Glutamate Dehydrogenase from the Hyperthermophile *Pyrococcus furiosus*

**THERMAL DENATURATION AND ACTIVATION**

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*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*₂ = 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≠₅₀ = 414 Kcal [mol GDH]⁻¹. *Tₚ₅₀ = 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≠₅₀ = 187 Kcal [mol GDH]⁻¹ and *Tₚ₅₀ = 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 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 seawater supplemented with a vitamin mixture, FeCl₂ (25 μM), elemental sulfur (5 g/liter, w/v), and Na₂WO₄ (10 μM) as described previously (5). The synthetic sea water medium (13), consisting of NaCl (24 g/liter), Na₂SO₄ (4 g/liter), KCl (0.7 g/liter), sodium HCO₃ (0.2 g/liter), H₂BO₃ (30 mg/liter), MgCl₂·6H₂O (10.6 g/liter), CaCl₂·2H₂O (1.5 g/liter), SrCl₂·6H₂O (25 mg/liter), sodium resazurin (0.2 mg/liter) was supplemented with 5 g/liter of elemental sulfur (S⁰) and 5 liter of tripsyne. Cells stored at 4 °C in this medium remained viable for at least a year. Large scale growth was carried out at 86 °C in the absence of sulfur but with titanium (III) nitrotateinate (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*₂ of GDH was estimated by gel filtration using a column (HR 10/30) of
The protein standards used were obtained from Bio-Rad and were as follows: bovine thyroglobulin (Mr = 670,000), bovine γ-globulin (Mr = 158,000), chicken ovalbumin (Mr = 44,000), equine myoglobin (Mr = 17,000), and vitamin B₁₂ (Mr = 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).

**Microcalorimetry and Thermobility 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 placed 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 lower trace is the pure GDH. Elution positions of the molecular weight markers are: 1, bovine thyroglobulin (Mr = 670,000); 2, bovine γ-globulin (Mr = 158,000); 3, chicken ovalbumin (Mr = 44,000); 4, equine myoglobin (Mr = 17,000); and 5, vitamin B₁₂ (Mr = 1350). A peak in the elution profile of the cell-free extract aligned with the position of elution of the GDH.

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.⁸

Table I shows how the activity of GDH decreases during incubation at increasing temperatures in aqueous solution. The enzyme is relatively stable (T₁/₂ = 0.5 h) at 107 °C.

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⁸ M. Kessel, unpublished data.
furiosus Glutamate Dehydrogenase

P. furiosus Glutamate Dehydrogenase

TABLE I

<table>
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<th>Temperature (°C)</th>
<th>Activity</th>
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<th>ΔS</th>
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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 105 to 125 °C. Inset, the effect of increasing temperature on the absorbance of P. furiosus GDH at 280 nm.

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, A280.

Fig. 6. Temperature dependence of the heat capacity change (ΔCp,act) 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

<table>
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<th>Temperature (°C)</th>
<th>Activity</th>
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° The ratio of ΔHcal/ΔHact=Max equals n° which is the number of independent folding units. Tm is the transition temperature. 1 cal = 4.18 J.

° Moles of independent cooperative folding unit. Spec, spectrophotometric data.

joints 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.

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 ΔHcal 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 dθ/dT at Tm, the van’t Hoff enthalpy can be determined according to the following equation,

$$\Delta H_{\text{vH}} = 4RTm^2(d\theta/dT)_{Tm}$$

where R is the gas constant. All of the ΔHvH values given in Table II are calculated from this equation. The van’t Hoff

H. Klump, unpublished data.

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The effect of high temperature on retention of enzyme activity, protease termed "S66" remains active for unfolding of large proteins is not unprecedented, since Shrake (ature 80°C), has recently been described (16, boxyl and amino termini unfold without disruption of the most thermoduric enzyme isolated from P. furiosus, as shown in Table I (see also Ref. 6). It is not, however, the true two-state process. Our data show that heat activation is a multistep process (ΔH_m/ΔH_m = 4.8), whereas denaturation involves relatively fewer intermediate states (ΔH_m/ΔH_m = 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), a-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 a-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.

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 (A280) 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 A280 that accompanies thermal denaturation. The relative change in A280 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 A280) 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 Gibberellic 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

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* M. W. W. Adams, unpublished data.
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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REFERENCES

**Glutamate dehydrogenase from the hyperthermophile Pyrococcus furiosus.**

**Thermal denaturation and activation.**

H Klump, J Di Ruggiero, M Kessel, J B Park, M W Adams and F T Robb


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