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Glutamate Dehydrogenase from the Hyperthermophile *Pyrococcus furiosus*

THERMAL DENATURATION AND ACTIVATION*

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Horst Klump‡, Jocelyne Di Ruggiero§, Martin Kessel¶, Jae-Bum Park‖, Michael W. W. Adams‖, and Frank T. Robb§¶**

From the ‡Biochemistry Department, University of Cape Town, Rondebosch 7700, South Africa, the §Center of Marine Biotechnology, University of Maryland, Baltimore, Maryland 21202, the ¶Department of Microbiology, University of Maryland, College Park, Maryland 20742, and the ‖Department of Biochemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602

Pyrococcus furiosus* is a marine hyperthermophile that grows optimally at 100 °C. Glutamate dehydrogenase (GDH) from *P. furiosus* is a hexamer of identical subunits and has an M_r = 270,000 ± 5500 at 25 °C. Electron micrographs showed that the subunit arrangement is similar to that of GDH from bovine liver (*i.e.* 3/2 symmetry in the form of a triangular antiprism). However, GDH from *P. furiosus* is inactive at temperatures below 40 °C and undergoes heat activation above 40 °C. Both NAD^+ and $NADP^+$ are utilized as cofactors. Apparently the inactive enzyme also binds cofactors, since the enzyme maintains the ability to bind to an affinity column (Cibacron blue F3GA) and is specifically eluted with $NADP^+$. Conformational changes that accompany activation and thermal denaturation were detected by precision differential scanning microcalorimetry. Thermal denaturation starts at 110 °C and is completed at 118 °C. Δ_{cal} = 414 Kcal [mol GDH]$^{-1}$. T_m = 113 °C. This increase in heat capacity indicates an extensive irreversible unfolding of the secondary structure as evidenced also by a sharp increase in absorbance at 280 nm and inactivation of the enzyme. The process of heat activation of GDH from 40 to 80 °C is accompanied by a much smaller increase in absorbance at 280 nm and a reversible increase in heat capacity with Δ_{cal} = 187 Kcal [mol GDH]$^{-1}$ and T_m = 57 °C. This absorbance change as well as the moderate increase in heat capacity suggest that thermal activation leads to some exposure of hydrophobic groups to solvent water as the GDH structure is opened slightly. The increase in absorbance at 280 nm during activation is only 12% of that for denaturation. Overall, GDH appears to be well adapted to correspond with the growth response of *P. furiosus* to temperature.

Recently, a number of marine isolates have been described that grow optimally around, or even above, 100 °C (1). Their existence raises the question of how metabolic processes are sustained at such extremely high temperature. These so-called hyperthermophiles are a diverse group, although all of them are Archaea, formerly known as Archaebacteria (2). Microorganisms that withstand extreme environments such as high salinity or extremes in pH generally exclude or modify the components of the environment that are inappropriate for biological processes by means of membrane barriers. Thermophilic microorganisms do not have this option of excluding heat. It follows that every aspect of their cellular metabolism must be adapted to function at the high growth temperatures of the organism. The study of key enzymes in their metabolism is clearly essential to understanding adaptations of hyperthermophiles. The organism used in this study, *Pyrococcus furiosus*, is a heterotroph that grows optimally at a temperature of 100 °C and can grow at 103 °C (3). Due to its unusual ability to grow without sulfur, large scale cultures of *P. furiosus* can be grown in conventional fermentors (4, 5). We have recently purified GDH[1] from the cytoplasmic fraction of cell-free extracts of *P. furiosus* (6). The enzyme utilizes either NAD or NADP as cofactors. In this study, we address structural and functional features of *P. furiosus* GDH and the conformational changes that accompany the heat activation and denaturation of this exceptionally heat stable enzyme.

MATERIALS AND METHODS

Bacterial Strain and Cultivation—*P. furiosus* (DSM 3638) was grown as closed static cultures in synthetic sea water supplemented with a vitamin mixture, $FeCl_3$ (25 μM), elemental sulfur (5 g/liter, w/v), and Na_2WO_4 (10 μM) as described previously (5). The synthetic sea water medium (13), consisting of NaCl (24 g/liter), Na_2SO_4 (4 g/liter), KCl (0.7 g/liter), sodium HCO_3 (0.2 g/liter), KBr (0.1 g/liter), H_3BO_3 (30 mg/liter), $MgCl_2 \cdot 6H_20$ (10.8 g/liter), $CaCl_2 \cdot 2H_2O$ (1.5 g/liter), $SrCl_2 \cdot 6H_2O$, (25 mg/liter), sodium resazurin (0.2 mg/liter) was supplemented with 5 g/liter of elemental sulfur (S^0) and 5/liter of tryptone. Cells stored at 4 °C in this medium remained viable for at least a year. Large scale growth was carried out at 88 °C in the absence of sulfur but with titanium (III) nitrilotriacetate (final concentration, 30 μM) as a reductant in a 500-liter stainless steel fermenter, as described previously (5). Cultures were sparged with argon at a rate of 7.5 liters/min.

Enzyme Purification and Chromatography—*P. furiosus* cells were grown under optimal conditions in a 500-liter fermenter and harvested and lysed as described previously (5, 6). GDH was purified using 600 g of cells (wet weight) as starting material (6). The M_r of GDH was estimated by gel filtration using a column (HR 10/30) of

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** To whom correspondence should be addressed: Center of Marine Biotechnology, 600 E. Lombard St., Baltimore, MD 21202. Tel.: 301-783-4821; Fax: 301-783-4806.

[1] The abbreviations used are: GDH, glutamate dehydrogenase; HPLC, high performance liquid chromatography.

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 protein standards used were obtained from Bio-Rad and were as follows: bovine thyroglobulin (M_r = 670,000), bovine γ-globulin (M_r = 158,000), chicken ovalbumin (M_r = 44,000), equine myoglobin (M_r = 17,000), and vitamin B_{12} (M_r = 1350). Affinity chromatography was performed on a 5 × 1-cm Cibacron blue F3GA column (19), in the presence of 5 mM of L-glutamate, at 24 °C. The enzyme eluted with a 3-ml pulse of 1 mM NADP.

Enzyme Assay—GDH activity was measured by the glutamate-dependent reduction of $NADP^+$ at 85 °C as described previously (6). The unit of activity of GDH is defined as micromoles of NADPH formed per min/mg of protein at 85 °C (6).

Electron Microscopy—A 4-μl sample of a 100 μg/ml solution of pure GDH was applied to a carbon-coated collodion covered grid for 30 s and removed by capillary action of filter paper. The grid was negatively stained with 1% aqueous uranyl acetate. Preparations were examined in a JEOL 100-CX electron microscope operating at 80 kV. Micrographs at nominal magnifications of × 58,000 were recorded on Kodak emulsion SO163.

The nomenclature for describing different views of the enzyme are those used by Josephs (7) for bovine liver GDH.

Microcalorimetry and Thermostability Determination—For determination of the thermostability of *P. furiosus* GDH at high temperatures, the purified enzyme was dialyzed against imidazole HCl buffer, pH 7.15, containing 10 mM dithiothreitol and placed in microcentrifuge tubes with O-ring sealed caps. Duplicate tubes were place in a Van Waters and Rogers heat block maintained at the indicated temperatures. Control experiments in which the imidazole buffer was incubated for similar time periods resulted in no change in the 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 assays.

For the temperature dependence of the heat capacity, the purified enzyme was scanned over a range of temperatures from 40 to 130 °C using the differential adiabatic scanning calorimeter DASM-4 (8). The area under the curve represents the enthalpy change due to the temperature-induced activation of 0.973 mg (2.04 mg/ml) of the enzyme dialyzed against 10 mM imidazole buffer, pH 7.29, containing 3.0 mM dithiothreitol. The heating rate applied was 1 K min^{-1}. The absorbance of pure GDH at 280 nm was recorded with a Pye Unicam model 1800 spectrophotometer using a thermostatted and pressurized cuvette compartment and a Hellma quartz cuvette with an optical path length of 1 cm. The heating rate was 1 K min^{-1}.

RESULTS

The binding and elution of pure GDH on an affinity column is shown in Fig. 1. The enzyme binds Cibacron blue F3GA tightly in the presence of 5 mM glutamate and is eluted specifically with 1 mM $NADP^+$ at 24 °C. NAD^+ is also effective in elution of the enzyme. In Fig. 2, the elution profile of cell-free extract and the pure enzyme eluted from a gel filtration column at 26 °C are shown. The pure enzyme eluted at a position corresponding to M_r 270,000, assuming a globular shape. The column was calibrated with bovine thyroglobulin (M_r = 670,000), bovine γ-globulin (M_r = 158,000), chicken ovalbumin (M_r = 44,000), equine myoglobin (M_r = 17,000), and vitamin B_{12} (M_r = 1350). A peak in the elution profile of the cell-free extract aligned with the position of elution of the GDH.

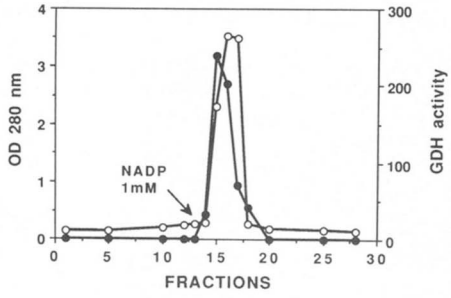

FIG. 1. **Affinity chromatography of *P. furiosus* GDH on a Cibacron blue F3GA column.** *Closed circles*, GDH activity in units/ml; *open circles*, optical density at 280 nm. Fractions were 3 ml each.

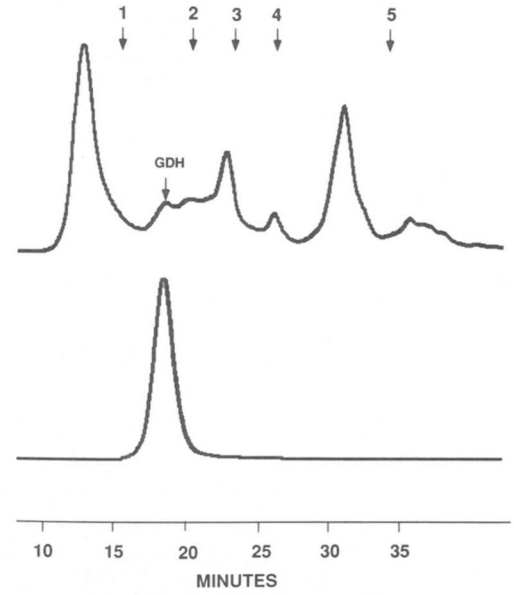

FIG. 2. **Gel filtration of *P. furiosus* GDH on Superose 12 HPLC.** The *upper trace* shows the elution profile of the cell free extract, and the *lower trace* is the pure GDH. Elution positions of the molecular weight markers are: *1*, bovine thyroglobulin (M_r = 670,000); *2*, bovine γ-globulin (M_r = 158,000); *3*, chicken ovalbumin (M_r = 44,000); *4*, equine myoglobin (M_r = 17,000); and *5*, vitamin B_{12} (M_r = 1350).

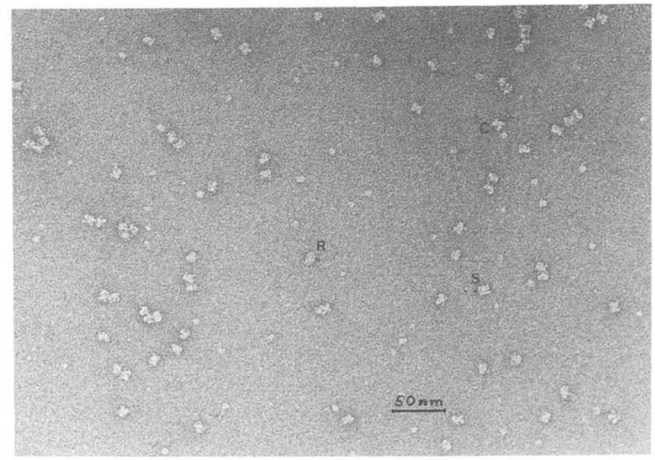

FIG. 3. **Transmission electron microscopy of *P. furiosus* GDH.** *S*, side view; *R*, ring view; and *C*, cloverleaf view. Magnification is × 58,000.

Electron micrographs of hexameric GDH (6) as it appears in electron microscopy after negative staining at room temperature is shown in Fig. 3. This is a typical field of purified GDH. The molecule presents a number of different orientations, labeled cloverleaf (*C*), ring (*R*), and side (*S*) views. The subunit arrangement is similar to that of GDH from bovine liver (7). The different shapes can be derived from the different orientations of a triangular antiprism. Experiments in which glutaraldehyde fixation was performed at 85 °C resulted in identical projections.[2]

Table I shows how the activity of GDH decreases during incubation at increasing temperatures in aqueous solution. The enzyme is relatively stable ($T_{1/2}$ = 0.5 h) at 107 °C. *P.*

[2] M. Kessel, unpublished data.

TABLE I

Inactivation of P. furiosus GDH at high temperatures

Temperature	$t_{1/2}$
°C	*h*
80	10.0
90	6.7
100	3.5
103	1.8
107	0.5

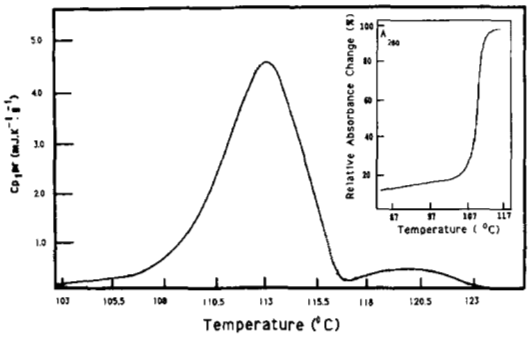

FIG. 4. **Temperature dependence of the heat capacity change (ΔCp_{denat}) of GDH from *P. furiosus* during denaturation.** A 2.04 mg/ml sample of pure GDH was scanned in the DASM4 microcalorimeter from 103 to 123 °C. *Inset*, the effect of increasing temperature on the absorbance of *P. furiosus* GDH at 280 nm.

furiosus GDH can be stored for several months at room temperature or at 4 °C without loss of activity (6).

Fig. 4 shows a typical heat capacity *versus* temperature scan of a 2.04 mg/ml solution of GDH between 103 and 120 °C, using the DASM4 scanning microcalorimeter (8). The thermal denaturation of the protein is accompanied by a steep rise in the heat capacity at about 108 °C. The heat capacity passes through a maximum at 113 °C and drops back to the instrumental base line. A minor peak of increased heat capacity occurs at 120 °C, but this was not present in experiments where the enzyme was dialyzed against 15 mM glycyl glycine buffer, pH 8.25.[3] Cooling the instrument and repeating the scans resulted in a stable base line, indicating that the thermal denaturation is irreversible. Briefly heating the protein to 110 °C does not destabilize the protein irreversibly. The unfolding transition of GDH was accompanied by a large increase in the absorbance at 280 nm (Fig. 1, *inset*). As far as we are aware, the exceptionally high denaturation temperature of 113 °C is the highest observed for any enzyme by microcalorimetric or any other physical techniques. There is a slight increase in absorbance at 300 nm due to aggregation, but the predominant contribution to the hyperchromic change results from the increase of the molar extinction coefficient when the irreversible unfolding of the protein is taking place.

Fig. 5 demonstrates that the moderate increase in absorbance between 37 and 97 °C is indeed parallel to the increase in enzyme activity. The spectral change (blue shift) and enzyme activity follow the same curves during repeated temperature cycles. Therefore, heat activation can be viewed as a reversible process. The change in absorbance at 280 nm during heat activation is only 12% of the change due to the heat denaturation of the protein. Fig. 6 shows the broad heat activation peak as obtained by differential scanning calorimetry after subtracting the instrumental base line. The heat capacity scan of the protein deviates from the buffer/buffer base line at 40 °C, rises to a maximum at about 57 °C, and joins the base line again at 74 °C. The excess heat capacity for the reversible activation is 11% of that observed for the denaturation of the same preparation of protein.

[3] H. Klump, unpublished data.

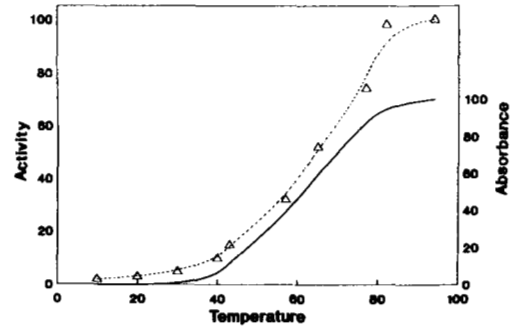

FIG. 5. **Comparison of the UV absorbance at 280 nm (in percent) and the relative increase of enzyme activity as a function of temperature.** *Broken line* with *open triangles*, enzyme activity; *solid line*, A_{280}.

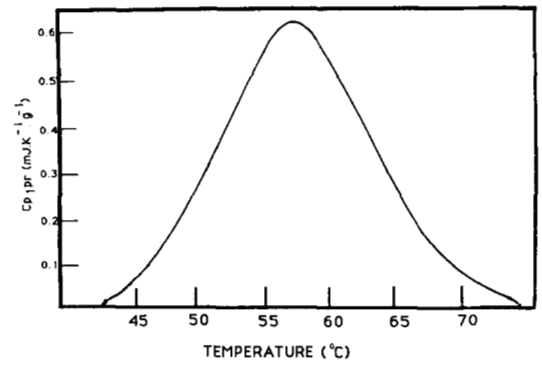

FIG. 6. **Temperature dependence of the heat capacity change (ΔCp_{activ}) of GDH during heat activation.** All of the experimental conditions are as described in the legend to Fig. 4, except the temperature range, which was 43–75 °C.

TABLE II

Heat activation and denaturation of P. furiosus GDH: values for thermodynamic transitions

	ΔH_{cal}	ΔH_{vH}		ΔS	T_m	n^a
		Cal	Spec[b]			
	kcal (mol GDH)$^{-1}$	*kcal (mol)*$^{-1}$			*cal (mol K)*$^{-1}$	°C
Activation	187	39	34	520	57	4.8
Denaturation	414	240	240	1072	113	1.7

[a] The ratio of $\Delta H_{cal}/\Delta H_{van't Hoff}$ equals n, which is the number of independent folding units. T_m is the transition temperature. 1 cal = 4.18 J.

[b] Moles of independent cooperative folding unit. Spec, spectrophotometric data.

From the heat capacity measurements, the thermodynamic parameters of the heat activation and the denaturation of the protein can be derived. These data are given in Table II. The calorimetric enthalpies ΔH_{cal} for the denaturation and the heat activation are calculated from the measured peak areas (excess heat capacity) and the protein concentration. Stepwise partial integration of the peaks gives the degree of thermal unfolding (Θ) as a function of the temperature (not shown). From the slope of $d\Theta T$ at T_m the van't Hoff enthalpy can be determined according to the following equation,

$$\Delta H_{vH} = 4RTm^2(d\Theta/dT)_{T_m}$$

where R is the gas constant. All of the ΔH_{vH} values given in Table II are calculated from this equation. The van't Hoff

enthalpy can also be derived from the differential scanning calorimetry data according to a procedure devised by Privalov and Khechinashvili (8), as follows.

$$\Delta H_{vH} = 4RTm^2(\Delta Cp_{max}/\Delta H_{cal})$$

The ratio of the calorimetrically determined enthalpies (ΔH_{cal}) over the corresponding van't Hoff enthalpies gives the number of cooperative subunits. The ratio becomes 1 for a true two-state process. Our data show that heat activation is a multistep process ($\Delta H_{cal}/\Delta H_{vH} = 4.8$), whereas denaturation involves relatively fewer intermediate states ($\Delta H_{cal}/\Delta H_{vH} = 1.7$). This indicates that heat activation cannot be described as a simple process and hence distinguishes this subtle structural change from the extensive unfolding that accompanies irreversible heat denaturation.

DISCUSSION

The study of enzymes from hyperthermophilic microorganisms has uncovered unusual biochemical adaptations that permit them to survive and grow at high temperatures. Several enzymes have been isolated recently from *P. furiosus*. These include a hydrogenase (4, 9), a ferredoxin (10), a novel tungsten-dependent aldehyde oxidoreductase (11, 12), α-glucosidase (13), and two proteases (14, 15). In each case, the enzymes are active near to or above 100 °C and have very little activity below 40 °C. In this work, we have examined the effect of high temperature on *P. furiosus* GDH, which is a large hexameric soluble enzyme with a molecular weight of 270,000. This GDH is the most thermostable dehydrogenase described to date, with respect to retention of enzyme activity, as shown in Table I (see also Ref. 6). It is not, however, the most thermoduric enzyme isolated from *P. furiosus*, since a protease termed "S66" remains active for 24 h at 100 °C even in the presence of SDS (15). Another GDH from the aerobic extreme thermophile, *Sulfolobus solfataricus* (growth temperature 80 °C), has recently been described (16, 17), but this GDH loses its activity five times faster at 100 °C than the enzyme described in our work.

P. furiosus GDH is relatively stable during brief exposure to temperatures up to 110 °C, as shown in Table I and in Fig. 4. The stability of the enzyme is strongly concentration-dependent at temperatures above 100 °C (6). Beyond 110 °C the enzyme denatures rapidly and irreversibly. The stepwise unfolding of large proteins is not unprecedented, since Shrake *et al.* (18) have described the partial heat denaturation of *Escherichia coli* glutamine synthetase, in which both the carboxyl and amino termini unfold without disruption of the subunit interactions.

As shown in Fig. 2, GDH is a major enzyme in extracts of *P. furiosus*. Homogeneity is reached with an enrichment of 75-fold over the cell free extract, indicating that GDH represents 1–2% of the soluble protein in the cell (6). The kinetic properties of *P. furiosus* GDH suggest that it functions *in vivo* in the catabolism of glutamate, since it has a low affinity for ammonia (6). This correlates well with the growth requirements of the strain; *P. furiosus* will grow only in the presence of proteins or peptides, which it ferments to organic acids with the production of hydrogen gas or hydrogen sulfide if it is grown in the presence of elemental sulfur. We conclude that GDH is a key enzyme in the pathway that ferments L-glutamate. An α-ketoglutarate oxidoreductase has been detected in *P. furiosus* that would provide the next step in the pathway, which could be a partial or complete tricarboxylic acid cycle.[4]

[4] M. W. W. Adams, unpublished data.

We have investigated the conformational flexibility of *P. furiosus* GDH as a function of increasing temperature. In order to follow the heat activation and the temperature-induced denaturation of GDH, we have measured two physical properties of the enzyme in aqueous solution, namely the excess heat capacity which is a measure of the increasing internal energy of the polymer and the optical density (A_{280}) which reflects the change in solvation of key chromophores of a protein, such as the exposure of Tyr and Trp to solvent water.

Heat activation of GDH is accompanied by a comparable small increase in the absorbance monitored at 280 nm (*cf.* Fig. 6) as compared with the large increase on A_{280} that accompanies thermal denaturation. The relative change in A_{280} in percent corresponds very well to the increase in enzyme activity measured over the same temperature interval (35–80 °C). We conclude from this that a conformational change that exposes some chromophores to the solvent is necessary to extend the initially inactive conformation of the protein to gain enzyme activity. The second physical parameter, the excess heat capacity, changes over the same temperature range (T_m = 57 °C) during activation. These changes are all reversible. This can be concluded from the relatively small changes of the physical parameters (ΔCp and A_{280}) at 57 °C, compared with the much more drastic changes at the temperature of irreversible denaturation (T_m 113 °C).

The thermally induced unfolding steps due to heat activation and to denaturation to a partially unfolded protein can be analyzed as a function of temperature, as described by Shrake *et al.* (18). The calculated van't Hoff enthalpies are listed in Table II. From the thermodynamic parameters in Table II, it is evident that heat activation is a multistate process that probably involves inter- and intrasubunit changes. It is clear from these results that neither the heat activation nor the thermal denaturation of *P. furiosus* GDH can be described by a simple two-state model. The physical changes that accompany activation are relatively minor compared with the heat denaturation, and the structural transition from an inactive to active enzyme in this case is relatively subtle.

In agreement with this, the results of gel filtration (Fig. 2) and electron microscopy (Fig. 3) confirm that the enzyme is a hexamer at room temperature. The ability of the inactive enzyme to bind cofactors and L-glutamate at room temperature is apparent from its behavior during affinity chromatography (Fig. 1). *P. furiosus* GDH does not bind the Cibacron F3GA resin unless L-glutamate is present and is eluted by the addition of $NADP^+$ or NAD^+. The hexameric structure of *P. furiosus* GDH appears to be identical in electron micrographs (Fig. 3) to bovine GDH (7). There is therefore no evidence for dissociation of the enzyme as a result of exposure to low temperature.

Notably, the effect of temperature on the enzyme activity of *P. furiosus* GDH reflects the relationship between temperature and the growth rate of *P. furiosus* reported by Fiala and Stetter (3), and a similar temperature response was reported for the activity of its hydrogenase-ferredoxin system (4). These are key enzymes of important pathways in *P. furiosus* (4, 6). Most enzymes from hyperthermophiles will likely display similar responses to temperature, and the sum of these responses may enable the cells to undergo rapid metabolic slowing in the event that these anaerobic organisms are flushed into cool aerobic environments. Stetter and co-workers (1, 3) have noted that the hyperthermophiles are capable of survival for long periods of time at low temperatures and that they may become more oxygen tolerant when they are

maintained at low temperature. This is certainly the case for *P. furiosus*, since cultures will retain viability for at least 1 year at 4 °C (5). This "shut-down" response may be a critically important factor for the survival and distribution of hyperthermophiles, all of which are strict anaerobes that occur in geothermally heated areas surrounded by cold aerated sea water.

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