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Extremely thermostable glutamate dehydrogenase (GDH) from the freshwater archaeon *Thermococcus waiotapuensis*: cloning and comparison with two marine hyperthermophilic GDHs

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Abstract Glutamate dehydrogenases (GDHs) from freshwater and marine hyperthermophilic Archaea were compared with respect to their responses to different salt concentrations. A gene encoding GDH from the terrestrial hyperthermophilic archaeon *Thermococcus waiotapuensis* (Twaio) was cloned, sequenced, and expressed at a high level in *Escherichia coli*. The deduced amino acid sequence, which consists of 418 amino acid residues, revealed a high degree of similarity with GDHs from related marine strains such as *Thermococcus litoralis* (Tl) and *Pyrococcus furiosus* (Pfu). Recombinant Twaio GDH was purified 27-fold to homogeneity. The enzyme is hexameric with a molecular weight of 259,000. The effects of several salts (KCl, $CaCl_2$, $MgSO_4$), temperature, and pH on enzyme activity were determined and compared in three hyperthermophilic GDHs, including *T. waiotapuensis*, and GDHs from two marine species, *T. litoralis* and *P. furiosus*. Kinetic studies suggested a biosynthetic role for the nicotinamide adenine dinucleotide phosphate- (NADP-) specific Twaio GDH in the cell. Interestingly, Twaio GDH revealed no salt responses, whereas the two marine GDHs showed substantial enhancement of activity as well as thermostability at increasing salt concentrations. Because electrostatic interactions between charged amino acid residues are thought to be a key feature of structural integrity and thermostability in hyperthermophilic GDHs, salt availability and its effects on marine enzymes could partially explain a higher thermal stability in marine species than in phyletically related freshwater species.

Key words *Thermococcus waiotapuensis* · Glutamate dehydrogenase · Hyperthermophile · Thermostability · Archaea · Salt · *Pyrococcus*

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Introduction

Hyperthermophilic microorganisms are able to grow optimally at temperatures above 90°C (Fisher et al. 1983; Fiala and Stetter 1986; Blochl et al. 1997; Stetter 1999). During the past two decades, great interest has been focused on the microbiological and biochemical properties of these microorganisms (Adams 1993, 1995; Erauso et al. 1993). Several hyperthermophiles thrive at temperatures well above the commonly accepted limits of stability for most cellular components, such as RNA, DNA, and proteins. For example, *Pyrolobus fumarii* has the highest recorded maximal growth temperature at 113°C. Novel mechanisms are presumed to confer stability on both proteins and nucleic acids under these extreme conditions. Most of the hyperthermophiles growing optimally at temperatures above 80°C have been isolated from marine geothermal areas and require elevated NaCl concentrations in their growth media.

Thermal stability studies have been performed on numerous proteins from several hyperthermophiles (Robb and Maeder 1998; Russell and Taylor 1995; Vieille and Zeikus 2001). Among these studies, glutamate dehydrogenase (GDH) is one of the most thoroughly studied enzymes and has been used as a model for understanding the molecular mechanisms involved in thermostability at around 100°C (Yip et al. 1995, 1998; Rice et al. 1996; Vetriani et al. 1998). Thermostable GDHs have been isolated and characterized during the past decade in several hyperthermophilic archaeal species including *Pyrococcus furiosus* (Consalvi et al. 1991b; Robb et al. 1992; Ohshima and Nishida 1993), *Pyrococcus woesei* (Ohshima and Nishida 1993), *Pyrococcus endeavori* (DiRuggiero et al. 1993), *Pyrococcus kodakaraensis* (Rahman et al. 1998b), *Thermococcus litoralis* (Ma et al. 1994), *Thermococcus zilligii* (Hudson et al. 1993), *Thermococcus profundus* (Kobayashi et al.

1995), *Sulfolobus solfataricus* (Schinkinger et al. 1991), *Archaeoglobus fulgidus* (Aalen et al. 1997), and *Pyrobaculum islandicum* (Kujo and Ohshima 1998).

GDH catalyzes the reversible nicotinamide adenine dinucleotide (phosphate)- (NAD(P)-) dependent oxidative deamination of L-glutamate to α-ketoglutarate and ammonia. Its primary role in the cell has been suggested to be biosynthetic (Consalvi et al. 1991b; Ohshima and Nishida 1993), but it is also thought to be responsible for glutamate catabolism in heterotrophic, proteolytic hyperthermophiles (Robb et al. 1992; Klump et al. 1992). Most hyperthermophilic GDHs are NADP (reduced) (NADP(H)) specific (DiRuggiero et al. 1993; Hudson et al. 1993; Ma et al. 1994; Kobayashi et al. 1995; Aalen et al. 1997), however. *Pyrococcus furiosus* (Consalvi et al. 1991b) and *S. solfataricus* (Consalvi et al. 1991a) GDHs have been reported to utilize both NAD(H) and NADP(H). *Pyrobaculum islandicum* GDH is unique among the Archaea because it utilizes only NAD(H) (Kujo and Ohshima 1998). Hyperthermophilic GDHs are hexameric (Consalvi et al. 1991b; Schinkinger et al. 1991; DiRuggiero et al. 1993; Oshima and Nishida 1993; Ma et al. 1994; Kobayashi et al. 1995; Aalen et al. 1997; Kujo and Ohshima 1998; Rahman et al. 1998b). Within the *Thermococcales* (*Pyrococcus* and *Thermococcus*), several closely related GDHs have been described. Most of these GDHs are of marine origin. Only two GDHs from freshwater hyperthermophiles have been reported (Hudson et al. 1993; Kujo and Ohshima 1998). Stimulation of GDH activity or thermostability by increased salt concentrations has been reported in the *Thermococcales* (Hudson et al. 1993; Ohshima and Nishida 1993; Ma et al. 1994). In contrast, the thermostability of the archaeon *Pyrobaculum islandicum*, isolated from a continental hot spring, appeared to be unaffected by salt (Kujo and Ohshima 1998).

The most thermostable GDH reported so far is the one found in *Pyrococcus furiosus*, and some molecular features of its thermostability were investigated through crystallographic study (Yip et al. 1995; Sedelnikova et al. 1996), comparison with mesophilic and less thermostable GDH species (Rice et al. 1996; Yip et al. 1998), and site-directed mutagenesis studies (Vetriani et al. 1998). *P. furiosus* GDH has relatively high charge density and possesses two major ion-pair networks (formed by 18 residues and 6 residues). These ion-pair networks are responsible for the thermal stability of *P. furiosus* GDH through intersubunit interactions and have been extensively described in recent publications (Yip et al. 1995, 1998; Rice et al. 1996; Vetriani et al. 1998). Although *P. furiosus* GDH is a clear example of thermal stabilization by ion pairs, so far there are trends but no exclusive mechanisms for thermostability among the hyperthermophilic enzymes studied (Vieille and Zeikus 2001).

In this study, we describe the cloning, expression, purification, and characterization of GDH from a hyperthermophilic archaeon, *Thermococcus waiotapuensis*, recently isolated by our group from a terrestrial hot spring in New Zealand (González et al. 1999). In addition, we compared the thermal stability and activity of *T. waiotapuensis* GDH with those of GDHs from two related marine species, *P. furiosus* and *T. litoralis*. This comparative analysis showed differential salt effects on the activity and thermostability of these GDHs that may yield new insights into the mechanisms of protein thermostability.

Materials and methods

Cloning of the *GDH* gene from *Thermococcus waiotapuensis*

Thermococcus waiotapuensis DNA was extracted from overnight cultures following the method described by Carbonnier and Forterre (1995). The *gdh* gene was amplified by PCR using a Perkin-Elmer/Cetus (Norwalk, CT, USA) model 2400 DNA thermal cycler. Forward and reverse primers were 5′-GAT GAC ATA TGG TTG AGC TTG ACC CAT TTG AAA TGG-3′ and 5′-AAT GTA CAT ATG TCA GTG CTT GAC CCA TCC-3′, respectively. Polymerase chain reaction (PCR) thermal conditions were 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 30 cycles. The amplified PCR product was purified using a Qiagen PCR purification kit (Qiagen, Valencia, CA, USA) and cloned into a TA vector (Invitrogen, Carlsbad, CA, USA). The primers used for amplification contained a *Nde*I restriction site (underlined) that allowed cloning of the gene at the corresponding site in the pET-11a expression vector (Novagen, Madison, WI, USA). *Nde*I-digested plasmid DNA was visualized by electrophoresis in a 1% agarose gel. The DNA bands corresponding to the digested *GDH* gene and pET-11a vector were excised, purified using a Qiagen gel extraction kit (Qiagen), and ligated together. The ligation mixture was used for the transformation of DH5α (Gibco, Invitrogen). Selection of the clones that had the correct orientation of the insert was performed by PCR using the T7 promoter primer (5′-TAA TAC GAC TCA CTA TAG GG-3′) and the backward primer listed above. Overexpression was obtained by transformation in the expression host *Escherichia coli* BL21 (Novagen). Overexpression was induced for 2.5 h by the addition of 0.4 mM isopropyl thiogalactoside (IPTG) to exponentially growing cultures of the recombinant strain (OD_{600} = 0.6).

DNA and protein sequencing

DNA was sequenced on an ABI 373A automated sequencer and the N-terminal amino acid sequences of native and recombinant GDHs were determined on a Beckman (Fullerton, CA, USA) LF 3000 protein sequencer. The nucleotide and amino acid sequences of the *T. waiotapuensis gdh* gene can be accessed under the accession number AF251788.

Purification of recombinant GDHs

Three recombinant GDHs were purified from *E. coli* BL21 strains harboring Twaio, Tl (Vetriani et al. 1998), and Pfu

(DiRuggiero and Robb 1995) GDH genes. *E. coli* BL21 cells overexpressing recombinant GDH genes were harvested by centrifugation (6,000 *g*, 20 min), washed in 0.9% NaCl, and resuspended in 2 ml TED buffer [50 mM Tris-HCl, pH 7.6, 1 mM ethylenediaminetetraacetic acid (EDTA0, 4 mM dithiothreitol (DTT)] per gram of cells. A single freezing and thawing step was used to lyse the cells. Cell lysate was heated at 75°C for 45 min to remove thermolabile *E. coli* proteins and assemble active GDH hexamers (DiRuggiero and Robb 1995). Denatured protein aggregates were removed by centrifugation at 48,000 *g* for 20 min at 4°C. Streptomycin sulfate was added dropwise to 1% w/v and the solution was incubated for 1 h at 4°C to precipitate nucleic acids. A clean supernatant was recovered by centrifugation at 48,000 *g* for 20 min at 4°C. This crude extract was diluted twofold with buffer A (50 mM Tris-HCl, pH 9.0, 1 mM EDTA, 1 mM DTT), and loaded onto a Q Sepharose (Pharmacia, Uppsala, Sweden) anion-exchange column (2.5 × 19.5 cm) equilibrated with the same buffer. The column was washed with buffer A, and the enzyme was eluted with a gradient from 0.1 to 0.6 M NaCl. GDH-containing fractions were combined, concentrated, and buffer-exchanged to buffer B (20 mM Tris-HCl, pH 8.0, 28 mM NaCl, 5 mM glutamate) by ultrafiltration in a stirred pressure cell (Amicon, Millipore, Bedford, MA, USA). Concentrated GDH fractions were loaded onto an affinity column (Matrix Red A; 2.5 × 11 cm, Amicon) equilibrated with buffer B. The column was washed with buffer B and followed by buffer C (20 mM Tris-HCl, pH 8, 28 m M NaCl). GDH was eluted by the direct injection of 2 mM NADP. All chromatographic procedures were conducted at room temperature.

Enzyme assays

GDH activity was routinely determined by oxidative deamination measuring the glutamate-dependent reduction of $NADP^+$ at 80°C using a Beckman DU 640 spectrophotometer equipped with a high-performance temperature controller. The reaction mixture contained 100 mM EPPS [*N*-(2-hydroxyethyl) piperazine-*N*′-(-3-propane-sulfonic acid)], pH 8.0, at 25°C, 2 mM L-glutamate, 0.5 mM NADP, and enzyme in a total volume of 325 µl. The reaction was initiated by the addition of NADP. One unit of the enzyme is defined as the amount required to produce 1 µmole of NADPH per minute. When required, reductive amination was measured at 80°C in a reaction mixture containing 100 mM EPPS, pH 8, at 25°C, 2 mM α-ketoglutarate, 50 mM NH_4Cl, and 0.5 mM NADPH.

Temperature and pH profiles

We compared the effects of temperature and pH on the oxidative deamination by recombinant Twaio, Tl, and Pfu GDHs. Optimum temperatures were determined in the standard assay mixture over a range from 50° to 97°C. In the pH dependence experiments, the following buffers were used: pH 5–6, 20 mM sodium acetate; pH 6–8, 20 mM sodium phosphate; pH 7–9, 100 mM EPPS; pH 9–10, 20 mM sodium carbonate-bicarbonate.

Thermostability

Thermostability of Twaio, Tl, and Pfu GDHs were estimated at 95°, 100°, and 104°C in an oil bath by measuring residual enzyme activity at various time points as described previously (Robb et al. 1992; Vetriani et al. 1998). Experiments were run at least in triplicate. Half-lives of enzyme activity were calculated from semilogarithmic plots of activity versus incubation time. Protein concentration during the thermostability assays was adjusted to 1.0 mg/ml in 100 mM EPPS (pH 8.0 at 25°C), and KCl was added at concentrations up to 1 M when indicated.

Protein analysis

Protein concentrations were determined by the Coomassie blue dye-binding method with bovine serum albumin (BSA) as a standard (Bio-Rad, Hercules, CA, USA). Molecular weight of the denatured monomer of GDH was estimated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and M_r of native GDH was estimated by gel filtration with a Sepharose S-300 (Pharmacia) column (2.5 × 113 cm) connected to a BioRad Econo system equilibrated with a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 50 mM NaCl.

Results and discussion

Sequence analysis of the gene encoding *Thermococcus waiotapuensis* GDH

The complete nucleotide sequence of the *T. waiotapuensis gdh* gene was determined and the deduced amino acid sequence was compared with GDHs from several hyperthermophilic archaeal species (Fig. 1). The deduced amino acid sequence of Twaio GDH consists of 418 amino acid residues and reveals a high degree of similarity and colinearity with other hyperthermophilic GDHs belonging to the *Thermococcales*. Strong ionic interactions through extensive ion-pair networks between subunits have been established as key features resulting in thermostability of Pfu and Tl GDHs near or above 100°C (Yip et al. 1995, 1998; Vetriani et al. 1998; Vieille and Zeikus 2001). Crystallography and comparative studies have led to characterization of two major ion-pair networks (Britton et al. 1995; Yip et al. 1995, 1998; Rice et al. 1996; Rahman et al. 1998a; Britton et al. 1999). The large one, which is conserved in Pfu and Tl GDHs, consists of 18 amino acid residues and the small one consists of 6 amino acid residues in Pfu and is reduced to 2 residues in Tl GDH. Twaio GDH retains the ion residues of the large network; however, residues Glu138 and Lys166 are replaced by Ser and Thr, respectively, reduc-

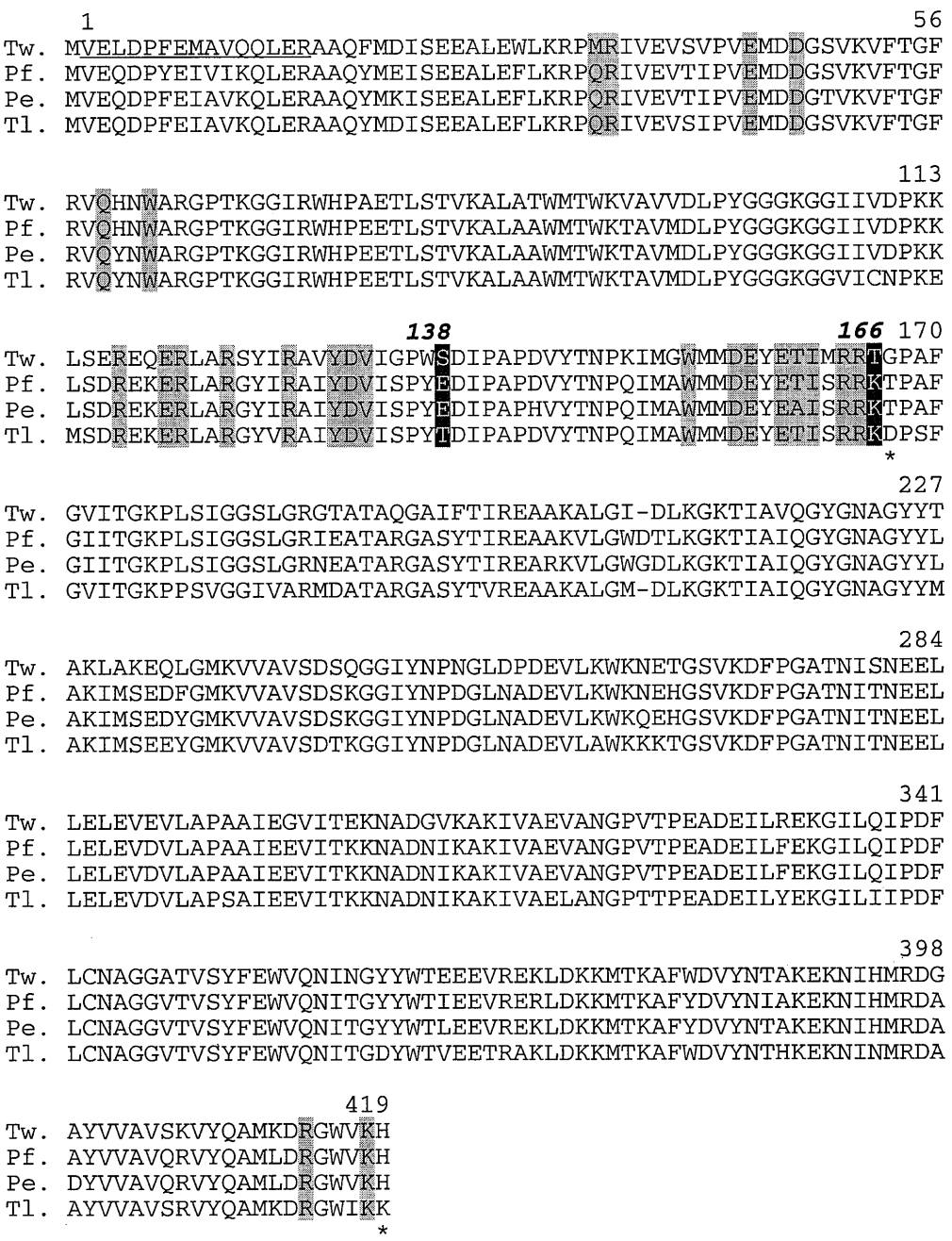

Fig. 1. Sequence alignment of several hyperthermophilic glutamate dehydrogenases (GDHs) belonging to the *Thermococcales*. Amino acid *numbering* is according to the sequence of native *Pyrococcus furiosus* GDH, which has been investigated intensively. *Thermococcus waiotapuensis* GDH retains the ion pairs (*gray shading*) in the large (18-residue) ion network of Pfu GDH, but in the small (6-residue) ion network, Glu138 and Lys166 are replaced by Ser and Thr, respectively (*black shading*). *Asterisks* indicate the additional sites of critical ionic interactions (residues 167 and 419 of Pfu GDH) derived from Vetriani et al. (1998). The N-terminal amino acid sequence of native Twaio GDH is *underlined*. *Tw*, *Thermococcus waiotapuensis*; *Pf*, *Pyrococcus furiosus*; *Pe*, *Pyrococcus endeavori*; *Tl*, *Thermococcus litoralis*

ing the small ion-pair network to 2 residues as found in Tl GDH. The amino-terminal sequence of native Twaio GDH showed a posttranslational modification in which the N-terminal methionine was removed as found in other hyperthermophilic GDHs (DiRuggiero et al. 1993; Ma et al. 1994; Kobayashi et al. 1995; Rahman et al. 1998b). Removal of the initial methionine is also thought to enhance thermal stability (DiRuggiero et al. 1993; Rahman et al. 1998b).

Purification of recombinant *Thermococcus waiotapuensis* GDH

Purification of the recombinant Twaio GDH is summarized in Table 1. Heat treatment was highly effective (8.8-fold purification) in removing *E. coli* proteins as shown by SDS-PAGE (Fig. 2A). Twaio GDH was purified 27-fold with 50% yield to homogeneity after two steps of column chromatography as confirmed by SDS-PAGE (Fig. 2A).

Molecular mass determination

Purified Twaio GDH produced a single band of 45,000 kDa on SDS-PAGE (Fig. 2A), and the molecular mass of the native protein determined by Sephacryl S-300 gel filtration chromatography was 259 kDa (Fig. 2B). This result reveals Twaio native GDH to be hexameric like other hyperthermophilic archaeal GDHs (Consalvi et al. 1991b; Schinkinger et al. 1991; Ohshima and Nishida 1993; Ma

Table 1. Purification of recombinant *Thermococcus waiotapuensis* glutamate dehydrogenase

Step	Total protein (mg)	Total activity (unit)[a]	Specific activity (unit/mg)	Yield (%)	Purification (fold)
Crude extract	707.9	4260	6.0	100.0	1
Heat treatment	85.0	4471	52.6	105.0	8.8
Q Sepharose	35.8	2720	76.0	63.8	12.7
Red A	13.4	2144	160.0	50.3	26.7

[a] One unit is defined as the amount of enzyme producing 1 µmole of nicotinamide adenine dinucleotide phosphate, reduced (NADPH) from NADP per minute

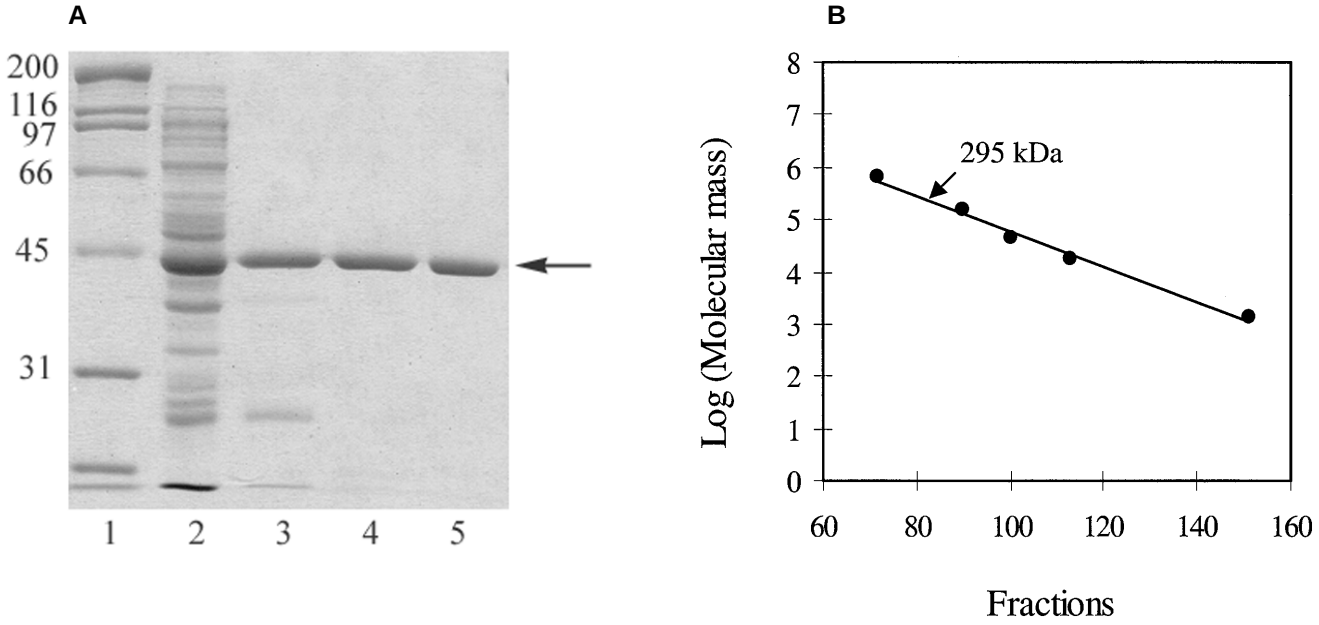

Fig. 2A,B. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purification steps and molecular weight estimation of native GDH by gel filtration chromatography. **A** SDS-PAGE profile. *Lane 1*, marker proteins; *lane 2*, crude extract; *lane 3*, supernatant after heat treatment; *lane 4*, Q Sepharose ion-exchange pool; *lane 5*, Red A affinity pool. Molecular standards were rabbit muscle myosin (200 kDa), *Escherichia coli* β-galactosidase (116.2 kDa), rabbit muscle phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), egg white ovalbumin (45 kDa), and bovine carbonic anhydrase (31 kDa). **B** Sephacryl S-300 gel filtration chromatography. Purified *T. waiotapuensis* recombinant GDH was eluted from the calibrated column, and the markers were bovine thyroglobulin (670 kDa), bovine gammaglobulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B_{12} (1.3 kDa). *Arrows*, *T. waiotapuensis* GDH

et al. 1994; Kobayashi et al. 1995; Aalen et al. 1997; Kujo and Ohshima 1998; Rahman et al. 1998b).

Effects of temperature and pH on enzyme activity

Temperature and pH profiles for the oxidative deamination of Twaio, Tl, and Pfu GDHs are shown in Fig. 3. Optimum growth temperatures for *Pyrococcus furiosus*, *Thermococcus litoralis*, and *T. waiotapuensis* are 100°, 88°, and 80°–85°C, respectively (Fiala and Stetter 1986; Neuner et al. 1990; González et al. 1999). These growth temperatures are related with the optimum reaction temperatures of the corresponding GDHs. Thus, Twaio GDH showed the lowest optimum temperature (84°C) among the tested species, and some decrease in specific activity was observed above 90°C, whereas the Tl and Pfu GDHs increased in specific activity at temperatures up to 97°C (Fig. 3A). At the low-temperature range below 65°C, Twaio GDH revealed the highest specific activity of the three GDHs. Molecular flexibility promotes substrate binding and catalysis, whereas the rigidity that accompanies thermostability apparently reduces low-temperature activity. Highly thermostable enzymes are significantly less flexible than their mesophilic counterparts (Daniel et al. 1996), which leads to an inverse relationship between stability and low-temperature catalytic function (Shoichet et al. 1995; Danson et al. 1996). Exceptions to this statement have also been reported (Hernández et al. 2000; Vieille and Zeikus 2001); however Tl GDH, which is significantly less stable than Pfu GDH (Rice et al. 1996; Vetriani et al. 1998; Britton et al. 1999), showed higher specific activity than Pfu GDH. Because the crystal structures of Pfu and Tl GDH have been reported to be very similar (Britton et al. 1995; Rice et al. 1996; Sedelnikova et al. 1996; Britton et al. 1999), the comparative study of these two enzymes can explain more clearly the

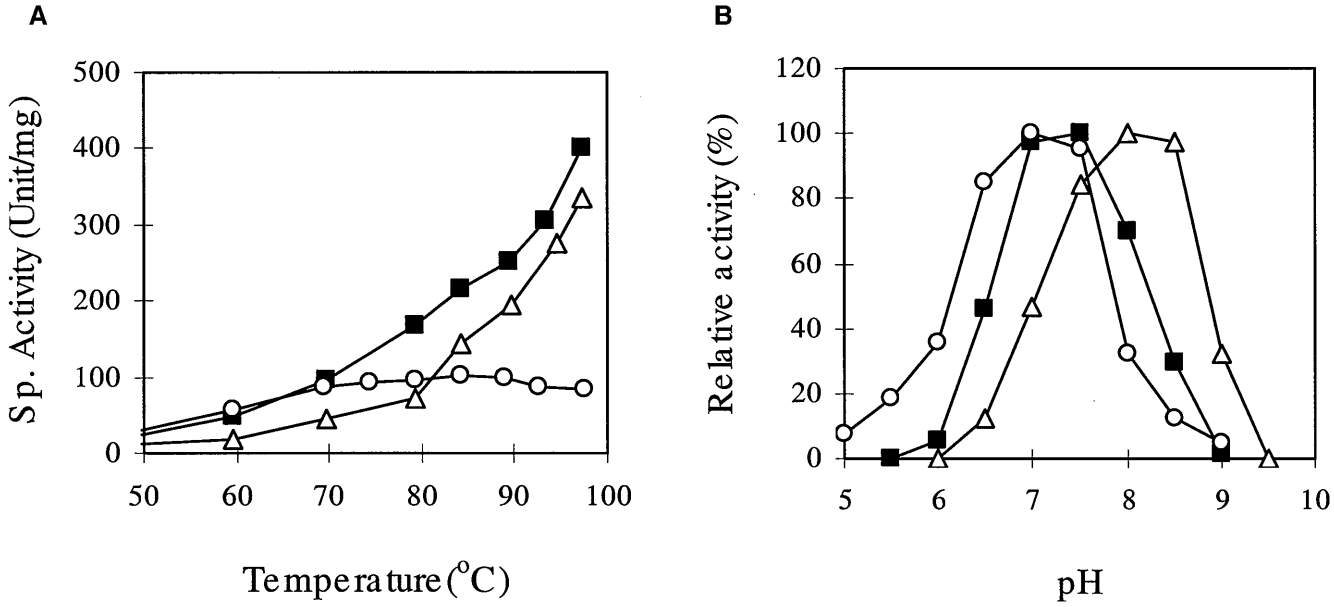

Fig. 3. Effects of temperature (**A**) and pH (**B**) on the activity of three hyperthermophilic GDHs from the *Thermococcales*. **A** GDH activity of *T. waiotapuensis* (*circles*), *T. litoralis* (*squares*), and *P. furiosus* (*triangles*). GDH activity was measured at various temperatures in the standard assay mixture. **B** GDH activity of *T. waiotapuensis* (*circles*), *T. litoralis* (*squares*), and *P. furiosus* (*triangles*)

relationship between rigidity or flexibility, thermal stability, and activity. The significantly different temperature ranges for the activity between Twaio GDH and those of Tl and Pfu GDHs confirm that trend.

The effects of pH on the activity of the three GDHs are shown in Fig. 3B. The optimum pH values for the oxidative deamination by Twaio, Tl, and Pfu GDH were pH 7.0, 7.5, and 8.0, respectively, at the assayed temperature. Twaio GDH showed the highest activity at neutral pH whereas most hyperthermophilic GDHs showed pH optima between 8 and 9.7 (Consalvi et al. 1991b; Hudson et al. 1993; Oshima and Nishida 1993; Kujo and Ohshima 1998; Rahman et al. 1998b). The optimum pH of Tl GDH was reported to be pH 8.0 (Ma et al. 1994), but the actual pH of the buffer used in that study (100 mM EPPS) at 80°C was 7.5.

Effects of ionic strength on GDH activity

Activity-enhancing effects of salts have been reported in a number of hyperthermophilic GDHs (Hudson et al. 1993; Ohshima and Nishida 1993; Ma et al. 1994; Aalen et al. 1997). Most of the studied species were of marine origin. *T. waiotapuensis* was isolated from a freshwater hot spring and grows optimally at low salt concentrations, failing to grow at NaCl concentrations greater than 1.8% (González et al. 1999). Interestingly, Twaio GDH showed no significant activity enhancement at increasing KCl concentrations (Fig. 4 A). Although *Thermococcus zilligii* was also isolated from a freshwater geothermal spring, *T. zilligii* GDH activity increased up to 4.3-fold in the presence of 0.3–0.4 M NaCl (Hudson et al. 1993). Pfu GDH activity showed salt dependence as previously reported (Ohshima and Nishida 1993). In our studies, Pfu GDH activity increased 6.2-fold in the presence of 0.5 M KCl, and 4.3-fold at 1 M KCl, compared with unsupplemented EPPS buffer. Tl GDH also showed enhanced activity at concentrations between 0.05 and 0.3 M KCl. GDH activity of *T. waiotapuensis* and *T. litoralis* decreased to 30% and 70%, respectively, at 1 M KCl.

Addition of $CaCl_2$ and $MgSO_4$ also showed enhancement of Pfu GDH activity (Fig. 4B). Pfu GDH showed a large increase of activity (3.5-fold) at 10 mM $CaCl_2$ or $MgSO_4$. GDH activity of *T. waiotapuensis* and *T. litoralis* increased very slightly (1.3-fold) at 10 mM concentration of either ion.

Thermostability

Half-lives ($t_{1/2}$) of Twaio and Tl GDHs were measured at three different temperatures with no addition of salt (Fig. 5A). Logarithmic plots of half-life versus temperature showed a similar pattern for these two GDHs. The half-life of Twaio GDH was about 85% of Tl GDH $t_{1/2}$ in 100 mM EPPS buffer (pH 8 at 25°C, pH 7.5 at 80°C). The half-life at 104°C was also determined with and without 1 M KCl (Fig. 5B). Half-lives of Twaio, Tl, and Pfu GDH were 4.2, 4.9, and 67 min, in the absence of KCl, and 3.3, 45, and 562 min, in the presence of 1 M KCl, respectively. In this study, increased thermostability in the presence of salts was found for *Pyrococcus furiosus* and *T. litoralis* (Fig. 5). A previous study (Ohshima and Nishida 1993) mentioned increased thermal stability of Pfu GDH at high KCl concentrations. The thermostability of Twaio GDH did not increase in the presence of salt (1 M KCl) and its half-life decreased to 79%. Tl and Pfu GDH were highly stabilized in the presence of KCl (Fig. 5C). This differential behavior can be related to the salt concentrations in the habitats

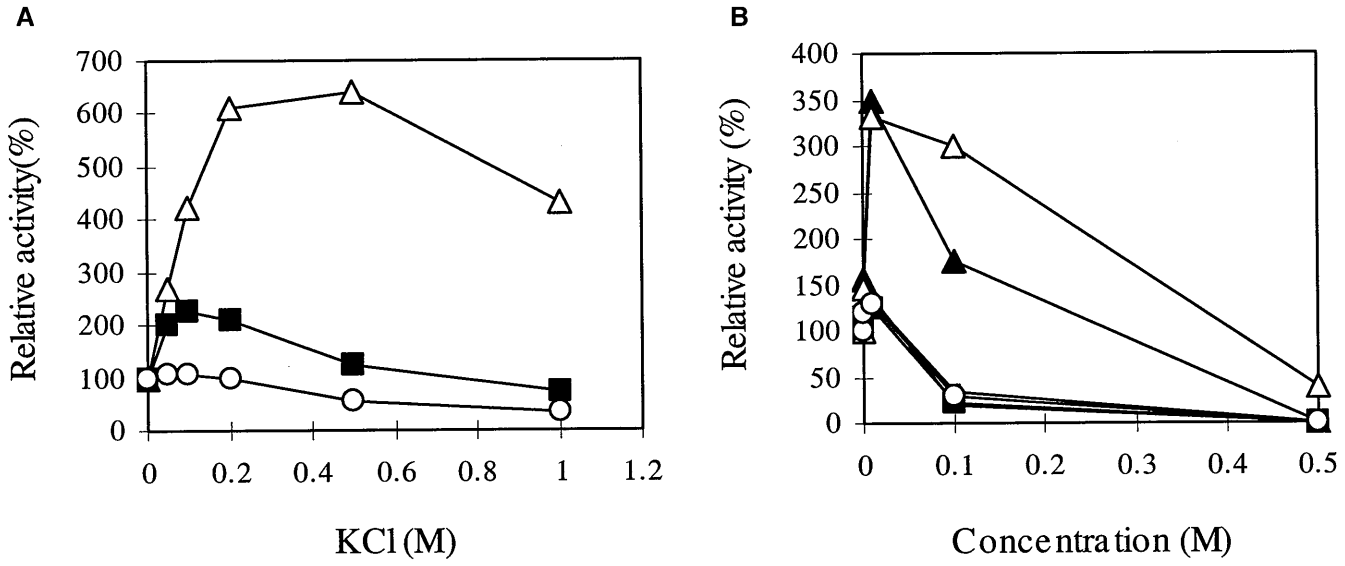

Fig. 4A,B. Effects of salts on GDH activity from three Thermococcales. **A** Oxidative deamination activity of *T. waiotapuensis* (*circles*), *T. litoralis* (*squares*), and *P. furiosus* (*triangles*). GDH activity was measured at 80°C at various KCl concentrations in 100 mM EPPS [*N*-(2-hydroxyethyl) piperazine-*N*′-(-3-propane-sulfonic acid)] buffer (pH 8.0 at 25°C). **B** Oxidative deamination activity of *T. waiotapuensis* (*circles*), *T. litoralis* (*squares*), and *P. furiosus* (*triangles*). GDH activity was measured at 80°C at various $CaCl_2$ (*solid symbols*) and $MgSO_4$ (*open symbols*) concentrations in 100 mM EPPS buffer (pH 8.0 at 25°C)

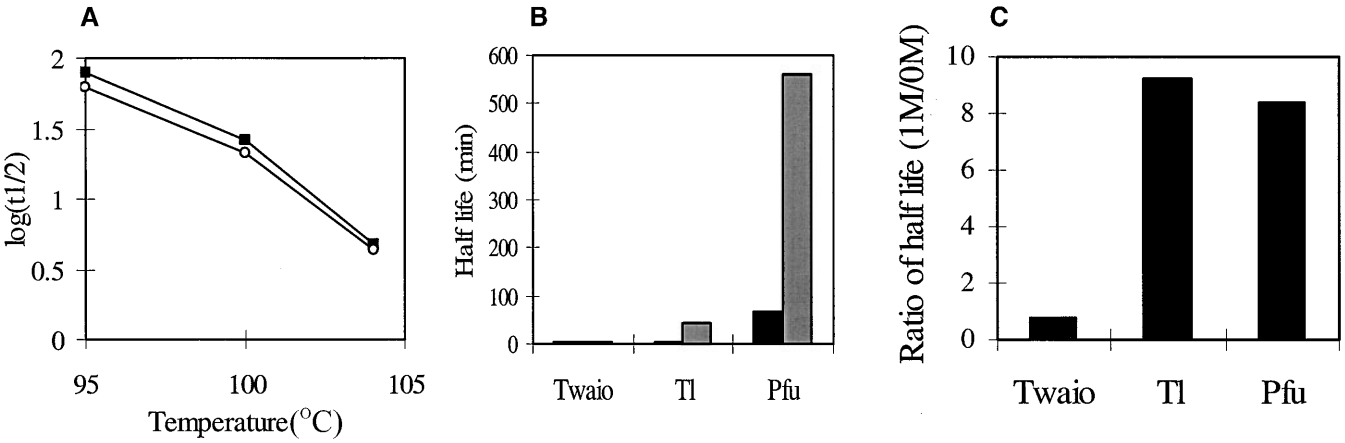

Fig. 5A–C. Thermostability of GDH. **A** Logarithmic plot of half-life for *T. waiotapuensis* (*circles*) and *T. litoralis* (*squares*) GDHs measured at 95°, 100°, and 104°C in 100 mM EPPS buffer with no additional salts. **B** Half-life of *T. waiotapuensis*, *T. litoralis*, and *P. furiosus* GDHs measured at 104°C in the absence (*black bars*) and presence (*gray bars*) of 1 M KCl. **C** Comparative effects of KCl on GDH thermostability represented by the ratio of half-lives (1 M/0 M KCl)

where these microorganisms were isolated. NAD-dependent GDH from the terrestrial hyperthermophilic archaeon *Pyrobaculum islandicum* also showed no difference in thermostability in the presence or absence of salts (Kujo and Ohshima 1998). In contrast, the GDH from the freshwater archaeon *T. zilligii* showed increased thermostability in the presence of NaCl (0.3–0.4 M) (Hudson et al. 1993). These findings imply that the mechanism of salt stabilization at high temperature is not only a reduction of protein solubility and water activity but a consequence of specific molecular interactions that vary for each protein and species.

Kinetic properties of *Thermococcus waiotapuensis* GDH

Twaio GDH utilized only NADP(H), as do most hyperthermophilic GDHs (DiRuggiero et al. 1993; Hudson et al. 1993; Ma et al. 1994; Kobayashi et al. 1995; Aalen et al. 1997). However, Pfu GDH (Consalvi et al. 1991b), *Pyrococcus kodakaraensis* GDH (Rahman et al. 1998b), and *S. solfataricus* (Consalvi et al. 1991a) GDH have been reported to utilize both NAD(H) and NADP(H). Kinetic constants of Twaio GDH are summarized in Table 2. The K_m value for α-ketoglutarate (0.27 mM) was lower than that of L-glutamate (1.3 mM). k_{cat}/K_m values for glutamate forma-

Table 2. Kinetic constants of recombinant *T. waiotapuensis* glutamate dehydrogenase

Substrate (mM)	K_m (mM)	k_{cat} [a] ($\times 10^4$ min^{-1})	k_{cat}/K_m ($\times 10^4$ min^{-1} mM^{-1})
L-Glutamate	1.3	1.1	0.9
α-Ketoglutarate	0.27	4.1	15.0
Ammonia	9.2	9.0	1.0
NADP	0.11	0.8	7.5
NADPH	0.17	15.0	90.0

[a] k_{cat} (molecular activity) was defined as molecules of product formed per min per molecule of enzyme, and calculated from V_{max} (μmoles product formed per minute per milligrams enzyme) and molecular weight of the enzyme

tion (15×10^4) and NADP formation (90×10^4) were higher than those for α-ketoglutarate formation (0.9×10^4) and NADPH formation (7.5×10^4). These results suggest that the reaction catalyzed by Twaio GDH is biased toward glutamate formation, indicating a probable biosynthetic role for GDH in *T. waiotapuensis*.

The structural basis of thermostability of hyperthermophilic GDH has been intensively investigated using Pfu GDH as a model system, and two major ion-pair clusters have been described that consolidate the hexameric structure through multiple intersubunit interactions. In the case of the small six-residue ion-pair cluster of Pfu GDH, this contribution has been confirmed experimentally (Vetriani et al. 1998). Twaio GDH retains all 18 residues in the large network, but in the small network Glu138 and Lys166 are replaced by Ser and Thr, respectively (see Fig. 1). These substitutions, which eliminate the network, explain the lower thermostability of Twaio GDH compared with Pfu and Tl GDHs (Rice et al. 1996; Vetriani et al. 1998). In the GDHs of *T. litoralis* and *P. kodakaraensis* (Rahman et al. 1998a, b; Britton et al. 1999), the noncharged residue, Thr138, replaces Glu138 of Pfu GDH, and site-directed mutagenesis has established a critical role of Glu138 in the ionic interactions (Vetriani et al. 1998). Besides maximizing electrostatic attractions, results from Vetriani et al. (1998) comparing Pfu and Tl GDHs also suggested the importance of reducing electrostatic repulsions.

Our results point to the existence of different strategies for achieving thermostability between marine and freshwater species. In marine species, the high availability of salts might contribute to stabilizing interactions by participating in electrostatic attractions within the protein complex. The intracellular concentration of KCl in *Pyrococcus furiosus* is known to be 700 mM (Zwickl et al. 1990). In the presence of salts (i.e., KCl), marine species (in this study, *Pyrococcus furiosus* and *Thermococcus litoralis*) showed increased thermal stability whereas a freshwater species, *Thermococcus waiotapuensis*, showed a decrease in thermostability. These results reflect sequence differences in the amino acid residues affecting one of the two major ion-pair networks. This finding may account partially for the general observation that marine hyperthermophiles are able to withstand and grow at higher temperatures than phyletically related freshwater strains.

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