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Glutamate dehydrogenase from the aerobic hyperthermophilic archaeon *Aeropyrum pernix* K1: enzymatic characterization, identification of the encoding gene, and phylogenetic implications

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Abstract NADP-dependent glutamate dehydrogenase (L-glutamate: NADP oxidoreductase, deaminating, EC 1.4.1.4) from the aerobic hyperthermophilic archaeon *Aeropyrum pernix* K1 (JCM 9820) was purified to homogeneity for characterization. The enzyme retained its full activity on heating at 95°C for 30 min, and the maximum activity in L-glutamate deamination was obtained around 100°C. The enzyme showed a strict specificity for L-glutamate and NADP on oxidative deamination and for 2-oxoglutarate and NADPH on reductive amination. The K_m values for NADP, L-glutamate, NADPH, 2-oxoglutarate, and ammonia were 0.039, 3.3, 0.022, 1.7, and 83 mM, respectively. On the basis of the N-terminal amino acid sequence, the encoding gene was identified in the *A. pernix* K1 genome, cloned, and expressed in *Escherichia coli*. Analysis of the nucleotide sequence revealed an open reading frame of 1257 bp starting with a minor TTG codon and encoding a protein of 418 amino acids with a molecular weight of 46 170. Phylogenetic analysis revealed that the glutamate dehydrogenase from *A. pernix* K1 clustered with those from aerobic *Sulfolobus solfataricus*, *Sulfolobus shibatae*, and anaerobic *Pyrobaculum islandicum* in Crenarchaeota, and it separated from another cluster of the enzyme from *Thermococcales* in Euryarchaeota. The branching pattern of the enzymes from *A. pernix* K1, *S. solfataricus*, *S. shibatae,* and *Pb. islandicum* in the phylogenetic tree coincided with that of 16S rDNAs obtained from the same organisms.

Key words Glutamate dehydrogenase · *Aeropyrum pernix* K1 · Thermostability · Sequence analysis · Phylogenetic analysis

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Introduction

During the past decade, many new hyperthermophiles growing at a temperature near or above 100°C have been isolated from marine and continental volcanic environments (Adams 1993). Most hyperthermophiles belong to Archaea, the third domain of life (Woese et al. 1990), and evolutionary attention has been paid to their biomolecules because they may be the most primitive group of microorganisms yet discovered. Most of these organisms growing at a temperature around the boiling point of water are known to be anaerobic organisms and to obtain energy by fermentation or a nonoxygenic respiratory system. This requirement has been commonly accepted because oxygen availability in the hydrothermal environments is low because of poor solubility at high temperatures. In 1993, an aerobic hyperthermophilic archaeon, *Aeropyrum pernix* K1, was isolated from a coastal solfataric vent at Kodakara-Jima Island, Japan (Sako et al. 1996). This archaeon is the first strictly aerobic organism growing optimally at a temperature above 90°C and is classified as a new member of the Crenarchaeota by phylogenetic analysis. Most species of the order *Sulfolobales* that belong to the same kingdom are known to be aerobic hyperthermophiles, but they are not able to grow at temperatures higher than 90°C.

We have so far investigated the structure and function of glutamate dehydrogenases (GluDHs) from marine and continental hyperthermophilic Archaea (Ohshima and Nishida 1993, 1994; Kujo and Ohshima 1998; Kujo et al. 1999). In general, GluDH is one of the enzymes for which the most abundant information concerning enzymological properties and the relationship between structure and function has

been obtained. Comparison of the properties and structure of the *A. pernix* K1 GluDH with those of the other GluDHs from hyperthermophiles may provide abundant information for elucidating the diversity and the evolutional relationship of hyperthermophilic archaeal GluDHs.

In the present study, we purified and characterized the GluDH from *A. pernix* K1. While this work was in progress, Kawarabayasi et al. (1999) determined the complete sequence of the genome of *A. pernix* K1. We identified the gene encoding the GluDH on the basis of the N-terminal amino acid sequence of the purified enzyme and expressed it in *Escherichia coli*. We compared the properties and the primary structure of the enzyme with those of the other GluDHs from hyperthermophiles. In addition, we performed a phylogenetic analysis originating from comparisons of the amino acid sequence among hyperthermophilic archaeal GluDHs.

Materials and methods

Chemicals and biochemicals

NADP, NADPH, NAD, and NADH were products of Kojin (Tokyo, Japan). All analytical grade reagents, such as L-glutamate monosodium salt and sodium 2-oxoglutarate, were purchased from Nacalai Tesque (Kyoto, Japan). Red Sepharose CL-4B (dye; reactive red 120, Sigma) was prepared as previously described (Ohshima and Sakuraba 1986).

Microorganism and growth conditions

The hyperthermophilic archaeon *A. pernix* K1 (JCM 9820) was obtained from the Japanese Collection of Microorganisms (JCM) (Wako, Saitama, Japan). The microorganism was cultured in the modified medium of Sako et al. (1996), which consists of natural seawater containing 5 g trypticase peptone, 3 g yeast extract, and 0.76 g $Na_2S_2O_3$ per liter (pH 7.0 adjusted with 0.5 N NaOH). Cells were grown by shaking (100 rpm) on an air-bath rotary shaker at 90°C in 700 ml of the medium in a 2-l flask. After 24 h cultivation, the evaporated water was replenished by addition of sterilized water (about 100 ml), and the cultivation was continued for a further 18 h. The cells were collected by centrifugation (7000 *g* for 15 min) and washed twice with 3% NaCl solution. The washed cells were suspended in 10 mM potassium phosphate buffer (pH 7.0) containing 10% glycerol and stored at –20°C until use.

Enzyme assay and protein determination

Enzyme activity of GluDH was assayed spectrophotometrically with a Shimadzu 160 A spectrophotometer equipped with a thermostat. The standard reaction mixture for the oxidative deamination was composed of 125 μmol glycylglycine/NaOH buffer (pH 8.3), 10 μmol L-glutamate (pH 8.3), 1.25 μmol NADP, and the enzyme in a final volume of 1.00 ml. For reductive amination, the mixture contained 125 μmol glycylglycine/NaOH buffer (pH 8.3), 200 μmol NH_4Cl (pH 8.3, adjusted with NaOH), 10 μmol sodium 2-oxoglutarate, 0.20 μmol NADPH, and the enzyme in a final volume of 1.00 ml. After the reaction mixture without the coenzyme was incubated at 50°C for 3 min, the reaction was started by the addition of the coenzyme, and the oxidation of NADPH was then monitored at 340 nm. One unit of the enzyme is defined as the amount catalyzing the formation of 1 μmol of NADPH per minute at 50°C in the oxidative deamination of L-glutamate. Protein was determined by the Bradford method (1976) using the standard assay kit from Bio-Rad (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard.

Purification of glutamate dehydrogenase from *A. pernix* K1

The entire operation was done at room temperature (about 25°C). All buffers used in the purification steps contained 10% glycerol, 1 mM ethylenediaminetetraacetic acid, and 0.1 mM dithiothreitol. The cells were washed twice with 3% NaCl solution, suspended in 10 mM potassium phosphate buffer (pH 7.2), and disrupted by ultrasonication. The cell debris was removed by centrifugation (15000 *g*, 15 min at 4°C), and the supernatant solution was used as the crude extract for the purification.

The crude extract (200 ml) was placed on a Red Sepharose CL-4B column (4 × 10 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.2). After the column was washed with the same buffer, the enzyme was eluted with a 1000-ml linear gradient of 0 to 1.0 M NaCl in the same buffer. The active fractions were pooled; the enzyme was dialyzed against 10 mM potassium phosphate buffer (pH 7.2) and applied to the Red Sepharose CL-4B column (4 × 10 cm). The column was washed with a bed volume of the same buffer and then equilibrated with the buffer supplemented with 5 mM L-glutamate (pH 7.2). The enzyme was eluted with a 900-ml linear gradient of NADP (0–1.0 mM) in the presence of 5 mM L-glutamate. The active fractions were collected and dialyzed against 10 mM potassium phosphate buffer (pH 7.2).

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE; 7.5% acrylamide gel) was carried out according to the method of Davis (1975), and SDS-PAGE (12% acrylamide slab gel, 1 mm thick) was performed by the procedure of Laemmli (1970). Activity staining was done at 50°C in a mixture containing 0.3 M Tris-HCl buffer (pH 8.0), 10 mM L-glutamate, 1.0 mM NADP, 0.04 mM phenazine methosulfate, and 0.05 mM *p*-iodonitrotetrazolium violet until a red band of sufficient intensity was visible. The protein band was stained with Coomassie brilliant blue G 250 (PAGE) and R 250 (SDS-PAGE).

Molecular mass determinations

The molecular mass of the native enzyme was measured using HPLC (Tosoh type CCPE) with a gel filtration column (TSK gel column G3000SWXL, 7.8 mm × 30 cm). The column was equilibrated with 0.1 M potassium phosphate buffer (pH 6.7) containing 0.1 M Na_2SO_4. The following marker proteins (Bio-Rad) were used to create a calibration curve: bovine thyroglobulin (molecular mass, 670 kDa), bovine β-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa). The subunit molecular mass of the enzyme was determined by SDS-PAGE. The marker proteins (New England BioLabs, Beverly, MA, USA) used were as follows: fusion of *E. coli* maltose-binding protein and β-galactosidase (molecular mass, 175 kDa), fusion of *E. coli* maltose-binding protein and paramyosin (83 kDa), bovine liver glutamate dehydrogenase (62 kDa), rabbit muscle aldolase (47.5 kDa), rabbit muscle triosephosphate isomerase (32.5 kDa), bovine milk β-lactoglobulin A (25 kDa), and chicken eggwhite lysozyme (16.5 kDa).

Steady-state kinetic analysis

The basic reaction mixtures for the oxidation and reduction were similar to those described under Enzyme assay and protein determination. Initial velocity experiments were done by varying the concentration of one substrate at a fixed concentration of other substrates as previously described (Cleland 1971; Ohshima and Nishida 1993). The K_m values were calculated from the secondary plot of the intercepts versus the reciprocal of the substrate concentration.

N-terminal amino acid sequencing

Approximately 2.5 μg of purified GluDH was subjected to SDS-PAGE as described earlier, followed by electroblotting onto a polyvinylidene difluoride membrane. The membrane was then stained with Ponceau S and destained. A protein band was excised and subjected to automated Edman degradation using a Shimadzu model PPSQ-10 protein sequencer (Shimadzu, Kyoto, Japan).

Cloning and expression of recombinant protein

The complete sequence of the genome of *A. pernix* K1 has been determined by the whole genome shotgun method (Kawarabayasi et al. 1999). On the basis of the N-terminal amino acid sequence of the native GluDH, the open reading frame of the GluDH homologue (ORF ID, APE1386) in the *A. pernix* K1 genome was identified using BLAST (Altschul et al. 1990). The plasmid DNA (pUAP-GDH, position 882874–885007 on the entire genome of *A. pernix* K1, has been inserted into the *Hin*cII site of pUC118) containing APE1386 was prepared from the shotgun clone A2GR6640 as described previously (Kawarabayasi et al. 1999). The *E. coli* strain JM109 was transformed with pUAP-GDH and plated on Luria-Bertani (LB) plates containing ampicillin (0.005%), β-D-thiogalactopyranoside (IPTG, 0.01%), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (0.02%). The transformants were grown at 37°C in LB medium containing ampicillin and IPTG. After 16 h cultivation, cells were collected, suspended in 10 mM potassium phosphate buffer (pH 7.2), and disrupted by ultrasonication. After centrifugation (16000 *g*, 15 min), the soluble fraction of the extract was heated at 85°C for 30 min. The denatured protein was removed by centrifugation (16000 *g*, 20 min). The recombinant enzyme was purified from the supernatant according to the same method as that used for the native enzyme. The N-terminal amino acid sequence of the purified recombinant enzyme was determined as described earlier.

Results

Purification

A typical result of purification of GluDH from the extract of *A. pernix* K1 is summarized in Table 1. The enzyme was purified about 37 fold with a 28% recovery by two successive Red Sepharose CL-4B affinity chromatography operations within a few days. In the first column chromatography, the enzyme was released from the affinity resin by the nonspecific elution method with an increase in NaCl concentration. This method was useful for the rapid removal of a large amount of contaminant proteins. In the second column chromatography, specific affinity elution by the ternary complex formation of NADP, enzyme and L-glutamate was used and achieved very high resolution. The purified enzyme was found to be homogeneous on the basis of SDS-PAGE. About 3 mg of the purified enzyme was obtained from 5 l of *A. pernix* K1 culture.

Table 1. Purification of glutamate dehydrogenase (GluDH) from *Aeropyrum pernix* K-1

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract[a]	398	56.6	0.142	100	1
First red sepharose	18.2	21.9	1.2	39	8.5
Second red sepharose	3.0	15.6	5.2	28	36.6

[a] Cells used were about 23 g (wet weight)

Molecular mass and subunit structure

The molecular mass of the *A. pernix* K1 GluDH was estimated to be about 270 kDa by gel filtration. SDS-PAGE of the purified enzyme gave only one band; the subunit molecular mass was determined to be about 46 kDa. These results show that the native enzyme has a hexamer structure composed of six identical subunits.

Effects of temperature and pH on enzyme activity

The effect of temperatures in the range of 40° to 100°C on the oxidative deamination was studied. The activity of the enzyme increased with an increase in temperature from 50° to 100°C. The highest activity was observed at 100°C (80.4 U/mg) and was about 15 times that at 50°C. The optimum temperature may be above 100°C. We were not able to assay at temperature above 100°C because of the instability of NADP under assay conditions. The pH optimum of the enzyme was determined at 50°C in 125 mM glycylglycine/NaOH buffer over the pH range 7.5–9.5. Buffer pH values were adjusted at this temperature. The enzyme showed maximal activity at the range of pH 8.3–8.7 for the deamination of L-glutamate and at pH 8.1–8.4 for the amination of 2-oxoglutarate.

Stability

The thermostability of the enzyme was examined. The enzyme retained its full activity on heating at 95°C for 30 min but lost 5% of the activity at 100°C after 30-min incubation. The enzyme completely lost activity on incubation at 115°C for 10 min. The enzyme was stable over a wide range of pH; on heating at 100°C for 30 min, the full enzyme activity was retained in the range of pH 5–10. The enzyme could be kept at a low temperature, around 4°C, without loss of activity for at least 2 months.

Substrate and coenzyme specificity and kinetic constants

The ability of the enzyme to catalyze the oxidative deamination of various α-amino acids and the reductive amination of various 2-oxo acids was examined. The enzyme was highly specific for L-glutamate in the oxidative deamination. None of the following amino acids — D-glutamate, L-norvaline, L-2-aminobutyrate, L-valine, L-alanine, L-aspartate, L-serine, L-cysteine, L-lysine, or L-phenylalanine (10 mM) – was the substrate. For reductive amination, the enzyme was highly specific for 2-oxoglutarate. No activity was detected with the following keto acids (10 mM): pyruvate, 2-oxovalerate, 2-oxoisocaproate, 2-oxobutyrate, or 2-oxoisovalerate. The enzyme requires NADP as the coenzyme for the oxidation of L-glutamate, which could not be replaced by NAD. For the reduction of 2-oxoglutarate, NADPH was the coenzyme and NADH was inert. The K_m values for NADP, L-glutamate, NADPH, 2-oxoglutarate, and ammonia were calculated to be 0.039, 3.3, 0.022, 1.7, and 83 mM, respectively.

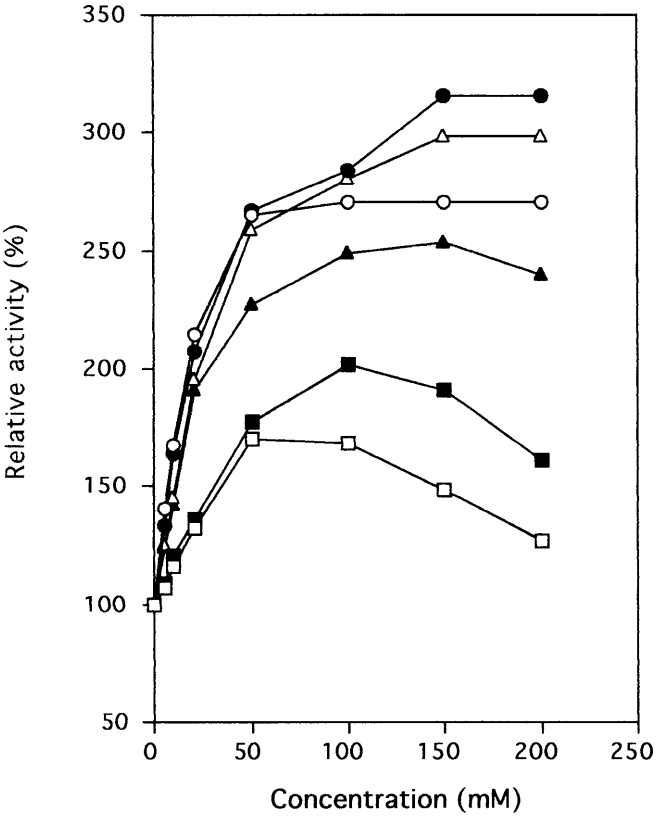

Fig. 1. Effect of salts on glutamate dehydrogenase (GluDH) activity. The reaction was started by the addition of the enzyme to the reaction mixture containing various concentrations of KCl (*solid squares*), NaCl (*open squares*), K_2SO_4 (*solid circles*), Na_2SO_4 (*open circles*), K_3PO_4 (*solid triangles*), and Na_3PO_4 (*open triangles*). pH of K_2SO_4, Na_2SO_4, K_3PO_4, and Na_3PO_4 solutions was adjusted to 8.3 with KOH, NaOH, KH_2PO_4, and NaH_2PO_4, respectively

Effect of salts on enzyme activity

The effect of salts on the oxidative deamination of L-glutamate was examined. The enzyme was markedly activated by the addition of KCl, NaCl, K_2SO_4, Na_2SO_4, K_3PO_4 and Na_3PO_4 (Fig. 1). The addition of KCl or NaCl gave a maximum enhancement of about 170–200% of the relative activity at a concentration of 50–100 mM. K_2SO_4 and Na_3PO_4 enhanced the activity by a maximum of about 280–300% at a concentration of 150–200 mM. $K_3PO_4$and Na_2SO_4 were less effective than K_2SO_4 and Na_3PO_4.

Identification and expression of the GluDH gene

The N-terminal sequence (15 amino acids) of the native enzyme was determined to be MQPTDPLEEARAQLR. On the basis of this sequence, the open reading frame (APE1386) of the GluDH homologue in the *A. pernix* K1 genome was identified as described in Materials and methods.

The N-terminal sequence of the native enzyme coincides with the underlined sequence of the deduced amino acid

sequence (MEVLALQPTDPLEEARAQLR, first 20 amino acids) (Swiss Prot accession number, Q9YC65) except for the first methionine. Kawarabayasi et al. (1999) assigned GTG as the start codon for this gene because the criterion used for the assignment of the potential coding region in the *A. pernix* K1 genomic sequence was the identification of the sense codons starting with ATG or GTG. However, the result of the N-terminal analysis of the native enzyme shows that the sense codon of the enzyme gene may start with a minor TTG codon, which is frequently used for the start codon in Cyanobacteria (Sazuka and Ohara 1996).

E. coli JM109 transformed with pUAP-GDH carrying the GluDH homologue produced hyperthermostable GluDH activity, which was not lost by incubation at 85°C for 30 min. The recombinant enzyme was purified according to the procedure described in Materials and methods. The apparent subunit molecular mass of the purified enzyme was estimated to be about 46 kDa by SDS-PAGE and coincides with that of the purified native enzyme. The N-terminal sequence of the recombinant enzyme was determined to be MQPTDPLEEARAQLRRAVDLLG YD, which was mixed with the sequence of ALQPTDPLEE ARAQLRRAVDLLGYD. These results suggest that TTG was also used for the start codon in *E. coli*. The origin of the contaminated sequence is unknown.

Sequence alignment and phylogenetic analysis

An amino acid sequence alignment of GluDHs from hyperthermophilic archaea is shown in Fig. 2. The GluDH sequences, from *Pyrococcus* sp. KOD1 (Rahman et al. 1998), *Pyrococcus furiosus* (Eggen et al. 1993), *Pc. abyssi* (GenBank accession number, AJ248284.1:234965–236227), *Pc. horikoshii* OT-3 (GenBank accession number, AP000006.1: 246255–247523), *Thermococcus* sp. ES4 (DiRuggiero et al. 1993), *Thermococcus litoralis* (Britton et al. 1995), *T. profundus* (Higuchi et al. 1997), *Pyrobaculum islandicum* (Kujo et al. 1999), *Sulfolobus solfataricus* (Maras et al. 1992), and *S. shibatae* (Benachenhou-Lahfa et al. 1994) were available. The proposed alignment was constructed with the multiple alignment program in GENETYX-SV/RC9.0 software (Software Development, Tokyo, Japan). The *A. pernix* K1 GluDH showed a sequence similarity of about 57% and 55% to GluDHs from *S. solfataricus* and *S. shibatae*, respectively. Comparison of the sequence with those of the enzymes from other hyperthermophilic Archaea showed a rather low similarity of about 49%–51%. The phylogenetic tree was constructed by the UPGMA method as shown in Fig. 3A. The GluDH from *A. pernix* K1 clustered with the GluDHs from *Pb. islandicum*, *S. solfataricus*, and *S. shibatae*, but it separated from another cluster of the GluDHs from *Thermococcales.* The two GluDH clusters clearly reflected the difference between Crenarchaeota and Euryarchaeota, the two kingdoms in Archaea.

Discussion

The genome sequencing project of *A. pernix* K1 was performed at the National Institute of Technology and Evaluation, Ministry of International Trade and Industry, Tokyo, Japan (Kawarabayasi et al. 1999). The information about the hyperthermostable enzyme genes identified in the *A. pernix* K1 genome may be a powerful tool to examine the relationship between their structure and function. In this study, NADP-dependent GluDH from the hyperthermophilic archaeon *A. pernix* K1 has been purified and characterized, and the gene encoding the enzyme was identified in the genome on the basis of N-terminal amino acid sequence and was cloned and expressed in *E. coli*. The hyperthermophilic GluDHs have been purified and characterized from several hyperthermophiles. Comparisons of the enzymological properties of the *A. pernix* K1 GluDH with those of GluDHs from *Pc. furiosus* (Ohshima and Nishida 1993), *Pc. woesei* (Ohshima and Nishida 1993), *T. litoralis* (Ohshima and Nishida 1994), *Pb. islandicum* (Kujo and Ohshima 1998), and *S. solfataricus* (Consalvi et al. 1991) are summarized in Table 2. The *A. pernix* K1 GluDH consists of six subunits with identical molecular masses, and the subunit structure is similar to those of other species of the hyperthermophiles. The optimum temperature for the oxidative deamination of the *A. pernix* K1 GluDH may be above 100°C and is similar to that of the *Pc. furiosus* and *Pc. woesei* enzymes (Table 2). The optimum pHs for oxidative deamination (8.3–8.7) and reductive amination (8.1–8.4) are similar to those of the *Pc. furiosus*, *Pc. woesei*, and *T. litoralis* enzymes but lower than those for the *Pb. islandicum* and *S. solfataricus* enzymes (Table 2). The *A. pernix* K1 GluDH retained its full activity on heating at 95°C for 30 min but lost 5% of the activity at 100°C after 30-min incubation. In thermostability, this enzyme is comparable to the *T. litoralis* GluDH and slightly less thermostable than the *Pb. islandicum*, *Pc. furiosus*, and *Pc. woesei* GluDHs. The remarkable characteristic of the enzyme is its absolute specificity for substrates. Most GluDHs from anaerobic hyperthermophiles specifically catalyze L-glutamate in oxidative deamination. On the other hand, the substrate specificity for 2-oxo acids is relatively low in reductive amination. For instance, the GluDHs from *Pc. furiosus, Pc. woesei*, *T. litoralis*, and *Pb. islandicum* catalyze the amination of pyruvate, 2-oxovalerate, 2-oxoisocaproate, 2-oxobutyrate, and 2-oxoisovalarate to a lesser extent than 2-oxoglutarate. However, the *A. pernix* K1 GluDH does not act on those 2-oxo acids and is highly specific for 2-oxoglutarate (Table 2). In addition, the enzyme is highly specific for NADP and NADPH as the coenzymes for deamination and amination, respectively. Although the enzymes from *Pc. furiosus, Pc. woesei*, and *T. litoralis* are NADP dependent, they have reactivity for NAD and NADH to a lesser extent. Therefore, the high specificity for NADP and NADPH is one of the characteristics of the enzyme. The K_m values for L-glutamate, 2-oxoglutarate, NADP, and NADPH are similar to those of other hyperthermophile GluDHs. However, a high K_m value for

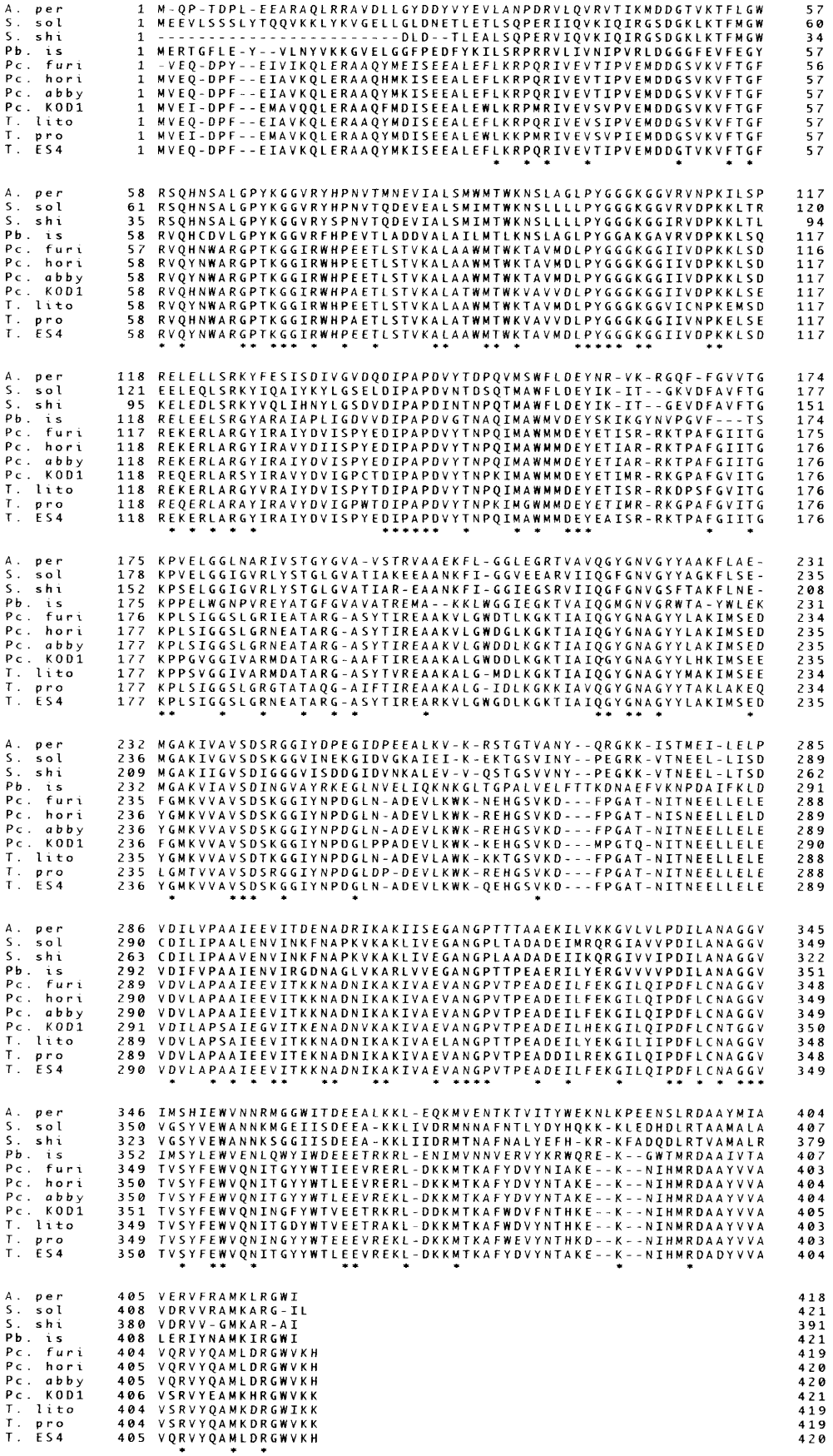

Fig. 2. Alignment of the amino acid sequences of GluDHs from *Aeropyrum pernix* K1 (A. per; GenBank AP000061), *Sulfolobus solfataricus* (S. sol; SWISS-PROT P80053), *S. shibatae* (S. shi; GenBank X73990), *Pyrobaculum islandicum* (Pb. is; GenBank AB027194), *Pyrococcus furiosus* (Pc. furi; GenBank M97860), *Pc. horikoshii* OT-3 (Pc. hori; GenBank AP000006.1), *Pc. abyssi* (Pc. aby; GenBank AJ248284), *Pyrococcus* sp. KOD1 (Pc. KOD1; GenBank D89911), *Thermococcus litoralis* (T. lito; GenBank L19995), *T. profundus* (T. pro; GenBank D87814), and *Thermococcus* sp. ES4 (T. ES4; GenBank L12408). *Asterisks* represent conserved residues among the 11 GluDHs

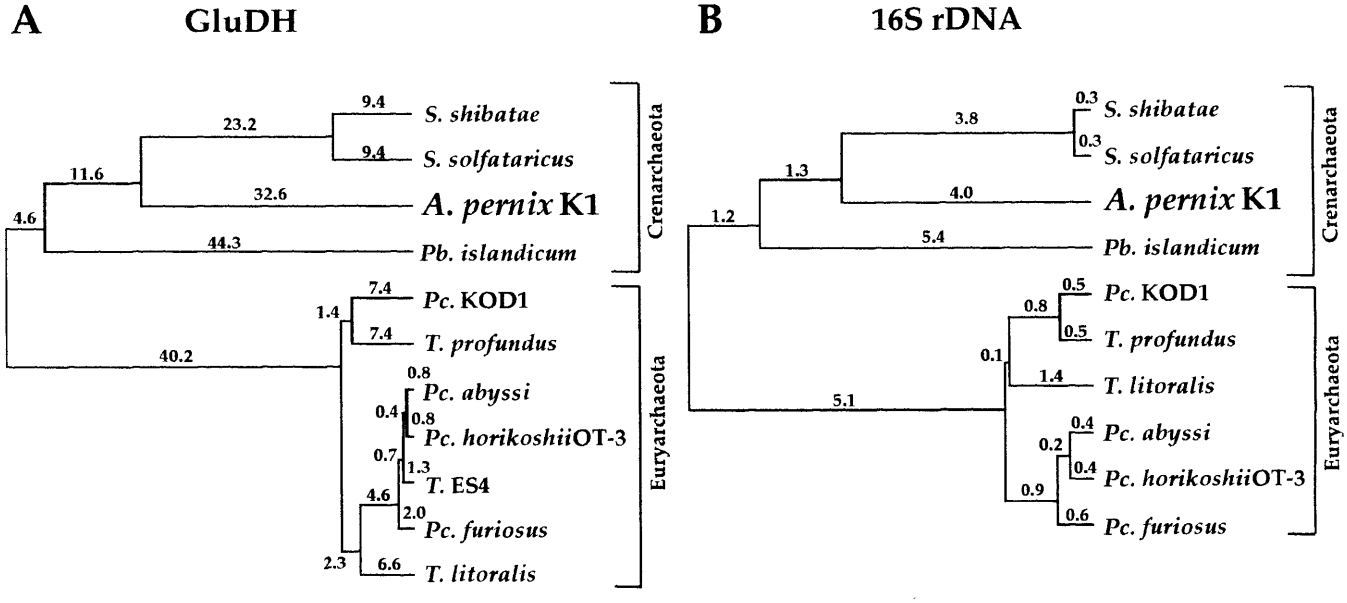

Fig. 3A,B. Phylogenetic trees for GluDH (**A**) and 16S rDNA (**B**) obtained from various hyperthermophilic Archaea. The branch lengths of the trees are drawn to scale and indicated by the *numbers*. GenBank accession numbers for the 16S rDNA sequences are as follows: *Pyrococcus* sp. KOD1 (D38650), *Pc. furiosus* (U20163), *Pc. abyssi* (Z70246), *Pc. horikoshii* OT-3 (D45214), *Thermococcus litoralis* (Z70252), *T. profundus* (Z75233), *Pyrobaculum islandicum* (L07511), *Aeropyrum pernix* K1 (D83259), *Sulfolobus solfataricus* (X03235), and *S. shibatae* (M32504). The l6S rDNA sequence of *Thermococcus* sp. ES4 could not be obtained

Table 2. Properties of GluDHs from hyperthermophilic Archaea

Parameter	Source of GluDHs					
	Crenarchaeota			Euryarchaeota		
	Aeropyrum pernix K1	*Sulfolobus solfataricus*	*Pyrobaculum islandicum*	*Pyrococcus furiosus*	*Pyrococcus woesei*	*Thermococcus litoralis*
Native molecular mass (kDa)	270	270	220	300	300	300
Subunit molecular mass (kDa)	46	45	36	47	47	47
Thermostability (°C)[a]	95	<80	100	105	105	95
Optimum activity temp (°C)[b]	>100	70	90	>100	>100	95
Optimum pH:						
For the oxidative deamination	8.3–8.7	10.0	9.7	8.2	8.2	7.8
For the reductive amination	8.1–8.4	9.0	8.7	7.4	7.4	7.8
Substrate:						
For the reductive amination	2-OG	2-OG, 2-OV	2-OG, 2-OV, 2-OB, 2-OIC	2-OG, 2-OV, 2-OB, 2-OC	2-OG, 2-OB, 2-OV	2-OG, 2-OC, 2-OV, 2-OB, 2-OIV
Coenzyme	NADP(H)	NADP(H)	NAD(H)	NADP(H)	NADP(H)	NADP(H)
K_m value (mM)						
L-glutamate	3.3	2.5	0.17	0.95	1.1	2.1
NAD(P)	0.039	0.05	0.025	0.035	0.017	0.045
2-Oxoglutarate	1.7	1.4	0.066	0.11	0.63	1.0
NH_3	83	4.2	9.7	6.3	19	5.6
NAD(P)H	0.022	0.01	0.005	0.007	0.024	0.044

2-OG, 2-oxoglutarate; 2-OB, 2-oxobutyrate; 2-OC, 2-oxocaproate; 2-OIC, 2-oxoisocaproate; 2-OV, 2-oxovalerate; 2-OIV, 2-oxoisovalerate

[a] Temperature at which the enzyme retains its full activity after incubation for 30 min

[b] Optimum temperature for the oxidative deamination

ammonia in the *A. pernix* K1 enzyme is recognized (Table 2). Another remarkable feature of the enzyme is the enhancement of the activity with NaCl, KCl, Na_2SO_4, K_2SO_4, Na_3PO_4, and K_3PO_4. The enzyme activity is enhanced about two- to threefold with these salts. Although the enhancement of the activity with NaCl and KCl has been described for GluDHs from other hyperthermophiles (Ma et al. 1994; Ohshima and Nishida 1993), activity enhancement with other salts, such as Na_2SO_4, K_2SO_4, Na_3PO_4, and K_3PO_4, has not been observed.

The phylogenetic relationship among the GluDHs from several hyperthermophilic Archaea was compared with that

depicted with the 16S rDNAs (Fig. 3). The branching patterns of the GluDHs from hyperthermophilic Euryarchaeota do not completely agree with those of the 16S rDNAs from the same organisms. On the other hand, the phylogenetic trees showed substantially similar branching patterns for the hyperthermophilic Crenarchaeota, suggesting that the GluDH genes from *A. pernix* K1, *S. solfataricus*, *S. shibatae*, and *Pb. islandicum* may not be transferred horizontally from the hyperthermophilic euryarchaeal species in the course of evolution. Each GluDH of those four strains has probably evolved separately from the euryarchaeal GluDHs according to the individual metabolic requirements of each strain. All the strains belonging to Euryarchaeota in Fig. 3 are marine anaerobic hyperthermophilic species and members of the order *Thermococcales.* GluDHs from the *Thermococcales* utilize NADP exclusively as a coenzyme, and their principal function has been suggested to be L-glutamate biosynthesis coupled with L-alanine production (Kengen and Stams 1994; Kobayashi et al. 1995; Ohshima and Nishida 1993).

We recently suggested that the physiological role of the NAD-dependent GluDH from *Pb. islandicum* is distinct from that of the GluDHs of *Thermococcales* (Kujo and Ohshima 1998). Selig and Schönheit (1994) have reported the presence of the citric acid cycle and its function for the oxidation of organic compounds to CO_2 with elemental sulfur or thiosulfate as the electron acceptor in *Pb. islandicum.* The presence of the citric acid cycle in cells of members of the *Thermococcales* has not yet been reported. Thus, we have predicted that GluDH may be linked to the citric acid cycle via 2-oxoglutarate in the cells of *Pb. islandicum* (Kujo and Ohshima 1998). The presence of the citric acid cycle has also been demonstrated in the aerobic *Sulfolobus* and in *Aeropyrum*. Most enzymes of the citric acid cycle have been found in the aerobic *Sulfolobus,* and the oxidation of acetyl-CoA to CO_2 via the citric acid cycle was demonstrated (Danson 1988, 1993). In the case of *A. pernix* K1, almost all genes of the enzymes in the citric acid cycle have been identified in the genome (Kawarabayasi et al. 1999). These observations and our results suggest that the GluDHs from *A. pernix* K1, *S. solfataricus*, *S. shibatae*, and *Pb. islandicum* may have evolved to fulfill the metabolic requirement of linkage to the citric acid cycle via 2-oxoglutarate.

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References

Adams MWW (1993) Enzymes and proteins from organisms that grow near and above 100°C. Annu Rev Microbiol 47:627–658

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Benachenhou-Lahfa N, Labedan B, Forterre P (1994) PCR-mediated cloning and sequencing of the gene encoding glutamate dehydrogenase from the archaeon *Sulfolobus shibatae*: identification of putative amino-acid signatures for extremophilic adaptation. Gene (Amst) 140:17–24

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Britton KL, Baker PJ, Borges KMM, Engel PC, Pasquo A, Rice DW, Robb FT, Scandurra R, Stillman TJ, Yip KSP (1995) Insights into thermal stability from a comparison of the glutamate dehydrogenases from *Pyrococcus furiosus* and *Thermococcus litoralis.* Eur J Biochem 229:688–695

Cleland WW (1971) Steady state kinetics. In: Boyer PD (ed) The enzymes, 3rd edn, vol 2. Academic Press, New York, pp. 1–65

Consalvi V, Chiaraluce R, Politi L, Gambacorta A, De Rosa M, Scandurra R (1991) Glutamate dehydrogenase from the thermoacidophilic archaebacterium *Sulfolobus solfataricus.* Eur J Biochem 196:459–467

Danson MJ (1988) Archaebacteria: the comparative enzymology of their central metabolic pathways. Adv Microb Physiol 29:166–231

Danson MJ (1993) Central metabolism of the Archaea. In: Kates M, Kushner DJ, Matheson AT (eds) The biochemistry of Archaea (Archaebacteria). New comprehensive biochemistry, vol 26. Elsevier, Amsterdam, pp. 1–24

Davis BJ (1975) Disc electrophoresis. 2. Method and application to human serum proteins. Ann NY Acad Sci 121:404–427

DiRuggiero J, Robb FT, Jagus R, Klump HH, Borges KM, Kessel M, Mai X, Adams MWW (1993) Characterization, cloning, and in vitro expression of the extremely thermostable glutamate dehydrogenase from the hyperthermophilic archaeon ES4. J Biol Chem 268:17767–17774

Eggen RIL, Geerling ACM, Waldkötter K, Antranikian G, de Vos WM (1993) The glutamate dehydrogenase-encoding gene of the hyperthermophilic archaeon *Pyrococcus furiosus*: sequence, transcription and analysis of the deduced amino acid sequence. Gene (Amst) 132:143–148

Higuchi S, Kobayashi T, Kimura K, Horikoshi K, Kudo T (1997) Molecular cloning, nucleotide sequence and expression in *Escherichia coli* of hyperthermophilic glutamate dehydrogenase gene from *Thermococcus profundus.* J Ferment Bioeng 83:405–411

Kawarabayasi Y, Hino Y, Horikawa H, Yamazaki S, Haikawa Y, Jin-no K, Takahashi M, Sekine M, Baba S, Ankai A, Kosugi H, Hosoyama A, Fukui S, Nagai Y, Nishijima K, Nakazawa H, Takamiya M, Masuda S, Funahashi T, Tanaka T, Kudoh Y, Yamazaki J, Kushida N, Oguchi A, Aoki K, Kubota K, Nakamura Y, Nomura N, Sako Y, Kikuchi H (1999) Complete genome sequence of an aerobic hyper-thermophilic crenarchaeon, *Aeropyrum pernix* K1. DNA Res 6:83–101

Kengen SWM, Stams AJM (1994) Formation of L-alanine as a reduced end product in a carbohydrate fermentation by the hyperthermophilic archaeon *Pyrococcus furiosus.* Arch Microbiol 161:168–175

Kobayashi T, Higuchi S, Kimura K, Kudo T, Horikoshi K (1995) Properties of glutamate dehydrogenase and its involvement in alanine production in a hyperthermophilic archaeon, *Thermococcus profundus.* J Biochem (Tokyo) 118:587–592

Kujo C, Ohshima T (1998) Enzymological characteristics of the hyperthermostable NAD-dependent glutamate dehydrogenase from the archaeon *Pyrobaculum islandicum* and effects of denaturants and organic solvents. Appl Environ Microbiol 64:2152–2157

Kujo C, Sakuraba H, Nunoura N, Ohshima T (1999) The NAD-dependent glutamate dehydrogenase from the hyperthermophilic archaeon *Pyrobaculum islandicum*: cloning, sequencing, and expression of the enzyme gene. Biochim Biophys Acta 1434:365–371

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the bacteriophase T4. Nature (Lond) 227:680–685

Maras B, Consalvi V, Chiaraluce R, Politi L, De Rosa M, Bossa F, Scandurra R, Barra D (1992) The protein sequence of glutamate dehydrogenase from *Sulfolobus solfataricus*, a thermoacidophilic archaebacterium. Is the presence of *N*-epsilon-methyllysine related to thermostability? Eur J Biochem 203:81–87

Ma K, Robb FT, Adams MWW(1994) Purification and characterization of NADP-specific alcohol dehydrogenase and glutamate dehy-

drogenase from the hyperthermophilic archaeon *Thermococcus litoralis.* Appl Environ Microbiol 60:562–568

Ohshima T, Nishida N (1993) Purification and properties of extremely thermostable glutamate dehydrogenases from two hyperthermophilic archaebacteria, *Pyrococcus woesei* and *Pyrococcus furiosus.* Biosci Biotechnol Biochem 57:945–951

Ohshima T, Nishida N (1994) Purification and characterization of extremely thermostable glutamate dehydrogenase from a hyperthermophilic archaeon, *Thermococcus litoralis*. Biocatalysis 11:117–129

Ohshima T, Sakuraba H (1986) Purification and characterization of malate dehydrogenase from the phototrophic bacterium, *Rhodopseudomonas capsulata.* Biochim Biophys Acta 869:171–177

Rahman RNZA, Fujiwara S, Takagi M, Imanaka T (1998) Sequence analysis of glutamate dehydrogenase (GDH) from the hyperthermophilic archaeon *Pyrococcus* sp. KOD1 and comparison of the enzymatic characteristics of native and recombinant GDHs. Mol Gen Genet 257:338–347

Sako Y, Nomura N, Uchida A, Ishida Y, Morii H, Koga Y, Hoaki T, Maruyama T (1996) *Aeropyrum pernix* gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100°C . Int J Syst Bacteriol 46:1070–1077

Sazuka T, Ohara O (1996) Sequence features surrounding the translation initiation sites assigned on the genome sequence of *Synechocystis* sp. strain PCC6803 by amino-terminal protein sequencing. DNA Res 3:225–232

Selig M, Schönheit P (1994) Oxidation of organic compounds to CO_2 with sulfur or thiosulfate as electron acceptor in the anaerobic hyperthermophilic archaea *Thermoproteus tenax* and *Pyrobaculum islandicum* proceeds via the citric acid cycle. Arch Microbiol 162: 286–294

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci USA 87:4576–4579