Purification and Characterization of Nitrate Reductase from the Halophile Archaebacterium Haloferax mediterranei

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Halophilic Nitrate Reductase, Enzyme Purification, Enzyme Characterization, Haloferax mediterranei

Nitrate reductase is induced in cells of Haloferax mediterranei by the presence of nitrate upon anaerobic conditions. This enzyme was purified more than 35-fold with a yield of 49%. Densitograms of polyacrylamide gel electrophoresis show the preparation to be 85% purity. The best enzyme preparation has a specific activity of 13.6 U/mg protein. It is the first halophilic nitrate reductase that has been purified near to homogeneity. The purification consists of five steps: an ammonium sulphate precipitation and four successive gel chromatographies with Sepharose CL-4B, calcium phosphate, DEAE-Sephacel and Sephacryl S-200. An average Mr of 170,000 was estimated by gel chromatography and non-denaturing gel electrophoresis. Effectiveness of electron donors, cofactors and inhibitors are reported. At low salt concentration the halophilic nitrate reductase was inactivated following first-order kinetics. The $K_m$ for nitrate depends on salt concentration and shows values in the range from 2.5 to 6.7 mM.

Introduction

Haloferax mediterranei is an extreme halophile archaebacterium able to grow on different organic compounds as source of carbon and consuming oxygen \cite{1}. Halophile bacteria are strict aerobes and therefore the low solubility of oxygen in concentrated salt solutions becomes a limiting factor at a rather low cell density \cite{2}. One of the alternative electron acceptors to oxygen is nitrate \cite{3}. It has been reported that under anaerobic conditions and in the presence of nitrate, nitrate reductase is induced in a culture of Halobacterium \cite{4}. It is also seen that formation of the enzyme was not suppressed by the presence of ammonia during growth, thus indicating that it belongs to the dissimilatory class \cite{5}.

Dissimilatory nitrate reductase from Halobacterium has been studied very little and only few characteristics, such as electron donors and heat stability at different salt concentrations, are known \cite{4, 5}. On the contrary, dissimilatory nitrate reductase from non-halophile bacteria are well known. Thus, the enzyme from Escherichia coli is a particulate protein linked to the cell membrane that presents a molybdo-iron protein structure with three subunits \cite{6-8}. Nitrate reductase from Paracoccus denitrificans have also isolated showing similar characteristics \cite{9}.

Taking into account the particular characteristics observed in the halophilic enzymes, such as malate dehydrogenase \cite{10, 11}, glutamate dehydrogenase \cite{12} or aspartate aminotransferase \cite{13}, the halophilic nitrate reductase should also present important differences with respect to the non-halophilic nitrate reductase, those doing very interesting the study of that enzyme.

In the present paper, we describe a method for the purification almost until homogeneity for the nitrate reductase from Haloferax mediterranei and some molecular and kinetic properties are shown.

Materials and Methods

\textbf{Growth conditions}

Haloferax mediterranei (strain R-4, ATCC 33500) isolated from seawater-evaporation ponds was grown aerobically in liquid shake culture as...
previously described by Rodríguez-Varela et al. [14]. After two days of growing at 37 °C, nitrate reductase was induced by anaerobiosis and the addition of KNO₃ 100 mM. The culture was maintained in shaking upon illumination with white light (15–20 W/m²).

**Enzyme purification**

Unless otherwise stated, the purification process was carried out at 15 °C. The halophilic nitrate reductase shows a high stability to this temperature.

**Step 1: Crude extract.** The freshly harvested bacteria were resuspended in 100 mM Tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.8, containing 3.5 mM NaCl. The cells were broken by ultrasonic desintegration followed of French press treatment as we reported previously [15]. The ultrasonic desintegration was carried out at 90 W for 4 min at 4 °C. The suspension was centrifuged at 45,000 × g for 60 min. The supernatant was recollected and the pellet with cell debris was treated in the French press at 19,200 psi and centrifuged as above. The supernatant together with the first supernatant was recollected and used in the following step.

**Step 2: Precipitation with ammonium sulphate.** The supernatant from step 1 was dialyzed against 20 volume of 1.73 mM (NH₄)₂SO₄ and the pH adjusted at 6.8. Then the solution was centrifuged at 45,000 × g for 60 min.

**Step 3: Chromatography in Sepharose CL-4 B.** The supernatant from step 2 was filtered through a Sepharose CL-4 B column (4 × 70 cm) equilibrated with 100 mM Tris-HCl buffer, pH 6.8, containing 1.73 mM (NH₄)₂SO₄. Elution was carried out with a decreasing linear gradient of 1.73–0.9 mM (NH₄)₂SO₄ in the same buffer at a flow rate of 50 ml/h. Fractions containing activity were pooled and concentrated by ultrafiltration and then dialyzed against 100 mM Tris-HCl buffer solution, pH 7.5, containing 4 mM NaCl.

**Step 4: Calcium phosphate chromatography.** The dialyzed solution from step 3 was filtered through a calcium phosphate column (4 × 40 cm) equilibrated with the above buffer solution. The enzyme was eluted with 100 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM sodium phosphate at a flow rate of 20 ml/h. Fractions containing activity were pooled and dialyzed overnight at 4 °C against Tris-HCl buffer 100 mM, pH 6.8, containing 2.4 mM (NH₄)₂SO₄.

**Step 5: DEAE-Sephacel chromatography.** The solution from step 4 was loaded on to a column (2.4 x 50 cm) of DEAE-Sephacel previously activated. The enzyme was eluted by the same Tris-HCl buffer at a flow of 20 ml/h with a decreasing linear gradient of 2.4–0 mM (NH₄)₂SO₄ (pH 6.8) and an increasing linear gradient of 0–4 mM NaCl (pH 7.5). Fractions containing activity were pooled and dialyzed against Tris-HCl buffer containing 4 mM NaCl.

**Step 6: Sephacryl S-200 chromatography.** The dialyzed solution from step 5 was applied to a Sephacryl S-200 filtration column (2.6 x 100 cm) equilibrated with Tris-HCl buffer, pH 7.5, and the enzyme eluted with the same buffer at a flow of 20ml/h. Fractions with activity were pooled and concentrated by ultrafiltration.

**Enzyme activity**

Nitrate reductase activity was measured by colorimetric determination of nitrite formed by the reduction of nitrate. The assay mixture contains in 1 ml: 0.1 mM Tris-HCl, pH 7.5, 3.5 mM NaCl, 10 mM KNO₃, 0.4 mM methyl viologen, 0.8 mg Na₂S₂O₄ in 0.1 ml NaHCO₃, 0.1 mM, and the enzyme. The assay was developed for 10 min at 60 °C and was initiated by the addition of dithionite and finished by the oxidation of this compound by shaking. 1 unit (U) of enzymatic activity corresponds to the reduction of 1 μmol KNO₃/min under standard assay conditions.

**Determination of molecular weight**

The molecular mass of the enzyme was calculated by gel filtration using Sephadex G-200. The following proteins were used as standards: apoferritin (443 kDa), catalase (240 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa) and peroxidase (49 kDa).

The molecular mass was also determined by electrophoresis under non-denaturing conditions. Relative mobilities of protein bands were calculated with respect to the following markers: bovine serum albumin (67 kDa), glucose-6-phosphate dehydrogenase (102 kDa), lactate dehydrogenase (140 kDa) and catalase (240 kDa).
Determination of Stokes radius

The Stokes radius (r_{s}) was determined using a Sephacryl S-200 column (2.6 × 90 cm) equilibrated with Tris-HCl 100 mM, pH 7.5, containing either 4 M or 0.6 M NaCl.

Protein determination

Protein was determined by the procedure of Schacterle and Pollack [16] in crude extract and eluents of column, and by the method of Lowry et al. [17] in other samples. Lysozyme or BSA were used as standard.

Chemicals and chromatography materials

(Ethylendinitrilito)tetraacetic acid (EDTA), NADH, NADPH, phenazine methosulphate (PMS), catalase, lysozyme, bovine serum albumin (BSA), bromophenol blue, amelite 1R-120, coomassie blue, p-hydroxymercubrizenoate (pHMB), tris(hydroxymethyl)aminomethane (Tris), methyl viologen (MV), benzyl viologen (BV), dithioerythritol (DTE), apoferritin, FAD and FMN were obtained from SIGMA (St. Louis, U.S.A.). Glycerine, sodium dodecyl sulfate (SDS), 2,6-dichlorophenol indofenol (DPIP), from Merck (Darmstadt, Germany). Aldolase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase and peroxidase from Boehringer (Mannheim, Germany). Sepharose CL-4 B, Sephacryl S-200 superfine, DEAE-Sephacel and blue dextran were purchased from Pharmacia (Uppsala, Sweden). Acrylamide, bis-acrylamide, tetramethylethlenediamine and ammonium persulphate from Bio-Rad (Richmond, U.S.A.). Yeast extract from Difco (Detroit, U.S.A.). All other reagents used were analytical grade.

Results

Induction of nitrate reductase

Experimental conditions for induction of nitrate reductase in a culture of *Haloferax mediterranei* are shown in Table I. The induction depends upon the presence of nitrate when the bacteria is grown in anaerobiosis. The addition of nitrate to a culture grown in aerobiosis induces less than 30% of the activity, thus indicating a requirement of anaerobic conditions for the appearance of new enzyme.

Purification of halophilic nitrate reductase

The enzyme from *Haloferax mediterranei* was purified to almost homogeneity. From electrophoresis, it is concluded that 85% of protein in the preparation corresponds to nitrate reductase.

The purification scheme is summarized in Table II. The purification involved (NH₄)₂SO₄ pre-

Table I. Nitrate reductase activity in a culture of *Haloferax mediterranei* grown under different conditions.

<table>
<thead>
<tr>
<th>Conditions of growth</th>
<th>Activity [mU/mg protein]</th>
<th>[%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobiosis with nitrate</td>
<td>348.4</td>
<td>100</td>
</tr>
<tr>
<td>Anaerobiosis without nitrate</td>
<td>8.0</td>
<td>2</td>
</tr>
<tr>
<td>Aerobiosis with nitrate</td>
<td>114.1</td>
<td>32</td>
</tr>
<tr>
<td>Aerobiosis without nitrate</td>
<td>66.7</td>
<td>19</td>
</tr>
</tbody>
</table>

*Haloferax mediterranei* was grown as described in Materials and Methods with 25% of salts and yeast extract 0.25% (w/v) under the indicated conditions. When indicated 100 mM KNO₃ was added to the culture. The enzymatic activity was measured in crude extract at the end of the exponential phase (about 120 h of growth).

Table II. Summary of purification of nitrate reductase from *Haloferax mediterranei*.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>864</td>
<td>330</td>
<td>0.38</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Supernatant of 1.7 m(NH₄)₂SO₄</td>
<td>584</td>
<td>327</td>
<td>0.56</td>
<td>99</td>
<td>1.5</td>
</tr>
<tr>
<td>Sepharose CL-4 B</td>
<td>204</td>
<td>299</td>
<td>1.52</td>
<td>90</td>
<td>4</td>
</tr>
<tr>
<td>Ca₃(PO₄)₂ Gel</td>
<td>99</td>
<td>238</td>
<td>2.62</td>
<td>72</td>
<td>6.9</td>
</tr>
<tr>
<td>DEAE-Sephacel</td>
<td>24</td>
<td>235</td>
<td>9.60</td>
<td>71</td>
<td>25.3</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
<td>12</td>
<td>163</td>
<td>13.60</td>
<td>49</td>
<td>35.8</td>
</tr>
</tbody>
</table>

For experimental details see the text.
Precipitation, fractionation on Sepharose CL-4 B and DEAE-Sephacel with decreasing gradients of \((\text{NH}_4)_2\text{SO}_4\), and gel-permeation chromatography on calcium phosphate and Sephacryl S-200. The enzyme was purified more than 35-fold with a 49% final yield.

Electron donors, cofactors and inhibitors

Table III shows the effectiveness as electron donors of some reduced cofactors for the enzymatic reduction of nitrate to nitrite. Nitrate reductase from *Haloferax mediterranei* can neither use the coenzymes NADH nor NADPH. Methyl and benzyl viologen kept in their reduced state by dithionite were the most effective electron donors, whereas reduced flavin nucleotides were almost ineffective. In the same way, the reduced electron intermediates DPIP or PMS were not able to reduce nitrate to nitrite.

Table III. Electron donors, cofactors and inhibitors for nitrate reductase from *Haloferax mediterranei*.

<table>
<thead>
<tr>
<th>Systeme</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{SO}_4^{2-} + \text{MV})</td>
<td>100</td>
</tr>
<tr>
<td>(\text{SO}_4^{2-} + \text{MV} + \text{pHMB})</td>
<td>5</td>
</tr>
<tr>
<td>(\text{SO}_4^{2-} + \text{MV} + \text{DTE})</td>
<td>5</td>
</tr>
<tr>
<td>(\text{SO}_4^{2-} + \text{MV} + \text{KCN})</td>
<td>16</td>
</tr>
<tr>
<td>(\text{SO}_4^{2-} + \text{MV} + \text{KCNO})</td>
<td>48</td>
</tr>
<tr>
<td>(\text{SO}_4^{2-} + \text{MV} + \text{KClO}_3)</td>
<td>56</td>
</tr>
<tr>
<td>(\text{SO}_4^{2-} + \text{MV} + \text{EDTA})</td>
<td>75</td>
</tr>
<tr>
<td>(\text{SO}_4^{2-} + \text{BV})</td>
<td>76</td>
</tr>
<tr>
<td>(\text{SO}_4^{2-} + \text{FMN or FAD})</td>
<td>5</td>
</tr>
<tr>
<td>(\text{SO}_4^{2-} + \text{DPPIP or PMS})</td>
<td>2</td>
</tr>
</tbody>
</table>

The activity was measured as described under Materials and Methods. The concentrations of different compounds were: dithionite 5 mM, EDTA 10 mM, flavin nucleotides, viologen dyes and redox mediators 0.2 mM, all other compounds 1 mM. The 100% of activity corresponds to 1.2 U/mg protein.

Table III also shows the effect of several classical inhibitors of nitrate reductase on the halophilic enzyme. It can be seen that \(\text{p-hydroxymercuribenzoate (pHMB)}\), dithioerythritol (DTE) or azide at the concentration of 1 mM inhibited almost completely nitrate reductase. Cyanide acted also as a potent inhibitor (85% inhibition at 1 mM). Other compounds, such as cyanate, potassium chlorate or EDTA, are not so strong inhibitor, diminishing partially the activity.

Molecular weight

The molecular weight of the halophilic nitrate reductase was determined by gel filtration (170,000 ± 2000) and by non-denaturing gel electrophoresis (172,000 ± 3000).

Stokes radius

The Stokes radius was determined by gel filtration in Sephacryl S-200 equilibrated with Tris-HCl 100 mM, pH 7.5. A value of 4.92 nm for \(r_s\) was obtained when the elution was carried out in the presence of 4 M NaCl, meanwhile 4.96 nm was the \(r_s\) with a lower (0.6 M NaCl) ionic strength.

Effect of pH and temperature

pH-Dependence of the enzymatic activity was examined in 0.5 M glycyl-glycine or Na-acetate buffers containing 3.5 M NaCl from pH 5.0 to pH 10.3. Maximum activity was observed at pH 7.5–7.7 (results not shown).

The enzyme exhibits a optimum temperature with a strong dependence on the concentration of NaCl present in the assay (Fig. 1). The maximum activity was found to be at 59, 68, 80 and 89 °C for 0.6, 1.2, 1.8 and 3.2 M of NaCl respectively. Arrhenius plots for the different concentrations of NaCl are shown in Fig. 1 (inset). Table IV sum-
Table IV. Arrhenius behavior and thermodynamic activation parameters for nitrate reductase from *Haloferax mediterranei* in the presence of several concentrations of NaCl.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0.6</th>
<th>1.2</th>
<th>1.8</th>
<th>3.2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arrhenius slope (K^−1)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(between 30 and 40 °C)</td>
<td>-2888</td>
<td>-3000</td>
<td>-8600</td>
<td>-10800</td>
</tr>
<tr>
<td><strong>Arrhenius slope (K^−1)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(between 50 and 60 °C)</td>
<td>-2111</td>
<td>-1666</td>
<td>-1620</td>
<td>-1111</td>
</tr>
<tr>
<td><strong>Ea (kJ/mol)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(between 30 and 40 °C)</td>
<td>23.9</td>
<td>24.9</td>
<td>71.4</td>
<td>89.7</td>
</tr>
<tr>
<td><strong>Ea (kJ/mol)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(between 50 and 60 °C)</td>
<td>17.5</td>
<td>13.8</td>
<td>13.4</td>
<td>9.2</td>
</tr>
</tbody>
</table>

These values were obtained from Fig. 1.

Table V. Effect of NaCl concentration on the $K_m$ for nitrate and $V_m$ of nitrate reductase from *Haloferax mediterranei*.

<table>
<thead>
<tr>
<th>NaCl [m]</th>
<th>$K_m$ app [mm]</th>
<th>$V_m$ app [mU/mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>2.5 ± 0.3</td>
<td>142 ± 3</td>
</tr>
<tr>
<td>2.0</td>
<td>5.0 ± 0.2</td>
<td>128 ± 4</td>
</tr>
<tr>
<td>3.4</td>
<td>6.7 ± 0.3</td>
<td>109 ± 3</td>
</tr>
</tbody>
</table>

The activity was measured as described in Materials and Methods using 5 mm dithionite and 0.4 mm MV as electron donor.

**Discussion**

The induction pattern of nitrate reductase in *Haloferax mediterranei* has been established. The results agree with those reported previously for *Halobacterium* [5]. The enzymatic activity is induced when the cells are grown in anaerobiosis in the presence of nitrate (Table I). The induction of the enzyme by nitrate, even when the cells are cultivated in the presence of amino acids as source of nitrogen, is a proof that the enzyme belongs to a dissimilatory way.

After the purification procedure, summarized in Table II, a preparation of nitrate reductase with a
grade of purity of 85% is obtained. Yield, purification factor and specific activity were much higher than those from the method of Marquez and Brodie [4] for Halobacterium and similar to those obtained in the purification procedures of the enzyme from non-halophile bacteria [6, 9].

In halophile bacteria under anaerobic conditions and using nitrate as oxidant, the nitrate reductase must be the last intermediate in the electron transfer chain. Since the only reductants which can effectively donate electrons in vitro to this dissimilatory enzyme are the artificial reduced dyes methyl and benzyl viologen (Table III), the question remains open as to the nature of the physiological electron donor for this enzyme. In this connection, experiments carried out using membrane particles from Haloferax containing nitrate reductase indicates that electrons from respiratory chain are transferred to nitrate reductase through cytochrome b (de la Rosa, personal communication), thus indicating this cytochrome as the immediate electron donor for the enzyme.

The classical inhibitors of nitrate reductase act on the enzyme from halophile bacteria in the expected way, thus indicating the molecular structure of the catalytic site in the halophilic nitrate reductase must be similar to that of the enzyme from other organisms.

The molecular mass of halophilic nitrate reductase, estimated by gel chromatography and non-denaturing electrophoresis in 170 kDa, seems to be similar to other enzymes from non-halophile bacteria, such as Rhodopseudomonas capsulata [18] or Micrococcus halodenitrificans [19]. Nevertheless, the molecular weight of nitrate reductase from bacteria shows a great variability, being possible to find values from 100,000, Azotobacter chroococcum [20], to 700,000, Escherichia coli [6, 8]. The small molecular weight observed in our case is in agreement with the small size observed for other halophilic enzymes such as dehydrogenases [21, 22]. This property of halophilic proteins could be explained by the tendency for the formation of aggregate in order to facilitate the solubility in media with high salt concentration [21, 23]. In fact, the Stokes radius of nitrate reductase from Haloferax, 4.92–4.96 nm, is also lower than the values obtained for the majority of nitrate reductase studied, e.g. 9.8 nm for Ankistrodesmus brauni [24], 7.05 nm for Rhodotorula glutinis [25], 8.9 nm for Chlorella vulgaris [26].

The halophilic nitrate reductase, as most halophilic enzymes, shows a remarkable gradient of thermophilicity, the optimum temperature being 89 °C when measured in the presence of 3.2 M NaCl. The optimum temperature has a direct dependence on salt concentration (Fig. 1), being in agreement with the previous results reported by Marquez and Brodie [4] for the enzyme from Halobacterium.

In general, the salts have a strong effect on the activity and stability of the halophilic enzymes, as it has been observed in all the cases analyzed (see reviews [27, 28]). The stability of the halophilic nitrate reductase at 21 °C is increased with the presence of salt in the medium. Also, the salts protect against heat inactivation. Those results indicate a stabilization of the second and higher structures of the halophilic enzyme by salts. However, this requirement seems to be not so strong as in other halophilic enzymes, such as aspartate aminotransferase [13], glutamate dehydrogenase [12] or malate dehydrogenase [29].

With respect to the kinetic parameters, the halophilic enzyme exhibits a hyperbolic kinetics with apparent $V_m$ and $K_m$ that depend on salt concentration, being the $K_m$ for nitrate 2.5 mM for 0.8 M NaCl and 6.7 mM for 3.4 M NaCl. These values are exceptionally higher in comparison with other non-halophilic nitrate reductases, which show values between 0.1 and 1.6 mM [9, 19, 20, 30].

In conclusion, nitrate reductase from Haloferax mediterranei is a dissimilatory nitrate reductase that is induced by nitrate in anaerobic conditions. The purified enzyme shares several characteristics with other halophilic enzymes, such as small size, thermophilicity, etc. However, the stabilization of the structure of this enzyme, unlike other halophilic proteins, does not require so very high salt concentrations.

Acknowledgements

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