Glutamate dehydrogenase (GDH) from the hyperthermophilic Archaean ES4 (optimal growth temperature 98°C and maximum growth temperature 110°C) was purified to homogeneity. The purified native enzyme had an $M_r$ of 270,000 $\pm$ 5,000 and was shown by gel filtration and SDS-polyacrylamide gel electrophoresis to be a hexamer with identical subunits of $M_r$ =46,000 $\pm$ 3,000. The hexameric subunit composition was also evident from electron micrographs, which show a triangular antiprism structure very similar to that of bovine GDH. The enzyme is exceptionally thermostable, with a half-time of inactivation of 3.5 h at 105°C. Differential scanning calorimetry revealed a $T_m$ for denaturation of 113°C and a $T_m$ for activation at 60°C. Antigenic cross-reaction with ES4 GDH was observed with the purified GDH from the thermophilic Archaean, Pyrococcus furiosus and Thermococcus litoralis as well as with bovine and yeast GDHs. The genome of ES4 was shown to contain a single copy of the $gdhA$ gene, and this was cloned and sequenced. The deduced amino acid sequence of the GDH from ES4 corresponded to the NH$_2$-terminal amino acid sequence obtained from the pure protein. From the nucleotide sequence the ES4 protein is composed of 420 residues. It has a relatively high hydrophobicity and a low number of sulfur-containing residues compared with mesophilic GDHs. Relatively high homology (52%) exists between the deduced amino acid sequence of ES4 GDH and Clostridium difficile GDH. Of the two distinct families of GDH sequences known, ES4 GDH belongs to the same family as vertebrates, $C$. difficile, and other Archaea. The $gdhA$ gene of ES4 was expressed in vitro in a rabbit reticulocyte cell-free lysate, thus providing a system for structural studies of the mechanisms of thermostability in hyper-thermophilic proteins.

A group of microorganisms have recently been discovered that have the remarkable property of growing optimally at or above 100°C. These so-called hyperthermophiles are all members of the Archaea (1, 2). The as yet unidentified archaeon, ES4, was isolated by Pledger and Baross (3). It is an anaerobic heterotroph that grows by the fermentation of peptides and carbohydrates to produce organic acids, CO$_2$, and H$_2$. ES4 is unusual among known hyperthermophiles as it is able to grow in a defined medium, albeit slowly, using L-glutamate as the sole carbon and nitrogen source. The optimal temperature and maximal temperature for growth are 98 and 110°C, respectively (3).

In order to investigate some of the metabolic properties of hyperthermophiles and mechanisms of protein “hyperthermostability,” we have focused on glutamate dehydrogenase (EC 1.4.1.3, GDH), which utilizes the nicotinamide-dependent oxidation of glutamate to α-oxoglutarate. This enzyme has been proposed by us to play a major role in amino acid utilization by Pyrococcus furiosus, another hyperthermophilic Archeon (4, 5). GDH is also an important enzyme because of its pivotal position between carbon and nitrogen metabolism. Recently, a number of other enzymes have been isolated from hyperthermophiles, and with the exception of the Pyrodictium brockii hydrogenase (6), these studies have been applied to $P$. furiosus (4, 5, 7–13), or the very closely related isolate $P$. woesei (14). However, GDH from $P$. furiosus is among the most thermostable of these enzymes (5, 13). ES4 GDH was of interest because its maximal growth temperature is 7°C higher than that of $P$. furiosus.

We describe here the characterization of ES4 GDH and the molecular cloning of the gene encoding this exceptionally thermostable enzyme, as well as its expression in vitro. ES4 GDH is the most thermostable dehydrogenase reported so far. Comparison of the ES4 GDH sequence with sequences from thermophilic and mesophilic organisms provides information on the structural adaptation to thermostability of these enzymes and on the molecular evolution of two families of GDH encoding genes.

### MATERIALS AND METHODS

NADP(H), NAD(H), and α-oxoglutarate were from Boehringer Mannheim. All other chemicals were of the highest available purity and were used without further purification.

**Bacterial Strain and Cultivation**—ES4 was grown as closed static cultures in synthetic sea water supplemented with a vitamin mixture, FeCl$_3$ (25 mM), elemental sulfur (5 g/liter, w/v), and Na$_2$WO$_4$ (10 mM). The abbreviations used are: GDH, glutamate dehydrogenase; kb, kilobase(s).
Hyperthermstable Glutamate Dehydrogenase

\( \mu m \) as previously described for \textit{P. furiosus} (15). The synthetic sea water medium (15), consisting of, per liter, NaCl (24 g), NaSO_4 (4 g), KCl (0.7 g), NaHCO_3 (0.2 g), KBr (0.1 g), H_2BO_3 (30 mg), MgCl_2·6H_2O (10.8 g), CaCl_2·2H_2O (1.5 g), SrCl_2·6H_2O (25 mg), sodium resazurin (0.2 mg), was supplemented with 5 g of elemental sulfur (S). Cells stored at 4 °C in this medium remained viable for at least 1 year. Large scale cultures were carried out in the absence of sulfur but with titanium (III) nitritotriacetate (final concentration, 30 mM) as a reductant in a 500-liter stainless steel fermenter, as previously described (15). Cultures were sparged with argon at a rate of 7.5 liters/min.

Purification of GDH—GDH was purified from 600 g of cells (wet weight) at 23 °C. The cell-free extract was prepared by sonication of a 20% (w/v) suspension in 50 mM Tris-HCl, pH 8.0, for 16 h in a Branson sonifying bath. The subsequent purification procedure was the same as for \textit{P. furiosus} GDH (4), up to and including the Q-Sepharose column. A gel filtration step using a Superdex 200 column (6 × 60 cm), equilibrated at 4 °C/mi/50 mM Tris, pH 8, containing 2 mM sodium dithionite, 2 mM dithiothreitol, 1 mM MgCl_2, 200 mM NaCl, and glycerol (10%, w/v) was employed instead of DEAE-Sephadex. These and all subsequent columns were controlled by a Pharmacia fast protein liquid chromatography system. The active fractions from the Superdex 200 column were combined, concentrated to about 200 ml using an Amicon ultrafiltration cell fitted with a PM30 membrane (Amicon, Beverly, MA), and applied to a column (5 cm × 1 cm) of Cibacron blue F3G-A (Bio-Rad), in the presence of 5 mM L-glutamate, at 24 °C. The enzyme was eluted with a 3-ml pulse of 1 mM NADP^+ (5). The pure GDH was established by SDS-gel electrophoresis. The M, of GDH was estimated by gel filtration using a 9 × 1.5-cm column of Sephacryl S-200HR (Pharmacia LKB Biotechnologies Inc., operated by a Bio-Rad Econo system, with 50 mM Tris-HCl buffer, pH 7.5, containing 2 mM sodium dithionite, 2 mM dithiothreitol, and 1 mM MgCl_2). The protein standards used were obtained from Bio-Rad and were as follows: bovine thyroglobulin (M, = 670,000), bovine albumin (M, = 185,000), chicken ovalbumin (M, = 44,000), equine myoglobin (M, = 17,000), and vitamin B12 (M, = 1,350). The GDH expressed was homogeneous when tested by SDS-PAGE. These results suggest that it was isolated as a hexameric species like \textit{P. furiosus} (4, 18). GDH utilizes NADP^+ exclusively as cofactor, whereas \textit{P. furiosus} utilizes both NAD^+ and NADP^+ (4).

Southern Hybridization—Genomic DNA from ES4 and \textit{P. furiosus} were digested with EcoRI, EcoRV, Sau3A, and BamHI. The fragments were separated on a 1% agarose gel and transferred to a nylon membrane (Magnagraph, MSI, MA). The hybridization was carried out with the 0.9-kb fragment of the ES4 gdhA gene following labeling by random primed-[alpha-^32P]dATP incorporation.

Expression of the GDH Gene—Polymerase chain reaction products with Ncol and BamHI sites at the 5' and 3' ends, respectively, were inserted into pTM1 (23) between the Ncol and BamHI restriction sites. A T7 promoter vector, pBluescript II, with translation initiation codon of gdhA was cloned into pBluescript plasmid (Stratagene). The hybridization was carried out with the 0.9-kb fragment of the ES4 gdhA following labeling by random primed-[alpha-^32P]dATP incorporation.

RESULTS

Enzyme Purification, Molecular Weight, and Enzymatic Properties of GDH—ES4 GDH was purified 75-fold from a cell-free extract and was homogeneous when tested by polyacrylamide gel electrophoresis (data not shown). The enzyme had an apparent molecular weight of 270,000 ± 5,000 M, as estimated by gel filtration on a calibrated Sephacryl S-200HR column, and a subunit molecular weight of 46,000 ± 3,000, as estimated by SDS-PAGE. These results suggest that it was isolated as a hexameric species like \textit{P. furiosus} GDH (4). However, in contrast ES4 GDH utilizes NADP^+ exclusively as cofactor, whereas \textit{P. furiosus} utilizes both NAD^+ and NADP^+ (4).

Fig. 1 is an electron micrograph of a typical field of ES4 GDH as seen after staining with uranyl acetate. The hexam-
eric structure of the molecule is derived by analysis of different orientations of the molecule lying on the specimen grid: cloverleaf (C), ring (R), and side (S) views, and is consistent with triangular antiprism. This analysis is similar to that of Josephs (17) for bovine liver.

Thermotolerance—ES4 GDH displayed total stability at room temperature or at 4 °C over a period of at least 6 months and did not require anaerobic conditions for stability. Fig. 2 shows the remarkable resistance to thermal denaturation exhibited by the pure enzyme. At a final concentration of 1.0 mg/ml, the half-life was 10.5 h at 100 °C, and 3.5 h at 105 °C. Addition of 250 mM potassium citrate improved the thermo- stability marginally (Fig. 2). The apparent half-life of the enzyme at 90 °C was 20 h.

Fig. 3 shows typical excess heat capacity versus temperature scans of ES4 GDH (1.5 mg/ml) between 45–75 °C and 103–120 °C. Activation of the enzyme was accompanied by a slight rise in heat capacity which was maximal at 60 °C (tmax) as shown in Fig. 3A. This transition was fully reversible. Thermal denaturation of the protein (Fig. 3B) was accompanied by a steep rise in the heat capacity beginning at 103 °C. The heat capacity passed through a maximum at 113 °C (tmax), and dropped back to the instrumental baseline at 117 °C (Fig. 3B). Total unfolding of the protein favored aggregation. Cooling the instrument and repeating the scan resulted in a stable base line, indicating that the thermal denaturation was irreversible. Briefly heating ES4 GDH to 110 °C in the calorimeter did not destabilize the protein irreversibly. The unfolding transition of GDH was accompanied by a large increase in the absorbance at 280 nm (data not shown). The relevant thermodynamic data of the heat activation and denaturation of ES4 GDH are listed in Table I. The ratio of ΔHca/ΔHdenat Hoff equals n, which is the number of independent folding units. The transition temperature (tca) is in °C. Values for ΔHca and ΔSca, which are the molar enthalpy and entropy changes are given in Kcal/mol and Kcal/(K·mol) respectively, assuming a molecular weight of 270,000 for the hexameric complex. 1.00 kcal = 4.18 KJ.

| Denaturation | Activation | n |
|--------------|------------|---|
| 414          | 120        | 1.9 |

An attempt was made to measure the ΔCp. Amino-terminal Sequencing—ES4 GDH was immobilized on a polyvinylidene difluoride membrane and subjected to amino-terminal microsequencing. In agreement with the physical studies, this resulted in a single unambiguous sequence, showing that GDH contains a single type of subunit. A comparison of amino-terminal sequences (below) shows strong homology among the thermophilic Archaea,

ES4  VEQDPFETAVKQLERAQYMIS
P. furiosus2  VEQDPFYEV1KQLERAQYMES
T. litoralis (29)  VEQDFFXTAVKQLXRAQYXDI

SEQUENCE I

2 Eggen, R. I. L., Geerling, A. C. M., Waldkotter, K., Antranikian, G., and de Vos, W. M. (1993) Gene (Amst.), in press.
where the bold letters denote conserved amino acid residues. The enzymes from ES4, P. furiosus, and T. litoralis do not have a methionine in the first position.

Western Blot—ES4 and P. furiosus enzymes have a hexameric structure with 46,000 ± 5,000 M, subunits; the bands detected on the Western blot (Fig. 4) corresponded to the GDH subunits visible on duplicate gels. The GDHs from ES4, T. litoralis, and P. furiosus all reacted strongly. Detectable, but much weaker, antigenic cross-reaction occurred with bovine and yeast GDHs.

Cloning and Sequencing of the ES4 gdhA Gene—Several clones carrying an identical 17-kb BamHI insert were identified by plaque hybridization of a lambda Fix library of ES4 genomic DNA. Southern hybridization of a digested X clone showed an XbaI, an EcoRI, and HindIII fragments containing the partial or complete gdhA gene. The fragments were subcloned into Bluescript KS+. The ES4 gdhA gene was sequenced using these subclones (Fig. 5). Hybridization of the digested genomic DNA from ES4 and P. furiosus with an ES4 gdhA clone showed a single band using several restriction enzymes (data not shown), corresponding to a single copy of the gdhA gene in the genomes of these two Archaea.

The complete nucleotide sequence of the ES4 gdhA gene, consisting of a single open reading frame of 1260 nucleotides, is shown in Fig. 6. The open reading frame is preceded by AT-rich regions in which a putative ribosome-binding site GAGGTG, at position -7, and a putative promoter consensus TTTATATA, at position -51, were found. The deduced amino acid sequence of the ES4 GDH consists of 420 residues and confirms the NH2-terminal amino acid sequence obtained from the pure protein, except that methionine is encoded at position 1. The calculated subunit M, = 47,169 corresponds well with the apparent molecular weight of the protein subunits determined on SDS-PAGE (46,000 M).

Expression of ES4 gdhA Gene—ES4 gdhA gene expression was obtained by in vitro transcription and translation. The transcription, using T7 RNA polymerase, gave a 1.8-kb messenger RNA for the gdhA gene (data not shown). The mRNA, at a concentration of 5–10 µg/ml, was translated using an mRNA-dependent translation system from rabbit reticulocytes. Approximately 34.5 mg of protein (740 pmol) were made per milliliter of reticulocyte lysate as calculated from the methionine content of GDH (13 mol methionine/mol GDH), the calculated final specific activity of [14C]methionine and the trichloroacetic acid-precipitable radioactivity. These results were consistent with the quantitation by Western blot analysis. The translation product was analyzed by SDS-PAGE and fluorography. Fig. 7 shows the accumulation of a 46-kDa polypeptide with maximum level obtained after 90 min of incubation. Gel filtration analysis was used to determine the molecular weight of the translation product. The hexameric form accounted for 12% of the total GDH accumulated, whereas the remaining 88% occurred as monomers with a molecular weight of 46,000. Apparently complete molecular assembly is not achieved under these conditions (see “Discussion”). Despite the ability of the subunits to form hexamers, the hexameric fraction did not show any enzyme activity when assayed as described for the native enzyme. Western blot analysis of the translation products (Fig. 8) showed a strong reaction of the 46-kDa polypeptide with the antisemur to P. furiosus GDH, and the in vitro product migrated identically to the subunits of the ES4 protein.

Comparison of ES4 GDH with Protein Sequences from Other Organisms—A sequence alignment of GDHs from ES4, other archaea, bacteria, and eucaryotes is presented in Fig. 9. The proposed alignment has been constructed with the Treealign program based on a Dayhoff matrix. The primary sequence of ES4 GDH is 40–52% similar to a group of organisms including Clostridium difficile (52%), Halobacterium salinarium (48%), Sulfolobus solfataricus (47%) and bovine (40%), and only 33% similar to another group containing Clostridium symbiosum, Escherichia coli, and the yeast Saccharomyces cerevisiae. A comparative analysis of amino acid composition among GDHs from mesophilic and thermophilic organisms revealed preferential changes of amino acid residues in the thermophiles, as follows: decreases were found in cysteine, methionine, and asparagine residues and increases in isoleucine and aspartic acid residues.

**DISCUSSION**

This paper describes the purification, cloning, and expression for the first time of a thermostable GDH from a hyperthermophile with a maximal growth temperature of 110 °C (3). We also describe the first enzyme to be characterized from this organism and the largest enzyme to be characterized and cloned from an organism that can grow above 100 °C. GDH is a major protein in the cytoplasm of ES4 as the yield of this enzyme during purification is comparable to that reported for the GDH (4), hydrogenase (8), and glyceraldehyde ferredoxin oxidoreductase (12) from P. furiosus. Our previous work with the GDH from P. furiosus established that it had a half-life of 1.8 h at 103 °C (5), the tmax for growth of the organism (28). The present study indicates that ES4 GDH has a half-life of 3.5 h at 105 °C and that it retained 16% activity after incubation for 23 h at 105 °C. It is therefore the most thermostable dehydrogenase reported to date. This extreme thermostability may relate to the maximal temperature for growth of the organism.

ES4 GDH has a hexameric structure with a molecular weight of 270,000. Microcalorimetry studies showed a denaturation temperature of 113 °C and an activation temperature of 60 °C. These values are very similar to those reported previously for P. furiosus GDH (5). The post-transitional rise reflects the exposure of nonpolar hydrophobic amino acids to the polar aqueous solvent during the unfolding process, indicating the contribution of hydrophobic interactions to protein stability (5).
The \( \text{NH}_2 \)-terminal amino acid sequence of ES4 GDH was determined and showed strong homology with sequences from two other hyperthermophiles, \textit{P. furiosus} (4) and \textit{T. litoralis} (29). The GDHs from these three hyperthermophiles do not possess methionine at the \( \text{NH}_2 \)-terminal, although methionine could allow the \( \text{NH}_2 \)-terminal of the protein to be locked by hydrogen bonding to internal \( \beta \)-sheet structures to prevent it from "unzipping" at high temperature (32). This has been described in the case of \textit{P. furiosus} rubredoxin, where a three-dimensional structure has been determined (31, 32). \textit{Sulfolobus solfataricus} GDH possesses an acetylated methionine at the \( \text{NH}_2 \)-terminal (30). This emphasizes the importance of obtaining direct amino acid sequencing data in addition to DNA deduced sequences, since the latter do not provide information about post-translational modification. The homology among thermophilic GDH amino acid sequences was confirmed by the Western blot analysis (Fig. 4) with ES4, \textit{P. furiosus}, and \textit{T. litoralis} enzymes. Bovine and yeast GDHs showed weak cross-reaction with the ES4 protein. This attests to the presence of highly conserved regions among GDH enzymes. Bovine and yeast GDHs showed weak cross-reaction with the ES4 protein. This attests to the presence of highly conserved regions among GDH enzymes.

The derived amino acid sequence is given in \textit{one-letter code} above the respective nucleotide sequence. The putative promoter is \textit{underlined}, and the putative ribosome-binding site is \textit{underlined} with asterisks.
control region shows an AT-rich region in which a putative promoter can be detected at position −51. The putative promoter sequence TTTATATA, referred to as Box A, resembles the eucaryotic TATA box and the consensus TTTA/TATA that polymerase is more closely related to the eucaryotic RNA polymerase 11 than to bacterial RNA polymerases (36, 37). The opposite bias observed in other hyperthermophiles, but to a smaller extent than for vertebrates, such as rabbits, does not grow below this temperature. Also, the cytoplasm of the hyperthermophile *P. furiosus*, which is closely related to ES4, contains 700 mM KCl (14). Therefore, extreme physicochemical conditions such as heat and high ionic strength may be required during molecular assembly of hyperthermstable enzymes. The participation of other factors such as chaperonins may also be required. Chaperone-like particles have been described from several thermophiles, for example *Thermus thermophilus* (40), *Sulfolobus bshatae* (41) and *Pyrdictium occultum* (42, 43). All possess ATPase activity and are induced by heat stress. These molecules resemble procaryotic (GroEL) and eucaryotic (Hsp60 and ribulose-bisphosphate carboxylase/oxygenase) chaperonins and are able to promote the refolding of several guanidine-HCl denatured enzymes from thermophilic bacteria in *vitro* (40). We are proceeding with further studies that may permit us to produce an active enzyme using the eucaryotic in *vitro* system.

A sequence alignment of GDHs from the three domains, Eucarya, Bacteria, and Archaea (1) revealed strong homology among thermophilic enzymes. It is interesting to note that ES4 GDH has 56% homology with GDH from the bacterium *C. difficile*, and 40% homology with bovine GDH.

The fact that ES4 GDH is closer to a bacterial GDH (*C. difficile*) than to *S. solfataricus*, a sulfur-dependent thermophile, or to *H. salinarium*, bespeaks the enormous phylogenetic distances within the Archaea. These results are in agreement with a GDH phylogenetic tree recently proposed by Porterre et al. (34), but do not agree with the universal tree of life previously presented by Woese et al. (1) or Rivera and Lake (44). The existence of two families of genes encoding hexameric GDHs has been deduced from the alignment of primary sequences and the use of percentage similarity between each pair of proteins (34). The appearance of these two gene families predates the divergence of the Bacteria, Eucarya, and the Archaea, since there are representatives of GDH from both families (I and II) in the Bacteria and the Eucaryotes. Thus, we find no evidence that the Archaea have a polyphyletic origin as suggested by Rivera and Lake (44). So far, only one family of GDH molecules has been found in the Archaea, so that it is possible that the ancestral split between the families occurred after the divergence of the Archaea. This would imply that the Archaea diverged very early in the time span of evolution. This must be treated with caution, however, since only three GDH sequences are available from Archaea, and none of these is from a methanogen.

The alignment of mesophilic and thermophilic GDHs was also used to establish specific structural features of the thermophilic enzymes. The primary sequence of GDH is similar to other GDHs and contains the strongly conserved regions observed in other GDH sequences (33). The conserved amino acids are grouped in clusters corresponding to the catalytic sites of the enzyme, namely substrate- and coenzyme-binding sites. The fingerprint motif Gly-Xaa-Gly-Xaa-Gly or Ala is completely conserved in the archaeal sequences. According to Wierenga et al. (45), the third conserved position in the motif is correlated to coenzyme specificity; Gly corresponds to NAD+ specificity and Ala to NADP+ specificity. However, the validity of assigning coenzyme specificity to a single
The sequences are as follow:
GenBank and ES4 sequence were
aligned using the Treealign program.

Fig. 9. Sequence alignment of bovine, H. sal, C. dif, C. sym, S. cer.
residue appears to be invalid. In particular, the C. symbiosum motif contains Ala in the third conserved position and the enzyme is NAD" specific (48), whereas the NAD" specific H. salinarium enzyme has glycine in this position (47).

Proteins from hyperthermophiles, including the multisubunit enzymes that we have described, display an extraordinary level of intrinsic thermostability in vitro (4, 5, 14, this work). There is no evidence for additional stabilizing factors to account for their function during growth at or near to 100 °C. Processes causing irreversible inactivation of enzymes at high temperatures include denaturation of asparagine residues, hydrolysis of peptide bonds at aspartic acid residues, destruction of disulfide bonds, and subsequent formation of incorrectly folded structures (48). ES4 GDH shows a decrease in the number of the sulfur containing residues, methionine, and cysteine, which is in agreement with several studies on hyperthermophilic enzymes (14, 49, 50). This characteristic might serve to stabilize covalent protein structures at high temperatures. The absence of initiating methionines in enzymes from several hyperthermophiles (4, 31, this work) may represent an extension of this adaptive feature. Zwickl et al. (14) found that the thermostable glyceraldehyde-3-phosphate dehydrogenase molecule had a "striking increase in the aromatic residue phenylalanine and a respective decrease in aspartic acid residues" through comparison with homologous mesophilic enzymes. In contrast, we find that GDH from ES4 exhibits a decrease in phenylalanine when compared with S. solfatarius (50) and C. difficile (51). Conversely, aspartic acid is indeed increased with increasing thermostability when GDH from these organisms is compared. It is therefore clear that the consideration of amino acid composition alone is insufficient to allow prediction of thermostability. Hensel et al. (49) show that most of the Asn residues that are hydrolyzed in M. fervidus GAPDH at 85 °C are replaced by more stable residues in P. woesei GAPDH. The conserved Asn residues are involved in catalytic sites and are probably protected by the rigid conformation of the molecule (49). Compared with mesophilic GDHS, ES4 GDH has an increased overall hydrophobicity. Similar tendencies have been reported for P. woesei GAPDH (44). Enhanced hydrophobic interactions therefore represent mechanisms for structural thermostability.

Further finding the thermoneutralizing the thermostability of this extremely stable enzyme must await the elucidation of its three-dimensional structure, which is in progress.

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