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ORIGINAL PAPER

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Gene cloning, heterologous overexpression and optimized refolding of the NAD-glutamate dehydrogenase from *Haloferax mediterranei*

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Abstract The NAD-dependent glutamate dehydrogenase (GDH) gene from the halophilic archaeon *Haloferax mediterranei* has been cloned. The analysis of the nucleotide sequence revealed an open reading frame of 1323 bp that encodes a NAD-GDH. The amino acid sequence displayed high homology with those from other sources, especially the highly conserved residues involved in 2-oxoglutarate binding. The expression of this gene in *Escherichia coli*, the refolding and further characterization, yielded a fully active NAD-GDH with the same features than those found for the wild-type enzyme. This halophilic NAD-GDH showed a highly dependence on salts for both stability and activity, being essential for the refolding of the recombinant enzyme.

Keywords Heterologous overexpression · Refolding · Characterization · Halophilic glutamate dehydrogenase

Introduction

Halophilic *Archaea*, such as *Haloferax mediterranei*, live in hypersaline environments and maintain an osmotic balance by accumulating high intracellular salt concentration, mainly KCl, which is isotonic with the exterior (Kushner 1985). The folding, stability and, therefore, the function of halophilic proteins highly depend on salt concentration, as well as on the interactions between these macromolecules and the solvent, since their environment may be considered a low water one. Thus, the enzymes from halophiles may serve as model proteins in such conditions, which are especially important for biotechnological applications, such as biotransformations where a low water environment is required (Danson and Hough 1997; Connaris et al. 1999).

L-glutamate dehydrogenases (EC 1.4.1.2-4, GDH) catalyze the inter-conversion between 2-ketoglutarate and L-glutamate reversibly, playing a key role since they provide a link between carbon and nitrogen metabolism (Smith et al. 1975). They are classified into three groups according to their coenzyme specificity: NAD or NADP-specific and NAD(P)-non specific dependent GDH. Generally, in prokaryotic organisms, NAD-GDH displays a catabolic role; meanwhile NADP-GDH is a biosynthetic enzyme (Veronese et al. 1974; Bonete et al. 1987; Ferrer et al. 1996; Ruiz et al. 1998). The halophilic archaeon *Hfx. mediterranei* has at least two different GDHs. The NADP-GDH (EC 1.4.1.4) is an hexameric enzyme composed of monomers with a molecular mass of 55 kDa and its activity was markedly dependent on the salt concentration, being optimal at 1.5 M NaCl or KCl at 60°C and pH 8.5 (Ferrer et al. 1996). It also displayed a high thermostability, requiring temperatures above 60°C for measurable rates of thermodenaturation. The thermophilic nature of halophilic enzymes has been noted in several systems (Dym et al. 1995; Camacho et al. 1995; Bonete et al.1996). Dym et al. (1995) pointed out, taking into account the crystal structure of *Haloarcula marismortui* malate dehydrogenase, several of the structural features concerning halophilicity are the same as those, which contribute to the stability of thermophilic enzymes. The amino acid composition for this NADP-GDH was determined, and it showed a higher cumulative amount of acidic amino acids than basic amino acids, similar to other halophilic proteins (Camacho et 1995; Dym et al. 1995; Pire et al. 1996). Another GDH, NAD-dependent (EC 1.4.1.2), displays a different behavior that may be related to the different and essential roles that GDHs should play in the metabolism of organisms (Smith et al. 1975), including extreme halophilic *Archaea*; although it still "raising

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questions'' as to the role of the distinct GDH proteins, as recently reported for *Halobacterium salinarum*, which had at least four putative GDH genes in its genome (Ingoldsby et al. 2005).

At one stage, it was believed that NADP and dual-specific GDHs were closely related but that NAD dependent GDHs were only distantly, if at all, related. However, the gene sequencing of GDHs from a wider range of microorganisms has showed there is not a sharp evolutionary division between NAD- and NADP-dependent GDHs and, there is also a strong similarity of sequences between some GDHs of opposite coenzyme preference (Lilley and Engel 1992; Hayden et al. 2002).

The NAD-GDH from *Hfx. mediterranei* seems to be very close to NADP-GDH, sharing some properties. Its cloning, sequence and characterization, reported here, may provide us useful insights for a better understanding of the different role of these two important enzymes and the structural bases for special behavior, both halophilic and thermophilic.

The cloning of NAD-GDH from *Hfx. mediterranei* has an additional interest since large amounts of protein are needed to achieve structural studies in this important enzyme. Moreover, the cloning and refolding of this enzyme provides useful information about the optimal conditions, temperature, salt concentrations, pH, buffers, and others factors, in order to success in getting a full active halophilic enzyme.

Materials and methods

Bacterial strains and vectors

Haloferax mediterranei strain R4(ATCC 33500) was used throughout this study. The strains used for cloning and expression were: *E. coli* strain XL1-Blue (P2) MRA, used as host for lambda EMBL3 vector (Stratagene, CA, Germany); *E. coli* NovaBlue (Novagen, WI, USA), used as clone for plasmids pCR 2.1 and pET3a and the expression host used was *E. coli* BL21 (DE3). The vector pCR 2.1 was used for cloning the PCR product, using the TA Cloning kit (Invitrogen, UK). The expression vector pET3a was purchased from Novagen.

PCR amplification of a part of NAD-GDH gene

DNA from *Hfx. mediterranei* was achieved as previously described by Dyall-Smith and Doolittle (1994) and Bright et al. (1993). The oligonucleotide primers designed for amplification of the gene were: NterGDH (5′-GCSCAGGAGGCSAACCCSTTCGAG-3′), designed based on a sequence of N-terminus from NADP-GDH from *Hfx. mediterranei* (AQEANPFE, Ferrer et al. 1996) and consensus sequence (5′GCSGTCTTCCAS-GTCATCCA-3′) designed based on a highly conserved inner amino acid sequence MWMTWK, usually located 300 pb downstream the ATG starting codon.

Enzyme assay

Glutamate dehydrogenase activity was routinely determined spectrophotometrically at 340 nm and 40°C. The reaction mixture for the amination reaction contained 10 mM 2-oxoglutarate, 200 mM ammonium acetate and 0.03 mM NADH, and for the deamination reaction: 50 mM L-glutamate and 0.3 mM NAD, dissolved in 0.1 M NaOH-glycine buffer pH 9.0, 4 M NaCl and 3.25 mM EDTA. For the pH, temperature and kinetic studies, buffer and substrates were varied as described in each case.

Library screening and DNA sequence

The *Hfx. mediterranei* DNA was partially digested with *Sau*IIIA and size fractionated by agarose gel electrophoresis. Restriction fragments ranging from 15 to 20 Kb extracted from the gel were ligated into lambda vector, using the lambda EMBL3/BamHI vector kit (Stratagene). Ligated DNA was packaged ''in vitro'' with a phage λ packaging extract (Stratagene) and phages were plated on XL1-Blue MRA(P2).

For library screening, plaques hybridization was performed using as a probe the PCR fragment labeled with digoxigenin (Boehringer-Mannheim, UK). The positive lambda phages were isolated after double screening and the DNA obtained was purified by a lambda DNA preparation kit (Quiagen, CA, Germany). Nucleotide sequencing was performed by the method of Sanger et al. (1977) for both strands, on an automated ABI PRISM^{TM} 3100 DNA sequencer (Applied Biosystems, UK).

Sequence comparisons

SwissProt and PIR databases were searched for sequence similarities using the computer program FASTA and alignments with the GDH sequence were obtained with BESTFIT and GAP (Wisconsin Package Version 10.1, Genetics Computer Group (GCG), Madison, WI, USA).

Expression of recombinant GDH in *E. coli*

The GDH gene was amplified from the lambda EMBL3 construct by using primers containing adequate restriction-sites. The forward and reverse primers (5′-GCCACTCCGA**CATATG**CAGAC-3′) and (5′-GAG-TGCAGA**CCTAGG**GAG-3′) were designed to incorporate the sites *Nde*I and *Bam*HI, respectively. The PCR-amplified product was inserted in the vector pCR 2.1. The resulting clone, designated as pCR 2.1-*Hm*GDH, was transformed in *E. coli* NovaBlue cells, the resulting plasmid digested with *Nde*I and *Bam*HI was ligated into the expression vector pET3a. This clone,

designated as pET3a- *Hm*GDH, was expressed in *E. coli* BL21(DE3).

Cells of *E. coli* BL21(DE3) containing pET3a-*Hm*GDH were grown in Luria-Bertani (LB) medium containing 50 μg/ml ampicillin and induced by the addition of 0.4 mM IPTG. Cells were harvested by centrifugation and cell pellets resuspended in 20 mM Tris–HCl buffer, pH 7.4, 2 M NaCl and 1 mM EDTA (buffer A), treated with 100 μg/ml lysozyme and 0.1% (v/v) Triton-X100, incubated at 30°C for 1 h, then the solution incubated on ice for 15 min. This suspension was then sonicated and centrifuged at 14,000× *g* for 10 min. The insoluble fraction was isolated and washed twice in buffer A, yielding the inclusion bodies and the insoluble cell fragments.

Inclusion body solubilization, refolding and purification of recombinant GDH

The insoluble fraction was resuspended in 20 mM Tris–HCl buffer, pH 8.0, containing 8 M urea, 2 mM EDTA and 50 mM dithiothreitol (DTT). The solubilization was carried out at 37°C until no pellet was visible. Refolding of recombinant GDH was achieved by rapid dilution. The optimal conditions were tested by varying pH, in a range from 7 to 9, salt concentration, both NaCl and KCl from 2 to 4 M and the nature of the buffer, Tris–HCl, phosphate and glycine-NaOH, in the presence of EDTA (3.25 mM) and without EDTA. The protein folding was monitored by measuring the enzymatic activity. The activity was routinely measured as described above and the protein concentration determined by the Bradford method (Bradford 1976).

The active enzyme was purified using precipitation with 2.5 M ammonium sulfate and the solution centrifuged at 30,000× *g* for 30 min at 4°C, and supernatant was loaded onto a DEAE-cellulose in 50 mM Tris–HCl buffer, 2.5 M ammonium sulfate, pH 7.5. The recombinant enzyme was eluted with 50 mM Tris–HCl pH 8.0 buffer, containing 4 M NaCl. The purified enzyme was stored at room temperature.

Purification of the wild-type NAD-glutamate dehydrogenase

Hfx. mediterranei (R4, ATCC 33500) cells were grown aerobically at 37°C in a medium containing 25% (w/v) salts and 0.5% yeast extract (Difco) (pH 7.0–7.3) as described by Rodríguez-Valera et al. (1983), and supplemented with 2% ammonium sulfate. First, the cells were collected by centrifugation and suspended in 50 mM Tris–HCl buffer, 2.5 M ammonium sulfate, pH 7.0, second, disrupted by sonication and finally centrifuged at 4°C. The supernatant was used as crude cell extract.

The cell extract was applied to a Sepharose-4B column, and eluted using 50 mM Tris–HCl buffers, with an ammonium sulfate gradient from 2.5 to 0.5 M, 20% (w/v) glycerol. The fractions displaying enzymatic activity were pooled and loaded onto a Reactive Blue-Sepharose column equilibrated with 20 mM Tris–HCl pH 8.0 containing 20% glycerol. The halophilic enzyme was eluted with a gradient from 0 to 3 M NaCl in the same buffer. The active fractions were pooled and further purified by gel filtration in Sephacryl-S300 equilibrated in 20 mM Tris–HCl buffer, pH 8.0, 3 M NaCl.

Once purified, samples were analyzed by SDS-PAGE and the molecular mass for both, wild-type and recombinant enzyme was determined by analytical gel filtration, on a Pharmacia FPLC Sephacryl-S300 column (1.6×60 cm), previously equilibrated in 20 mM Tris–HCl buffer pH 8.0, 2 M NaCl and calibrated with different proteins as molecular mass markers from 425 kDa (ferritine) to 12.5 kDa (cytochrome *c*).

Effect of salt concentration on the activity and the stability of glutamate dehydrogenase

Both, wild type and recombinant enzymes, were tested for activity at different salt concentrations in a range of NaCl and KCl concentrations, from 0 M to 4 M in 50 mM Tris–HCl, pH 8.0 buffers.

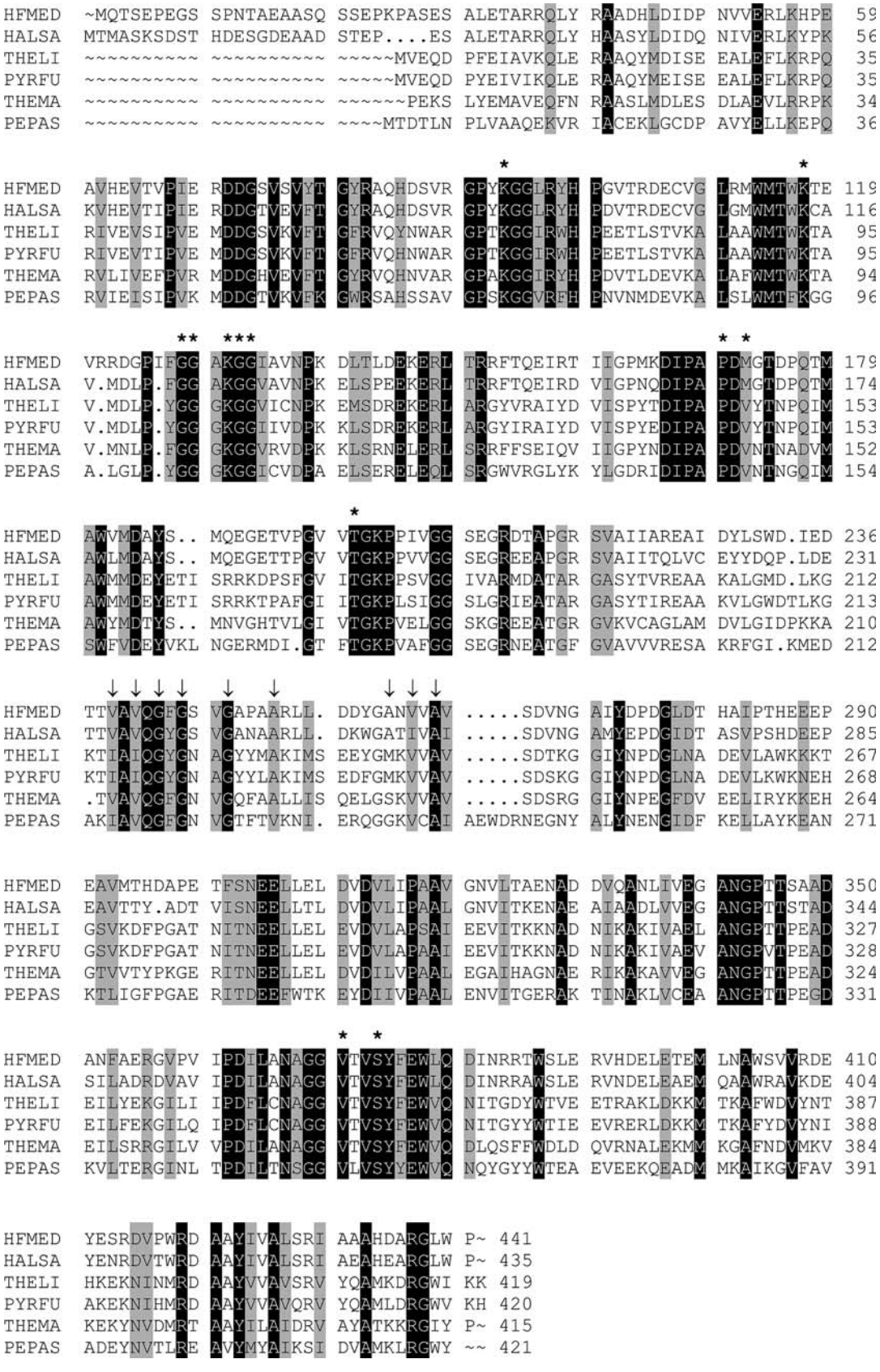
For stability assays, a stock of highly concentrated sample of each enzyme was used, reaching the salt concentration studied by adjusting it with 20 mM Tris–HCl, pH 8.0 buffer containing the salt concentration needed in each case, or no salts added. Aliquots of the enzyme incubated at each salt concentration were taken at different time intervals. The activity for each time was expressed as the logarithm of the residual activity related to the initial one, at zero time. The logarithm of relative activity obtained was plotted versus time to determine the pseudo first-order constant for the time-dependent lost of activity, as well as the half-life for the enzyme at each salt concentration.

Optimal pH and temperature

The GDH activities were tested for optimal pH in 100 mM Bis–Tris propane buffers for pHs from 6.5 to 10.0, all containing 4 M NaCl. The substrates NADH, 2-oxoglutarate and ammonium acetate were added in concentrations 0.3, 2.0 and 30 mM, respectively, for the amination reaction. The pH was checked after each reaction and showed no changes.

Data processing

Reciprocal initial velocities plotted versus reciprocal substrate concentrations were linear. Initial velocities (v) obtained at each salt concentration, pH or temperature, respectively, by varying the substrate concentration (S) were fitted to Eq. 1 to obtain values for maximum velocity (V), the Michaelis constant (K) for the substrate and the apparent first-order constant for the interaction of enzyme and substrate (V/K).


HFMED ~MQTSEPEGS SPNTAEAASQ SSEPKPASES ALETARRQLY RAADHLDIDP NVVERLKHPE 59
HALSA MTMASKSDST HDESGDEAAD STEP....ES ALETARRQLY HAASYLDIDQ NIVERLKYPK 56
THELI ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~MVEQD PFEIAVKQLE RAAQYMDISE EALEFLKRPQ 35
PYRFU ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~MVEQD PYEIVIKQLE RAAQYMEISE EALEFLKRPQ 35
THEMA ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~PEKS LYEMAVEQFN RAASLMDLES DLAEVLRRPK 34
PEPAS ~~~~~~~~~~ ~~~~~~~~~~ ~~~~MTDTLN PLVAAQEKVR IACEKLGCDP AVYELLKEPQ 36
HFMED AVHEVTVPIE RDDGSVSVYT GYRAQHDSVR GPYKGGLRYH PGVTRDECVG LRMWMTWKTE 119
HALSA KVHEVTIPIE RDDGTVEVFT GYRAQHDSVR GPYKGGLRYH PDVTRDECVG LGMWMTWKCA 116
THELI RIVEVSIPVE MDDGSVKVFT GFRVQYNWAR GPTKGGIRWH PEETLSTVKA LAAWMTWKTA 95
PYRFU RIVEVTIPVE MDDGSVKVFT GFRVQHNWAR GPTKGGIRWH PEETLSTVKA LAAWMTWKTA 95
THEMA RVLIVEFPVR MDDGHVEVFT GYRVQHNVAR GPAKGGIRYH PDVTLDEVKA LAFWMTWKTA 94
PEPAS RVIEISIPVK MDDGTVKVFK GWRSAHSSAV GPSKGGVRFH PNVNMDEVKA LSLWMTFKGG 96
HFMED VRRDGPIFGG AKGGIAVNPK DLTLDEKERL TRRFTQEIRT IIGPMKDIPA PDMGTDPQTM 179
HALSA V.MDLP.FGG AKGGVAVNPK ELSPEEKERL TRRFTQEIRD VIGPNQDIPA PDMGTDPQTM 174
THELI V.MDLP.YGG GKGGVICNPK EMSDREKERL ARGYVRAIYD VISPYTDIPA PDVYTNPQIM 153
PYRFU V.MDLP.YGG GKGGIIVDPK KLSDREKERL ARGYIRAIYD VISPYEDIPA PDVYTNPQIM 153
THEMA V.MNLP.FGG GKGGVRVDPK KLSRNELERL SRRFFSEIQV IIGPYNDIPA PDVNTNADVM 152
PEPAS A.LGLP.YGG GKGGICVDPA ELSERELEQL SRGWVRGLYK YLGDRIDIPA PDVNTNGQIM 154
HFMED AWVMDAYS.. MQEGETVPGV VTGKPPIVGG SEGRDTAPGR SVAIIAREAI DYLSWD.IED 236
HALSA AWLMDAYS.. MQEGETTPGV VTGKPPVVGG SEGREEAPGR SVAIITQLVC EYYDQP.LDE 231
THELI AWMMDEYETI SRRKDPSFGV ITGKPPSVGG IVARMDATAR GASYTVREAA KALGMD.LKG 212
PYRFU AWMMDEYETI SRRKTPAFGI ITGKPLSIGG SLGRIEATAR GASYTIREAA KVLGWDTLKG 213
THEMA AWYMDTYS.. MNVGHTVLGI VTGKPVELGG SKGREEATGR GVKVCAGLAM DVLGIDPKKA 210
PEPAS SWFVDEYVKL NGERMDI.GT FTGKPVAFGG SEGRNEATGF GVAVVVRESA KRFGI.KMED 212
HFMED TTVAVQGFGS VGAPAARLL. DDYGANVVAV .....SDVNG AIYDPDGLDT HAIPTHEEEP 290
HALSA TTVAVQGYGS VGANAARLL. DKWGATIVAI .....SDVNG AMYEPDGIDT ASVPSHDEEP 285
THELI KTIAIQGYGN AGYYMAKIMS EEYGMKVVAV .....SDTKG GIYNPDGLNA DEVLAWKKKT 267
PYRFU KTIAIQGYGN AGYYLAKIMS EDFGMKVVAV .....SDSKG GIYNPDGLNA DEVLKWKNEH 268
THEMA .TVAVQGFGN VGQFAALLIS QELGSKVVAV .....SDSRG GIYNPEGFDV EELIRYKKEH 264
PEPAS AKIAVQGFGN VGTFTVKNI. ERQGGKVCAI AEWDRNEGNY ALYNENGIDF KELLAYKEAN 271
HFMED EAVMTHDAPE TFSNEELLEL DVDVLIPAAV GNVLTAENAD DVQANLIVEG ANGPTTSAAD 350
HALSA EAVTTY.ADT VISNEELLTL DVDVLIPAAL GNVITKENAE AIAADLVVEG ANGPTTSTAD 344
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PYRFU GSVKDFPGAT NITNEELLEL EVDVLAPAAI EEVITKKNAD NIKAKIVAEV ANGPVTPEAD 328
THEMA GIVVTYPKGE RITNEELLEL DVDILVPAAL EGAIHAGNAE RIKAKAVVEG ANGPTTPEAD 324
PEPAS KTLIGFPGAE RITDEEFWTK EYDIIVPAAL ENVITGERAK TINAKLVCEA ANGPTTPEGD 331
HFMED ANFAERGVPV IPDILANAGG VTVSYFEWLQ DINRRTWSLE RVHDELETEM LNAWSVVRDE 410
HALSA SILADRDVAV IPDILANAGG VTVSYFEWLQ DINRRAWSLE RVNDELEAEM QAAWRAVKDE 404
THELI EILYEKGILI IPDFLCNAGG VTVSYFEWVQ NITGDYWTVE ETRAKLDKKM TKAFWDVYNT 387
PYRFU EILFEKGILQ IPDFLCNAGG VTVSYFEWVQ NITGYYWTIE EVRERLDKKM TKAFYDVYNI 388
THEMA EILSRRGILV VPDILANAGG VTVSYFEWVQ DLQSFFWDLD QVRNALEKMM KGAFNDVMKV 384
PEPAS KVLTERGINL TPDILTNSGG VLVSYYEWVQ NQYGYYWTEA EVEEKQEADM MKAIKGVFAV 391
HFMED YESRDVPWRD AAYIVALSRI AAAHDARGLW P~ 441
HALSA YENRDVTWRD AAYIVALSRI AEAHEARGLW P~ 435
THELI HKEKNINMRD AAYVVAVSRV YQAMKDRGWI KK 419
PYRFU AKEKNIHMRD AAYVVAVQRV YQAMLDRGWV KH 420
THEMA KEKYNVDMRT AAYILAIDRV AYATKKRGIY P~ 415
PEPAS ADEYNVTLRE AVYMYAIKSI DVAMKLRGWY ~~ 421

◀

Fig. 1 Alignments of amino acidic residues sequences for GDH from *Haloferax mediterranei* (HFMED), *Halobacterium salinarum* (HALSA), *Thermococcus litoralis* (THELI), *Pyrococcus furiosus* (PYRFU), *Thermotoga maritima* (THEMA) and *Peptostreptococcus asaccharolyticus* (PEPAS). Amino acid identities among the different GDHs are typed in *black boxes*. Homologous residues among the sequences are typed in *gray boxes*. Residues involved in binding the coenzyme are marked (*downarrow*) and residues, which are implicated in 2-oxoglutarate binding are marked (*asterisks*)

$$v = VS/(K + S) \quad (1)$$

using the algorithm of Marquardt–Levenberg with the SigmaPlot program (Jandel Scientific, Version 1.02, Germany).

Nanoelectrospray LC/MS analysis

Wild-type purified enzyme (30 mg/ml) was dialyzed against 100 mM ammonium hydrogen carbonate pH 8.5 at 4° overnight. The protein was then digested with trypsin 1:20 (enzyme: substrate) in 1 mM HCl. The digestion was incubated at 37°C for 18 h. After lyophilization, the sample was sent to Agilent Technology (Germany), where it was dissolved in 40 µl of 0.1% formic acid in water. The sample was analyzed in a nanoLC/Trap with an injection volume of 5 µl. Digested sample was processed using the SpectrumMill Bioinformatics software with the NCBI database. Pre-processing for SpectrumMill database search was done by Data-extractor implemented in SpectrumMill directly.

Results

Identification of the gene encoding for NAD-glutamate dehydrogenase

In order to identify the possible genes encoding GDH enzymes, a DNA fragment of 203 bp was amplified using two oligonucleotides based on the N-terminal sequence of *Hfx. mediterranei* NADP-dependent GDH and a conserved sequence for GDH from different organisms. This fragment was sequenced using the same oligonucleotide primers employed in its generation. The labeled PCR fragment was obtained and used as a probe for screening in a lambda library of *Hfx. mediterranei*. All the plaques from the second screening gave a strong hybridization signal. The DNA from positive phages was isolated and the coding region of *Hm*GDH sequenced. This region corresponded to an open reading frame of 1321 bp (AJ315142), encoding a protein of 441 amino acid residues (Q977U6), and identified as GDH by database searches. The molecular mass of the enzyme was estimated to be 47,950 Da. The sequence also contained the putative archaeal TATA box, (TTATC) located about 74 bp and Shine-Dalgarno sequence (GAGG) located a 16 bp upstream the starting codon ATG (Palmer and Daniels 1995).

Amino acid sequence analysis

The amino acid sequence of NAD-GDH (HFMED) was aligned with the sequences of *Hbt. salinarum* (HALSA) AN. P29051, *Thermococcus litoralis* (THELI) AN. Q56304, *Pyrococcus furiosus* (PYRFU) AN. P80319, *Thermotoga maritima* (THEMA) AN. P96110 and *Peptostreptococcus asaccharolyticus* (PEPAS) AN. P28997 (Fig. 1). This alignment displayed the high homology found for the regions containing the conserved residues, most of them identical in all compared GDHs.

Expression of NAD-GDH gene in *E. coli*

The gene encoding GDH was ligated to *Nde*I/*Bam*HI digested pET3a, as described in Materials and methods. Recombinant genes are transcribed in this vector by the T7 RNA polymerase (Studier et al. 1990; Pire et al.

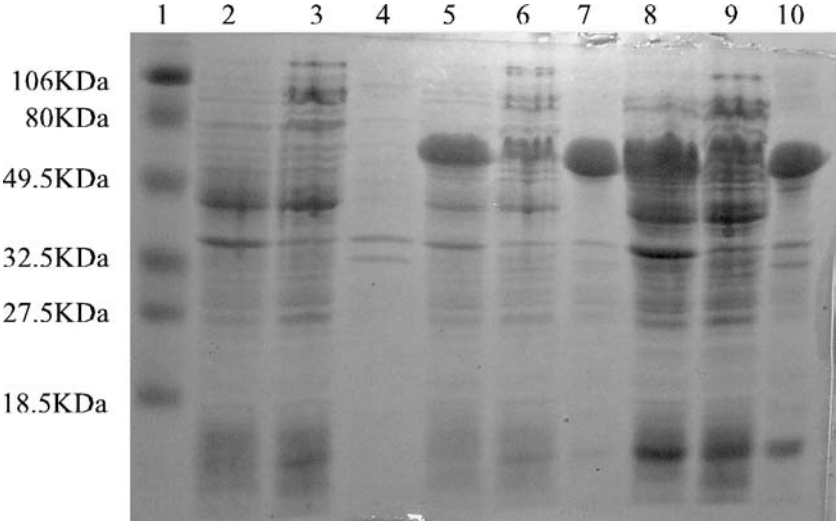

Fig. 2 Expression of the recombinant NAD-GDH from *Haloferax mediterranei* in *E. coli*. The fractions obtained, analysed by SDS-PAGE, are shown in the gel stained with Commassie Blue. *Lane 1* was for the standards, *Lanes 2– 4* for the sonicated extract from transformed *E. coli* BL21 with pET3a without insert, *Lanes 5– 7*, total proteins, soluble fraction and insoluble, respectively, they all are IPTG induced. *Lanes 8– 10* no induced fractions

2001). The gene encoding for T7 RNA polymerase is under the control of the *lac* promoter and may, therefore, be induced by IPTG. A high expression of recombinant GDH was obtained by addition of IPTG to a culture of *E. coli* BL21(DE3) transformed with the recombinant plasmid pET3a- *Hm*GDH. The protein samples obtained from the induced cultures were analyzed by SDS-PAGE. The gel, as shown in Fig. 2, displayed a very strong band in the insoluble fraction corresponding in size (subunit molecular mass) to the NAD-GDH from *Hfx. mediterranei*. This band was absent in the control cells, containing the plasmid pET3a without insert.

Solubilization and refolding of recombinant NAD-GDH

In order to obtain active enzyme, inclusion bodies were dissolved in 20 mM Tris–HCl buffer pH 8.0, containing 50 mM DTT, 2 mM EDTA and 8 M urea. NAD-GDH activity was restored optimally by 20-fold rapid dilution. The optimum-refolding buffer was 100 mM glycine-NaOH pH 8.5, containing 4 M NaCl and 3.25 mM EDTA. The refolding was also tried with KCl but the results were optimal with NaCl, as shown in Fig. 3. The plot of activity versus time indicates that enzyme refolding was also time dependent, with the maximum of activity about 30 h after the rapid dilution.

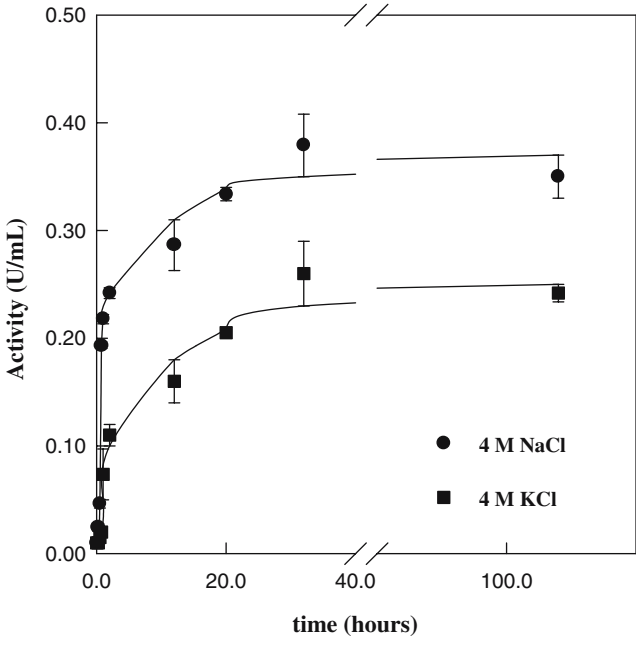

Fig. 3 Refolding of the recombinant NAD-GDH from *Hfx. mediterranei*. The solubilized inclusion bodies were refolded by rapid dilution 1:20 in 100 mM glycine-NaOH buffer, pH 8.5, 3.25 mM EDTA and 4 M NaCl (*filled circle*) or 4 M KCl (*filled square*), and the activity measured at different times to follow the refolding process

Purification and characterization of wild-type and recombinant NAD-GDH

The chromatogram for the purification on Sepharose 4B (Fig. 4) displays two different GDH activities. The first peak corresponds to the NADP-dependent GDH and was determined as described by Ferrer et al. (1996). The second one displayed NAD-dependent GDH activity. The fractions with higher NAD-GDH activity were pooled and purified as indicated in Table 1.

On the other hand, the refolded recombinant enzyme was purified as displayed in Table 2. The halophilic behavior of the NAD-GDH allowed the use of ammonium sulfate precipitation, as a first step, still being soluble even at 2.5 M ammonium sulfate. The chromatography in DEAE-cellulose produced a highly concentrated sample that was further purified by gel filtration.

The enzymes, wild type and recombinant, were applied to a Sephacryl-S300 column, yielding apparent molecular masses: 310 ± 40 kDa for the wild-type NAD-GDH and 290 ± 38 kDa for the recombinant.

The molecular mass for the subunits, estimated by SDS-PAGE, was 54 ± 4 kDa, value very close to 47,959 kDa, calculated from the sequence of the recombinant NAD-glutamate dehydrogenase.

Substrate specificity

In order to determine the coenzyme specificity for the GDH, activity was measured for the amination reaction varying the concentration of NADH and NADPH, in a range from 0.01 to 0.1 mM.

Both native and recombinant enzymes displayed high activity for NADH as coenzyme, but no activity was detected for NADPH as coenzyme, thus indicating its high specificity for NADH. In order to determine the

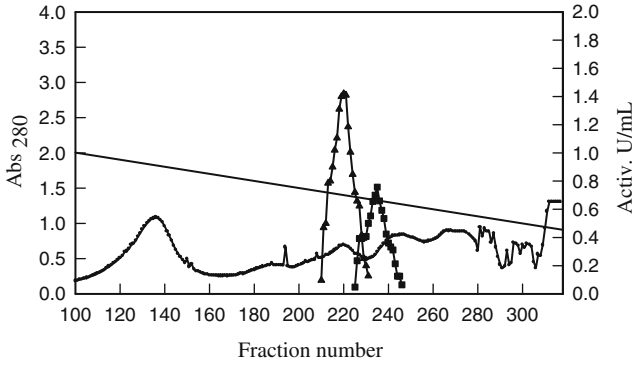

Fig. 4 Separation of the NADP- and NAD- GDHs from *Hfx. mediterranei* by chromatography on Sepharose 4B column, using an ammonium sulfate gradient from 2.5 to 0.5 M, 20% glycerol, as displayed by the straight line. The NADP-GDH (*filled triangle*) was measured in Tris–HCl buffer pH 8.5, 2 M NaCl as described in Ferrer et al. (1996). The NAD-GDH (*filled square*) was measured in 100 mM glycine-NaOH buffer pH 8.5, 4 M NaCl and 3.25 mM EDTA

Table 1 Purification of native NAD-GDH from *Hfx. mediterranei*

Purification step	Volume (ml)	Activity (U/ml)	Yield (%)	Protein conc. (mg/ml)	Specific activity (U/mg)	Purification factor
Supernatant	60	4.12	100	9.19	0.448	1
Sepharose-4B A.S gradient	89	1.74	63	3.65	4.77	10.6
Blue-Sepharose	32	4.21	54	0.070	60.5	135
Sephacryl-S300	36	0.81	12	0.0078	104	232

Table 2 Purification of recombinant NAD-GDH from *Hfx. mediterranei*

Purification step	Volume (ml)	Activity (U/ml)	Yield (%)	Protein conc. (mg/ml)	Specific activity (U/mg)	Purification factor
Solubilized inclusion bodies	50	0.33	100	0.051	6.39	1
Amonium sulphate precipitation supernatant	50	0.32	83	0.047	6.75	1.1
Amonium sulphate precipitation pellet	10	0.055	–	0.012	4.8	–
DEAE-cellulose	5	2.5	77	0.028	89.3	5.4

Table 3 Kinetic parameters for native and recombinant NAD-GDH determined for the amination reaction in 0.1 M Bis–Tris Propane buffer pH 8.0 and 4 M KCl

Substrate	NAD-GDH Native			NAD-GDH Recombinant		
	V_{app} (U/ml)	K_{app} (mM)	V/K	V_{app} (U/ml)	K_{app} (mM)	V/K
2-KG	0.671 ± 0.010	2.33 ± 0.11	288 ± 10	0.77 ± 0.03	2.5 ± 0.2	306 ± 22
NH_4^+	1.14 ± 0.15	90 ± 20	12.4 ± 1.2	1.06 ± 0.07	53 ± 6	20.0 ± 1.3
NADH	0.591 ± 0.006	$13.3 \times 10^{-3} \pm 0.8 \times 10^{-3}$	44 ± 2	0.728 ± 0.005	$13.1 \times 10^{-3} \pm 0.5 \times 10^{-3}$	55 ± 1.9

specificity for 2-oxoglutarate, the activity was measured in the presence of 2-oxobutirate and 2-oxovalerate, instead of 2-oxoglutarate in a range from 2 to 40 mM. The activity displayed by the enzyme with these oxoacids was too low to determine the kinetic parameter, again confirming the specificity for 2-oxoglutarate.

The kinetic parameter in the amination reaction for both, native and recombinant NAD-GDH are summarized in Table 3. The results obtained for recombinant NAD-GDH were very close to those obtained for the wild-type enzyme, thus indicating the same affinity for substrates, including the specificity for the coenzyme, and the same catalytic efficacy.

The enzyme, wild-type and recombinant, was also tested for the deamination reaction. The substrates L-glutamate and NAD were varied in a range from 10 mM to 100 mM and from 0.1 mM to 1 mM, respectively. The in vitro activities measured for this reaction were much lower than those found for the amination reaction, with Km for L-glutamate and V_{max} of about 40 mM and 0.05 U/ml, respectively. Other amino acids, L-Asp, L-His, L-Ala, L-Ser and L-Leu, were tested as possible substrates for the deamination reaction, but no activity was found at all.

The possible effect of these amino acids, 2-oxobutirate and 2-oxovalerate, added in the reaction mixture at 50 mM, on the activity of NAD-GDH for the amination reaction was also tested, and no significant changes in the activity were observed.

Effect of salt concentration on the activity and stability

The wild-type and recombinant NAD-GDH activities, determined in 100 mM Bis–Tris propane buffers pH 8.0 at different NaCl and KCl concentrations, are dependent on salt concentration reaching maximal activity at 4 M NaCl and KCl. The activity with KCl was slightly higher than that measured with NaCl.

The (residual) activity has been measured after incubating the enzyme at different salt concentrations and different times. The stability of both NAD-GDHs was tested at different NaCl concentrations. The half-life for each salt concentration, tabulated in Table 4, reached higher values as NaCl concentrations were increased. No significant loss of enzyme activity, wild type and recombinant, was observed when enzyme was stored both at 4 M NaCl or 2.5 M ammonium sulfate.

Activity dependence with pH and temperature

Both NAD-GDH activities, wild type and recombinant, were tested for optimal pH in the reactions they are able

Table 4 Effect of salt concentration on the stability of the wild-type and recombinant NAD-GDH. Half-life was determined from the plot of the logarithm of the residual activity versus time at each NaCl concentration

	wild-type	Recombinant
NaCl (M)	$t_{1/2}$ (h)	$t_{1/2}$ (h)
0.5	2.0 ± 0.2	4.9 ± 0.4
1.0	9.6 ± 0.9	13.4 ± 0.8
2.0	100 ± 6	80 ± 5
3.0	442 ± 23	927 ± 74

to catalyze. The activity for both enzymes, measured in 100 mM Bis–Tris propane, 4 M NaCl or KCl, and pHs between 6.3 and 9.5, reached maximal values at pHs in a range from 8.0 to 8.5. The results were the same at 4 M NaCl than using 4 M KCl. The optimal temperature, determined for both wild type and recombinant, in the presence of 4 M NaCl was 60°C, and it was also the same at 4 M KCl.

Nanoelectrospray LC/MS analysis

The sequences determined by mass spectrometry for the peptides obtained by Nanoelectrospray LC, were: RFTQEIRT, KDLTLDEKERL, KDLTLDEKE and RAADHLDIDPNVVERL. No sequence was obtained for N-terminal. The score of these peptides, obtained with the database, was 50.87 and the mean of peptide spectral intensity was 1.08×10^8.

Discussion

Halophilic enzymes have potential applications in biotransformations where low water environments are required. For this purpose, it would be necessary to produce these enzymes in sufficient amounts for large-scale use. The amounts obtained by purifying the enzyme directly from the native halophilic organism are far lower than those obtained by the expression of cloned genes in an halophilic host or a mesophilic host such as *E. coli*. The Archaeon *Hfx. volcanii* has been used as a host for the homologous overexpression of proteins such as dihydrolipoamide dehydrogenase from the same organism (Jolley et al. 1996). The other possibility, the heterologous overexpression in mesophilic organisms such as *E. coli*, overcomes the problems associated with growing halophiles. However, despite the obvious advantages of using *E. coli*, rapid growth, high biomass levels and its well known genetics, there may be difficulties in expressing halophilic genes due to the lack of the salt concentrations, in the internal environment, which these enzymes need to get their correct folding. Due to the advantages of heterologous expression, NAD-GDH from *Hfx. mediterranei* has been cloned and overexpressed in *E. coli*.

Initially, the sequence obtained showed high homology with GDHs from different sources. Identity was 73.3% with NAD-GDH from *Hbt. salinarum*, 47% with NADP-GDH from *Pyrococcus horikoshii*, 45.6% with NAD(P)-GDH from *Pyrococcus endeavori* and 46% with NAD-GDH from *Pyrococcus furiosus* (sequences obtained from the databases).

NAD-GDH from *Hfx. mediterranei* shows high similarity with the GDHs from hyperthermophilic archaea, especially for those residues implicated in 2-oxoglutarate binding site and NADH binding domain (Wang et al. 2003).

Wierenga et al. (1985) identified a conserved sequence characteristic of the nucleotide binding βαβ fold in dehydrogenases (Rossman-folding). In this motif, comprising about 26 amino acids, there were three conserved glycine residues with the sequence Gly-X-Gly-X-X-Gly, and there were six conserved hydrophobic residues, forming a hydrophobic core between the helix and β strands. For the halophilic NAD-GDH here reported, this motif was present at residues G243, G245 and G248, the hydrophobic core formed by the residues V239, V241, A252, A260, V262 and the last position occupied by the A264.

The GDH from *Hfx. mediterranei* was an enzyme with NAD-specificity, with a secondary structure formed by 16 α helix and 14 β strands. Generally, in NAD binding enzymes there is a negatively charged residue at C-terminus of the second β strand. The residues involved in binding 2-oxoglutarate were: K93, K117, K131, A169, P170, T199, V371 and S374, and the binding core was GGXXGGXXVNPK139. These residues are conserved in all sequences compared, with the exception of P170, which may be substituted by Gly in some cases. Comparing with GDH from *Clostridium symbiosum* (Teller et al. 1992), residues D166, I167/V, P168, A169, P170/G and D171 seems to be important in the stabilizing the face of the catalytic pocket.

The overexpressed protein was reactivated from inclusion bodies. Accumulation of inclusion bodies has been related to the kinetics of translation (De Bernardez 2001) when the rate of translation exceeds the rate of folding, resulting in an aggregated of protein. It may be a problem in the heterologous expression but sometimes, as in our case, it can be used to advantage given that it facilitates the purification from the cell lysate. Host proteins may be easily removed prior to solubilization (Connaris et al. 1999) and further eliminated in the purification process precipitating them in ammonium sulfate.

The method used for refolding the enzyme included rapid dilution of the solubilized inclusion bodies. The buffer conditions are critical in this step. The optimal buffer for refolding was glycine-NaOH including 4 M NaCl pH 8.5, 3.25 mM EDTA. The pH, salt concentration and nature of the buffer have to be carefully chosen to get the correct folding of the enzyme. It has been already reported the stimulating effect that amino acids display on the NAD-GDH from *Hbt. salinarum*

(Pérez-Pomares et al. 1999). The presence of glycine in the refolding buffer improves the correct folding of the enzyme. Even more important is the presence of high salt concentrations in the buffer. The optimal results were obtained with NaCl instead of KCl and at concentrations near saturation (4 M). The dependence on salt concentration for getting the correct folding is a common feature for halophilic enzymes. For example, refolding of dihydrolipoamide dehydrogenase from *Hfx. volcanii* was also achieved by dilution of the solubilized inclusion bodies in a buffer containing 2 M KCl, and moreover, citrate synthase from the same organism, produced in a soluble but inactive form, was reactivated simply by incubating with 2 M KCl (Connaris et al. 1999).

The archaeon *Hfx. mediterranei* has another GDH that differs in its specificity for the coenzyme. This NADP-GDH has been reported to be active in the amination reaction and possess a similar values of Km for 2-oxoglutarate, but far different for ammonium. The Km for ammonium displayed by the NADP-GDH was 4.2 mM (Ferrer et al. 1996), far different than that found for the NAD-GDH, 53–90 mM. However, this high Km is lower to that found for the NAD-GDH from *Hbt. salinarum* (450 mM) (Bonete et al. 1986), very likely due to the role of these enzymes may play when high ammonium concentrations are available to the organism. The recombinant NAD-GDH was also tested for substrates and coenzyme specificity, and again it was confirmed as an NAD specific GDH, that just shows significant activity when is measured in the reductive amination reaction, just as the wild-type one.

The characterization of both, recombinant and wild-type enzymes, showed the same dependence on salts for both activity and stability. The halophilic enzymes usually need high salt concentration for stability and optimal activity (Bonete et al. 1986, 1987, 1996; Dym et al.1995; Ferrer et al.1996; Danson and Hough 1997; Serrano et al. 1998; Martínez-Espinosa et al. 2001). SDS-PAGE revealed a subunit with an apparent molecular mass of 54 ± 4 kDa, slightly higher than 47,959 kDa calculated from the gene sequence. This feature is very common for halophilic enzymes and has been attributed to the intrinsic negative charge of these proteins (Bonete et al. 1996; Hayden et al. 2002). The NAD-GDH also shows this "excess" of negatively charged amino acidic residues that are located in its external surface as we saw by molecular modeling.

The calculated molecular mass of 310 kDa for wild type and 290 for recombinant NAD-GDH corresponds to a hexamerical enzyme. This feature is consistent with the GDH structures reported in available literature for this enzyme. The NAD-GDHs have either four identical subunits, as found in *Neurospora crassa* (Veronese et al. 1974) or six identical subunits (Baker et al. 1992).

The purification protocol followed for the wild-type NAD-GDH was very similar to those developed in our laboratory and used successfully in the purification of other halophilic enzymes as glucose dehydrogenase (Bonete et al. 1996), NADP-GDH (Ferrer et al. 1996), nitrate reductase (Martínez-Espinosa et al. 2001), α-amylase (Pérez-Pomares et al. 2003) and ferredoxin (Martínez-Espinosa et al. 2003) from *Hfx. mediterranei*, isocitrate dehydrogenase (Camacho et al. 1995) from *Hfx. volcanii* and *Sulfolobus solfataricus*, NAD and NADP-GDH (Bonete et al. 1986, 1987) from *Hbt. salinarum*. It has also been used for enzymes from Bacteria, as the NAD-GDH I and II (Bonete et al. 2003) from *Salinibacter ruber* and NAD-GDH from *Thermus thermophilus* HB8 (Ruiz et al. 1998). Moreover, the catalytic properties for the wild type and the recombinant enzyme were nearly identical, so the structure of the active site has to be similar, indicating a successful refolding process.

Wild type and recombinant NAD-GDH were very stable at salt concentrations, as high as 3 or 4 M, but they lost their stability dramatically when the salt concentration was lower than 2 M NaCl. For optimal activity, concentrations between 3 and 4 M NaCl or KCl were required. The halophilic nature of the enzyme was observed for both, activity and stability. This important feature allows the enzyme to work optimally at the high KCl concentrations that this enzyme has to endure in the interior of the halophilic organism they belong (Kushner 1985). The halophilic enzymes appear to be stabilized by salts according to the Hofmeister series ($SO_4^{=} > Cl^- >> NO_3^-$ and $Na^+ > K^+ > Cs^+$) (Werber et al. 1986); in fact NAD-GDH from *Hfx. mediterranei* was fully active for months in 4 M NaCl or 2.5 M ammonium sulphate. The amino acid sequence of NAD-GDH from *Hfx. mediterranei* reveals that this enzyme contains a high excess of acidic over basic residues that have to be counteracted by cations like potassium and sodium. These cations would be playing an essential role in the stability and the activity of this enzyme, although the role of cations and anions in the stabilization of halophilic enzymes is not always identical. For example, enzymes such as isocitrate dehydrogenase from *Hfx. volcanii* were found to be mainly sensitive to cations and very little sensitive to anions. However, other halophilic enzymes such as malate dehydrogenase from *Hfx. volcanii* were very sensitive to the effect of anions (Madern et al. 2004).

The high optimal temperature is also a common feature among halophilic enzymes. Other enzymes from *Hfx. mediterranei* such as NADP-GDH (60°C) (Ferrer et al. 1996), the extracellular α-amylase (50–60°C) (Pérez-Pomares et al. 2003) or the NAD and NADP GDHs from *Hbt. salinarum* (70°C) (Bonete et al. 1986), are also examples of the high thermophilicity of these halophilic enzymes and it may be an adaptive response to the extreme temperatures that they may endure in their natural environments. Although this behavior versus temperature may be related to similar structural features common to halophilic and thermophilic enzymes (Dym et al. 1995; Britton et al. 1998), the thermal stability might be achieved by totally different ways, even within the same family proteins (Knapp et al.

1997). Nevertheless, it can be found similarities between halophilic and thermophilic GDHs sequences, which may related to this behavior. In fact, the primary structure of halophilic GDH (*Hm*) displays similarities when it is compared with the GDHs from *T. maritima Tm*) and *P. furiosus Pf*) (Knapp et al. 1997). As the authors state, glycine residues are drastically reduced in the thermostable enzymes with regard to the mesophilic GDH from *C. symbiosum Cs*). The amino acid sequence of *Cs* GDH contains 48 glycine residues, whereas *Pf* and *Hm* contains only 34 and 35, respectively. Moreover, these authors suggest that a decrease in the sulphur content may be related with thermostability. The primary structures of *Hm* and *Pf* GDHs contain 10 and 12 methionine residues, respectively, in contrast to 17 methionine residues present in *Cs* GDH. Furthermore, the number of cysteine residues decreases from two in *Cs* GDH to one in *Pf* and *Hm* GDHs.

The structure determination of the *Pf* GDH has pointed out a potential role of ionic pairs clusters in the thermal stability of this enzyme, a feature shared by halophilic malate dehydrogenase (Dym et al. 1995; Rice et al. 1996; Britton et al. 1998). Ion pairs may be also implicated in the thermostability of *Hm* GDH as occurs in thermostable enzymes such as *Pf* GDH and *Tm* GDH (Rice et al. 1996; Knapp et al. 1997), but the 3D structure of *Hm* GDH would be necessary to confirm the implication of ion pairs in the thermostability of this enzyme.

The studies on the NAD-GDH from *Hfx. mediterranei* we report in this paper, and the recent overexpression of GDH from *Hbt. salinarum* are very useful to elucidate the controversy about the cofactor specificity of GDH from *Hbt. salinarum* since, not only two but four different GDH genes can be found into the *Hbt. salinarum* genome (Ingoldsby et al. 2005), so the comparison between different enzymes does not deal only on the substrate specificity but in similarities in kinetic behavior, structure and the role it may play in the organism. The NAD-GDH from *Hbt. salinarum* seems to be very similar in some sequence motives, but very different in its behavior, such as ''in vitro'' regulation. L-amino acids and other metabolites are reported to be activators and inhibitors of this GDH (Pérez-Pomares et al. 1996), but these metabolites, tested on the activity of the NAD-GDH here reported, displayed no detectable effect. Moreover, the NAD-GDH from *Hbt. salinarum* displays very high activity in the deamination reaction being supposed to be an enzyme with a catabolic role; meanwhile, *Hfx. mediterranei* enzyme displays very low activity in this way of reaction. Although these are in vitro assays, this is a clear difference to assure that the role of these two halophilic enzymes may be not the same in vivo.

On the other hand, the key position of GDHs in metabolism makes essential the right assignation of the roles that each GDH plays. The study of this metabolic regulation would be more readily achieved in *Hfx. mediterranei*, instead of *Hbt. salinarum*, because *Hfx. meditrranei* is able to grow in minimal mediums easily controlled.

Furthermore, since the wild-type and recombinant enzyme displays the same features and behavior, we can conclude that the overexpression of halophilic enzymes in a non halophilic organism such as *E. coli* may be used as an easy and quick method to get high concentration of enzyme that also can be easily refolded and purified following the method we are proposing here for the NAD-GDH.

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