Thus, saturating concentrations have been used standardly. The alternative substrate of *Haloferax denitrificans* nitrate reductase (Hochstein and Lang, 1991), chlorate, had only a minor effect on the reduction of nitrate by *Haloferax volcanii*, with respect to the employed concentration range from 0 to 10 mmol/l. In Table II, line 5 is shown that 10 mmol/l potassium chlorate resulted in about 20% inhibition.

Table II. Nitrate-reduction in presence of different cofactors, stimulating agents and inhibitors. Conditions: About 300 µg of membranes were added to a final volume of 2 ml. The reaction medium of the control contained 1.75 mol/l NaCl, 50 mmol/l Tris-buffer pH 7.5, 10 mmol/l KNO_3 and the indicated electron donors. The incubation temperature was 60 °C; each rate is calculated from three-point kinetics (5, 10, 15 min). All indicated compounds are tested in the following concentration ranges (generally 6 steps): MV from 0 to 1.2 mmol/l, S_2O_4 from 0 to 5 mmol/l, $KClO_3$ from 0 to 10 mmol/l, KCN from 0 to 1 mmol/l, thiocyanate from 0 to 20 mmol/l.

Compounds	Rate of nitrate-reduction (µmol nitrite/mg protein per min)	% of activity
Control (0.4 mmol/l MV, 1.2 mmol/l S_2O_4)	1.76	100
0.1 mmol/l MV, 1.2 mmol/l S_2O_4	0.79	44.9
0 mmol/l MV, 1.2 mmol/l S_2O_4	0	0
0.4 mmol/l MV, 0 mmol/l S_2O_4	0	0
1.2 mmol/l S_2O_4, 0.4 mmol/l MV, 10 mmol/l $KClO_3$	1.4	79.5
1.2 mmol/l S_2O_4, 0.4 mmol/l MV, 0.2 mmol/l KCN	0.8	45.5
1.2 mmol/l S_2O_4, 0.4 mmol/l MV, 1 mmol/l KCN	0	0
1.2 mmol/l S_2O_4, 0.4 mmol/l MV, 5 mmol/l thiocyanate	1.2	68.2
1.2 mmol/l S_2O_4, 0.4 mmol/l MV, 10 mmol/l thiocyanate	0.35	19.9
1.2 mmol/l S_2O_4, 0.4 mmol/l MV, 20 mmol/l thiocyanate	0	0

Cyanide, a potent inhibitor of the reduction reaction (Fig. 4), acts noncompetitively with respect to nitrate. The apparent inhibition constant is 300 µmol/l. Furthermore, NR activity was inhibited by relatively high concentrations of thiocyanate, using preincubation times of 30 min (Table II): under these conditions the C_{150} value was estimated to be 7 mmol/l. Azide, which is described to be a very effective competitive inhibitor

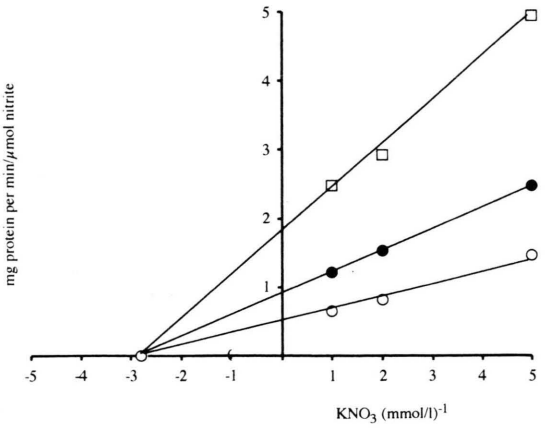

Fig. 4. Lineweaver-Burk plot calculated for the substrate dependence of nitrate reduction in presence of 0.1 mmol/l KCN (●), 0.3 mmol/l KCN (□) and without KCN (control) (○). The K_M value for nitrate under all three conditions is 0.357 mmol/l, V_{max} (control) is 2 µmol nitrate·mg protein^{-1}·min^{-1}. The samples contained 200 µg of membranes, KCN was 30 min. preincubated with the membranes. Each point in the diagram was calculated by 3 point kinetical analysis. All other conditions as described in Fig. 1.

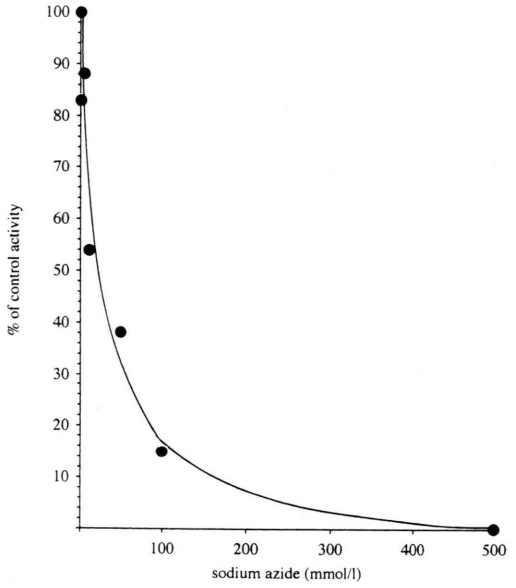

Fig. 5. Influence of sodium azide on the nitrate reduction by *Haloferax volcanii* membranes. Prior to addition of sodium dithionite, the samples were incubated for 5 min in incubation medium containing the indicated concentrations of azide and membranes corresponding to 200 µg of protein. Control activity: 1.1 µmol nitrate·mg protein^{-1}·min^{-1}.

for *Haloferax denitrificans* nitrate reductase, was effective in inhibition of the *Haloferax volcanii* enzyme, too: We measured 50% inhibition at 60 µmol/l NaN_3 (Fig. 5).

Kinetic constants of nitrate reductase

The kinetic parameters of halophilic nitrate reductase have been determined using saturating concentrations of methyl viologen and dithionite and using the usual NaCl concentration of 1.75 mol/l. Under these conditions a K_M (nitrate) of 0.36 mmol/l and V_{max} of 1.8 µmol/mg protein per min have been determined (Fig. 4). These values are in the same order of magnitude as the already determined constants for different dissimilatory nitrate reductases. Using the indicated standard conditions and 10 mmol/l KNO_3, kinetics were hyperbolic with a linear phase up to 20 minutes ($t_{1/2}$ = 8 min). Every rate given in the paper in hand has been calculated by three point kinetics at 5, 10, and 15 min.

Cellular location and subunit composition of Haloferax volcanii nitrate reductase

Nitrate reductase activity could be found associated with the membrane fraction of the archaea. From our results, we conclude that the protein might be located on the surface of the cytoplasma membranes in the periplasmic space. The reasons for this conclusion are: i. stirring of the isolated membranes in buffer containing 1.75 mol/l NaCl resulted in migration of most of the activity to the soluble phase. ii. Neither stirring of the membrane vesicles in destillated water (lysis) nor membrane solubilization by detergents (octylglucoside plus cholic acid, alternatively triton x-100 in concentrations up to 6%) was able to induce better solubilization. iii. Treatment of the membranes with pressures of 20,000 psi (French press) was not more effective than stirring. Thus, at least the part of the enzyme which contains the active nitrate reducing site may be excluded to occur in a tightly membrane-bound form.

Studying the protein contents of the fractions shown in Table III by SDS gel electrophoresis (not shown), we found that the active fractions each contained one accumulated band of 61 kDa, one of 31 kDa, which seemed to be the most accumulated peptide in the gel and additionally a 12 kDa

Table III. Isolation and partial purification of *Haloferax volcanii* nitrate reductase. Activity test conditions: 1.75 mol/l NaCl, 50 mmol/l Tris pH 7.5, 10 mmol/l KNO_3, 0.4 mmol/l methyl viologen, 2.3 mmol/l sodium dithionite, 35 µg of protein. The temperature was 60 °C. The purification factor was calculated by the increasing specific activity. All other conditions are described in Methods.

Fractions	Total yield of protein [mg]	Specific activity [µmol nitrite/ mg protein per min]	Total activity [µmol nitrite/min]	Yield [%]	Purification factor
Membranes	1320	0.17	224.4	100	1
Supernatant	942	0.28	263.8	118	1.7
Pellet	285	0.04	11.4	5	0.2
Precipitate,					
5% PEG	429	0.06	27.1	12	0.4
10% PEG	253	0.16	40.8	18	0.9
15% PEG	110	1.57	172.9	77	9.2
20% PEG	55	0.54	29.8	13	3.2
30% PEG	33	0.11	3.5	2	0.7

band; whereas no accumulation could be found in height of the reported subunit α (about 120 kDa). The supernatant after the stirring and centrifugation process contained additionally to the described bands one of about 100 kDa. Since the samples have been stored at 4 °C before electrophoresis was carried out, it cannot be excluded that the α subunit underwent degradation to smaller peptides.

Discussion

Halophilic enzymes usually react very sensible on the lack of NaCl. *Haloferax mediterranei* ATPase, *e.g.* is instable and disaggregates to single subunits if concentrations of less than 1 mol/l NaCl were employed (Dane *et al.*, 1992). Since we were able to isolate the nitrate reductase activity by stirring the membranes in pure water and the enzyme showed stability and activity in buffer without NaCl (Fig. 1), we have to conclude that the *Haloferax volcanii* enzyme is not halophilic. On the other hand, our isolation protocols showed that the enzyme most possibly is located on the surface of the cytoplasma membrane.

The amino acid composition of halophilic enzymes consist of about 20% of glutamic acid and aspartic acid. Recently, we were able to deduce the amino acid sequences of the *Haloferax volcanii* ATPase A-and B-subunits from their genes (Steinert *et al.*, 1995). The A-subunit contains 20% and the B-subunit 21% acidic amino acids. The resulting negative charges are proposed to form clusters which are stabilized by positively charged counter ions (Böhm and Jaenicke, 1994). The cells of halophilic archaea are known to accumulate high concentrations of K^+-ions, which might serve as such counter ions. This mechanism is proposed to be responsible for the stability of proteins under high salt conditions and on the other hand it causes the instability of the same enzymes in low salt media.

The nitrate reductase of *Haloferax mediterranei,* an extremely halophilic bacterium living in about 4 mol/l NaCl in saltern ponds, is reported to be a halophilic enzyme (Alvarez-Ossorio *et al.*, 1992), whereas that one of *Haloferax denitrificans* shows no salt dependency (Hochstein and Lang, 1991). Enzymes without salt dependency may not own the described high amount of negative charges within their amino acid chains. The respective enzymes must have different mechanisms to protect their active conformation against the high ion concentration in the sourrounding medium. Hochstein and Lang (1991) assume that their halobacterial nitrate reductase may have its origin in nonhalophilic bacteria, since nitrate respiration most probably has been developed in the evolution later than oxygen respiration. This question, however, cannot become solved without knowing more data, especially of the amino acid sequences of different nitrate reductases. We are forcing our studies in this direction.

Since the periplasm of different bacteria contains negatively charged oligosaccharides like β-D-glucans (for review see Ferguson (1991)) and lots of different proteins, one of the roles of this com-

partment may be protection of the cell against differences in the osmolarity of the sourrounding medium. Thus, the electron transport proteins in the periplasm of halophilic organisms may be well protected against loss of water by a high amount of osmotically active solutes in this area. Different mechanisms of stabilization and protection against dehydration under high salt conditions may not be necessary. So far, however, nothing is known about the contents of the periplasm of halophilic archaea.

Like other nonhalophilic and halophilic enzymes, the NR of *Haloferax volcanii* is inhibited by cyanide and azide. The latter effect may be interpreted to be due to the binding of the negatively charged azide ion to positive charged metal ions within the nitrate reductase. Azide is described to inhibit the NR of *Haloferax denitrificans* competitively with respect to nitrate, thus it may well be that it acts directly in the molybdenum containing site of the NR, most probably by metal chelation (van't Ried *et al.*, 1975) The here described C_{I50} value of 60 µmol/l corresponds well with that of *Pseudomonas aeruginosa* (Carlson *et al.*, 1982) in the order of magnitude.

Cyanide, which blocks electron transfer between cytochrome oxidase and O_2 in oxygen respiration, acts as a noncompetitive inhibitor of nitrate reduction. In general, halophilic archaea are proposed to own a normal respiratory chain (Cheah, 1970). The chain includes flavoproteins, quinone and different cytochromes (for references see Skulachev (1993)) and there is evidence for a specific *b*-type cytochrome that is required as electron donor for the nitrate reductase *in vivo* (Ferguson, 1982; Taniguchi and Itagaki, 1960). The specificity of electron transfer pathways to the periplasm is not yet understood.

Like the halophilic nitrate reductase from *Haloferax mediterranei*, the *volcanii* enzyme shows a remarkable grade of thermophilicity. The enzyme works well up to 85 °C. It is a well known fact, that halophilic organisms have developed strategies to live in very warm salt ponds, *e.g.* in the Death Sea or in saltern ponds at the coast of southern Spain. One of these strategies is the enlarged stability of their enzymes under the influence of high temperatures. The NR of *Haloferax mediterranei* shows a very similar temperature dependence (Alvarez-Ossorio *et al.*, 1992), though this is described to be salt-dependent, in contrast to the *Haloferax volcanii* enzyme.

A vide variety of (apparent and/or deduced from nucleotide sequences) molecular weights of the holoenzyme have been found for different organisms. The values range from 170,000 to 800,000 Da (McGregor *et al.*, 1974; Craske and Ferguson, 1986; Hochstein and Lang, 1991; Alvarez-Ossorio *et al.*, 1992). The enzyme with the highest molecular mass (800,000 kDa) has been isolated from *E. coli* (McGregor *et al.*, 1974). It contains about 4 mol of molybdenum per mol of enzyme and thus is concluded to be a tetramer of 4 large and 4 small subunits. The *Haloferax* enzymes are reported to be smaller, 170,000 Da for *Haloferax mediterranei* (Alvarez-Ossorio *et al.*, 1992) and 380,000 Da for *Haloferax denitrificans*, which behaved as a dimer during gel filtration (Hochstein and Lang, 1991). The *Haloferax volcanii* enzyme appears to be as small as the *mediterranei* enzyme (about 170,000 Da). We have no doubt, that these enzymes are very closely related, despite of the difference in their salt dependency.

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