

# Pressure-induced thermostabilization of glutamate dehydrogenase from the hyperthermophile *Pyrococcus furiosus*

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## Abstract

In this paper, elevated pressures up to 750 atm (1 atm = 101 kPa) were found to have a strong stabilizing effect on two extremely thermophilic glutamate dehydrogenases (GDHs): the native enzyme from the hyperthermophile *Pyrococcus furiosus* (*Pf*), and a recombinant GDH mutant containing an extra tetrapeptide at the C-terminus (rGDH<sup>t</sup>). The presence of the tetrapeptide greatly destabilized the recombinant mutant at ambient pressure; however, the destabilizing effect was largely reversed by the application of pressure. Electron spin resonance (ESR) spectroscopy of a spin-label attached to the terminal cysteine of rGDH<sup>t</sup> revealed a high degree of mobility, suggesting that destabilization is due to weakened intersubunit ion-pair interactions induced by thermal fluctuations of the tetrapeptide. For both enzymes, the stabilizing effect of pressure increased with temperature as well as pressure, reaching 36-fold for rGDH<sup>t</sup> at 105 °C and 750 atm, the largest pressure-induced thermostabilization of an enzyme reported to date. Stabilization of both native GDH and rGDH<sup>t</sup> was also achieved by adding glycerol. Based on the kinetics of thermal inactivation and the known effects of glycerol on protein structure, a mechanism of pressure-induced thermostabilization is proposed.

**Keywords:** electron spin resonance; glutamate dehydrogenase; glycerol; pressure; *Pyrococcus furiosus*; stability; temperature

Pressure is an increasingly useful tool for studying the structure and function of proteins (Weber & Drickamer, 1983; Balny et al., 1989; Gekko & Yamagami, 1991; Gross & Jaenicke, 1994), including proteins from thermophilic organisms. High pressure (>300 MPa) is typically viewed as a denaturant and has thus been used in several studies of enzyme denaturation (Weber & Drickamer, 1983; Jaenicke, 1991; Samarasinghe et al., 1992). However, there is increasing evidence that moderate pressures ( $\leq 100$  MPa), below those normally needed for pressure-induced denaturation can cause subtle, but important, changes in protein structure and dynamics (Heremans & Heremans, 1989a, 1989b; Frauenfelder et al., 1990; Gross et al., 1993; Cioni & Strambini, 1994). Moreover, recent studies have shown that moderate pressures can stabilize proteins against thermoinactivation (Michels & Clark, 1992, 1997; Hei & Clark, 1994; Michels et al., 1996; Mozhaev et al., 1996), particularly thermophilic enzymes at very high tempera-

tures (Michels & Clark, 1992, 1997; Hei & Clark, 1994; Michels et al., 1996). For example, it was found that application of 500 atm increased the thermal half-life of hydrogenase from *Methanococcus jannaschii* 4.8-fold at 90 °C (Hei & Clark, 1994), and increased the half-life of a protease from the same organism 2.7-fold at 125 °C (Michels & Clark, 1997).

In this paper, we examine the thermostability and pressure-induced thermostabilization of glutamate dehydrogenase (GDH) from the hyperthermophilic archaeon *Pyrococcus furiosus* (*Pf*). *Pf* is one of the most thermophilic organisms known, with an optimum growth temperature of 100 °C (Fiala & Stetter, 1986). *Pf* GDH is a hexamer composed of six identical subunits, each containing 419 amino acids (Yip et al., 1995). The enzyme has a melting temperature ( $T_m$ ) for denaturation of 113 °C (Klump et al., 1992), placing it among the most thermostable GDHs studied to date.

The present study compares the thermostabilities of two *Pf* GDHs, the native and a recombinant GDH mutant. The two enzymes differ only in that the recombinant GDH mutant, designated rGDH<sup>t</sup>, contains an extra tetrapeptide (Gly-Ser-Gly-Cys), at the C-terminus. The presence of the tetrapeptide destabilized the enzyme markedly; electron spin resonance (ESR) spectroscopy of a spin-label attached to the C-terminal cysteine identified rapid motion

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**Abbreviations:** ESR, electron spin resonance; GDH, glutamate dehydrogenase; MTSSL, methanethiosulfonate spin-label; *Pf*, *Pyrococcus furiosus*; rGDH<sup>t</sup>, recombinant GDH tetrapeptide mutant.

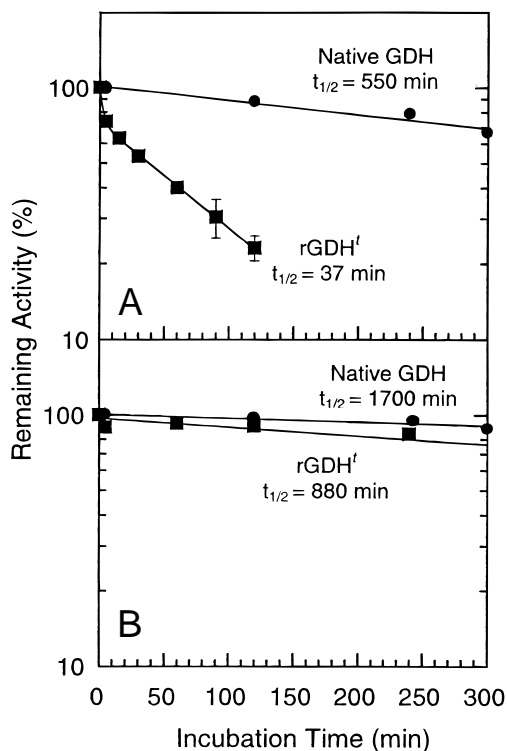
of the tetrapeptide, as a possible cause of the destabilization. However, the destabilizing effect was largely offset by increased pressure. In fact, pressure greatly stabilized both enzymes against thermostabilization. Stabilization to a lesser extent was also achieved by adding glycerol, suggesting that compression and/or rigidification of the protein's structure plays a role in pressure-induced thermostabilization.

## Results

### Pressure-induced thermostabilization

Figure 1 shows thermostabilization trajectories of both native GDH and rGDH' at 103 °C and at 5 and 500 atm. At 5 atm (Fig. 1A), native GDH was much more stable than rGDH', with half-lives of 550 and 37 min, respectively, indicating that the additional tetrapeptide at the C-terminus greatly destabilizes rGDH' compared to the native enzyme. However, the destabilizing effect of the tetrapeptide was largely reversed by the application of 500 atm, which increased the half-life of rGDH' 24-fold (Fig. 1B). As a control, recombinant GDH without the C-terminal tetrapeptide was also cloned and expressed. Thermostabilization studies with this enzyme showed it to be about 20% more thermostable than native GDH at 103 °C (results not shown), confirming that the extra tetrapeptide lowers the thermostability of rGDH'.

The effects of pressure on both enzymes as a function of temperature are summarized in Table 1. The degree of pressure stabilization is given by the ratio of thermal half-lives at 500 and



**Fig. 1.** Thermostabilization trajectories of native GDH (●) and rGDH' (■) at 103 °C, and (A) 5 atm and (B) 500 atm, showing half-lives. Half-lives are averages of at least two trials. Error bars represent mean deviations. Some points have error bars smaller than the symbols.

**Table 1.** Effect of pressure on thermal half-lives of rGDH' and native GDH at different temperatures<sup>a,b</sup>

Enzyme	Temperature (°C)	$t_{1/2}$ (5 atm) (min)	$\frac{t_{1/2} (500 \text{ atm})}{t_{1/2} (5 \text{ atm})}$
rGDH'	103	37 ± 1.5	24
rGDH'	105	13 ± 0.5	28
Native GDH	103	550 ± 25	3.1
Native GDH	106	115 ± 5.5	4.0
Native GDH	109	12 ± 0.1	18

<sup>a</sup>Half-lives are averages of at least two trials.

<sup>b</sup>Errors represent mean deviations.

5 atm (last column). Pressure stabilized both enzymes greatly; rGDH' was stabilized by 28-fold at 105 °C and native GDH by 18-fold at 109 °C. These results and those at other temperatures (Table 1) indicate that pressure has a greater stabilizing effect on rGDH' than native GDH, and that pressure-induced thermostabilization increases with temperature.

Pressure stabilization was also studied as a function of pressure. Table 2 gives the magnitude of pressure-induced thermostabilization of rGDH' at 105 °C and various pressures. The thermal half-life increased from 13 min at 5 atm to 470 min at 750 atm. The degree of pressure stabilization therefore increased from 13-fold at 275 atm to 36-fold at 750 atm, the largest pressure-induced thermostabilization of an enzyme reported to date.

### Dynamics of the C-terminal tetrapeptide

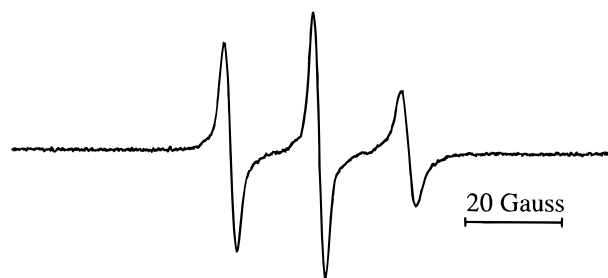
To investigate the mechanism by which the tetrapeptide destabilizes rGDH', a cysteine-specific spin-label (MTSSL) was attached to the C-terminal cysteine, and the dynamics of the C-terminal tetrapeptide were probed using ESR spectroscopy at 21 °C. The crystal structure of the native GDH hexamer (Yip et al., 1995) shows that the C-terminus and presumably the tetrapeptide are located at the surface of the enzyme and extend away from intersubunit surfaces (structure not shown). The ESR spectrum of rGDH'-MTSSL at 21 °C is shown in Figure 2. rGDH' contains only two cysteines per subunit: the C-terminal cysteine and a mostly buried cysteine at position 343. To ensure that the ESR spectrum corresponds to the label attached at the C-terminus, native GDH was spin-labeled in a control experiment. The native GDH control gave

**Table 2.** Pressure-induced thermostabilization of rGDH' at 105 °C as a function of pressure<sup>a,b</sup>

Pressure (atm)	Half-life (min)	$\frac{t_{1/2} (P \text{ atm})}{t_{1/2} (5 \text{ atm})}$
5	13 ± 0.5	1
275	170 ± 11	13
500	360 ± 13	28
750	470 ± 30	36

<sup>a</sup>Half-lives are averages of at least two trials.

<sup>b</sup>Errors represent mean deviations.



**Fig. 2.** ESR spectrum of spin-labeled rGDH<sup>I</sup> in 100 mM EPPS buffer (pH 8.0) at 21 °C. Protein concentration was 24  $\mu$ M.

a very weak ESR signal that accounted for less than 2% of the spectrum of rGDH<sup>I</sup>-MTSSL. Quantifying the ESR signal by double integration revealed an overall labeling stoichiometry of 4.8 spin-label molecules per GDH hexamer.

The narrow line widths of the spectrum (Fig. 2) indicate a highly mobile spin-label; analysis of the line widths yields rotational correlation times for  $\tau_{iso}(B)$  and  $\tau_{iso}(C)$  of 0.62 and 0.79 ns, respectively (Schreier et al., 1978). For completely isotropic motion,  $\tau_{iso}(B)$  and  $\tau_{iso}(C)$  are equivalent; therefore, our results indicate that rotation of the spin-label is very rapid and nearly isotropic (Schreier et al., 1978).

#### Aggregation of GDH during thermoinactivation

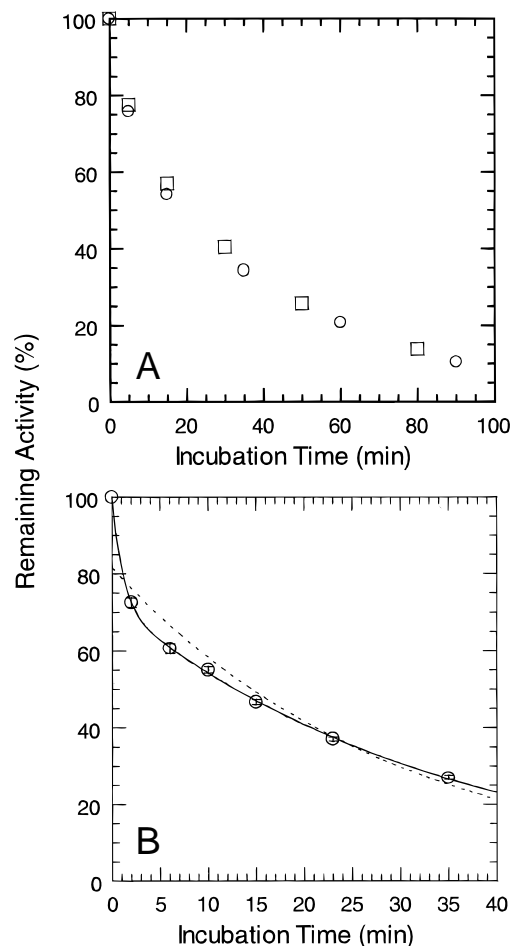
An increase in solution turbidity accompanied inactivation of both native and rGDH<sup>I</sup>, indicating that aggregation occurred during thermoinactivation. The insoluble aggregated protein could be removed by centrifugation for 45 min at 14,000  $\times$  *g*. However, as shown in Figure 3A, aggregation is rapid relative to the rate-limiting step(s) of thermoinactivation because a 10-fold decrease in concentration of native GDH did not affect the overall inactivation rate.

#### Glycerol-induced thermostabilization

The effect of glycerol on the thermoinactivation of rGDH<sup>I</sup> at 105 °C and 5 atm is summarized in Table 3. Glycerol stabilized the enzyme against thermoinactivation, with a half-life of 175 min at the optimum stabilizing concentration of 25% (v/v) glycerol. Increasing the glycerol concentration to 50 and 75% (v/v) produced lower half-lives of 120 and 80 min, respectively. As shown in the last column in Table 3, the maximum degree of stabilization by glycerol was 14-fold. The native GDH was similarly stabilized by 25% (v/v) glycerol (data not shown), exhibiting 6.6- and 11-fold stabilization at 107 and 109 °C, respectively. These results show that glycerol is also effective in stabilizing both rGDH<sup>I</sup> and native GDH against thermoinactivation. Furthermore, as with pressure-induced stabilization, the results for the native GDH show that glycerol-induced stabilization increases with temperature.

#### Combined effect of pressure and glycerol

Figure 4 shows thermoinactivation trajectories of rGDH<sup>I</sup> at 105 °C and different conditions. Curves A–D represent thermoinactivation of rGDH<sup>I</sup> under four different conditions: (1) 5 atm, no glycerol;



**Fig. 3. A:** Thermoinactivation trajectories of native GDH at enzyme concentrations of 5  $\mu$ g/mL ( $\circ$ ) and 50  $\mu$ g/mL ( $\square$ ). **B:** Single (---) and double-exponential (—) fits of thermoinactivation data of rGDH<sup>I</sup> at 105 °C and 5 atm.

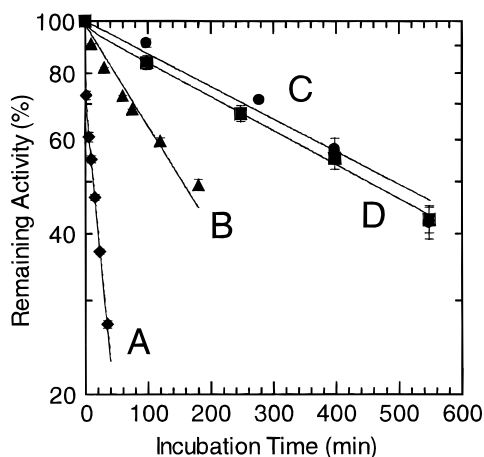
(2) 5 atm, 25% glycerol; (3) 750 atm, no glycerol; and (4) 750 atm, 25% glycerol. Independently, glycerol and high pressure extended the half-life of rGDH<sup>I</sup> to 175 and 470 min, respectively. However, the combination of both (curve D) gave nearly the same half-life, 460 min, as for 750 atm alone. This result suggests that pressure and glycerol stabilize rGDH<sup>I</sup> by similar mechanism(s).

**Table 3.** Effect of glycerol on the thermoinactivation of rGDH<sup>I</sup> at 105 °C and 5 atm<sup>a,b</sup>

Glycerol (% v/v)	$t_{1/2}$ (5 atm) (min)	$t_{1/2}$ (glycerol) $t_{1/2}$
0	13 $\pm$ 0.5	1
25	175 $\pm$ 7	14
50	120 $\pm$ 3	9.2
75	80 $\pm$ 5	6.3

<sup>a</sup>Half-lives are averages of two trials.

<sup>b</sup>Errors represent mean deviations.



**Fig. 4.** Effect of combining pressure and glycerol on the thermostability of rGDH' at 105 °C. Curves A–D represent thermostability trajectories at different conditions: (A) 5 atm, no glycerol (◆); (B) 5 atm, 25% (v/v) glycerol (▲); (C) 750 atm, no glycerol (●); and (D) 750 atm, 25% glycerol (■). The half-lives are 13, 175, 470, and 460 min for curves A–D, respectively. Half-lives are averages of two trials. Error bars represent mean deviations.

## Discussion

### Destabilizing effects of the C-terminal tetrapeptide

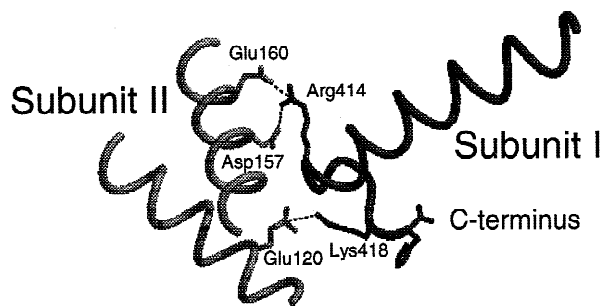
The dynamics of the extra C-terminal tetrapeptide were probed by ESR spectroscopy. Analysis of the ESR spectrum (Fig. 2) of rGDH'-MTSSL yielded rotational correlation times for  $\tau_{iso}(B)$  and  $\tau_{iso}(C)$ , of 0.62 and 0.79 ns, respectively. The rotational correlation time is governed by the following motions: (1) motion of the spin-labeled side chain relative to the peptide backbone due to rotations about the chemical bonds of the cysteine side chain and the spin-label, (2) motion of the peptide backbone (primarily the tetrapeptide) to which the MTSSL is tethered, and (3) tumbling of the entire rGDH'-MTSSL complex. The rotational correlation time of the entire rGDH'-MTSSL complex (motion (3) above) estimated from the Stokes–Einstein equation is  $\sim 160$  ns, indicating that the short correlation time measured from ESR spectroscopy reflects much more rapid motion of the tetrapeptide backbone and/or rotations about the bonds of the side chain and spin-label.

The relationship between the motion of MTSSL spin-labeled side chains and local protein dynamics was investigated previously by a series of single site-directed cysteine labelings at different positions within T4 lysozyme (Mchaourab et al., 1996). The conclusion from these studies was that rotations about the bonds connecting the MTSSL nitroxide to the peptide backbone are highly responsive to motions of the backbone itself (Mchaourab et al., 1996). Therefore, the short correlation times corresponding to spin-labeled rGDH' should reflect largely the rapid motion of the tetrapeptide. Furthermore, Mchaourab et al. (1996) found that the spectra of MTSSL attached to sites where the label conflicts sterically with nearby side-chain and main-chain atoms indicated both constrained and anisotropic motion. The high and nearly isotropic mobility of rGDH'-MTSSL suggests that the tetrapeptide is not involved in such tertiary interactions, but instead is moving about freely. This is consistent with the crystal structure of *Pf* native GDH, which shows that the C-terminus is located at the surface of the enzyme (not shown).

ESR spectroscopy was also used previously to probe the dynamics of the C-terminal cysteine of thymidylate synthase (Carreras et al., 1994). In this case, the C-terminal tetrapeptide is “relatively disordered” with crystallographic *B*-factors between 40 and 60 Å<sup>2</sup>, compared to an average *B*-factor of about 16 Å<sup>2</sup> for the remainder of the enzyme (Carreras et al., 1994). The rotational correlation time obtained for this system was 1 ns; by comparison, the C-terminal tetrapeptide of rGDH' is even more disordered. In a related study, Qu et al. (1997) examined the dynamics of the penultimate amino acid of yeast iso-1-cytochrome *c*, cysteine 102, spin-labeled with MTSSL. The ESR spectrum of the labeled protein denatured by 2 M guanidinium hydrochloride yielded rotational correlation times for  $\tau_{iso}(B)$  and  $\tau_{iso}(C)$  of 0.44 and 0.45 ns, respectively. Comparison of these results with the correlation times of rGDH'-MTSSL further supports the notion of the C-terminal tetrapeptide undergoing rapid isotropic motion that is exceptionally rapid for such a large protein. As a final comparison, the correlation time for the unattached tetrapeptide GSGC tumbling freely in solution estimated from the Stokes–Einstein equation is  $\sim 0.15$  ns.

The emerging picture of freely fluctuating C-terminal tetrapeptides at the surface of the GDH hexamer suggests that destabilization may be due to increased thermal fluctuations in the region near the C-termini of all six subunits. At high temperatures, thermal fluctuations of the extra tetrapeptide should increase fluctuations of the polypeptide backbone to which it is covalently attached. Increased fluctuations in the C-terminal region may, in turn, destabilize rGDH' by weakening key intra and intersubunit interactions near the C-terminus. Figure 5 shows three such intersubunit ion-pair interactions involving two residues, Lys418 and Arg414, near the C-terminus of a *Pf* GDH subunit (Yip et al., 1995) (designated as Subunit I).

The ion-pair interactions shown in Figure 5 are part of the largest ion-pair network (composed of 18 residues) at the subunit interface that contribute to the hyperstability of *Pf* GDH (Yip et al., 1995). Both Asp157 and Glu120 are involved in additional ion-pair interactions with other residues that are part of the 18-residue ion-pair network (not shown). Recent structure-stability studies of the highly homologous GDHs (sharing 87% sequence identity) from *Pf* and *Thermococcus litoralis* indicate that ion-pair networks are crucial to the stability of both enzymes (Vetriani et al., 1998). In that paper, three ion-pair interactions (two of which are intersubunit) that are part of a six-residue ion-pair network found in the



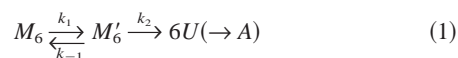
**Fig. 5.** Intersubunit ion-pair interactions (indicated by dashed lines) near the C-terminus of a single subunit (Subunit I, darker) of native *P. furiosus* GDH. The side chains of amino acid residues involved in ion-pair interactions, as well as the C-terminal histidine, are shown explicitly. Figure drawn with coordinates (1GTM) from the PDB.

*Pf* but not the *T. litoralis* GDH were engineered by site-directed mutagenesis, producing a more thermostable mutant *T. litoralis* GDH. In addition to the intersubunit ion pairs shown in Figure 5, an intrasubunit ion-pair interaction between the terminal His419 and Glu25 (not shown) could also be important to the thermostability of *Pf* GDH. Furthermore, if cooperativity between interactions in the ion-pair networks in *Pf* GDH is important, as suggested by Yip et al. (1995, 1998), the weakening of even a few such interactions (Fig. 5) is expected to lower the stability of *Pf* GDH.

Comparative studies have suggested that immobilization of both N- and C-termini is an adaptive feature for increasing the thermostability of several hyperthermophilic proteins over their mesophilic counterparts (Day et al., 1992; Macedo-Ribeiro et al., 1996; Hennig et al., 1997; Russell et al., 1997; Robb & Maeder, 1998). Our results with GDH from the hyperthermophile *P. furiosus* suggest that increasing the flexibility of the C-terminus by a tetrapeptide extension significantly reduces the stability of the enzyme at temperatures above 100 °C. Furthermore, the decrease in thermostability likely results from the weakening of ionic interactions, especially between subunits, involved in ion-pair networks.

#### Mechanism of thermal inactivation

A mechanistic interpretation of pressure-induced thermostabilization requires at least partial understanding of the mechanism of thermal inactivation. In the case of GDH, a model of enzyme denaturation has been described by Singh et al. (1996), who found that thermal denaturation of GDH from bovine-liver was consistent with the following mechanism:



where  $M_6$  is the active native hexamer,  $M'_6$  is an inactive hexameric intermediate (referred to as a molten-globule-like intermediate by Singh et al., 1996),  $U$  is the unfolded monomer, and  $A$  is a final aggregated state. The denaturation process was found to be kinetically controlled (i.e., thermal inactivation included at least one nonequilibrium step) from the dependence of melting temperature on the scan rate during differential scanning calorimetry (DSC). Furthermore, the existence of an inactive hexameric intermediate,  $M'_6$ , was suggested by following the change in particle size during thermal denaturation. Using light scattering, no significant change in the average particle size (of the hexamer) was detected even after a substantial fraction of the initial activity was lost. With further inactivation, the average particle size dropped abruptly, suggesting dissociation of the hexamer to monomers. The last step in the model, aggregation, occurred very rapidly and did not affect the overall rate of GDH denaturation.

GDHs from *Pf* and bovine-liver are both hexameric (Yip et al., 1995; Singh et al., 1996) and possess high sequence identity to the GDH from *C. symbiosum* (37 and 28%, respectively (Teller et al., 1992; Britton et al., 1995)), indicating a high degree of structural homology between the three enzymes. In addition, inactivation of GDH and rGDH<sup>f</sup> exhibits characteristics consistent with the inactivation scheme proposed for bovine-liver GDH. For example, like bovine-liver GDH, thermoinactivation of both native GDH and rGDH<sup>f</sup> led to the appearance of aggregation. However, as shown in Figure 3A, aggregation is not the rate-limiting step, because a 10-fold decrease in concentration of native GDH did not affect the overall inactivation rate. Consalvi et al. (1991) have also shown

that the thermostability of *Pf* native GDH is independent of enzyme concentration in the range of 1–15,000 µg/mL.

As discussed by Singh et al. (1996), further insight into the inactivation mechanism can be obtained by fitting the inactivation data to the kinetic equations for various inactivation models. The inactivation mechanism proposed here (Equation 1), involving a reversible (nonequilibrium) step followed by an irreversible step, leads to a two-exponential inactivation trajectory (see Supplementary material in Electronic Appendix). In contrast, a mechanism consisting of either two consecutive irreversible steps, or an equilibrium step followed by an irreversible step, yields a single-exponential decline in the active hexamer,  $M_6$ , and hence the observed activity. As shown in Figure 3B, the inactivation trajectory of rGDH<sup>f</sup> could be fitted more closely with a double-exponential than a single-exponential curve. A two-exponential fit derived from Equation 1 led to equal or better fits of all inactivation data at high and low pressures, consistent with the proposed mechanism of thermal inactivation. Therefore, this inactivation model describes the available data for *Pf* GDH, suggesting a mechanism whereby pressure reduces the inactivation rate by stabilizing the native hexamer and/or inhibiting dissociation of the hexameric intermediate.

#### Mechanism of pressure-induced thermostabilization

A possible mechanism for pressure-induced thermostabilization can be proposed based on the comparison of pressure and glycerol effects on GDH thermostability. It is well known that the addition of glycerol and other polyols to aqueous solutions can stabilize proteins against thermoinactivation (Back et al., 1979; Gekko & Timasheff, 1981a, 1981b; Timasheff, 1993). As with all stabilizing co-solvents studied so far, glycerol causes preferential hydration around proteins; in other words, glycerol is preferentially excluded from surface regions of the protein (Timasheff & Arakawa, 1997). Glycerol appears to be excluded from protein surfaces by the solvophobic effect; that is, contact between nonpolar regions of proteins and the glycerol–water mixture is even more unfavorable than contact with water alone (Gekko & Timasheff, 1981b; Timasheff, 1993; Timasheff & Arakawa, 1997). Glycerol is not completely absent near the surface of the protein, however. In fact, glycerol has an affinity for polar regions of proteins (Timasheff & Arakawa, 1997), and as shown by Gekko & Timasheff (1981a) binds to proteins in direct proportion to its concentration. An increasing amount of glycerol bound to polar and charged residues of rGDH<sup>f</sup> may help explain why glycerol concentrations higher than 25% (v/v) are less effective at stabilizing the rGDH<sup>f</sup> (Table 3).

It has been proposed that the solvophobic effect induced by glycerol causes the protein molecule to adopt a more compact structure to minimize the nonpolar surface area exposed to solution (Gregory, 1988; Cioni & Strambini, 1994). The tendency to assume a compact structure is consistent with findings from several studies of protein structure in the presence of glycerol. For example, glycerol has been shown to reduce the apparent specific volume of proteins in aqueous solutions (Gekko & Timasheff, 1981a), and to decrease both the volume and compressibility of protein interiors (Prieve et al., 1996). Compression of the protein structure has been rationalized through the reduction of voids or cavities within the protein (Gekko & Hasegawa, 1986, 1989; Prieve et al., 1996).

The compression of voids was also used to explain the effect of both glycerol and pressure on the interior flexibility of apoazurin as probed by the kinetics of tryptophan phosphorescence decay

(Cioni & Strambini, 1994, 1996). In this case, glycerol and pressure (250–2,750 bars) both reduced the flexibility of the protein interior at relatively high temperatures, consistent with a more closely packed, more rigid structure. Further effects of glycerol on protein dynamics include reduced thermal backbone fluctuations in proteins (Butler & Falke, 1996), and a decreased rate of hydrogen exchange from buried, slow-exchanging protons (Calhoun & Englander, 1985; Gregory, 1988; Wang et al., 1995). Our results show that both pressure and glycerol stabilized native GDH and rGDH' substantially; furthermore, the degree of pressure and glycerol-induced stabilization both increased with temperature. Thermostabilization of both rGDH' and native GDH by pressure and glycerol may therefore result from similar mechanism(s), whereby the enzyme adopts a more compact and rigid structure, and/or thermal fluctuations away from the native state are diminished.

To investigate this hypothesis, we examined the combined effect of glycerol and pressure on the thermostability of rGDH'. If pressure and glycerol stabilize rGDH' via the same mechanism(s), the presence of glycerol would not be expected to have an additional stabilizing effect on rGDH' at high pressures. As shown in Figure 4, glycerol and high pressure independently extended the half-life of rGDH' to 175 and 470 min, respectively. However, the combination of both (curve D) gave nearly the same half-life, 460 min, as for 750 atm alone. Therefore, pressure and glycerol appear to stabilize rGDH' by similar mechanism(s).

While it has been shown previously that a reduction in thermal fluctuations can increase protein stability (Vihinen, 1987; Daniel et al., 1996), why compression should increase stability warrants closer examination. The compression of proteins occurs through the collapse of intra and intersubunit cavities created from the imperfect packing of amino acid residues (Gekko & Hasegawa, 1986, 1989). Compression of such cavities may increase thermostability by increasing intra and/or intersubunit contacts and interactions (Priev et al., 1996), and by decreasing the amplitude of thermal motions in proteins (Gregory, 1988; Cioni & Strambini, 1994, 1996; Butler & Falke, 1996; Priev et al., 1996). Moreover, it has been found that filling or creating cavities by site-directed mutagenesis can lead to an increase or decrease of thermostability, respectively (Kellis et al., 1988, 1989; Eriksson et al., 1992a, 1992b). The effects on thermostability have been attributed to a strengthening or weakening of van der Waals interactions through the increase or decrease of molecular contacts (Pakula & Sauer, 1989; Hubbard et al., 1994).

The two proposed mechanisms of pressure stabilization are not mutually exclusive. From statistical thermodynamics, the mean-square volume fluctuation of a protein,  $\overline{\delta V^2}$ , is given by Cooper (1976):

$$\overline{\delta V^2} = kTV\beta_T \quad (2)$$

where  $k$  is the Boltzmann constant,  $T$  is the absolute temperature,  $V$  is the total volume of the protein, and  $\beta_T$  is the isothermal compressibility. The mean-square volume fluctuation is thus proportional to the temperature, volume, and compressibility of a protein. Because compression of a protein leads to a decrease in volume and compressibility, it will also reduce fluctuations resulting from high temperatures and create a more rigid and stable structure. Therefore, Equation 2 shows that compression can stabilize proteins by reducing thermal fluctuations as well as by increasing intra and/or intersubunit interactions.

Pressure-induced stabilization via reduced thermal fluctuations and/or compression is consistent with the model of GDH inactivation presented in Equation 1. Both mechanisms could decrease the overall rate of inactivation by stabilizing the native hexamer  $M_6$ , relative to the hexameric intermediate  $M'_6$ , and by retarding the irreversible dissociation and unfolding of the hexameric intermediate into unfolded monomers  $U$ . Furthermore, the proposed mechanisms of pressure stabilization are also consistent with the observation that both pressure and glycerol-induced stabilization increased with temperature. As shown by Equation 2, thermal fluctuations of proteins increase with temperature. Likewise, cavities also expand with increasing temperature (Frauenfelder et al., 1990). Thus, both the reduction of fluctuations and compression of GDH by pressure and glycerol are expected to be more pronounced at higher temperatures, inducing a greater degree of stabilization. In accord with our observation, the effect of pressure in reducing the flexibility of apoazurin was also found to be more pronounced at higher temperatures (Cioni & Strambini, 1994).

An important point to consider is the difference in the effect of pressure between native GDH and rGDH'. As shown in Table 1, 500 atm stabilized rGDH' more than native GDH at all temperatures studied. As mentioned earlier, the extra C-terminal tetrapeptide may destabilize rGDH' by increasing fluctuations in the region near the C-terminus. If so, pressure should stabilize rGDH' to a greater degree by reducing the additional thermal fluctuations due to the presence of the tetrapeptide. Therefore, a mechanism of pressure stabilization involving the reduction of thermal fluctuations is consistent with the greater stabilization of rGDH' compared to that of native GDH.

Previous investigations (Hei & Clark, 1994; Michels et al., 1996) identified a potential mechanism of pressure stabilization based on the volume change of solvation of hydrophobic residues (i.e., a positive  $\Delta V$  for transfer of hydrophobic side chains from an apolar environment to an aqueous phase). Unfolding and denaturation of very hydrophobic proteins are thus expected to be opposed by pressure, because pressure favors the state occupying the smaller volume (i.e., the native state). However, it is unlikely that this mechanism of pressure stabilization plays a major role in the dramatic pressure stabilization of *Pf* GDH. For example, the glyceraldehyde-3-phosphate dehydrogenase from *Thermotoga maritima* is slightly stabilized by pressure (with a  $t_{1/2}[500 \text{ atm}]/t_{1/2}[10 \text{ atm}]$  of 1.6), and has an average Kyte–Doolittle amino acid hydrophobicity of  $-0.039$  (Kyte & Doolittle, 1982; Hei & Clark, 1994). In comparison, *Pf* native GDH is stabilized by 500 atm up to 18-fold, with a lower (less hydrophobic) average amino acid hydrophobicity of  $-0.279$ . Furthermore, the mechanism of hydrophobic solvation cannot explain the significantly greater pressure stabilization of rGDH' over that of native GDH.

In summary, we examined the effect of pressure on the stability of two glutamate dehydrogenases from the hyperthermophilic archaeon *P. furiosus*. In the case of *Pf* rGDH', the additional C-terminal tetrapeptide may weaken crucial intersubunit ion-pair interactions by increasing the flexibility of the region around the C-terminus, and thus reduce the protein's native thermostability. On the other hand, application of high pressure stabilizes the protein to a remarkable degree. Based on the kinetics of thermoinactivation and the observed effects of glycerol on GDH stability, a possible mechanism of pressure stabilization involving the compression of voids and/or decreased thermal fluctuations has been proposed. These results provide insights into the structural determinants of protein stability at high temperatures and illustrate that

protein structure-stability relationships at extreme temperatures can be strongly influenced by pressure.

## Materials and methods

### Purification of recombinant GDH

Native GDH from *Pf* was kindly supplied by M.W.W. Adams (University of Georgia). *Escherichia coli* strain BL21 carrying the pPGDH plasmid was grown, induced, harvested, and resuspended as described previously (DiRuggiero & Robb, 1995). Cells were lysed by a single freezing and thawing sequence and the lysate was treated with streptomycin sulfate (7.4 mg/mL) for 70 min at 4 °C. The supernatant fraction was recovered by centrifugation at  $31,000 \times g$  for 85 min at 4 °C. The extract containing recombinant GDH was heated at 70 °C for 40 min and centrifuged at  $31,000 \times g$  for 40 min to precipitate *E. coli* proteins. The supernatant fraction was applied to an anion-exchange column (Q-Sepharose FF; Pharmacia Biotech, Piscataway, New Jersey) ( $1.6 \times 12$  cm), equilibrated in TED buffer (50 mM Tris, 1 mM DTT, 1 mM EDTA, pH 9.0), and GDH was eluted at 0.4 M NaCl. The eluent was then buffer-exchanged and concentrated with buffer A (20 mM Tris, 28 mM NaCl, 5 mM L-glutamate, pH 8.0) to remove NaCl. The concentrated eluent was loaded onto an affinity column (Dye-Matrx Red A; Millipore, Beverly, Massachusetts) ( $1.6 \times 10$  cm), previously equilibrated with buffer A. Recombinant GDH was eluted with 20 mM Tris, 28 mM NaCl (pH 8.0) containing 1 mM NADP. All chromatography steps were carried out at 4 °C. The final protein preparation was pure according to SDS-PAGE and Coomassie blue staining.

### Protein concentration determination

Protein concentrations were measured by the Bradford Bio-Rad (Hercules, California) protein microassay (Bradford, 1976), using bovine serum albumin as a standard.

### Identification of C-terminal tetrapeptide

The mass of the rGDH<sup>r</sup> was determined by electrospray mass spectrometry (solvent system H<sub>2</sub>O:CH<sub>3</sub>CN, 1:1) performed at the College of Chemistry Mass Spectrometry Facility at the University of California Berkeley, on a Micromass BioQ triple quadrupole mass spectrometer (sample introduction by direct injection). Comparison with the calculated mass using the nucleotide sequence of *Pf* GDH (Eggen et al., 1993) showed that rGDH<sup>r</sup> was heavier by ~300 amu. Sequencing of the plasmid *gdhA* gene (ABI 373A DNA Sequencer, dye terminator reactions) encoding the rGDH<sup>r</sup> revealed an extra tetrapeptide of Gly-Ser-Gly-Cys (304 amu) at the C-terminus, consistent with the mass spectrometry results.

### Enzyme assays

GDH activity was measured by monitoring the glutamate-dependent reduction of NADP<sup>+</sup> at 85 °C as described previously (Robb et al., 1992), except that assays were carried out in 100 mM EPPS buffer (pH 8.0 at room temperature).

### Determination of thermal half-lives

Thermostability experiments were performed using a high-pressure, high-temperature bioreactor described previously (Hei &

Clark, 1994). Temperatures of the thermostability experiments were accurate to within  $\pm 0.1$  °C. Experiments were initiated by injecting 7.0 mL of a solution of the enzyme in incubation buffer (50  $\mu$ g/mL) into the bioreactor, followed by 10 mL of nitrogen to purge the injection line. The incubation buffer was 100 mM EPPS (pH 8.0 at room temperature), with the appropriate concentrations of glycerol when called for. The bioreactor was immediately pressurized hyperbarically (with helium) to the appropriate pressure by opening it to the gas reservoir and then sealing the reactor; enzyme injection and pressurization were usually complete within 30 s. Samples were withdrawn from the bioreactor at various times (e.g., 10, 30, 75, 120, and 180 min) and assayed at ambient pressure to determine residual enzyme activity.

### Spin-labeling and ESR spectroscopy

The C-terminal tetrapeptide of rGDH<sup>r</sup> was spin-labeled at the terminal cysteine with [1-oxyl-2,2,5,5-tetramethyl-D-pyrroline-3-methyl] methanethiosulfonate (Toronto Research Chemicals, Canada). A fivefold molar excess of the methanethiosulfonate spin-label (MTSSL) from a stock solution in acetonitrile was added to a solution of rGDH<sup>r</sup> in 100 mM EPPS, pH 8.0, and incubated for 1.75 h (with gentle mixing) at room temperature. The final volume fraction of acetonitrile in the spin-labeling solution was 0.9%. Excess label was removed and the labeled enzyme concentrated by washing five times in a Microcon-100 microconcentrator (Millipore) at  $3,000 \times g$  for 8 min. ESR spectra were obtained at 21 °C and ambient pressure on a Bruker ESP300 (Bruker, Germany) spectrometer, equipped with a loop gap resonator using the following instrument settings: microwave power, 1 mW; modulation amplitude, 2.1 G; and a scan width of 120 G.

## Supplementary material in Electronic Appendix

Contains the mathematical solution of the glutamate dehydrogenase denaturation model proposed by Singh et al. (1996).

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