Glucose dehydrogenase from the halophilic Archaeon *Haloferax mediterranei*: Enzyme purification, characterisation and N-terminal sequence

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Abstract An NAD(P)-glucose dehydrogenase from the extremely halophilic Archaeon, *Haloferax mediterranei*, has been purified to electrophoretic homogeneity. The purified enzyme has been characterised with respect to its cofactor specificity, subunit composition and its salt and thermal stability. The N-terminal amino acid sequence has been determined and N-terminus alignment with sequences of other glucose dehydrogenases shows that the halophilic enzyme most closely resembles the NAD(P)-linked glucose dehydrogenase from the thermophilic Archaeon *Thermoplasma acidophilum*. However, the halophilic glucose dehydrogenase appears to be a dimeric protein, in contrast to the tetrameric enzyme from the thermophile.

Key words: Glucose dehydrogenase; Archaea; Halophile; Purification

1. Introduction

The Archaea [1] are recognized as a separate branch of ancient organisms that have evolved under conditions of high temperature, anaerobic atmosphere and high salinity. Three groups of Archaea exist: the extreme halophiles, the methanogens and the thermophiles. Extremely halophilic Archaea, which require 10–20% NaCl for optimal growth, were considered a rather homogeneous group of heterotrophic microorganisms predominantly using amino acids as their source of carbon and energy. However, it has been shown that some halophilic Archaea are able to use not only amino acids but different sugars as well [2,3], and *Haloferax mediterranei* for example grows in a minimal medium containing glucose as the only source of carbon [4]. It is thought that *H. mediterranei* uses a modified Etner-Doudoroff pathway for the catabolism of glucose [5]. The first enzyme of this unusual metabolic route is glucose dehydrogenase (EC 1.1.1.47), which catalyses the oxidation of β-D-glucose to gluconic acid using NAD or NADP as a coenzyme.

$$\text{Glucose} + \text{NAD(P)}^+ \rightarrow \text{gluconate} + \text{NAD(P)H} + \text{H}^+$$

Glucose dehydrogenases from the thermophilic Archaea, *Thermoplasma acidophilum* [6] and *Sulfolobus solfataricus* [7], are also dual-cofactor specific, and are able to oxidise a number of sugars. The gene encoding the *T. acidophilum* glucose dehydrogenase has been cloned, sequenced and over-expressed in *E. coli* [8], and the 3D structure of the recombinant enzyme has been determined [9]. Therefore, we are now embarking on a detailed study of the enzyme from the halophilic Archaea.

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Enzymes from the extreme halophiles are often difficult to purify because they usually require high salt concentrations for stability and hig-resolution purification procedures are therefore limited. The present paper describes the purification to homogeneity, characterisation and N-terminal amino acid sequence of a dual coenzyme specificity glucose dehydrogenase from *H. mediterranei*.

2. Materials and methods

2.1. Culture of microorganism and purification of glucose dehydrogenase from H. mediterranei

H. 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), as described earlier [4]. The medium was supplemented with 1% (w/v) glucose. *H. mediterranei* cells were resuspended in 50 mM sodium phosphate, pH 6.6, containing 2.5 M $(NH_4)_2SO_4$ (buffer 1) (1 g wet wt/5 ml) and lysed by sonication at 4°C. The disrupted suspension was clarified by centrifugation (60 min at 105 000 × *g*). All subsequent steps were carried out at room temperature. The crude extract was applied to a Sepharose 4B column (2.6 × 53 cm) equilibrated with buffer 1. Glucose dehydrogenase activity was eluted with a decreasing gradient of ammonium sulfate (750 ml of buffer 1 and 750 ml 0.5 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer, pH 6.6). The fractions with activity were pooled and applied to a DEAE-cellulose column (1.5 × 8.0 cm), previously equilibrated with buffer 1. After washing, glucose dehydrogenase activity was eluted with 50 mM phosphate buffer, pH 7.3, containing 2 M NaCl. Active fractions were pooled and dialysed three times against a 100-fold volume of 20 mM sodium phosphate, pH 7.4, containing 20% (v/v) glycerol and 10 mM $MgCl_2$ (buffer 2) at 4°C. They were then applied to a Blue-Sepharose CL-6B column (2.5 × 10 cm) equilibrated with buffer 2. After washing, glucose dehydrogenase was eluted with the buffer 2 without absorption. The fractions with activity were pooled and the phosphate was removed by dialysis against a 100-fold volume of 20 mM Tris-HCl buffer, pH 7.4, containing 20% glycerol and 10 mM $MgCl_2$ (buffer 3). Active fractions were applied to a column of Reactive Red-120 (Sigma) equilibrated with buffer 4. Non-bound protein was removed by washing the column with buffer 3. Glucose dehydrogenase activity was eluted with the same buffer but containing 2 M NaCl. The purified enzyme was stable for several months when stored at 4°C.

2.2. Enzyme assay and protein determinations

Glucose dehydrogenase was routinely assayed spectrophotometrically at A_{340} and 40°C in 20 mM Tris-HCl buffer, pH 8.8, 25 mM $MgCl_2$, containing 2 M NaCl, 0.22 mM $NADP^+$ and 25 mM glucose. One unit of enzyme activity is the reduction of 1 μmol of $NADP^+$ per min. The NAD^+-dependent glucose dehydrogenase activity was measured in the same way substituting NAD for NADP. Kinetic data were analysed according to Cleland [10]. Protein concentrations were determined by the method of Bradford [11].

2.3. Determination of M_r

The M_r value for the native glucose dehydrogenase was performed by gel filtration on a Sepharose CL-6B column as described in [12]. The filtration was carried out in 20 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, 10 mM $MgCl_2$ and 1 M NaCl. After desalting by acetone precipitation, the subunit M_r of the purified glucose

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dehydrogenase was determined by SDS-PAGE [13] and by CTAB-PAGE [14]. Discontinuous gel electrophoresis at pH 8.5 was performed as in [15]. Glucose dehydrogenase was visualised by coupling the glucose-dependent reduction of $NADP^+$ to dioformazan production.

2.4. Salt and thermal stability analysis

The salt stability was studied in unpurified preparations of halophilic glucose dehydrogenase. The enzyme solutions in 4 M NaCl (20 mM Tris-HCl buffer, pH 8.5) were diluted quickly with 20 mM Tris-HCl buffer, pH 8.5, to obtain 0.6 and 0.05 M NaCl concentrations, and 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 0.5, 1.3, 2.0 or 3.0 M NaCl, at various temperatures over a period of up to 48 h. Samples were removed at known time intervals, rapidly cooled in ice, and the remaining activity determined by the standard assay procedure.

2.5. N-terminal amino acid sequencing

After SDS-PAGE [13], the glucose 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 of glucose dehydrogenase

The level of glucose dehydrogenase in *H. mediterranei* was influenced by the composition of the culture medium. The specific activity obtained in a culture with 0.5% yeast extract was 0.16 U/mg, whereas in 1% glucose minimum medium was 1.18 U/mg. On the other hand, if the first medium was supplemented with 1% glucose, a similar specific activity (0.97 U/mg) was obtained. The purification of glucose dehydrogenase from *H. mediterranei* is summarised in Table 1. All steps were performed in buffers of high salt concentration or in the presence of glycerol (20% w/v) to stabilize the protein structure. The DEAE-cellulose step we did not achieve an increase in the purification but served to concentrate the enzyme for the next step. The halophilic enzyme was found to be homogeneous as judged by electrophoresis on SDS-PAGE and by native gel electrophoresis where the single band observed with Coomassie Blue corresponded with that after an enzymatic activity stain.

3.2. Molecular weight and subunit structure

Electrophoresis of the purified glucose dehydrogenase from *H. 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 M_r of 53 ± 3 kDa. However, the M_r value determined by CTAB-PAGE was 39 ± 4 kDa. As in the cases of *H. salinarium* arginine deaminase [16], *Haloarcula vallismortis* glyceraldehyde-3-phosphate dehydrogenase [17], and halophilic serine proteinase [18], the subunit M_r of *H. mediterranei* glucose dehydrogenase determined by SDS-PAGE is about 25% higher than that obtained by CTAB-PAGE. The value from latter technique is regarded as more reliable for highly-acidic halophilic proteins, and with a native M_r of 89 kDa from gel filtration, we conclude that *H. mediterranei* glucose dehydrogenase is a dimeric enzyme.

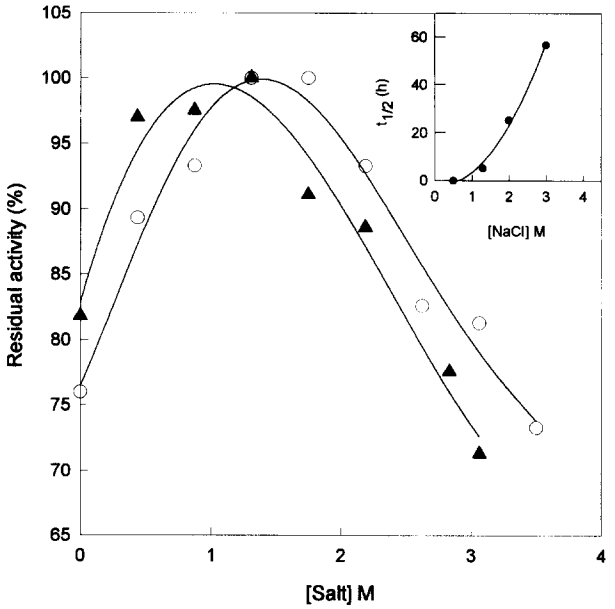

Fig. 1. Effect of NaCl (○) and KCl (▲) on the activity of glucose dehydrogenase from *Haloferax mediterranei*. (Inset) Salt stability of *Haloferax mediterranei* glucose dehydrogenase at 65°C. For each salt concentration, the first-order rate constants and $t_{1/2}$ (●) were determined in the presence of 0.5, 1.3, 2.0 and 3.0 M NaCl.

3.3. Catalytic properties

The catalytic properties of the purified halophilic glucose dehydrogenase were similar to those reported for the enzyme from other Archaea, such as *T. acidophilum* [6,19], *S. solfataricus* [7], *Thermoproteus tenax* [20]. *H. mediterranei* glucose dehydrogenase is catalytically active with both coenzymes NAD^+ and $NADP^+$. The kinetic parameters of the forward reaction were determined under optimal conditions to be: K_m $[NAD^+] = 1.2 \pm 0.3$ mM, K_m $[NADP^+] = 0.024 \pm 0.002$ mM and K_m [β-D-glucose/$NADP^+$] $= 3.9 \pm 0.2$ mM. A number of carbohydrates have been tested as possible substrates for the dehydrogenase (Table 2). When $NADP^+$ served as electron acceptor, D-glucose was oxidized at the highest rate (106 U/mg) among the products tested. When NAD^+ served as a coenzyme, among a number of sugars examined, only D-xylose and D-glucose were oxidized at a high rate by the halophilic glucose dehydrogenase. These results are similar to those obtained for *S. solfataricus* glucose dehydrogenase [7]. It appears that any configuration change at C2, C3 and C4 of

Table 1
Purification of NAD(P)-glucose dehydrogenase from *Haloferax mediterranei*

Enzyme fraction	Total protein (mg)	Specific activity (U/mg)	Enrichment (-fold)	Yield (%)
Cell extract	790	0.7	–	100
Sepharose-4B	25	17	23	72
DEAE-cellulose	23	17	23	66
Blue-Sepharose	1	190	271	33
Red-Sepharose	0.2	550	786	19

Table 2
Substrate and coenzymes specificities of *Haloferax mediterranei* glucose dehydrogenase

Substrate (mM)	Coenzyme	Relative rate (%)	
		$NADP^+$	NAD^+
D-Glucose		100	26
D-Mannose		7	7
D-Galactose		13	1
D-Fructose		3	1
D-Xylose		88	79
D-Fucose		18	3
D-Glucosamine		6	5
D-Ribose		3	–

All sugars were tested at 0.1 M concentration. $NADP^+$-dependent D-glucose oxidation rate was taken as 100. NAD^+ and $NADP^+$ concentration were 5 times the K_m values.

Table 3
Alignment of N-terminal sequences of glucose dehydrogenases

	1 10 20
T. acidophilum	TEQ**KAI**VTDAP K**G**GVKYTT**I**DMPE
H. mediterranei	M**KAI**AVKR--**G**EDRPVV**I**E

Identities involving the archaeal sequences are indicated in boldface. The numbers refer to the positions of amino acids in the alignment.

the glucose molecule markedly reduced the enzymes affinity for the sugar.

3.4. Effect of salt on activity and stability of glucose dehydrogenase

The activity of *H. mediterranei* glucose dehydrogenase was markedly dependent on the concentration of NaCl or KCl in the activity buffer, being optimal at 1.3 M NaCl/KCl (Fig. 1). At lower concentrations, the stimulatory effect of KCl is slightly higher than the effect of identical concentrations of NaCl. Besides KCl/NaCl, the activity also depends on presence of bivalent cations. In the absence of such ions, glucose dehydrogenase preparations possessed low enzymic activity; thus, a significant increase of activity was caused by the addition of metal ions ($Mn^{2+} > Mg^{2+} > Ni^{2+}$), whereas the addition of 2 mM EDTA in the standard mixture decreased enzyme activity even further.

In addition to increasing the catalytic activity of the halophilic glucose dehydrogenase, the presence of molar concentrations of KCl or NaCl markedly increased the thermostability of the enzyme. The effect of temperature on glucose dehydrogenase stability was studied by incubating the enzyme at different temperatures in absence of substrates. *H. mediterranei* glucose dehydrogenase was fairly stable, requiring temperatures above 60°C for measurable rates of thermodenaturation. In the presence of 3 M NaCl, the first-order rate constant for thermal inactivation at 65°C was 0.0122 h^{-1} ($t_{1/2}$ = 56.8 h^{-1}), whereas the stability decreased significantly as the salt concentration was lowered (Fig. 1, inset). The thermophilic nature of halophilic enzymes has been noted in several systems [21,22], and Dym et al. [21] 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.

3.5. N-terminal sequence

Based on alignment of complete amino acid sequences of glucose dehydrogenase, it is clear that the eubacterial and archaeal enzymes belong to different families. Therefore, the N-terminal sequence data obtained for the glucose dehydrogenase from *H. mediterranei* is shown in comparison only with that of the enzyme from the thermophilic Archaeon, *T. acidophilum* (Table 3). An identity of approx. 30% is observed over this short sequence.

4. Concluding remarks

We have characterised glucose dehydrogenase from an extremely halophilic Archaeon and showed it to have a broad substrate specificity, ability to use either NAD^+ or $NADP^+$ as cofactor, and requirement for a divalent metal ion for full enzymic activity.

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