NADP-glutamate dehydrogenase from the halophilic archaeon Haloferax mediterranei: enzyme purification, N-terminal sequence and stability

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Abstract

An NADP(H)-specific glutamate dehydrogenase of Haloferax mediterranei has been purified to apparent homogeneity and characterised. The purified enzyme was stabilized by glycerol in absence of salt. Glutamate dehydrogenase from Hf mediterranei is a hexameric enzyme with a native molecular mass of 320 kDa composed of monomers each with a molecular mass of 55 kDa. At pH 8.5 the enzyme has \( K_{\text{m}} \) of 0.018, 0.34 and 4.2 mM for NADP\(^+\), 2-oxoglutarate and ammonium, respectively. Amino acid composition and sequence of the first 16 residues of the N-terminus have been determined.

Keywords: NADP-glutamate dehydrogenase; Archaea; Halophilic; Haloferax mediterranei; Thermostability

1. Introduction

L-Glutamate dehydrogenases (EC 1.4.1.2-4, GDH), as a class, catalyse the interconversion of 2-oxoglutarate and L-glutamate, using \( \text{NAD}^+ \) or \( \text{NADP}^+ \) as a cofactor:

\[
2\text{-oxoglutarate} + \text{NAD(P)}H + \text{NH}_4^+ + \text{H}^+ \leftrightarrow \text{glutamate} + \text{NADP}^+ + \text{H}_2\text{O}
\]

\( \text{NAD}^+ \)-dependent enzymes are part of glutamate catabolism, whereas \( \text{NADP}^+ \)-dependent glutamate dehydrogenases are involved in ammonia assimilation. Enzymes purified from vertebrates are able to use both coenzymes [1]. Glutamate dehydrogenase is a well-studied enzyme, evolutionary conserved in all three primary domains: Eucarya, Bacteria and Archaea. The 3-dimensional structure of the hexameric glutamate dehydrogenase from Clostridium symbiosum has been recently determined, providing considerable insights for structure-function relationship studies of this class of enzymes [2]. Bacterial and fungal \( \text{NADP}^+ \)-dependent and vertebrate dual-specificity GDHs have a hexameric structure (\( M_r 48 000-53 500 \)) [1], and \( \text{NAD}^+ \)-dependent enzymes have either four identical subunits (\( M_r \approx 115 000 \)), as found in Neurospora crassa [3], or six identical subunits (\( M_r 48 000 \)), as found in C. symbiosum [2]. Glutamate dehydrogenase offers a model to study the quaternary assembly and interaction between...
protein subunits at high salt concentrations. The study of halophilic adaptation of oligomeric proteins is particularly interesting since subunit interactions may be additionally involved in protein stabilization.

In the present paper we report the purification, characterisation and N-terminal sequencing of NADP-glutamate dehydrogenase from the extreme halophilic Archaeon, *Haloferax mediterranei*, which grows at 2–4 M NaCl with isotonic concentrations of KCl internally.

2. Materials and methods

2.1. Culture of microorganisms

*Haloferax mediterranei* (R4, ATCC 33500) cells were grown aerobically at 37°C, pH 7.0, in a medium containing 25% (w/v) salts and 0.5% (w/v) yeast extract (Difco).

2.2. Purification of glutamate dehydrogenase

*Hf. mediterranei* cells (10 g wet weight) from a 2 l culture were resuspended in 50 ml of 50 mM sodium phosphate buffer, pH 6.6, containing 2.5 M (NH₄)₂SO₄ (buffer 1) and lysed by sonication at 4°C. The disrupted suspension was clarified by centrifugation (60 min at 105000 × g). All subsequent steps were carried out at room temperature. The crude extract was applied to a Sepharose-4B (Pharmacia) column (2.6 × 53 cm) equilibrated with buffer 1. Glutamate dehydrogenase activity was eluted with a decreasing gradient of ammonium sulfate (750 ml of buffer 1 and 750 ml 0.5 M (NH₄)₂SO₄ in 50 mM phosphate buffer, pH 6.6). The fractions with activity were pooled and applied to a DEAE-cellulose (Fine mesh, Sigma) column (1.5 × 8.0 cm), previously equilibrated with buffer 1. After washing, glutamate dehydrogenase activity was eluted with 10 mM sodium phosphate buffer, pH 7.3, containing 20% (v/v) glycerol, 0.5 M NaCl and 2 mM EDTA (buffer 2). Active fractions were pooled and applied to a Hydroxyapatite HT (Bio-Rad) column (2.5 × 3 cm) equilibrated with buffer 2. Glutamate dehydrogenase activity was eluted with an increasing gradient of sodium phosphate (75 ml of buffer 2 and 75 ml of 0.5 M sodium phosphate buffer, pH 7.3, containing 20% (v/v) glycerol, 0.5 M NaCl and 2 mM EDTA). Active fractions were applied to a Sepharose CL-6B column (2.5 × 36 cm) equilibrated and eluted with buffer 2.

2.3. Enzyme assay and protein determinations

Glutamate dehydrogenase was routinely assayed spectrophotometrically at 40°C in 20 mM Tris-HCl buffer, pH 8.5, containing 2 mM EDTA, 2 M NaCl, 100 mM ammonium acetate, 0.15 mM NADPH and 20 mM 2-oxoglutarate (both from Boehringer Mannheim). One unit of the enzyme is the oxidation of 1 μmol of NADPH min⁻¹. Protein concentrations were determined by the method of Bradford.

2.4. Determination of subunit and native M, values

After desalting by acetone precipitation, the subunit M, of the purified glutamate dehydrogenase was determined by SDS-PAGE and CTAB-PAGE [4]. The M, value for the native glutamate dehydrogenase was obtained by gel filtration chromatography on a Sepharose CL-6B (Pharmacia) column as described in [5]. The filtration was carried out in 20 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, 10 mM MgCl₂ and 1 M NaCl.

2.5. Salt and thermal stability analysis

The salt stability was studied in purified preparations of halophilic glutamate dehydrogenase. The enzyme solution in 2.0 M KCl (20 mM Tris-HCl buffer, pH 8.5) was diluted quickly with 20 mM Tris-HCl buffer, pH 8.5 to obtain 0.5, 1.0 and 1.5 M KCl concentrations. To obtain 2.5, 3.0 and 4.0 M, solid KCl was added. The stability was determined as the percent original activity remaining at the end of specified time intervals. The thermal stability was determined by incubating the enzyme in 20 mM Tris-HCl buffer, pH 8.5, containing 2 mM EDTA, 20% (v/v) glycerol, and 0.5 M NaCl, at various temperatures. Samples were removed at known time intervals, rapidly cooled in ice, and the remaining activity determined by the standard assay procedure. The effect of salt in thermal stability was examined at 67°C in a range of 0.5–4.0 M KCl.
2.6. Amino acid composition and N-terminal amino acid sequence

The sample was hydrolysed in 6 M HCl, 0.1% (v/v) phenol and 0.05% (v/v) β-mercaptoethanol under vacuum and analysed in a Waters HPLC system after derivatization with o-phthaldialdehyde. The procedure required 8-20 µg of the enzyme.

After SDS-PAGE, the glutamate dehydrogenase protein was electrophoretically transferred to PVDF membrane (Immobilon P, Millipore) and stained with Coomassie Blue R-250. The stained band was excised and its N-terminal amino acid sequence determined, using an Applied Biosystems 470A gas-phase sequencer, coupled to an Applied Biosynthesis 120 phenylthiohydantoin analyser.

3. Results and discussion

3.1. Purification and characterisation

To establish appropriate growth conditions prior to purification, the levels of NADP(H)-specific glutamate dehydrogenase activity in *Hf. mediterranei* cells cultivated with different nitrogen sources were examined. *Hf. mediterranei* was grown on a minimal medium containing ammonium sulfate, potassium nitrate or monosodium L-glutamate as nitrogen source. The total cellular levels of NADP(H)-specific glutamate dehydrogenase (NADP-GDH) activity were highest in cells grown with ammonium sulfate (0.54 U mg⁻¹) and lowest in cells grown in potassium nitrate (0.25 U mg⁻¹). The purification of NADP-GDH from *Hf. mediterranei* is summarised in Table 1. Electrophoresis of the purified GDH from *Hf. mediterranei* with anionic (SDS) and cationic (CTAB) detergents revealed that the enzyme migrated as a single protein band. The mobility of the enzyme in SDS-PAGE corresponds to a subunit Mₐ of 55±5 kDa and 48±4 kDa in CTAB-PAGE. Although the movement of NADP-GDH on CTAB-PAGE was slower, the difference between its mobilities was not so striking. The native Mₐ was 320±26 kDa from gel filtration. We conclude that *Hf. mediterranei* NADP⁺-GDH, like *Pyrococcus furiosus* GDH [6], is a hexameric protein composed of identical subunits. This result is consistent with the observation of two families of GDHs: a hexameric and a tetrameric family.

3.2. Catalytic properties

The catalytic properties of the purified halophilic NADP-GDH were similar to those reported for the enzyme from other Archaea, such as *Halobacterium salinarum* [5], *Pyrococcus furiosus* [6] and *Thermococcus litoralis* [7]. *Hf. mediterranei* GDH is catalytically active only with NADPH. The kinetic parameters of the amination reaction were determined under optimal conditions to be: *Kₐ* [NADPH] = 0.018±0.002 mM, *Kₐ* [2-oxoglutarate] = 0.34±0.04 mM and *Kₐ* [ammonium] = 4.2±0.4 mM. The optimal temperature and pH were 60°C and 8.5, respectively. The activity of *Hf. mediterranei* NADP-GDH was markedly dependent on the concentration of NaCl or KCl (Fig. 1), being optimal about 1–1.5 M NaCl or KCl. At lower concentrations, the stimulating effect of KCl is slightly higher than the effect of identical concentrations of NaCl.

3.3. Salt stability and thermostability of glutamate dehydrogenase

In the presence of 4 M NaCl the purified NADP-GDH remained fully active at room temperature
Pal (W)

Fig. 1. Effect of NaCl and KCl on the activity of purified NADP-GDH from H. mediterranei. (20–25°C) for months. However, the stability is rapidly lost upon lowering the salt concentration. On the other hand, the replacement of the salt for glycerol, a major carbon and energy source for natural communities of halophilic Archaea [8], greatly increased enzyme stability (at 20–25°C) from t1/2 = 4.3 h (in 1 M NaCl) up to t1/2 = 476 h (in 20% glycerol).

In addition to increasing the catalytic activity of the halophilic NADP+ GDH, the presence of molar concentrations of KCl or NaCl markedly increased the thermostability of the enzyme. The effect of temperature on GDH stability was studied by incubating the enzyme at different temperatures in absence of substrates. Hf: mediterranei GDH was fairly stable, requiring temperatures above 60°C for measurable rates of thermodenaturation. The thermostability decreased significantly as the salt concentration was lowered (Table 2). The half-life (t1/2) of halophilic GDH in presence of 2 M KCl (at 67°C) was about 27 min, while in 4 M KCl the first-order rate constant for thermal inactivation was 9.85 × 10^-3 h^-1 with a t1/2 of 70 h. These results show that Hf: mediterranei GDH is more thermostable than halophilic malate dehydrogenase from Haloarcula marismortui [9], which at 63°C in 4 M NaCl showed a t1/2 of 8.5 h.

The kinetics of thermal inactivation of Hf. mediterranei GDH were also analysed by incubating samples of GDH in Tris/EDTA buffer containing either 0.5 M KCl or 3 M KCl or 0.5 M KCl plus 20% glycerol at different temperatures. The temperature dependence of the rate constant for inactivation was analysed according to the Arrhenius equation. The thermal stability of the enzyme is substantially reduced at the lower KCl concentration, with concomitant differences in the activation energies (Ead) for the thermal inactivation process (364 kJ mol^-1 and 47 kJ mol^-1 at 3 M and 0.5 M KCl, respectively). The presence of 20% (v/v) glycerol in the incubation buffer with lower KCl concentration decreases the rate of denaturation process, increasing the Ead (187 kJ mol^-1). Thermodynamic parameters (ΔH°, ΔS°, ΔG°) of the inactivation reaction were also calculated, and the values for 3 M KCl (ΔH° = 360 kJ mol^-1, ΔS° = 710 J mol^-1 and ΔG° = 119 kJ mol^-1 at 340°K) are very close to those for isopropylmalate dehydrogenase from Thermus thermophilus [10]. The thermophilic nature of halophilic enzymes has been noted in several systems [11,12], and Dym et al. [11] point out, from the crystal structure of Haloarcula marismortui malate dehydrogenase, that several of the structural features conferring halophilicity are the same as those contributing to the stability of thermophilic enzymes.

### 3.4. Amino acid composition and N-terminal sequence

The amino acid composition of Hf. mediterranei GDH is more similar to those of GDHs from other Archaea [6,13] than Eubacteria [14]. As in other Archaea, in Hf. mediterranei GDH the cumulative amount of acidic amino acids is much higher than the cumulative amount of basic amino acids (Lys plus Arg) and the content of valine was remarkably high, probably related to its thermophilic character.

<table>
<thead>
<tr>
<th>KCl (M)</th>
<th>t1/2 (h)</th>
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<tr>
<td>0.5</td>
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<tr>
<td>1.0</td>
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Values obtained from the first-order kinetics of thermodenaturation.
The N-terminal sequence of the \textit{Hf. mediterranei} (16 amino acids) was determined to be AQEANP-FESQEAIDD. This sequence was compared with the N-terminal amino acid sequence of GDHs from other organisms. The alignment was not very good. This result is in accordance with the observation of Baker et al. \cite{2} that the similarity between hexameric GDHs is low for the N-terminal 50 residues. However, seven (A1,Q2,E3,N5,S9,I14,D16) of the 16 amino acids in the \textit{Hf. mediterranei} enzyme are substituted for similar amino acids (V1,E2,Q3,D5,19,L14,E16) in four archaeal enzymes \cite{7,15-17} and three conserved amino acid residues (PFE). Little amino acid similarity was observed with other GDHs from \textit{Escherichia coli}, \textit{Sulfolobus solfataricus}, \textit{Saccharomyces cerevisiae} and \textit{Hf. salinarum}.

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References


