Overexpression in a non-native halophilic host and biotechnological potential of NAD+-dependent glutamate dehydrogenase from Halobacterium salinarum strain NRC-36014

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ORIGINAL PAPER

Overexpression in a non-native halophilic host and biotechnological potential of NAD^+-dependent glutamate dehydrogenase from *Halobacterium salinarum* strain NRC-36014

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Abstract Enzymes produced by halophilic archaea are generally heat resistant and organic solvent tolerant, and accordingly important for biocatalytic applications in 'green chemistry', frequently requiring a low-water environment. NAD^+-dependent glutamate dehydrogenase from an extremely halophilic archaeon *Halobacterium salinarum* strain NRC-36014 was selected to explore the biotechnological potential of this enzyme and genetically engineered derivatives. Over-expression in a halophilic host *Haloferax volcanii* provided a soluble, active recombinant enzyme, not achievable in mesophilic *Escherichia coli*, and an efficient purification procedure was developed. pH and salt dependence, thermostability, organic solvent stability and kinetic parameters were explored. The enzyme is active up to 90 °C and fully stable up to 70 °C. It shows good tolerance of various miscible organic solvents. High concentrations of salt may be substituted with 30 % DMSO or betaine with good stability and activity. The robustness of this enzyme under a wide range of conditions offers a promising scaffold for protein engineering.

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Keywords Halophilic enzyme · Halophilic expression host · Glutamate dehydrogenase · *Halobacterium* · Organic solvents · Thermostability · Salt substitution

Introduction

The increasing demand for enzymes for industrial applications prompts the search for novel biocatalysts that are stable under harsh industrial conditions. Extremophiles are a natural source of such stable enzymes (de Champdoré et al. 2007). Each group of extremophiles has unique features, and their enzymes are adapted to work under the particular extreme conditions of their environmental niche, offering a wide range of application possibilities (Van den Burg 2003).

For halophilic microorganisms, the natural habitat is characterized by multi-extreme conditions such as extremely high ionic strength, up to saturated salt water, elevated temperature (49–58 °C, Robinson et al. 2005) and high levels of UV radiation (sunlight). Thus, enzymes from halophiles typically withstand both high salt conditions and elevated temperatures. Stability at high temperatures and ability to work in aqueous/organic media (low water environment) make halophilic enzymes attractive candidates for industrial application.

Our own enzymological studies have focused on the amino acid dehydrogenase family and on the possibility of engineering these for biotechnological application. These enzymes have applications in biosensors and diagnostic kits (Rinaldi et al. 2004), in synthesis of chiral amino acid synthons for the pharmaceutical industry (Engel and Paradisi 2010), for regeneration of reduced or oxidized cofactors (Bolivar et al. 2008) etc. Thus far, our starting points for protein engineering have been mesophilic amino

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acid dehydrogenases (Wang et al. 2001; Seah et al. 2002; Busca et al. 2004; Paradisi et al. 2007). For the reasons mentioned above, a halophilic starting point would be potentially attractive, and in this paper we assess the potential of one of the glutamate dehydrogenases of *Halobacterium salinarum* for this purpose. This is the so-called GDHX of the colourless strain NRC-36014. The corresponding gene was first sequenced by Benachenhou and Baldacci (1991) and initially attributed to an $NADP^+$-dependent GDH. However, Hayden et al. (2002) clearly showed that this gene encodes an NAD^+-dependent GDH, corresponding, in fact, to the enzyme first isolated and described by Bonete et al. (1986). It later emerged that this strain of *Hbt. salinarum* has four distinct GDH genes (Ingoldsby et al. 2005), and the gene encoding GDHX is not one of the three to be found in the complete genome sequence of *Hbt. salinarum* NRC-1 (Ng et al. 2000).

Our objective in this study was to establish (a) whether GDHX is indeed robust; (b) whether efficient recombinant expression is possible. Although active halophilic enzymes can be obtained from parent organisms, the quantity is not usually adequate for bulk production. Also, since the potential to make multiple, mutated variants is essential for our purpose, heterologous/homologous over-expression in a suitable host is critically important. *Escherichia coli* is usually the first choice for recombinant gene expression, but the requirement of high salinity for stability and activity make halophilic enzymes a difficult target for mesophilic expression in a native, active form. No doubt halophilic proteins can be produced on a large scale in *E. coli* but, owing to lack of high ionic strength in the cellular environment, this tends to be in the form of inclusion bodies, which need to be solubilised and refolded in vitro to recover functionally active enzyme (Diaz et al. 2006). During this study, we first attempted to produce halophilic GDHX in *E. coli* and to refold it in vitro. The literature describes a number of methods for the redirection of proteins into the soluble and active fraction (Tsumoto et al. 2003; Singh and Panda 2005) but in vitro refolding conditions have to be optimized for every protein as there is no universal method available. The right refolding conditions for halophilic proteins are especially difficult to establish owing to lack of understanding of the competition between folding and aggregation of these proteins. Soluble expression in a halophilic host is therefore an attractive alternative to in vitro refolding procedures and was also explored here.

Haloferax volcanii (*Hfx. volcanii*), an extremely halophilic archaeon, is thought to be an appropriate host for the expression of recombinant halophilic proteins. The presence in *Hfx. volcanii* of an efficient transformation system (Cline et al. 1989, 1995), several integrating and shuttle vectors and selectable markers (Allers et al. 2004, 2010; Holmes et al. 1994; Holmes and Dyall-Smith 1990) provides an opportunity to obtain soluble and active halophilic proteins using the native-like internal environment of the host cell. The homologous expression of recombinant dihydrolipoamide dehydrogenase from *Hfx. volcanii* (Jolley et al. 1996) and of ferredoxin from the extremely halophilic archaeon *Haloarcula japonica* strain TR-1 (Matsuo et al. 2001) in the parent organism are previous examples of successful homologous expression of recombinant halophilic proteins in their native hosts. In the present paper, however, we report the heterologous expression of GDHX (Ingoldsby et al. 2005) from *Halobacterium salinarum* strain 36014 in a halophilic host (*Hfx. volcanii*) using the pRV1-tna shuttle vector which has a tryptophan-inducible promoter (Large et al. 2007). We also describe a novel procedure for purification from the halophilic host and assess the enzyme's biotechnological potential by exploring thermostability and organic solvent stability.

Materials and methods

All chemicals used during this study were of analytical grade. Bovine serum albumin (BSA), salts, polyethylene glycol 600 (PEG-600), organic solvents, novobiocin, agarose, betaine, glycerol, amino and keto acid substrates, Sepharose 4B and DEAE-cellulose were purchased from Sigma-Aldrich (Ireland). DNA ladder, protein markers, restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (UK). Cofactors (>98 % purity) were from Apollo Scientific (UK).

Strains, plasmids, media and cultural conditions

Haloferax volcanii DS70 strain and pRV1-tna vector were kindly provided by Dr. Peter Lund, University of Birmingham. *Hfx. volcanii* DS70 cells were cultivated in sterilized 18 % modified growth medium (MGM) (Dyall-Smith 2006) at 42 °C with (transformed cells) or without (non-transformed cells) 0.3 μg/ml novobiocin. The pET5a vector was obtained from Novagen. *E. coli* BL21DE3 Δ*gdh* strain was kindly provided by Dr. Michael Sharkey (Sharkey and Engel 2009) and *E. coli* XL10 Gold strain from Stratagene was grown in LB medium at 37 °C (Sambrook et al. 1989) with the addition of 100 μg/ml ampicillin for cells containing the plasmid.

Plasmid construction and purification

An empty pRV1-tna vector and pET5a vector containing the desired GDHX gene (Hayden et al. 2002) were both digested with *Nde*I and *Eco*RI restriction enzymes. The pRV1-tna vector backbone and the GDHX gene were excised from agarose gel, using an Agarose Gel DNA

extraction kit from Roche, mixed together and ligated using T4 DNA ligase. The resultant vector pRV1-tnaGDHX was transformed into *E. coli* XL10 Gold cells. Recombinant plasmids were purified from 3 to 4 ml of an overnight culture of *E. coli* XL10 Gold strain using a QIA Prep Spin Mini-prep Kit. The presence and size of the insert were confirmed by digesting recovered plasmids with restriction enzymes, and positive plasmids were sequenced by Eurofins MWG Operon. Plasmid stocks were kept at −20 °C.

Heterologous over-expression in a mesophilic host (*E. coli*) and preparation of inclusion bodies

Overexpression of recombinant GDHX in *E. coli* BL21(DE3) Δ*gdh* strain using the pET5a vector was performed as described previously (Hayden et al. 2002). Expression was induced with 1 mM final concentration of IPTG for 4–6 h. Cells were harvested by centrifugation (22,600*g*) and the pellet was resuspended in 50 mM HEPES-NaOH buffer, pH 7.5, containing 0.5 M NaCl, 5 mM EDTA, 0.35 mg/ml lysozyme, and incubated at 20 °C for 30 min. 1 % Triton X-100 was added to the suspension and followed by sonication. The lysate was centrifuged and the supernatant was collected in a fresh tube. The pellet (inclusion bodies) was washed twice with TBS containing 1 % (*v/v*) Triton X-100 and both pellet and supernatant were analysed on SDS-PAGE for expression.

Solubilisation and in vitro renaturation of inclusion bodies

Inclusion bodies were solubilised in 50 mM HEPES-NaOH buffer pH 7.5 containing 8 M urea and 25 mM DTT, and renaturation of protein was attempted using both rapid dilution and gradual dialysis methods. During dialysis, different final concentrations (20, 100 and 500 µg/ml) of solubilised protein were placed in a dialysis tube and immersed in refolding buffer, 50 mM Tris–HCl, pH 7.5, containing 2 or 4 M NaCl with and without 40 mM glutamate at 4 °C, room temperature (∼18 °C) and 37 °C, and gradual buffer exchange was facilitated by slow agitation. Separately, using a syringe and vigorous agitation, solubilised protein was rapidly diluted to final concentrations of 20, 25 or 100 µg/ml in different refolding buffers (50 mM Tris–HCl, 50 mM KPi, 50 mM HEPES-NaOH and/or 80 mM glycine-NaOH containing 0–4 M NaCl, pH 6–10 with intervals of 0.5 units according to buffering capacity of buffers, with and without 40 mM glutamate, 2 mM NAD^+, 20 mM 2-oxoglutaric acid or 0.4 M arginine at 4, 18 (room temperature) and 37 °C). After adding protein solution to refolding buffer, in order to precipitate *E. coli* proteins, solid ammonium sulphate was added to a final concentration of 2.5 M immediately, after 5 min or after 30 min. Refolding was monitored by measuring GDH activity.

Haloferax volcanii transformation and over-expression of GDHX

Haloferax volcanii DS70 competent cells were transformed with pRV1-tnaGDHX (PRV1-GDHX) vector according to the method described by Cline et al. (1989) and transformants were selected on 18 %MGM/novobiocin plates incubated at 37 °C for 5–8 days. A single transformant colony was selected to inoculate 5 ml 18 %MGM/novobiocin medium and incubated for 72 h at 42 °C. These freshly grown cells of *Hfx. volcanii* containing the pRV1-GDHX vector were used to inoculate 500 ml 18 %MGM/novobiocin medium, induced with filter-sterilized tryptophan at a final concentration of 4 mM and incubated at 42 °C for 72 h for over-expression.

Preparation of *Hfx. volcanii* cell extract

The late exponential *Hfx. volcanii* cells were harvested by centrifugation at 11,500*g* for 45 min and resuspended in 50 mM potassium phosphate (KPi) buffer (pH 6.6) containing 2.5 M $(NH_4)_2SO_4$. Cells were broken by sonication using a Sonicator^R Ultrasonic Processor XL (Misonix, Farmingdale, NY), debris was removed by centrifugation (45 min, 37,400*g*, 4 °C) and the supernatant was used for further analysis. Protein concentrations were estimated by the Bradford (1976) method, using BSA as a standard.

Activity assay of GDHX

The catalytic activity of glutamate dehydrogenase was assayed by measuring the change in concentration of NAD(P)H via absorbance measurements at 340 nm in a 1 cm cuvette using a Cary 50 UV-Visible spectrophotometer (Varian Inc., Palo Alto, CA, USA). The assays were performed at 40 °C as described by Bonete et al. (1987). The reaction was started by adding enzyme to 1 ml of reaction mixture. The buffers were 80 mM glycine-NaOH, pH 9.0, containing 0.9 M NaCl, 2.6 mM EDTA, 50 mM glutamate and 3 mM NAD^+ for oxidative deamination, and 80 mM glycine-NaOH buffer, pH 8.5, containing 3.2 M NaCl, 2.6 mM EDTA, 105 mM ammonium acetate, 20 mM 2-oxoglutarate and 0.15 mM NADH for reductive amination.

MS analysis of recombinant GDHX

For identification of recombinant GDHX by mass spectrometry, the over-expressed GDHX band on an SDS-PAGE gel was cut out and digested with trypsin. Digests

were fractionated by HPLC and peaks were analysed by data-dependent LCMS/MS on an LCQ Orbitrap XL mass spectrometer (ThermoScientific).

Thermal stability

To check the thermostability of recombinant GDHX, duplicates of 500 µl crude extract were incubated for 30 min in sealed vials at different temperatures from 50–90 °C, at intervals of 10 °C. After incubation, the samples were cooled on ice for 5 min and centrifuged at 12,300*g* for 15 min to remove precipitates formed during heating. GDH activity in the supernatant was assayed. Unheated enzyme was used as a control.

Purification of recombinant GDHX from *Hfx. volcanii* by three-step method

All purification steps were performed at room temperature (18–20 °C). The crude extract was loaded on a pre-equilibrated Sepharose 4B column (1.5 × 20 cm) with 50 mM potassium phosphate buffer, pH 6.6, containing 2.5 M $(NH_4)_2SO_4$. The column was washed with 1.5 column volumes of the same buffer at a flow rate of 0.5 ml per minute. The proteins were separated with a decreasing gradient of ammonium sulphate (2.5–0.5 M), in the same buffer, as described by Bonete et al. (1986). The active fractions (having NAD^+-GDH activity) were pooled and loaded on a 10 ml DEAE-cellulose column, pre-equilibrated with 50 mM KPi buffer, pH 6.6, containing 2.5 M $(NH_4)_2SO_4$, at a flow rate of 0.5 ml min^{-1}. The column was washed with 10 column volumes of the same buffer. The enzyme was eluted and concentrated by washing with 4 M NaCl in 50 mM KPi, pH 7.3. Active fractions were pooled and enzyme was further purified by heating at 90 °C for 30 min in sealed tubes followed by 5 min cooling on ice and centrifugation at 12,300*g* for 15 min. Purification was monitored by analysing fractions from each step by SDS-PAGE.

Optimum salt concentration for GDHX activity

Recombinant GDHX was tested for activity at different salt concentrations in the range 0–4 M NaCl and 0–3 M KCl using standard assay conditions for both amination and deamination. Two sets of reaction mixtures, with salt (4 M NaCl or 3 M KCl) and without salt (0 M NaCl or KCl), were prepared and incubated at 40 °C for spectrophotometric assay. The assay buffer without salt was used to dilute the assay buffer with salt to get different final concentrations of salt in the cuvette. The solutions were mixed in the cuvette by pipetting and reactions were monitored for 1 min after adding the enzyme.

Effect of pH on GDHX activity

Amination and deamination activities were assayed at different pH values from 6.5 to 10.5. 80 mM KPi buffer (pH 6.5, 7.0, 7.5 and 8.0), 80 mM Tris–HCl buffer (pH 8.0, 8.5 and 9.0) and 80 mM glycine-NaOH buffer (pH 8.5, 9.0, 9.5, 10.0 and 10.5) containing 2.6 mM EDTA and 0.9 M NaCl (for forward reaction) or 3.2 M NaCl (for reverse reaction) were used. The overlap of buffer ranges was used to check for specific buffer effects.

Temperature profile

The effect of elevated temperature on the catalytic activity of the enzyme was assessed by measuring activity at 40–90 °C. Reaction mixtures were prepared for both oxidation and reduction activities and assays were performed for 1 min at each particular temperature. The reaction mixtures (without coenzyme) were equilibrated in a water bath at the chosen temperature before starting the reaction. Coenzyme, in a minimal volume, was added at the last moment in order to avoid chemical breakdown. At 70 °C and above, the reactions were performed in covered cuvettes to avoid evaporation at such high temperatures.

Effect of organic solvents on the activity and stability of GDHX

Water-miscible organic solvents [methanol (MeOH), ethanol (EtOH), acetone, acetonitrile (CH_3CN), 2-propanol, dimethylsulfoxide (DMSO), dimethylformamide (DMF) and glycerol] were chosen to determine their effect on stability and activity of GDHX. For stability assays, enzyme solution was incubated in 5 and 10 % (*v/v*) organic solvents for 3 days at room temperature (18–20 °C). Enzyme solution was also incubated at 70 °C for 24 h in the presence of 10 % MeOH and EtOH. Controls were set up without organic solvent under the same experimental conditions. Aliquots were withdrawn at different time intervals and residual activities were measured spectrophotometrically using standard reverse assay solution. For activity assay, the reaction mixture for reductive amination was supplemented with the above-mentioned organic solvents (5 and 10 % *v/v*). Catalytic efficiency of GDHX was determined in aqueous-organic mixtures at 40 °C in the usual way.

Stability of GDHX in 0.5 M NaCl in the presence of different additives

The possible effect of different additives on GDHX stability under suboptimal salt concentration was investigated. The enzyme solution in 50 mM KPi, pH 7.3 containing

4 M NaCl was diluted eightfold with the same buffer without salt to get 0.5 M final concentration of NaCl, and supplemented with different additives such as glutamate (100 mM), 2-oxoglutarate (40 mM), NAD^+ (3 mM), 2-oxoglutarate + NAD^+ (40 mM + 3 mM), glycerol, DMSO, DMF and betaine (30 %). All samples were incubated at 4 °C and the retained activity was determined at intervals for 10 days under standard deamination assay conditions. Samples in 4 M NaCl and 0.5 M NaCl without any additive were used as controls.

Optimal DMSO concentration for GDHX activity in the absence of added salt

Reaction mixtures for both amination and deamination assays were supplemented with 20 and 30 % DMSO instead of 0.9 or 3.2 M NaCl, respectively, and rates of reaction were measured at 40 °C for 1 min. Assay buffer without DMSO and salt was used for dilution to get different final concentrations of DMSO in the reaction mixture.

Kinetic parameters in salt and DMSO

Kinetic parameters (K_m and k_{cat}) were calculated from initial rates that were determined in 80 mM glycine-NaOH buffer, either at pH 8.5 and containing 2.6 mM EDTA and 3.2 M NaCl or 20 % DMSO for reductive amination reactions or at pH 9.0 with 0.9 M NaCl or 10 % DMSO, for oxidative deamination reactions. In each instance, the concentration of the substrate of interest was varied, while the other appropriate substrates were maintained at fixed concentrations, 50 mM glutamate and 3 mM NAD^+ for oxidative deamination and 20 mM 2-oxoglutarate, 0.15 mM NADH and 450 mM ammonium acetate for reductive amination. The change of absorbance was recorded spectrophotometrically at 340 nm and readings were taken in triplicate. All data and their standard errors were calculated by a non-linear regression method (Wilkinson 1961) with the Enzpack Version 3.0 program (Biosoft Ltd., UK).

Results

Expression in *E. coli* and in vitro refolding of recombinant GDHX

The gene encoding GDHX of *Halobacterium salinarum* NRC-36014 strain was cloned in the pET5a expression vector and overexpressed successfully in *E. coli* strain BL21(DE3) as inclusion bodies. As shown in Fig. 1a, the SDS-PAGE analysis of supernatant and corresponding pellet displayed a strong band in the insoluble fraction corresponding in size to the GDHX (M_r ~47.5 kDa) from *Hbt. salinarum* NRC-36014 (Ingoldsby et al. 2005). Recombinant protein was solubilised with 8 M urea and in vitro renaturation was initiated by the removal of the denaturant either by dialysis or rapid dilution. Unfortunately, despite exploration of multiple permutations of conditions and additives, all in vitro refolding experiments provided either insoluble aggregates or soluble inactive

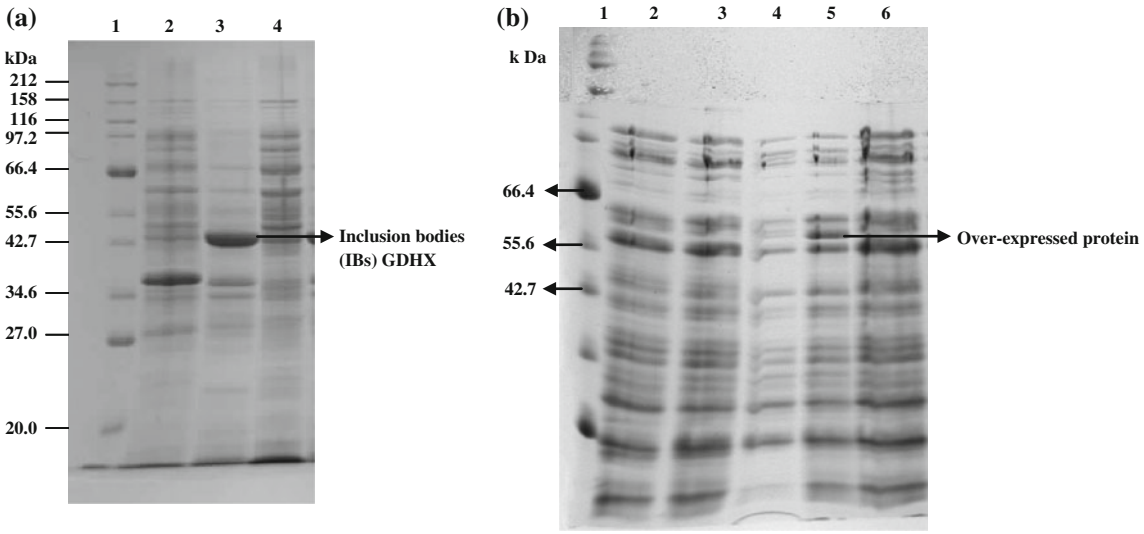

Fig. 1 SDS-PAGE of overexpression of GDHX. **a** Over-expression in *E. coli* as insoluble fraction: *lane 1* M_r markers, *lane 2 E. coli* crude extract without pET5a vector (control), *lane 3* GDHX (pellet), *lane 4* GDHX (supernatant). **b** Soluble expression in halophilic host using 4 mM tryptophan: *lane 1* M_r markers, *lane 2 Hfx. volcanii* (crude extract), *lane 3 Hfx. volcanii* + pRV1-tna + 4 mM tryptophan (crude extract), *lane 4 Hfx. volcanii* + pRV1-GDHX (crude extract), *lane 5 Hfx. volcanii* + pRV1-GDHX + 4 mM tryptophan (crude extract), *lane 6 Hfx. volcanii* + pRV1-GDHX + 4 mM tryptophan (pellet)

protein, suggesting that none of the conditions tested were suitable for refolding of protein in its native active state.

Expression of recombinant GDHX in halophilic host, *Hfx. volcanii*

Haloferax volcanii DS70 strain, a halophilic host, was used for over-expression of NAD^+-dependent GDHX to get a soluble and active enzyme. *Hfx. volcanii* competent cells were transformed with pRV1-GDHX and a few colonies were picked for the expression using 4 mM tryptophan for induction. *Hfx. volcanii* cells containing empty pRV1-tna plasmid with or without tryptophan were used as a control. The analysis of the expression by SDS-PAGE (Fig. 1b) showed an over-expressed band corresponding to an approximate apparent M_r of 60 kDa in the soluble fraction. This band was absent from the control cells without plasmid and also from cells with empty plasmid (without insert) induced with 4 mM tryptophan.

All cell extracts were used to measure NAD^+-dependent GDH activity by cuvette assay using standard GDHX assay buffer. A very low activity was found in control samples (i) *Hfx. volcanii* crude extract (0.006 U/mg), (ii) *Hfx. volcanii* + pRV1-tna vector without insert + 4 mM tryptophan (0.008 U/mg). Since *Hfx. volcanii* DS70 is not a GDH knock-out strain and has three endogenous genes for GDH (Hartman et al. 2010) of unknown coenzyme specificity, some NAD^+-GDH activity was expected in control cell lysates. Approximately the same NAD^+-GDH activity values in both controls and the same protein profile (Fig. 1b, lanes 2 and 3) showed that the presence of tryptophan did not markedly change the expression of any gene of the host cells. However, NAD^+-GDH activity was ~800 times higher (6.52 U/mg) in the induced sample as compared to controls, corresponding to the observed over-expressed recombinant GDHX band. Slight activity detected in control samples owing to endogenous GDH was subtracted when activity was measured in over-expressed samples.

Identification of overexpressed band by MS

Though the activity from the endogenous *Hfx. volcanii* gene(s) was very low as compared to the total activity of the over-expressed samples, it was important to identify if the over-expressed band belonged to GDHX. The gene sequences for all four *Hbt. salinarum* GDH(s) have been reported already (Ingoldsby et al. 2005) and it was hoped that mass spectrometric analysis would allow unambiguous identification of the overexpressed protein band. To obtain stretches of internal sequence, the over-expressed band on the previously performed SDS-PAGE was subjected to trypsin digestion. The results indicated trace contamination with keratins and with the trypsin used for digestion. However, data base searching showed perfect matching of digested peptides with NAD^+-GDH of *Halobacterium* species in Genbank entry P29051 (GDHX sequence) (Fig. 2). None of the peptides matched the amino acid sequence(s) of endogenous *Hfx. volcanii* GDH(s) indicating that the overexpressed band belonged to GDHX.

Purification of recombinant protein

An efficient method, modified from the previously described procedure by Bonete et al. (1986), was developed for purification of recombinant GDHX from the halophilic host (*Hfx. volcanii*). Cell-free extract was loaded on a pre-equilibrated Sepharose 4B column and eluted with a decreasing gradient of 2.5–0.5 M $(NH_4)_2SO_4$. The partially purified (1.8-fold) enzyme eluted at around 1.6 M $(NH_4)_2SO_4$ in 69 % yield. SDS-PAGE analysis of pooled fractions from this hydrophobic interaction step revealed a major protein band with many contaminant bands (Fig. 3, lane 3), and therefore another purification step was attempted with DEAE-cellulose chromatography. As shown in Fig. 3, lane 4, active fractions pooled from DEAE-cellulose column still had many contaminants which needed to be removed before further characterization of this enzyme. The pooled fractions were heated at 90 °C for 30 min yielding ~95 % pure protein with ~56 % retention of activity (Fig. 3, lane 5; Table 1). This purification procedure, summarized in Table 1, gave an overall recovery of 30 % of the total over-expressed enzyme protein**.** Purified enzyme was found to be stable for several months at 4 °C.

Optimum salt concentration for activity

GDHX activity in both directions of reaction was determined by cuvette assay with increasing concentrations of NaCl and KCl. The salts had different effects on GDHX activity. The enzyme showed activity in the absence of the added salt which initially increased with increasing concentrations of salt, showing optimum activity in the forward direction at 0.6 M for both KCl and NaCl (Fig. 4a). For reductive amination, on the other hand, the enzyme showed unusual behaviour (Fig. 4b): the activity obtained in the absence of added salt decreased with increasing KCl concentration. However, with NaCl stimulation was observed, with optimum activity being achieved at 3–3.5 M.

Optimum pH

The effect of pH on GDHX activity was studied for both oxidative deamination and reductive amination reactions. The assay mixture was adjusted to various pH values

```
H.V.GDH1      -MASAAPSTATDDEPT-------EETALETARRQLERAAAHLDVDPGVIERLRHPNQVHR 52
H.V.GDH2      -MMNEDGKNRNGEAVTDGGSGSEPETALATARRQLERAASHADVDDGVVARLKHPTRVQQ 59
GDHX          MTMASKSDSTHDESGDEAADSTEPESALETARRQLYHAASYLDIDQNIVERLKYPKKVHE 60
H.V.GDH3      --MAQEAN-----------------PFESLQEQIDDAATYLDVRDDVIERLKNPERVLE 40
                    .                        .: : :.*:  **:: *:  .:: **: * :* .
H.V.GDH1      VSVPLERDDGSTAVYTGYRAQHDSVRGPYKGGLRFHPDVTEAECIGLSMWMTWKCAVMDL 112
H.V.GDH2      VSVPLRRDDGSLDVFTGFRAQHDDVRGPYKGGLRYHPEVTADECIGLSMWMTWKCAVMDL 119
GDHX          VTIPIERDDGTVEVFTGYRAQHDSVRGPYKGGLRYHPDVTRDECVGLGMWMTWKCAVMDL 120
H.V.GDH3      TNLAVEMDDGDVELFRAYRSQFNGDRGPYKGGIRYHPNVSRDEVKALSGWMVYKCAVVDI 100
              ..:.:. ***   :: .:*:*.:. *******:*:**:*:  *  .*. **.:****:*:
H.V.GDH1      PFGGGKGGVVVDPKDLSTDEKERLTRRFAEELRPFIGPTKDIPAPDMGTDAQTMAWFMDA 172
H.V.GDH2      PFGGGKGGVAVDPKTLSDEERERLTRRFAEEIRNVVGPKKDVPAPDMGTGPQEMAWFMDA 179
GDHX          PFGGAKGGVAVNPKELSPEEKERLTRRFTQEIRDVIGPNQDIPAPDMGTDPQTMAWLMDA 180
H.V.GDH3      PYGGGKGGIVIDPKAYSESELERITRSFAKELRPLVGESRDIPAPDVNTGQREMNWIKDT 160
              *:**.***:.::**  * .* **:** *::*:* .:* .:*:****:.*. : * *: *:
H.V.GDH1      YSMQEGETKAGVVTGKPPVVGGSEGRDTAPGRSVAIIARETIDDLGWDIEDTTVAVQGFG 232
H.V.GDH2      YSMQQGETTPGVVTGKPPVIGGSYGREEAPGRSVAIVTREAVDFYDWDIEDTTVAVQGFG 239
GDHX          YSMQEGETTPGVVTGKPPVVGGSEGREEAPGRSVAIITQLVCEYYDQPLDETTVAVQGYG 240
H.V.GDH3      YETLENTTAPGVITGKALSNGGSEGRVEATGRSTMLTAREAFDYLGRDIEGATVAVQGYG 220
              *.  :. * .**:***.   *** **  *.***. : :: . :  .  :: :******:*
H.V.GDH1      SVGAPAARLLDDEGATVVAVSDVNGAIYDPDGLDTHDVPTHEEEPEAVMKYDAPRKLSNE 292
H.V.GDH2      SVGANAARLLDEWGAKVVAVSDVDGAIYDPDGLDTQDVEGHDERPGMVSGYDAPESLSNE 299
GDHX          SVGANAARLLDKWGATIVAISDVNGAMYEPDGIDTASVPSHDEEPEAVTTY-ADTVISNE 299
H.V.GDH3      NAGSIAARLVEDLGASVVAVSDSSGGIYDPDGLDTRAVKDFKNETGTVSDYEGTEAITNE 280
              ..*: ****::. **.:**:** .*.:*:***:**  *  ..:..  *  * .   ::**
H.V.GDH1      ELLELDVDVLIPAAVGNVLTAENADDVRADLVVEGANGPTTSAADEIFEARGILVVPDIL 352
H.V.GDH2      ELLELDVDVLIPAAIGNVITTENVDSISAEMVVEGANGPTTFAADAVLEERGIPVIPDIL 359
GDHX          ELLTLDVDVLIPAALGNVITKENAEAIAADLVVEGANGPTTSTADSILADRDVAVIPDIL 359
H.V.GDH3      ELLTLDVDLLVPAALENAIDGELAHDVKADVIVEAANGPLTPDADDVLTEREIHVFPDIL 340
              *** ****:*:***: *.:  * .. : *:::**.**** *  ** ::  * : *.****
H.V.GDH1      ANAGGVTVSYFEWLQDLNHRSWSLERVHDELETEMLRAWTAVRERVEEHDVT-WRDAAYM 411
H.V.GDH2      ANAGGVTVSYFEWLQDINRRQWSLERVHEELESEMLKAWNAVREHVEERDLT-WRDAAYV 418
GDHX          ANAGGVTVSYFEWLQDINRRAWSLERVNDELEAEMQAAWRAVKDEYENRDVT-WRDAAYI 418
H.V.GDH3      ANAGGVTVSYFEWVQNRQRFYWTEQRVNDELERVIVDAFEELVDACESHDLPNFRTAAYV 400
              **************:*: ::  *: :**::***  :  *:  : :  *.:*:. :* ***:
H.V.GDH1      VALERVSEAHEHRGLWP 428
H.V.GDH2      VALSRIGGAKETRGLWP 435
GDHX          VALSRIAEAHEARGLWP 435
H.V.GDH3      VAIRRVVDSYDSAGNWP 417
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Fig. 2 Detection of GDHX by mass spectrometry. Crude extract of *Hfx. volcanii* potentially containing GDHX, was loaded on SDS-PAGE and the over-expressed band was digested with trypsin. GDHX peptides identified by LCMS/MS, shown in bold and underlined, are different from the corresponding aligned sequences for the three endogenous GDH(s) from *Hfx. volcanii* (sequences are aligned using CLUSTALW)

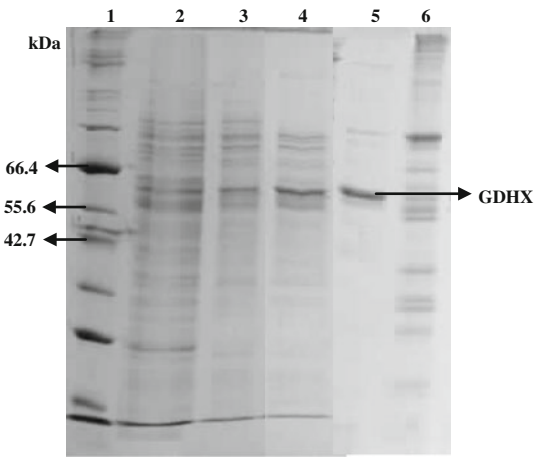

Fig. 3 SDS-PAGE of GDHX purification**.** *Lane 1* M_r markers, *lane 2* *Hfx. volcanii* + pRV1-GDHX + 4.0 mM tryptophan (crude extract), *lane 3* pooled Sepharose 4B fractions, *lane 4* pooled DEAE-cellulose fractions, *lane 5* 90 °C (supernatant), *lane 6* 90 °C (pellet)

between 6.5 and 10.5 using 80 mM KPi pH 6.5–8.0, 80 mM Tris–HCl pH 7.5–9.0 and 80 mM glycine-NaOH pH 8.5–10.5. These three buffers provided overlaps as a control for buffer-specific effects. The optimum pH was 9.2 for oxidative deamination and 8.5 for reductive amination (Fig. 5a). Under standard assay conditions at the optimum pH of each reaction, the amination rate was twofold higher than the deamination rate. Deamination activity increased 2.4-fold and amination activity 2.7-fold when Tris–HCl buffer was replaced by glycine-NaOH (Fig. 5b).

Temperature dependence

The purified GDHX was assayed at temperatures ranging from 40 to 90 °C in order to determine optimum temperature for catalysis. The activity increased with the increase in temperature (Fig. 6), and this enzyme remained functional even at 90 °C. The temperature stability of GDHX

Table 1 Purification yield of recombinant GDHX from *Hfx. volcanii* cells

Purification step	Volume (ml)	Total protein (mg)	Activity (U/mg)	Purification (-fold)	Yield (%)
Crude extract	17	120.7	3.1	1	100
Pooled Sepharose 4B	39	46.9	5.6	1.8	69
Pooled DEAE-cellulose	19	17.1	9.0	2.9	40.7
Heat treatment: 90 °C for 30 min	18.5	3.12	35	11.3	29.6

The activity was measured in the reverse direction (amination assay). Protein concentration was estimated by Bradford assay (BioRad). Most active eluted fractions were pooled from columns and directly assayed before the next purification step

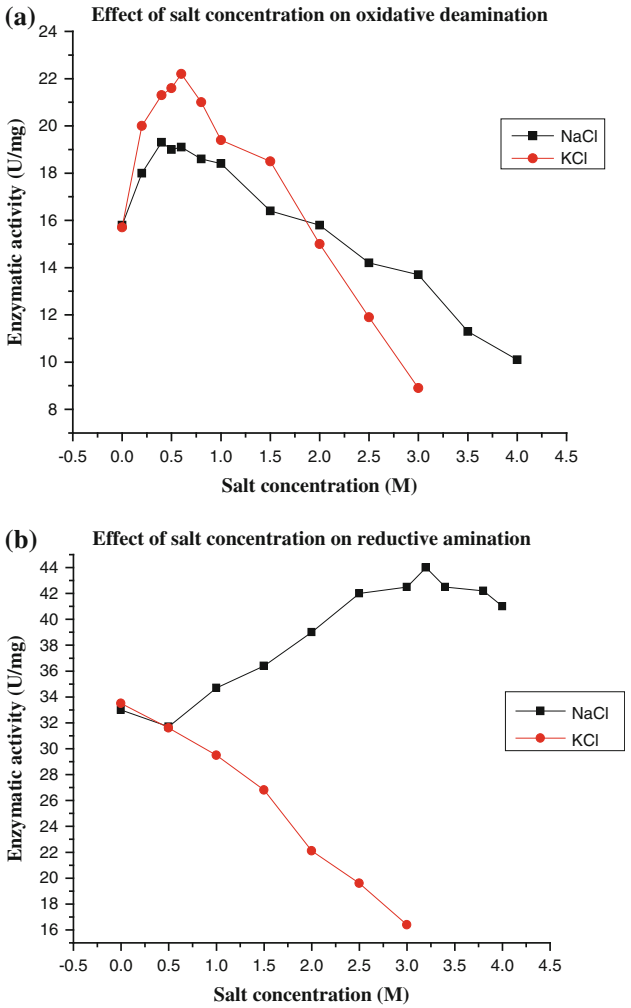

Fig. 4 Salt dependence of activity. The effect of increasing NaCl and KCl concentrations on **a** oxidative deamination and **b** reductive amination reactions

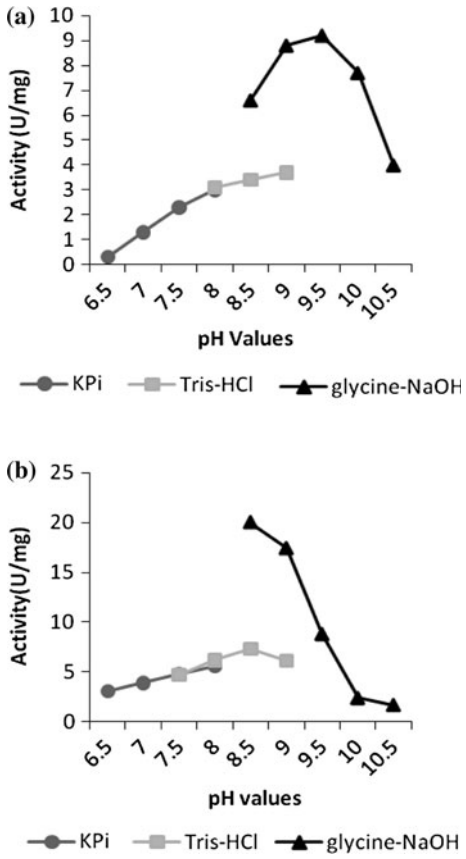

Fig. 5 pH dependence of activity. **A**ctivity was assayed at various pH(s) using standard method for **a** oxidative deamination and **b** reductive amination

was studied by incubating the enzyme for 30 min at temperatures from 50 to 90 °C, then removing and cooling immediately on ice before measuring the residual activity at 40 °C. The relative activity was expressed as a percentage based on the maximum activity of the unheated control as 100 %. The enzyme was entirely stable from 50 to 70 °C (Fig. 6) and lost only 12 % activity at 80 °C. The thermostability of this enzyme is quite remarkable as 55 % activity was retained even after 30 min at 90 °C.

Stability and activity in organic solvents

Because salt has the effect of reducing water activity, enzymes from the halophilic archaea are thought to be important biocatalysts in aqueous/organic and non-aqueous media (Adams et al. 1995). It was of interest, therefore, to investigate the effects of organic solvents on the activity and stability of halophilic GDH. Under optimal salt concentrations GDHX was, if anything, slightly activated in

presence of 10 % glycerol or DMSO (Table 2). However, the same concentrations (10 %) of ethanol, acetone, acetonitrile and 2-propanol in the reaction mixture inhibited catalytic activity by 65–70 %. Methanol and dimethyl formamide gave lesser extents of inhibition (26 and 49 %, respectively).

The effect of organic solvents on the stability of GDHX was analysed by pre-incubating it at room temperature for 72 h in aqueous buffer solutions containing 5 or 10 % organic solvent. GDHX retained 100 % activity in the aqueous DMSO mixtures, and more than 80 % activity was found in 10 % MeOH and 10 % acetonitrile. Moreover, 73 and 63 % activities were retained in 10 % glycerol and DMF respectively (Table 2). On the other hand, 2-propanol was found to be a bad organic cosolvent for GDHX as 94 % of activity was lost over 3 days in 10 % solution. In general and as expected, less activity was lost in 5 % organic solvents as compared to 10 % (Table 2).

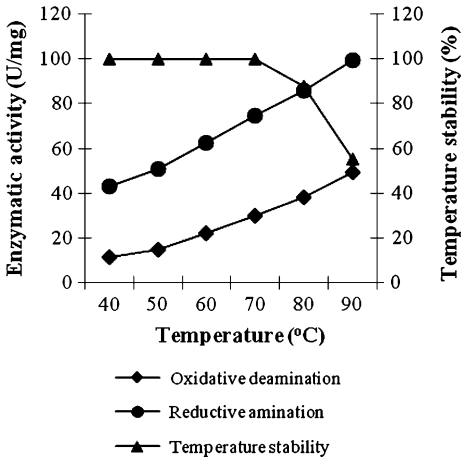

Fig. 6 Effect of temperature on GDHX activity and stability. Activity was studied by carrying out the enzyme reactions at different temperatures in the range 40–90 °C. The thermostability was measured by incubating GDHX solutions at various temperatures ranging from 50 to 90 °C for 30 min and the remaining activity was measured at 40 °C

As described above, GDHX was 100 % stable at 70 °C in aqueous buffer. Stability at this elevated temperature was also investigated in the presence of 10 % MeOH and EtOH over 24 h as MeOH and EtOH are commonly used to dissolve many water-insoluble compounds. To obtain measurable rates, the enzyme solution was diluted to a final concentration of 0.086 mg/ml in 50 mM KPi buffer pH 7.3, containing 4 M NaCl and 10 % MeOH or EtOH. The control had the same enzyme concentration in aqueous buffer plus salt. Duplicate samples were incubated at 70 °C and assayed periodically using the reductive amination assay. As shown in Fig. 7a, adding MeOH and EtOH to the enzyme solution resulted in 10 and 33 % loss in activity, respectively, at zero time. Unexpectedly, 20 % loss in activity was found in the control sample (diluted enzyme in 4 M NaCl buffer without any organic solvent) after 5 min incubation at 70 °C. However, GDHX retained the remaining 80 % activity for 4 h. In brief, after 24 h incubation at 70 °C, the control sample enzyme retained 56 %

Table 2 Stability and activity of GDHX in the presence of organic solvents

Organic solvent	Polarity index (I)[a]	Residual activity (%)[b] in 5 % organic solvents		Residual activity (%) in 10 % organic solvents		Relative activity (%)[c] in 10 % organic solvent buffers
		0 time	3 days	0 time	3 days	
None (control)		100	100	100	100	**100** (43)
DMSO	6.4	103	103	104	100	**102** (44)
DMF	7.2	100	86	94	63	**51** (22)
Glycerol		105	87	103	73	**109** (46.5)
Methanol (MeOH)	5.1	100	96	96	88.5	**74** (32)
Ethanol (EtOH)	4.3	87.3	68	66	40	**34** (14.6)
Acetone	5.1	89	56	72.3	38	**38** (16.4)
Acetonitrile	5.8	95	95	91.3	82	**30** (12.7)
2-propanol	3.9	71	20	42.2	6.4	**33** (14.1)

The figure in bold is a relative activity (%) compared to activity in non-solvent medium while the figure in bracket is a specific activity (U mg^{-1})

[a] Polarity index (I) is a measure of the ability of the solvent to interact with various polar test solutes (from Gupta et al. 1997)

[b] GDHX was pre-incubated in the presence and absence of organic solvents at room temperature in 50 mM potassium phosphate buffer containing 4 M NaCl. The residual activity was measured in standard conditions. The percentage of remaining activity relative to the non-solvent control is shown

[c] GDHX activity was measured in 80 mM Glycine-NaOH buffer containing 2.6 mM EDTA and 3.2 M NaCl (pH 8.5) with and without 10 % organic solvents. The figure in bold is a relative activity (%) compared to activity in non-solvent medium while the figure in bracket is a specific activity (Umg^{-1})

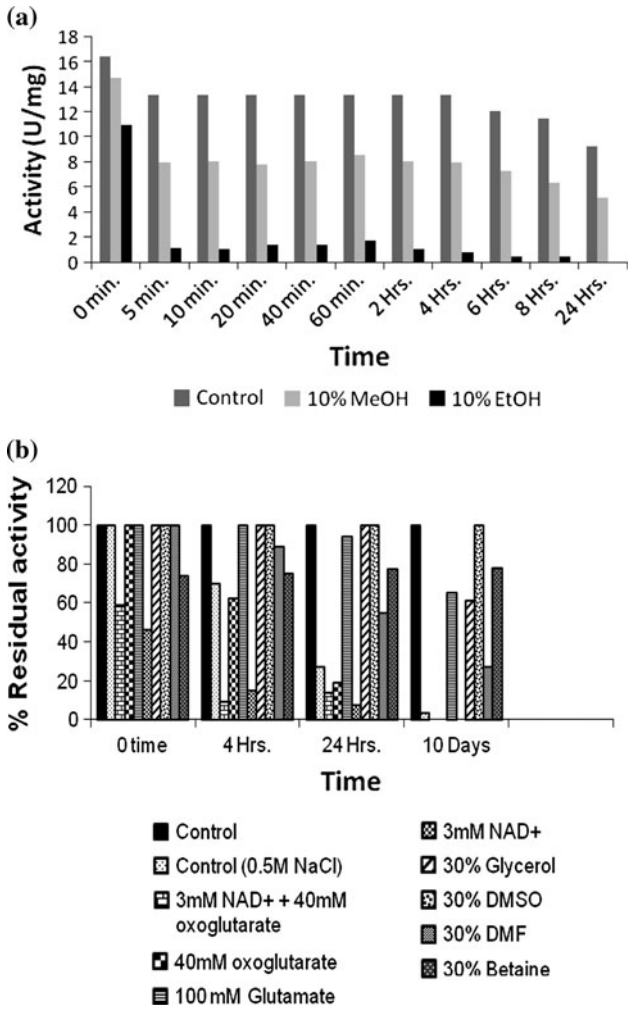

Fig. 7 Stability of GDHX in organic solvents and under suboptimal salt conditions. **a** Stability of GDHX in 10 % MeOH and EtOH at 70 °C. Enzyme was pre-incubated with and without 10 % MeOH and EtOH at 70 °C for 24 h and remaining activity relative to control was measured under standard assay conditions. **b** Stability of GDHX in 0.5 M NaCl in the presence of different additives. Enzyme solution was incubated under suboptimal salt concentrations in the presence of different additives at 4 °C and the remaining activity relative to controls was measured after known intervals of time

activity when compared with the original activity, more than 35 % of the activity was retained in 10 % MeOH, but no activity was detected in 10 % EtOH (Fig. 7a).

Protection of GDHX by additives against inactivation at suboptimal salt concentrations

Since the stability and activity of GDHX was observed to be 100 % in 10 % DMSO and it is also reasonably stable and active in glycerol and DMF (Table 2), it was decided to study the possible stabilizing effect of these solvents at suboptimal salt concentrations. The enzyme solution was incubated at 4 °C in 0.5 M NaCl containing 30 % (*v*/*v*) of the above-mentioned osmolytes. As the presence of substrates frequently enhances folding and stability of enzymes, e.g. NAD^+-dependent glutamate dehydrogenase from *Clostridium symbiosum* (Aghajanian and Engel 1997), the stabilizing effect of glutamate and 2-oxoglutarate on GDHX was also studied under suboptimal salt concentrations. The residual activity was measured in duplicate samples. GDHX in 4 M NaCl and in 0.5 M NaCl without any additive was used as control.

As shown in Fig. 7b, incubation in 0.5 M NaCl without additives resulted in the 75 % loss of activity in 24 h. However, after 24 h in 30 % DMSO the enzyme showed more than 100 % of the initial activity. Surprisingly, 22 % immediate loss in activity was observed when enzyme was diluted in the buffer containing 0.5 M NaCl and 30 % betaine. However, remarkably, after this initial loss, the enzyme retained the remaining 78 % activity throughout the 10-day period of observation. This stability reflects both on the intrinsic properties of the protein itself and on the purity of the preparation in terms of freedom from proteinase contamination. Among the four osmolytes tested, DMF had least stabilizing effect with only 27 % activity remaining after 10 days of incubation. Interestingly, 65 % GDHX activity was retained in the presence of 100 mM glutamate in low salt concentration.

Effect of DMSO on GDHX activity in the absence of salt

DMSO is one of the most extensively used solvents in chemistry, medicine and biotechnology. Therefore, following the above-mentioned experiment where enzyme showed 100 % stability in DMSO at a suboptimal salt concentration, it was decided to observe the effect on enzymatic activity of GDHX of entirely replacing sodium chloride with different concentrations of DMSO. Results for amination and deamination reactions are illustrated in Fig. 8a, b**.** In the forward direction, the enzyme showed highest activity in 10 % DMSO, but in the reverse direction maximum activity was observed in the presence of 20 % DMSO. It is evident from the figures that in both directions of reaction the enzyme showed higher activity than control over the range of DMSO concentrations. Interestingly, 26 % decrease in activity was observed when 3.2 M NaCl and 20 % DMSO were added together in the amination assay buffer (Fig. 8b, lane 4). However, increase in activity was observed when the same concentration of DMSO (20 %) was used in the reaction mixture without NaCl, suggesting that an appropriate hydrophobic environment is needed to stabilize the hydrophobic core and ultimately to achieve maximum catalytic activity of the enzyme. A slight decrease in activity was observed in 30 % DMSO without NaCl. The corresponding comparison in the presence of

3.2 M NaCl could not be made because it is not possible to dissolve NaCl to such a high concentration in 30 % DMSO.

Kinetic parameters

The apparent K_m and k_{cat} values displayed in Table 3 show the affinity and efficiency of the recombinant GDHX during amination and deamination reactions. A comparison of K_m values of pure recombinant enzyme with values reported for the partially purified enzyme from native organism by Bonete et al. (1986, 1987) shows approximately the same K_m value for NAD^+, but our K_m value for glutamate is 3.5-fold higher (14 mM) than that previously reported. On the other hand, K_m values for oxoglutarate, and ammonia are lower for recombinant GDHX than previously reported for these substrates.

Kinetic properties of the enzyme were also analysed after replacing NaCl with optimum DMSO concentrations for deamination (10 % DMSO) and amination (20 % DMSO) activity. As shown in Table 3, K_m values for all substrates except NADH decreased with overall increase in catalytic efficiency (k_{cat}/K_m) when NaCl was replaced with DMSO. The enzyme seems to have higher affinity for its substrates without salt but lower k_{cat} values for glutamate, NAD^+ and oxoglutarate in DMSO when compared with values in the presence of salt. A high salt concentration is thus still needed for optimal catalytic activity.

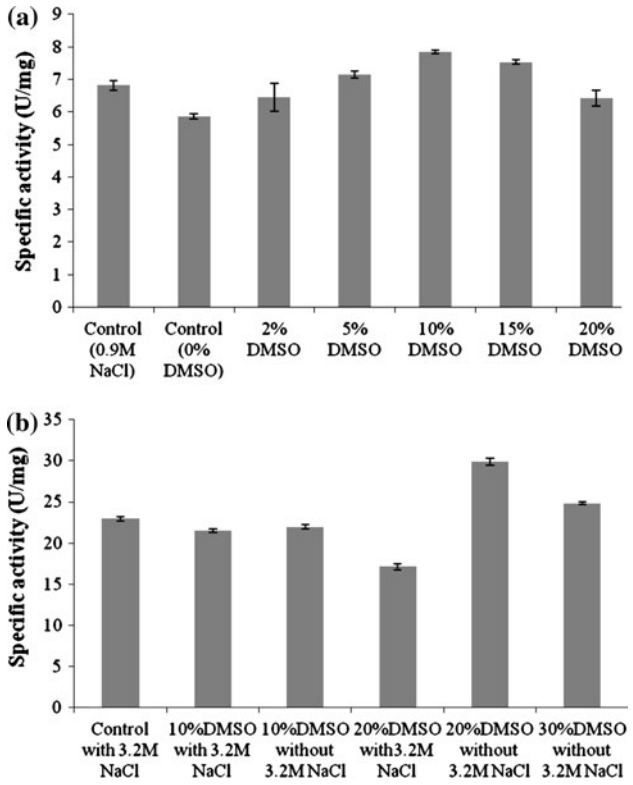

Fig. 8 Effect of DMSO on GDHX activity in the absence of additional salt. Rate of reaction was measured for 1 min. at 40 °C in increasing concentrations of DMSO in both directions under the conditions indicated **a** for the forward reaction and **b** for the reverse reaction

Discussion

Because the halophilic enzymes, in general, need high salt concentration to maintain their structure and biological activity, the heterologous expression of halophilic genes in a mesophilic host such as *E. coli* results mostly in insoluble expression products (Diaz et al. 2006) or sometimes a soluble, inactive product (Connaris et al. 1999) owing to the low ionic strength in the host cell. However, in view of our group's experience in refolding enzymes, we attempted first of all to take advantage of high levels of expression in *E. coli* and rely on the ability to reclaim soluble enzyme from the inclusion bodies. As shown above, in spite of exhaustive exploration of conditions and additives, this was a failure. To avoid all the above-mentioned problems, therefore, GDHX has been overexpressed in a halophilic

Table 3 Kinetic parameters calculated for recombinant GDHX in NaCl (normal font) and in DMSO (bold)

Substrate	Concentration range (mM)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Glutamate	0.5–100	14 ± 1.51	24 ± 0.89	1.7×10^3
		9.0 ± 0.7	**17 ± 0.4**	**1.9×10^3**
NAD^+	0.03–3	0.35 ± 0.015	16 ± 0.26	45×10^3
		0.13 ± 0.001	**15 ± 0.34**	**115×10^3**
2-oxoglutarate	1–40	3.4 ± 0.84	56 ± 3.8	16.5×10^3
		0.8 ± 0.04	**46 ± 0.67**	**57.5×10^3**
NH_4^+	25–800	118 ± 14.7	85 ± 3.5	0.72×10^3
		112 ± 12.8	**84 ± 3.42**	**0.75×10^3**
NADH	0.006–0.3	0.011 ± 0.002	39 ± 0.98	3.5×10^6
		0.025 ± 0.003	**47 ± 2.01**	**1.8×10^6**

host, *Haloferax volcanii,* using the pRV1-tna vector with its tryptophan-inducible promoter. In contrast to the expression of the ferredoxin gene from *Haloarcula japonica* strain TR-1 in *Haloferax volcanii* (Matsuo et al. 2001), the soluble and active expression of halophilic enzyme GDHX in a non-native halophilic host was here obtained successfully. It is noteworthy that the apparent subunit molecular weights estimated by SDS-PAGE were substantially different for the solubly expressed enzyme and for the inclusion bodies. It has been shown for other halophilic proteins (Hayden et al. 2002; Diaz et al. 2006) that an anomalously high subunit M_r is frequently obtained. This could possibly result from the high density of surface negative charge leading to low SDS binding (Tokunaga et al. 2008) and incomplete unfolding. It may be that the misfolded protein in the inclusion bodies can be more completely unfolded by the detergent, thus giving an M_r value closer to the true figure.

Overexpression of halophilic enzymes in a halophilic host could be useful to study the characteristics of many enzymes which are usually difficult to express in a soluble form in *E. coli* and/or to refold in vitro like GDHX in this case. Although it is not impossible to refold halophilic enzymes in vitro (Pire et al. 2001; Diaz et al. 2006; Hayden et al. 2002) optimizing the refolding conditions for every single protein is a time-consuming procedure. Expression in a halophilic host is a good solution of this problem allowing the protein to acquire native structure in a more or less natural environment. There is still, in theory, a question as to whether the recombinant enzyme expressed in this way will be identical to the normal biological product in the parent organism. Other work in our laboratory has focused on the expression of the various GDH genes in *Hbt. salinarum* (Ibrahim 2010) and we have not detected any significant differences in stability or other properties between recombinant GDHX and the naturally occurring GDHX.

On the other hand, purification of recombinant protein from halophilic host could be tricky when compared with purification from a mesophilic host (*E. coli*) where contaminant proteins can easily be precipitated out by the addition of ammonium sulphate (Connaris et al. 1999). It is important to maintain the high salt concentration during the purification of halophilic proteins as they are not stable in low salt concentrations. To make purification simple and fast, the five-step method described by Bonete et al. (1986) has been modified by reducing the number of steps. Conditions that bring about irreversible thermal inactivation of proteins are specific for each protein and this underlies the use of heat treatment as a purification tool for thermotolerant proteins (Ingoldsby 2005; Carrigan et al. 2005; Ibrahim 2010). The thermostability of GDHX was exploited, in combination with two chromatographic steps, to get rid of all contaminant proteins. In the new three-step procedure, the incorporation of the heat step at the end provided the best final yield of protein as compared to a sequence with the heat step at the beginning or in the middle of the procedure. The optimum pH values for oxidative deamination (pH 9.2) and for reductive amination (pH 8.5) are in good agreement with the general concept that GDHs catalyse the oxidative deamination of glutamate optimally at alkaline pH (8-10), while the pH optimum for reductive amination will generally be 0.5–2 pH units lower (Hudson and Daniel 1993). The K_m value for NAD^+ is similar to that achieved by Bonete et al. (1986) previously for this enzyme, while the values for other substrates appear to differ. Low K_m and high k_{cat}/K_m values for substrates in DMSO show great potential of this enzyme and possible derived mutants for application in chemical synthesis. With the added organic solvent, this enzyme is able to catalyse its reaction efficiently in the absence of any salt additional to the 80 mM glycine-NaOH buffer. $NADP^+$-dependent GDH from *Haloferax mediterranei* also showed low K_m values for substrates when 3 M KCl was replaced with 20 % (*v/v*) glycerol ((Ferrer et al. 2001) suggesting that replacement of salt with certain organic solvents may provide a more active conformation in the binding pocket of halophilic enzymes.

The remarkably high thermostability and catalytic activity of GDHX at elevated temperatures underline the biotechnological potential of this enzyme. A high optimal temperature for several halophilic enzymes has already been reported (Diaz et al. 2006; Ferrer et al. 1996; Perez-Pomares et al. 2003; Bonete et al. 1986) and it is thought to be a common feature among halophilic enzymes owing to the extreme temperatures the parent microorganisms sometimes face in their natural environment. In addition to thermostability, another highly desirable property for industrial applications of biocatalysts is stability in organic solvents. GDHX has been found to be more stable and active in more polar solvents. It is described in the literature that polar organic solvents can replace water in the essential water layer without damaging the protein structure (Reslow et al. 1992). The catalytic activity of GDHX is more than double in 10 %MeOH (74 %) when compared with EtOH (34 %). The difference in polarity index values (MeOH 5.1 and EtOH 4.3) for these solvents could explain this behaviour. Previously, *Haloferax mediterranei* protease was studied in organic solvent media, giving greater activity in solvents of increasing polarity (Stepanov et al. 1992). Moreover, higher activity in 5 % organic solvents when compared with 10 % (Table 2) reflects less competition of the organic solvents with water for the protein surface (Gupta et al. 1997). High stability and activity of recombinant GDHX under suboptimal salt concentrations in the presence of DMSO could be related to the salting out

properties of this organic solvent. DMSO was shown to stabilize the halophilic extracellular protease from *Natrialba magadii* (Ruiz and De Castro 1997) and also from *H. halobium* by Kim and Dordick (1997) who suggested that DMSO acts to satisfy the salting out requirement for protein stability. In this study, enhancement of the activity of the enzyme by replacement of NaCl with DMSO is another proof of this principle. Halophilic proteins require a salting-out environment because of limited hydrophobic interactions in their cores, and additives with salting-out properties increase the apparent hydrophobicity of the protein's core, in the same way that salt promotes a catalytically active conformation. Accordingly, salt can be replaced by those organic co-solvents that may act as salting-out additives (Kim and Dordick 1997). Stability of these enzymes in low salt concentrations in the presence of organic solvents would provide several advantages since (i) the corrosive effect of the salt on industrial reaction vessels is minimized, (ii) salt-sensitive reactions can be performed in a low-salt environment, (iii) organic solvents are likely to improve the solubility of many industrial substrates.

In conclusion, our data show the possibility of obtaining soluble and active halophilic proteins in good yield using a non-native halophilic host and reveal the robust properties of this enzyme under a variety of industrially relevant conditions. This enzyme could therefore offer an appropriate scaffold for protein engineering to construct novel biocatalyst(s) for industrial application.

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