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Expression and In Vitro Assembly of Recombinant Glutamate Dehydrogenase from the Hyperthermophilic Archaeon *Pyrococcus furiosus*†

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The *gdhA* gene, encoding the hexameric glutamate dehydrogenase (GDH) from the hyperthermophilic archaeon *Pyrococcus furiosus*, was expressed in *Escherichia coli* by using the pET11-d system. The recombinant GDH was soluble and constituted 15% of the *E. coli* cell extract. The N-terminal amino acid sequence of the recombinant protein was identical to the sequence of the *P. furiosus* enzyme, except for the presence of an initial methionine which was absent from the enzyme purified from *P. furiosus*. By molecular exclusion chromatography we showed that the recombinant GDH was composed of equal amounts of monomeric and hexameric forms. Heat treatment of the recombinant protein triggered in vitro assembly of inactive monomers into hexamers, resulting in increased GDH activity. The specific activity of the recombinant enzyme, purified by heat treatment and affinity chromatography, was equivalent to that of the native enzyme from *P. furiosus*. The recombinant GDH displayed a slightly lower level of thermostability, with a half-life of 8 h at 100°C, compared with 10.5 h for the enzyme purified from *P. furiosus*.

Pyrococcus furiosus is a marine hyperthermophilic archaeon that grows optimally at 100°C (10). Its glutamate dehydrogenase (GDH; EC 1.4.1.3) catalyzes the reversible oxidative deamination of L-glutamate to α-ketoglutarate and ammonia. GDHs are ubiquitous enzymes that occupy a pivotal position between carbon and nitrogen metabolism (12). In *P. furiosus*, GDH represents up to 2% of the total soluble protein, indicating that it plays a major role in metabolism (22). *P. furiosus* GDH and the GDH from the deep-sea isolate ES4 are the most thermostable dehydrogenases currently known (7, 8, 19, 22). These enzymes have optimal activity above 85°C and a half-life of 10 h at 100°C (8, 22). The *P. furiosus* enzyme possesses a dual coenzyme specificity, NAD^+ and $NADP^+$, while GDHs isolated from several other hyperthermophilic archaea are $NADP^+$ specific (8, 14, 17, 22). GDHs from *P. furiosus*, *Thermococcus litoralis*, and ES4 have a hexameric structure and a M_r of 270,000 (8, 15, 17), whereas GDH from *Thermococcus* strain AN1 apparently has a tetrameric structure (14). Bacterial and fungal $NADP^+$-linked and vertebrate dual-specificity GDHs have a hexameric structure (24), and NAD^+-linked enzymes have either four identical subunits, as found in *Neurospora crassa* (27), or six identical subunits, as found in *Clostridium symbiosum* (21).

Above 40°C, *P. furiosus* GDH undergoes reversible heat activation which is accompanied by a conformational change (15). The enzyme is totally inactive at room temperature but remains in its hexameric form (15, 22). Differential scanning microcalorimetry of *P. furiosus* GDH also revealed thermal denaturation at 113°C, irreversible unfolding of the secondary structures, and inactivation of the enzyme (15); 113°C is the highest denaturation temperature recorded for a protein of this size. Similar results were obtained with GDH from ES4 (8).

The *gdhA* genes of *P. furiosus* (9), ES4 (8), and *T. litoralis* (7b) have been cloned and sequenced. The deduced GDH amino acid sequences are highly homologous to each other (7a) and have only 48% homology with GDHs from the archaea *Sulfolobus solfataricus* and *Halobacterium salinarium* (4, 8). As with glyceraldehyde-3-phosphate dehydrogenase from *Pyrococcus woesei* (29), the amino acid composition of hyperthermophilic GDHs has slightly increased overall hydrophobicity and decreased average chain flexibility compared with mesophilic GDHs (8). However, amino acid compositions and sequence analyses provide little insight into protein-stabilizing features for GDH (and hyperthermophilic enzymes in general). To elucidate protein thermostability, detailed structural information is needed in conjunction with mutagenesis studies with recombinant enzymes.

As a preamble to these studies, we have expressed GDH from the hyperthermophilic archaeon *P. furiosus* in *Escherichia coli*. We also report in vitro assembly of GDH hexamers from monomers produced by overexpression at 37°C in *E. coli.*

MATERIALS AND METHODS

Chemicals and enzymes. NADP(H), NAD(H), and 2-oxoglutarate were from Boehringer Mannheim, Indianapolis, Ind. Restriction enzymes and *Taq* polymerase were from Promega (Madison, Wis.). All other chemicals were of the highest purity available and were used without further purification.

Cultures and media. *P. furiosus* DSM 3638 was grown as described by Robb et al. (22). *E. coli* BL21 (DE3)pLysS [F^- *ompT hsdsB* (r_B^- m_B^-) *gal dcm* (DE3) pLysS (Cam^r)] (Novagen, Madison, Wis.) referred to as *E. coli* BL21, was used to clone and express the *P. furiosus gdhA* gene. All *E. coli* cultures were inoculated from a stock culture in glycerol (stored at −80°C) made from a single colony. Ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) were included in Luria-Bertani medium as required.

Nucleic acid extraction. A *P. furiosus* culture (1 liter) was filtered through 3MM paper (Whatman) to remove most of the elemental sulfur. Cells were collected by centrifugation at 4,500 × *g* for 15 min at 4°C, and the pellet was washed twice with 2 ml of TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA). DNA from *P. furiosus* was extracted by the hexadecyltrimethylammonium bromide-cesium chloride method (2). Plasmid DNA was isolated by standard methods (23).

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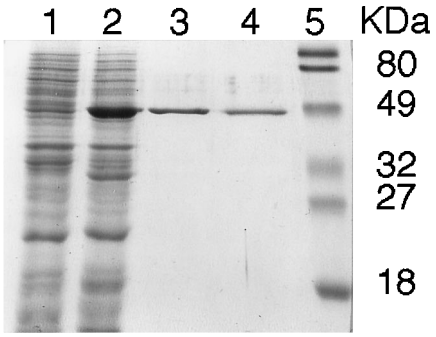

FIG. 1. SDS-PAGE of *P. furiosus* and recombinant GDHs. Samples of cell extracts (5 μl) and purified enzymes (1.5 μg) were applied to an SDS–12% polyacrylamide gel. Lanes: 1, *E. coli* BL21 extract; 2, *E. coli* BL21(pPGDH) extract containing recombinant GDH; 3, recombinant GDH; 4, GDH from *P. furiosus*; 5, molecular weight markers.

Expression of the GDH gene. Plasmid pPGDH was constructed by inserting a PCR product of *P. furiosus* genomic DNA into the pET-11d vector (Amp^r) (Novagen) (25). The product contained the *gdhA* gene with *Nco*I and *Bam*HI restriction enzyme sites at the 5′ and 3′ ends, respectively. *E. coli* BL21 cells containing plasmid pPGDH [referred to as *E. coli* BL21(pPGDH)] were induced by 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at midexponential phase and incubated for 2.5 h at 37°C. The cells (from a 1-liter culture) were centrifuged, and the pellet was washed once in 0.9% NaCl and resuspended in 4 ml of TED buffer (50 mM Tris HCl [pH 7.6], 1 mM EDTA, 1 mM dithiothreitol). A single freezing-and-thawing step was used to lyse the cells. The lysate was treated with DNase I (100 μg/ml) for 15 min at 37°C. The supernatant fraction was recovered by centrifugation at 48,000 × *g* for 45 min at 4°C.

Protein purification. GDH was purified from *P. furiosus* cells as described previously (8). *E. coli* BL21(pPGDH) extract containing recombinant GDH was heated either at 75 or 90°C for 15 min and centrifuged at 48,000 × *g* for 20 min at 4°C. The supernatant fraction was applied to an anion-exchange resin (Econo-pac Q cartridge; Bio-Rad, Richmond, Calif.) equilibrated in TED buffer, and GDH was eluted at 0.24 M NaCl. GDH was further purified by affinity chromatography on a Cibacron blue F3GA column (5 by 1 cm; Bio-Rad) in 20 mM Tris (pH 8.0)–28 mM NaCl containing 5 mM L-glutamate. The enzyme was eluted in the same buffer with a 3-ml pulse of 1 mM $NADP^+$ (15). All steps were carried out at 22°C. The homogeneity of purified GDH was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide).

Protein analysis. Protein concentrations were determined by the bicinchoninic acid colorimetric assay with bovine serum albumin as a standard (Pierce, Rockford, Ill.). After SDS-PAGE, protein bands were scanned and quantified with a densitometer (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, Calif.) equipped with image-quantitating software (ImageQuaNT; Molecular Dynamics). The M_r of recombinant GDH forms was estimated by gel filtration with a Sephacryl S-200HR column (90 by 1.5 cm; Pharmacia, Piscataway, N.J.) operated by a Bio-Rad Econo system with 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl as the eluent, and by gel filtration with a column (HR 10/30) of Superose 12 operated by an LKB Bromma 2151 HPLC system with 50 mM Tris HCl buffer (pH 8.0) containing 0.2 M NaCl as the eluent. The standards used were obtained from Bio-Rad: bovine thyroglobulin (M_r 670,000), bovine globulin (M_r 158,000), chicken ovalbumin (M_r 44,000), equine myoglobin (M_r 17,000), and vitamin B_{12} (M_r 1,350). Chromatography was carried out at 22°C. Heat pulses were applied to recombinant GDH fractions with a TEMPTRONIC Thermocycler (Thermolyne). Immunodetection was performed as described by DiRuggiero et al. (8).

Enzyme assay. GDH activity was measured by monitoring the glutamate-dependent reduction of $NADP^+$ at 85°C as described previously (22). One unit of activity is defined as 1 μmol of NADPH formed per min per mg of protein (22).

Thermostability determination. For determination of GDH thermostability at 100°C, the enzyme was equilibrated in 0.05 M glycylglycine buffer (pH 7.5) with repeated filtration in Centriprep-30 units (Amicon, Lexington, Mass.) and placed in microcentrifuge tubes with O-ring-sealed caps. Duplicate tubes were set in a heat block maintained at 100 ± 1°C. Control experiments in which glycylglycine buffer alone was incubated for similar periods resulted in no change in pH, indicating that the buffer system was stable under these conditions. Tubes were removed at hourly intervals, chilled on ice, centrifuged briefly, and sampled for enzyme activity. The enzyme concentration was 1 mg/ml.

Protein sequencing. Purified GDH was resolved by SDS-PAGE in 20 mM Tricine buffer (pH 8.2) and electroblotted onto a polyvinylidene difluoride membrane (18). The microsequencing was performed by cyclic Edman degradation.

ES4	**VEQDP**FEI**A**V**KQLERAAQY**MIS
Thermococcus litoralis	**VEQDP**FX**I**A**V**K**QL**X**RAAQY**XDI
Pyrococcus furiosus	**VEQDP**YEI**V**I**KQLERAAQY**MES
Recombinant GDH	M**VEQDP**YEI**V**I**KQLERAAQY**MES

FIG. 2. Alignment of N-terminal amino acid sequences of ES4 (8), *T. litoralis* (17), *P. furiosus* (9), and recombinant GDH (see the text).

RESULTS

Expression of GDH in *E. coli*. SDS-PAGE analysis of protein extract obtained during in vivo expression of GDH is shown in Fig. 1. As expected, no specific products were seen in the sample from *E. coli* BL21 (lane 1). An abundant product with an M_r of 46,000 (the molecular weight of the *P. furiosus* GDH subunit [22]), was found in the soluble fraction of the sample from *E. coli* BL21(pPGDH) ($gdhA^+_{pfu}$) (lane 2). Densitometric analysis of several identical gels showed that the recombinant GDH accounted for about 15 ± 3.3% of the total soluble proteins in *E. coli* BL21(pPGDH) extract. On SDS-PAGE analysis, purified protein obtained from *E. coli* BL21(pPGDH) extract (lane 3) was identical in size to the enzyme purified from *P. furiosus* (lane 4). Immunodetection with an antiserum raised against *P. furiosus* GDH yielded a strong cross-reaction with the recombinant GDH (result not shown).

N-terminal amino acid sequence. The N-terminal amino acid sequence of the recombinant GDH was compared with amino acid sequences of GDHs from *P. furiosus*, *T. litoralis*, and the deep-sea isolate ES4 (Fig. 2). The recombinant GDH had an identical sequence to the enzyme from *P. furiosus*, except that it contained an initial methionine not present in the enzyme from *P. furiosus* or in any of the other GDHs. The deduced *P. furiosus* GDH amino acid sequence is 86 and 95% homologous to deduced GDH sequences of *T. litoralis* and ES4, respectively (8, 9, 17).

Effect of heat treatment on recombinant GDH activity. *P. furiosus* GDH is usually assayed at 85°C. However, to analyze the effect of temperature on the activity of the recombinant enzyme, we assayed the *E. coli* extracts at 45°C. Aliquots of *E. coli* BL21(pPGDH) extract were subjected to 15-min heat pulses from 50 to 95°C, and 250 μg of total protein was assayed for 1.2 min at 45°C for GDH. Control experiments in which *E. coli* BL21 extract was assayed under the same conditions did not yielded detectable activity (results not shown). The GDH activity in the aliquot containing the recombinant enzyme increased with temperature pulses that ranged from 50 to 90°C (Fig. 3A). Application of a 15-min heat pulse at 90°C to the *E. coli* BL21(pPGDH) extract resulted in a twofold increase in GDH activity at 45°C.

The effect of heat treatment at 85°C for different incubation times is shown in Fig. 3B. The enzyme reached a plateau of activity at 12 min, after which no change in GDH activity was detected (Fig. 3B).

Structural analysis of the recombinant GDH. *E. coli* BL21(pPGDH) extract was analyzed by molecular exclusion chromatography on a calibrated Sephacryl S-200 HR column. GDH activity in the fractions was assayed at 85°C. An extract heated for 15 min at 75°C, an intermediate temperature, was applied to the column. This experiment showed that recombinant GDH from *E. coli* was active at 85°C and that all of the activity was located in the high-molecular-weight fractions (fractions 52 to 55), which corresponded to the 270,000 M_r hexameric form (Fig. 4A). No detectable activity was found in low-molecular-weight fractions (fractions 66 to 69) corresponding to the 46,000 M_r monomeric form (Fig. 4A). An

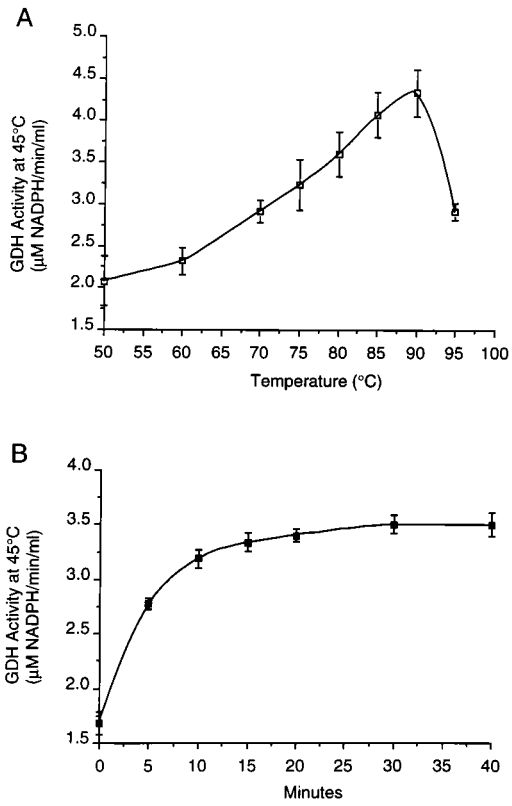

FIG. 3. Effect of heat treatments on the activity at 45°C of *E. coli* BL21(pPGDH) extract containing the recombinant GDH. Heat pulses from 50 to 95°C at 15-min intervals (A) or heat treatments at 85°C at different incubation times (B) were applied to the extract.

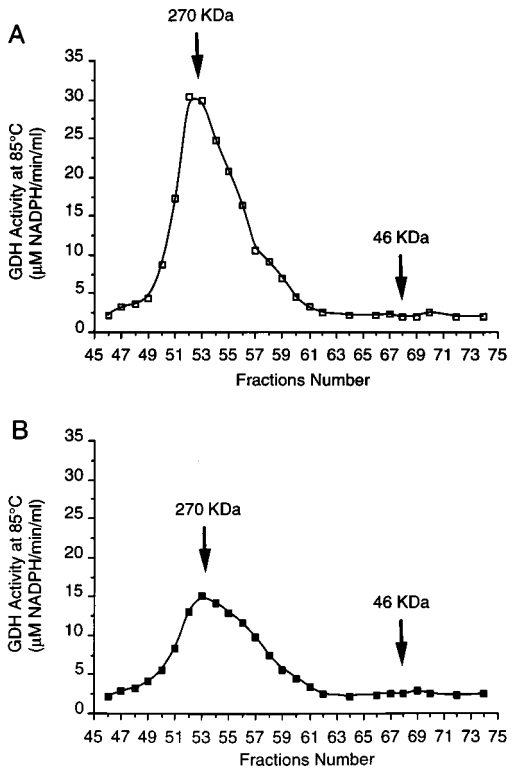

FIG. 4. GDH activity at 85°C of fraction collected after gel filtration chromatography on a calibrated Sephacryl S-200 HR column. Two types of *E. coli* BL21(pPGDH) extracts containing the recombinant GDH were applied to the column: extract heated for 15 min at 75°C (A) and untreated extract (B).

untreated extract was applied to the same column, and GDH activity was found only in high-molecular-weight fractions (fractions 52 to 55) (Fig. 4B). However, the activity of the peak fractions was only 50% of that of the heated extract. The molecular weights of these fractions were confirmed by rechromatography on a Superose 12 column (results not shown).

Fractions obtained by molecular exclusion chromatography were analyzed by SDS-PAGE. Both high-molecular-weight fractions (fractions 52 to 55 combined) (lanes 3 and 5) and low-molecular-weight fractions (fractions 66 to 69 combined) (lanes 4 and 6) contained GDH, visualized as 46,000 M_r subunits on SDS-PAGE (Fig. 5). Fractions from the unheated extract (lanes 3 and 4) contained *E. coli* proteins, which were removed by heat treatment as shown for the heat-treated extract fractions (lanes 5 and 6). Densitometric analyses performed on several identical gels showed that the untreated extract contained equal amounts of hexameric and monomeric GDH. This is also apparent from the SDS-PAGE gel shown in Fig. 5. Heat treatment (15 min at 75°C) increased the proportion of GDH hexamers in the extract from 50 to 85% (Fig. 5).

Subunit assembly of recombinant GDH. Fractions obtained by molecular exclusion chromatography of an untreated *E. coli* BL21(pPGDH) extract (Fig. 4B) were heated individually at 75°C for 15 min. After heat treatment, GDH activity was assayed at 85°C and compared with the activity of the fractions before heat treatment. The high-molecular-weight fractions (fractions 52 to 55) did not show any change in activity, but the previously inactive monomeric fractions (fractions 66 to 69) displayed a peak of activity after the heat treatment. The heat-treated fractions 66 to 69 were combined and analyzed by molecular exclusion chromatography on a calibrated Sephacryl S-200 HR column. Analysis of the elution profile by SDS-PAGE (Fig. 6) showed that after heat treatment the high-molecular-weight fractions were composed entirely of hexameric GDH. A minor component made up of unassembled monomers could be detected as shown in Fig. 6, lane 3.

Specific activity and thermostability. Recombinant GDH was purified by heat treatment for 15 min at 75 or 90°C and affinity chromatography. The specific activity of these samples at 85°C was identical to that of the enzyme purified from *P.*

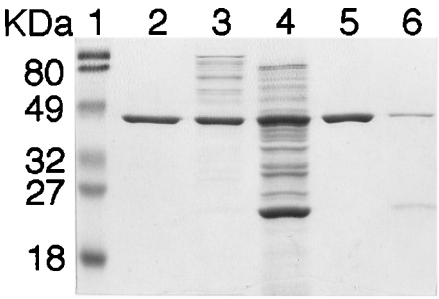

FIG. 5. SDS-PAGE of molecular exclusion chromatography fractions of *E. coli* BL21(pPGDH). Samples of chromatography fractions (6 μl each) and purified enzyme (1.5 μg) were applied to an SDS–12% polyacrylamide gel. Lanes: 1, molecular weight markers; 2, recombinant enzyme; 3, high-molecular-weight fractions of an untreated extract applied to the gel filtration column; 4, low-molecular-weight fractions of the same extract; 5, high-molecular-weight fractions of a extract heated for 15 min at 75°C and applied to the gel filtration column; 6, low-molecular-weight fractions of the same extract.

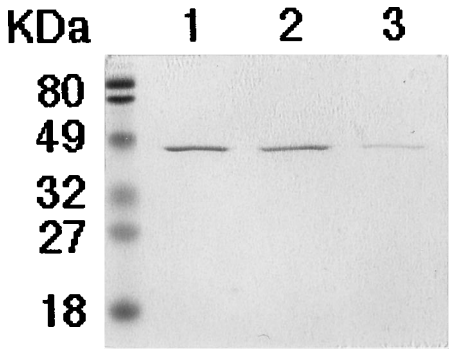

FIG. 6. SDS-PAGE of molecular exclusion chromatography fractions of *E. coli* BL21(pPGDH) after heat treatment. Fractions 67, 68, and 69 from the experiment showed in Fig. 4B were heated individually at 75°C for 15 min, combined, and sized by gel filtration chromatography. The fractions thus obtained were analyzed on an SDS–12% polyacrylamide gel. Lanes: 1, purified recombinant enzyme (1.5 μg); 2, concentrated sample of a high-molecular-weight fraction (200 μl); 3, concentrated sample of a low-molecular-weight fraction (200 μl).

furiosus (Table 1). The thermostability of these purified enzymes was determined at 100°C over a period of 25 h. The calculated half-lives at this temperature are shown in Table 1. A slight difference was observed between the recombinant enzyme preparations, both of which had $t_{1/2}$ of 7.0 to 8.0 h, and the *P. furiosus* GDH, which had a $t_{1/2}$ of 10.5 h, as reported previously (22).

DISCUSSION

P. furiosus GDH was overexpressed in *E. coli*, constituting 15% of the soluble proteins in the cell extract. The purified recombinant enzyme had a specific activity equivalent to that of the native enzyme. Very few enzymes from hyperthermophilic archaea have been expressed in *E. coli*; citrate synthase from *Thermoplasma acidophilum* (26) is an M_r 43,000 monomer, the α-amylase (16) and ferredoxin (13) from *P. furiosus* are an M_r 150,000 homodimer and an M_r 7,500 monomer, respectively, while the glyceraldehyde dehydrogenase (GAPDH) from *P. woesei* (29) is an M_r 150,000 homotetramer. The *P. furiosus* GDH is therefore the most complex hyperthermostable protein to be expressed in a heterologous system. In most of the previous studies, the intrinsic thermostability of the enzymes allowed drastic heat treatment to be used as an initial step in purification of the enzyme from heat-labile *E. coli* proteins, and the recombinant proteins were obtained in a soluble, fully active conformation. It occurred to us that these heat treatments could potentiate the maturation of the enzymes, and this is indeed true for *P. furiosus* GDH.

TABLE 1. Specific activity and thermostability at 100°C of native and recombinant GDHs

GDH[a]	Sp act (U)[b]	$t_{1/2}$[c]
P. furiosus	411 ± 33	10.5 ± 0.5
Recombinant (75°C)	419 ± 30	7.0 ± 0.3
Recombinant (90°C)	423 ± 13	8.1 ± 0.6

[a] *P. furiosus* GDH; recombinant GDHs heat treated at 75 and 90°C, respectively.

[b] The activity was measured in triplicate at 85°C; 1 U of activity was defined as 1 μmol of NADPH formed per min per mg of protein. Values are means ± standard deviations.

[c] The thermostability was measured at 100°C in 0.05 M glycylglycine buffer (pH 7.5). The enzyme concentration was 1 mg/ml.

Overproduction of GDH in *E. coli* at 37°C results in recovery of 50% of the recombinant GDH in monomeric form. It is important to point out that the purified GDH from *P. furiosus* and the hexameric fractions of the recombinant GDH retain their hexameric structure at ambient temperature, even though these enzymes are inactive at 22°C (15, 22; also see above). These results suggest strongly that monomers accumulate in the *E. coli* host cells and not during the purification steps. In addition, in *P. furiosus*, the enzyme is always produced at temperatures higher than 70°C, since the organism does not grow below this temperature (10), and the intracellular concentration of KCl is 700 mM (29). Therefore, we hypothesize that extreme physicochemical conditions such as heat and high ionic strength may be required for rapid molecular assembly of this hyperthermostable enzyme. As far as we know, this hypothesis has not been tested for other multimeric enzymes from hyperthermophiles. Interestingly, half of the recombinant enzyme assembled spontaneously into hexamers in *E. coli* cells. We propose that the rate of spontaneous assembly into hexamers at 37°C in the host cell cytoplasm was slow because of the relatively low temperature compared with the growth temperature of *P. furiosus*. The intervention of other factors such as chaperones must also be considered, since it has been shown that GroEL can enhance the assembly of incompletely folded subunits of a recombinant luciferase from *Vibrio harveyi* (11). We are presently exploring the possibility that *E. coli* chaperones are involved in the assembly of GDH at low temperature.

In previous studies, we have shown by calorimetry and optical density (A_{280}) measurements that *P. furiosus* GDH undergoes reversible heat activation (15, 22). This event, starting at 40°C, is accompanied by a progressive conformational change of the protein from an initially inactive conformation. In the present work, two processes are involved: first, the oligomerization of recombinant monomers into hexamers, and second, the reversible heat activation of the enzyme to achieve an active conformation at 85°C. The recombinant hexameric GDH was inactive at 22°C but showed activity at 85°C, indicating that reversible activation could occur with GDH assembled in vitro. We have demonstrated that the increase in recombinant GDH activity was related to heat treatment and that it was the result of in vitro assembly of inactive monomers into the active hexameric conformation of GDH. GDHs from *Clostridium*, yeast, and bovine cells (28), as well as the archaeon *Sulfolobus solfataricus* (6), have been found to be inactive in their monomeric forms. Our results are in agreement with these findings. The assembly process was dependent on the protein concentration, since a diluted solution of monomers, used to assay activity at 85°C for 1.2 min, did not show any GDH activity (Fig. 4B). Dependence on protein concentration has also been shown for thermostability in *P. furiosus* GDH (22) and *S. solfataricus* GDH (6).

Our results are the first instance of in vitro oligomeric GDH assembly of monomers produced in an in vivo system. In previous studies, West and Price (28) showed that the reassociation of GDH subunits from bovine, yeast, and *C. symbiosum* cells was performed after dissociation by guanidine-HCl and progressive dilution of the chaotropic agent. In that instance, only trimeric subunits were able to reassemble into hexamers, while dissociation to monomers was accompanied by secondary-structure changes and irreversible loss of activity. The major problem associated with most, if not all, protein assembly studies is the need to dismantle mature proteins by the use of denaturants that are present during reassembly. The system we describe here has the advantage that proteins are blocked at a stage prior to assembly at ambient temperature

and can be assembled under defined conditions. The processes of assembly of multimeric protein and the folding of individual subunits are hard to differentiate, because of the difficulty in separating these events temporally during the renaturation of proteins. However, as shown in Fig. 3B, in vitro assembly of recombinant GDH is a very slow event at low temperatures and this could allow a variety of processes to be studied, e.g., the accumulation of intermediates during assembly of the enzyme at suboptimal temperatures. The conformation of monomers when produced in *E. coli* at 37°C has not been investigated. The unassembled monomers may be in a cold intermediate state, as found with the GAPDH of *Thermotoga maritima* (20).

The N-terminal amino acid sequence of the recombinant GDH was compared with amino acid sequences of GDHs from *P. furiosus*, *T. litoralis*, and ES4 (Fig. 2). The sequence of the recombinant GDH is identical to that of the native GDH from *P. furiosus*, except that it contains an N-terminal methionine that is not present in other hyperthermophilic GDHs. Presumably, the initial methionine is cleaved during posttranslational modification in *P. furiosus*, and its absence has been observed in several hyperthermostable proteins, notably *P. furiosus* rubredoxin. The absence of the N-terminal methionine has been shown to influence the rubredoxin structure (5). In that instance, the three-dimensional structure is known (5), and it is hypothesized that the major determinant of thermostability is the absence of the initial methionine in the enzyme, allowing the N terminus of the protein to be locked by hydrogen bonding to internal, β-sheet structures to prevent it from unzipping at high temperature (1, 5). The *P. furiosus* recombinant GDH is slightly less thermostable than the protein isolated from *P. furiosus* cells (Table 1). Although the cluster of helices α1 to α5 at the N terminal is not thought to be functionally crucial within the GDH family (3), the presence of methionine could be one of the factors contributing to the slight destabilization of the recombinant enzyme at 100°C.

Expression of the extremely thermostable GDH from *P. furiosus* has provided fully active recombinant GDH. Site-directed mutagenesis studies are now possible, and they will provide insights into the structural basis for extreme thermostability. Moreover, because of the slow process of assembly of these inactive subunits into the active hexameric conformation of the enzyme, this expression system has great potential for kinetic studies of the assembly of multimeric proteins; the effect of various ions, substrates, and solvent polarity; and the role of chaperones in these processes.

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