Mechanism of pressure-induced thermostabilization of proteins: Studies of glutamate dehydrogenases from the hyperthermophile *Thermococcus litoralis*

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(RECEIVED January 26, 2001; FINAL REVISION April 25, 2001; ACCEPTED May 30, 2001)

Abstract

In this study, we investigated the effect of pressure on protein structure and stability at high temperature. Thermoinactivation experiments at 5 and 500 atm were performed using the wild-type (WT) enzyme and two single mutants (D167T and T138E) of the glutamate dehydrogenase (GDH) from the hyperthermophile *Thermococcus litoralis*. All three GDHs were stabilized, although to different degrees, by the application of 500 atm. Interestingly, the degree of pressure stabilization correlated with GDH stability as well as the magnitude of electrostatic repulsion created by residues at positions 138 and 167. Thermoinactivation experiments also were performed in the presence of trehalose. Addition of the sugar stabilized all three GDHs; the degree of sugar-induced thermostabilization followed the same order as pressure stabilization. Previous studies suggested a mechanism whereby the enzyme adopts a more compact and rigid structure and volume fluctuations away from the native state are diminished under pressure. The present results on the three GDHs allowed us to further confirm and refine the proposed mechanism for pressure-induced thermostabilization. In particular, we propose that pressure stabilizes against thermoinactivation by shifting the equilibrium between conformational substates of the GDH hexamer, thus inhibiting irreversible aggregation.

Keywords: Pressure; pressure stabilization; *Thermococcus litoralis*; glutamate dehydrogenase; mutants; trehalose

Though not used as often as temperature or denaturants, pressure has nonetheless become an increasingly useful tool for the analysis of protein structure and function (Frauenfelder et al. 1990; Gross and Jaenicke 1994; Heremans and Smeller 1998). In particular, pressure has helped to elucidate protein-folding pathways. For example, pressure-jump studies of staphylococcal nuclease revealed that the volume of the protein-solvent system in the transition state of folding/unfolding is larger than in either the folded or unfolded states, consistent with a molten-globulelike intermediate

(Vidugiris et al. 1995). In another recent study, high-pressure, stopped-flow measurements provided evidence for a dry molten-globulelike transition state; furthermore, the structure of the transition state varied with the concentration of denaturant, i.e., with the solution environment (Pappenberger et al. 2000). Besides its use as a research tool, pressure has both potential and proven practical applications. Pressures around 1–2 kilobars have been shown to dissociate aggregates and assist proteins to refold into their native structures (Gorovits and Horowitz 1998; St. John et al. 1999). Application of pressure in combination with low concentrations of guanidine hydrochloride was effective in reversing the effect of aggregation and promoting refolding of several proteins (St. John et al. 1999). Remarkably high recoveries (up to 100%) of properly folded proteins were obtained from inclusion bodies of human growth hormone,

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Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1101/ps.4001>.

lysozyme, and β -lactamase (St. John et al. 1999). Other related applications of pressure include its use in the food industry and in vaccine development and virus inactivation (Hayashi and Balny 1996; Heremans 1997; Silva et al. 1996). In particular, high-pressure processes such as pascalization have been used to sterilize food stuffs because pressure inactivates microorganisms by destabilizing their biomembranes (Heremans and Smeller 1998).

Until recently, pressure generally has been viewed and employed as a destabilizer of protein structure; for example, in studies of pressure-induced unfolding and dissociation of monomeric as well as multimeric proteins (Jaenicke 1991; Panick et al. 1998; Weber and Drickamer 1983). However, moderately high pressures (<1000 atm) have been shown to stabilize proteins against both reversible unfolding and irreversible inactivation resulting from high temperatures (Hawley 1971; Millar et al. 1974; Mozhaev et al. 1996). In particular, there are many examples of pressure stabilizing thermophilic and hyperthermophilic proteins against thermal inactivation (Hei and Clark 1994; Michels et al. 1996; Sun et al. 1999). One example was with the glutamate dehydrogenase (GDH) from the hyperthermophile *Pyrococcus furiosus* (Pf), where Sun et al. (1999) showed that pressures in the range of 250–500 atm stabilized the GDH against thermoinactivation. The thermal half-life at 105°C as measured by remaining activity increased from 13 min at 5 atm to 360 min at 500 atm, the largest pressure-induced thermostabilization recorded for any protein. Based on the known effects of glycerol on protein structure and stability, a mechanism of pressure stabilization involving the compression of cavities and reduction of volume fluctuations in the native structure was proposed (Sun et al. 1999).

As part of our continuing effort to elucidate the effect of pressure on enzyme structure, flexibility, and stability at high temperatures, we have carried out studies with the highly homologous GDH from the hyperthermophile *Thermococcus litoralis* (Tl) (sharing 89% sequence identity with the Pf GDH). Tl is an archaeon isolated from marine thermal springs and grows optimally at 88°C (Neuner et al. 1990). The GDH from Tl is very thermostable, with a melting temperature of 109°C (Vetriani et al. 1998). Like Pf GDH, Tl GDH is a hexamer composed of six identical subunits.

In this paper, we describe results for the wild-type (WT) enzyme and two single mutants of Tl GDH: D167T and T138E. The single mutations originally were designed to alter electrostatic interactions at a particular intersubunit region of the GDH (Vetriani et al. 1998). Figure 1 shows half of a WT Tl GDH hexamer (i.e., a trimer) viewed along the trimer-trimer axis, exposing the trimer-trimer interface. The Thr138s and Asp167s are highlighted, illustrating their proximity to each other and to the subunit-subunit interfaces. As shown previously (unpubl.), ion-pair interactions as well as long-range electrostatic interactions involving residues at positions 138 and 167 are important to the ther-

Fig. 1. *Thermococcus litoralis* wild-type glutamate dehydrogenase viewed along the threefold (trimer-trimer) axis with the top trimer removed, thus exposing the trimer-trimer interface. Subunits A, B, and C are colored gray, blue, and pink, respectively, and the Thr138s and Asp167s are colored yellow and red, respectively. The distance between any two Asp167s is ~10 Å, while that between any two Thr138s is ~20 Å. Figure created using the program RasMol.

mostability of the GDH. In particular, long-range electrostatic repulsion among and between positions 138 and 167 was shown to correlate with the thermostability of the three GDHs: WT, D167T, and T138E.

The goal of this study was to further examine the thermostabilization affected by moderately high pressures and to extend our understanding of the pressure effects on protein structure and stability at high temperatures. To this end, we investigated the effect of pressure on the kinetic thermostability of the WT GDH and the two single mutants D167T and T138E. All three enzymes were stabilized by pressure against thermoinactivation. Interestingly, the degree of pressure stabilization differed significantly between the three enzymes, suggesting changes in the structure and/or dynamics of GDH caused by the mutations. Thermoinactivation experiments performed in the presence of trehalose indicated that stabilization by a stabilizing sugar also differed for the three GDHs. Our results, along with the known mechanism of protein stabilization by sugars and recent studies of protein structure and dynamics under pressure, provide further insights into the mechanism of pressure stabilization of GDH.

Results

Most hyperthermophilic proteins studied to date (Hiller et al. 1997), including Tl GDH, undergo irreversible thermoinactivation. Therefore, the thermoinactivation experiments

performed in this work were analyzed in terms of the kinetics and not the thermodynamics of GDH inactivation. The time course of GDH thermostability was followed by activity assays of samples withdrawn at fixed time intervals from a bioreactor as described previously (Sun et al. 1999). The percentage of GDH activity remaining in solution then was plotted as a function of incubation time. The remaining activity was confirmed to be proportional to the amount of nondenatured GDH remaining in solution (unpubl.).

We showed previously that the single mutations D167T and T138E altered the kinetic stability of *Tl* GDH (unpubl.). Specifically, the D167T and T138E single mutants are, respectively, slightly more and much less thermostable than the WT GDH. However, all three GDHs assembled into hexameric forms that were indistinguishable by native polyacrylamide gel electrophoresis (data not shown). Furthermore, the activity-temperature profiles of both the D167T and T138E mutants are very similar to that of the WT GDH (Fig. 2). Therefore, neither mutation appears to grossly alter the conformation of GDH.

Effect of pressure on stability

Thermostability experiments at low and high pressures were performed using the WT, D167T, and T138E GDHs at different temperatures. Thermostability trajectories were fitted as described in Materials and Methods, from which apparent first-order rate constants, k_{obs} , were determined. Figure 3 shows Eyring plots of the first-order inactivation rate constants for the thermostability of the GDHs at 5 (open symbols) and 500 atm (filled symbols). Application of 500 atm stabilized all three GDHs against thermostability as evidenced by the downward shifts in the respective Eyring plots (reflecting a decrease in the rate

Fig. 2. Specific activity of wild-type (○), D167T (◇), and T138E (□) glutamate dehydrogenases as a function of temperature. Error bars represent average deviations of two or more duplicate assays.

Fig. 3. Eyring plots showing the effect of pressure on the thermostability of *Tl* T138E (□, ■), wild type (○, ●), and D167T (◇, ◆) glutamate dehydrogenases (GDHs). The open and filled symbols represent thermostability experiments at 5 and 500 atm, respectively. As shown for the T138E GDH at 90°C, the difference in the $\Delta G^\ddagger$ of inactivation at low and high pressure (i.e., $\Delta\Delta G^\ddagger$) is proportional to the vertical distance on an Eyring plot.

constants of inactivation). In addition, the downward shifts are different in magnitude, indicating different degrees of pressure stabilization. Because of the large difference in stability between the T138E mutant and the other two GDHs, thermostability of all three enzymes at a common temperature was not possible. Instead, thermostability experiments for each GDH were performed over as wide a range of temperature as was experimentally feasible, resulting in half-lives between 10 and 1000 min. The lower limit on the half-life (10 min) was constrained by the time required to pressurize the sample and reach thermal equilibrium, whereas the upper limit was a matter of practicality. Nevertheless, over this range of half-lives, the degree of pressure stabilization follows the order: D167T < WT < T138E, with the largest stabilization corresponding to the T138E mutant.

The inactivation rate constants were further analyzed using transition-state theory and presented as the difference in the free energy of activation (for the inactivation process), $\Delta\Delta G_p^\ddagger$, between thermostability at 5 and 500 atm (Table 1). Positive values of $\Delta\Delta G_p^\ddagger$ indicate stabilization by pressure. Table 1 confirms the order in the degree of pressure stabilization shown graphically in Figure 1. In addition, the values of $\Delta\Delta G_p^\ddagger$ indicate that the degree of pressure stabilization for each individual GDH tends to increase with the experimental temperature; for example, the $\Delta\Delta G_p^\ddagger$ values for the WT are 3.8, 4.9, and 6.3 kJ/mol at 98.8, 100, and 101.4°C, respectively. This trend suggests that the stabilizing effect of pressure increases as the stability decreases. The same trend was observed for the pressure stabilization of the highly homologous native *Pf* GDH at various temperatures (Sun et al. 1999).

Table 1. Quantifying the effect of pressure on the thermostability of D167T, WT, and T138E GDHs

Enzyme	Temp. (°C)	$t_{1/2}$ [5 atm] (min) ^a	$\Delta\Delta G_p^\ddagger$ (kJ/mol) ^{a,b}
D167T	101.5	80	3.4
D167T	102.9	67	3.4
D167T	104	32	3.9
WT	98.8	97	3.8
WT	100	79	4.9
WT	101.4	29	6.3
T138E	86	68	8.0
T138E	87	60	7.6
T138E	90	22	8.6
T138E	92	10	9.6

^a Half-lives and $\Delta\Delta G_p^\ddagger$ were determined based on k_{obs} (for further details, see Materials and Methods).

^b $\Delta\Delta G_p^\ddagger = \Delta G_{500\text{ atm}}^\ddagger - \Delta G_{5\text{ atm}}^\ddagger$.
GDH, glutamate dehydrogenase; WT, wild type.

Effect of trehalose on stability

The effect of a stabilizing sugar, trehalose, on the thermostability of the GDHs also was investigated. Table 2 gives the half-lives of all three GDHs in the absence and presence of 0.5 M trehalose. Because of differences in stability between the enzymes, thermoinactivation temperatures were chosen to give approximately the same half-life in the absence of trehalose. The results indicate that trehalose stabilized all three GDHs against thermoinactivation. Furthermore, the degree of stabilization conferred by trehalose was dependent on the enzyme. Trehalose had the largest stabilizing effect on the T138E mutant, with a $\Delta\Delta G_{tre}^\ddagger$ of 6.0 kJ/mol. The effect on the WT and D167T enzymes was more modest, with $\Delta\Delta G_{tre}^\ddagger$ values of 2.6 and 1.5 kJ/mol, respectively.

Discussion

Previous experiments demonstrated that the single mutations D167T and T138E affected the stability of *Tl* GDH (unpubl.). Because both positions 138 and 167 are located on the surface of the GDH monomer and occupy loop re-

gions between secondary structural elements, it is unlikely that either mutation induces major changes in the secondary or tertiary structure of the monomer. Figure 2 shows that the activity-temperature profiles of the three GDHs are very similar up to 95°C. The similarity in specific-activity profiles is further evidence that all three GDHs are structurally alike at least at temperatures below the onset of thermal denaturation.

Like the GDH from *Pf*, Figure 3 shows that pressure has a substantial stabilizing effect on all three GDHs from *Tl*. The vertical distance between the 5 and 500 atm data points is proportional to the difference in the $\Delta G^\ddagger$ s for the denaturation process between 5 and 500 atm (Fig. 3). It is clear that the degree of pressure stabilization is different between the three GDHs, with the degree of pressure stabilization following the order: D167T < WT < T138E, where the largest stabilization corresponds to the T138E mutant.

Table 1 quantifies some of the data points in Figure 3 by listing low-pressure half-lives and $\Delta\Delta G_{ps}^\ddagger$ at several temperatures. For each GDH, pressure has a somewhat larger stabilizing effect as the temperature is increased. However, the degree of pressure stabilization is not solely a function of the thermoinactivation temperature, as the largest pressure stabilization is observed for T138E at temperatures more than 10°C lower than those used for D167T or WT. Any molecular model explaining the effect of pressure must be consistent with the following observations: (1) pressure stabilizes all three GDHs against thermoinactivation, (2) the degree of pressure stabilization follows the order D167T < WT < T138E, and (3) pressure stabilization increases with temperature for each GDH.

Studies of the effects of temperature and pressure on the structure and dynamics of proteins pointed to the existence of substates within a given state of the protein (Frauenfelder et al. 1988, 1990, 1991). These substates, termed conformational substates (CS), are similar, but not identical in structure and energy, and perform the same function (Frauenfelder et al. 1990). The arrangement of CS is hierarchical, resulting in different levels or tiers of CS. For example, myoglobin contains three CS of tier 0 (i.e., CS⁰); each of these CS⁰s is, in turn, more finely divided into further CS of tier 1 (i.e., CS¹) (Frauenfelder et al. 1990). The existence of CS² also has been shown, and it is likely that CS³ and CS⁴ also are present; however, differences in properties (e.g., structure, energy, volume) are greatest between members of CS⁰ tier (Frauenfelder et al. 1990). Above a characteristic temperature (200 K in the case of myoglobin) all CS within all tiers are in equilibrium (Frauenfelder et al. 1990, 1991). Most significant for our work, it was found that pressure shifted the equilibrium between two members of the CS⁰ tier of myoglobin, indicating a difference in volume. The difference in volume was a function of temperature with a value of about 15 cm³/mol at room temperature (Frauenfelder et al. 1990). By means of

Table 2. Effect of trehalose on the thermoinactivation of GDHs

TCH Enzyme	Temp. (°C)	Half-life (min) ^{a,b}		$\Delta\Delta G_{tre}^\ddagger$ (kJ/mol) ^{a,c}
		No trehalose	0.5 M trehalose	
D167T	101.5	80 ± 5	129 ± 9	1.5 ± 0.3
WT	100.0	80 ± 1	185 ± 20	2.6 ± 0.3
T138E	86.0	69 ± 2	504 ± 39	6.0 ± 0.2

^a Half-lives and $\Delta\Delta G_{tre}^\ddagger$ were determined based on k_{obs} (for further details, see Materials and Methods).

^b Errors are average deviations of two experiments.

^c $\Delta\Delta G_{tre}^\ddagger = \Delta G_{tre}^\ddagger$. Errors were propagated following Equation 9. GDH, glutamate dehydrogenase; WT, wild type.

an equilibrium between CS, Frauenfelder and coworkers (1990) have demonstrated a mechanism that explains the observed effect of pressure on the structure, dynamics, and function (e.g., binding of carbon monoxide to myoglobin) of proteins.

Our own previous study on the effect of pressure on GDH from *Pf* suggested a possible model of pressure stabilization whereby the enzyme adopts a more compact and rigid structure and volume fluctuations away from the native state are diminished (Sun et al. 1999). The results obtained in this study with the WT and mutant GDHs, along with recent findings on the mechanism of protein aggregation (Kendrick et al. 1998), have led to further confirmation and refinement of the molecular mechanism for pressure stabilization of GDH previously put forth by us (Sun et al., 1999). Specifically, we now propose that pressure stabilizes by modulating the equilibrium between CS of the GDH hexamer, thus reducing unfolding and eventual aggregation. This refined model combines the idea and existence of CS (Frauenfelder et al., 1988, 1990, 1991) with the recent mechanism proposed for the aggregation of human interferon- γ (Kendrick et al. 1998). Under this model, the apparent first-order inactivation of GDH is modified to:

where H_{CS0} and H_{CS1} are CS that comprise the native-state ensemble of the GDH hexamer, H_U is a partially unfolded hexamer that then is able to aggregate to form the aggregated species A, $K_1 (= H_{CS1}/H_{CS0})$ is the equilibrium constant for the conversion between H_{CS0} and H_{CS1} , and k_i is the first-order inactivation constant for the formation of H_U . For simplicity, we have assumed the existence of only two CS, with H_{CS0} being the lower energy and volume CS. Equation 1 assumes that only H_{CS1} can irreversibly unfold into an aggregate-competent (term used by Kendrick et al. 1998) species H_U . The mechanism shown by Equation 1 is consistent with the observation that thermoinactivation of all *Tl* GDHs follows apparent first-order kinetics and that the rate-limiting step precedes aggregation (unpubl.). Kendrick and coworkers also found that aggregation of recombinant human interferon- γ follows apparent first-order kinetics, leading the authors to the same conclusion that a poly-molecular process such as aggregation was not rate-limiting (Kendrick et al. 1998).

Assuming that both H_{CS0} and H_{CS1} are active at the non-denaturing assay temperature of 85°C, Equation 1 can be solved for the remaining GDH activity as a function of time:

$$Act(t) = Act_i \exp[-k_{obs}t] \quad (2)$$

$$k_{obs} = \left(\frac{k_1 K_1}{1 + K_1} \right) \quad (3)$$

where Act_i and $Act(t)$ are, respectively, the GDH activity measured initially and at time t . The rate constant k_{obs} is the

first-order inactivation constant determined from fits to thermoinactivation experiments (see Materials and Methods). According to Equations 1–3, pressure can stabilize GDH by shifting the equilibrium between CS towards that of H_{CS0} . The relation between the equilibrium constant K and pressure is characterized by a volume difference:

$$\left(\frac{\partial \ln K_1}{\partial P} \right)_T = - \left(\frac{\Delta V_1}{RT} \right) \quad (4)$$

where P is pressure, ΔV_1 is the difference in volume between H_{CS1} and H_{CS0} (i.e., $V[H_{CS1}] - V[H_{CS0}]$), R is the universal gas constant, and T is the temperature. We propose that GDH inactivation follows Equation 1 and that the volume of H_{CS1} is greater than that of H_{CS0} (i.e., $\Delta V_1 > 0$). Therefore, according to Equation 4, an increase in pressure will result in a decrease of K_1 ; this, in turn, will stabilize GDH against thermoinactivation by decreasing k_{obs} (Equation 3). This is consistent with Le Châtelier's principle, which predicts that pressure will shift the equilibrium towards the state of lowest volume (i.e., H_{CS0}), thus inhibiting formation of H_U and aggregation.

The proposed model of pressure stabilization also should be able to explain the varying degree of pressure stabilization between the three GDHs (Fig. 3). Figure 4 is a schematic of positions 138 and 167 as viewed along the trimer-trimer axis (same line-of-sight as Fig. 1) for the D167T, WT, and T138E GDHs. The number of negatively charged residues occupying these positions increases from D167T (none) to WT (Asp167s) to T138E (Asp167s + Glu138s). Thermostability experiments with KCl in concert with theoretical calculations have shown that the electrostatic repulsion among these positions weakens intersubunit interactions near the core of the GDH hexamer (unpubl.). In particular, with no added salts in the thermoinactivation solution, stability was inversely correlated with the magnitude of electrostatic repulsion and follows the order: T138E < WT < D167T.

We propose that the destabilizing electrostatic repulsion originating around positions 138 and 167 also creates larger conformational fluctuations of the entire hexamer. In terms of Equation 1, one way that larger fluctuations can be manifested is by an increase in the volume of the higher-energy CS H_{CS1} . Assuming this, stronger electrostatic repulsion will result in larger values of ΔV_1 s for the equilibrium step between CS. The conformational fluctuations should be smallest in the case of the D167T mutant because positions 138 and 167 are both occupied by uncharged threonines (Fig. 4). On the other extreme, both positions are negatively charged in the T138E mutant, resulting in the largest conformational fluctuations (Fig. 4). Fluctuations of the WT GDH should be intermediate because only positions 167 are occupied by negatively charged residues (Fig. 4). Thus, the magnitude of ΔV_1 s is predicted to follow the same order as

Fig. 4. Schematic representation of positions 138 and 167 within a glutamate dehydrogenase trimer looking along the trimer-trimer axis (same line-of-sight as Fig. 1). (a) D167T: all six residues are occupied by uncharged Thr. (b) Wild type: Asp at positions 167 are negatively charged. (c) T138E: Asp at positions 167 and Glu at positions 138 are negatively charged. The distance between any two closest neighbors is about 10 Å.

the electrostatic repulsion — D167T < WT < T138E — with the largest volume difference corresponding to the T138E enzyme. And because the effect of pressure is governed by ΔV_f , the observed order in the magnitude of pressure stabilization between the GDHs can be rationalized by differences in the electrostatic repulsion around positions 138 and 167.

In support of our model of pressure stabilization, we took advantage of the well-known effects of sugars on protein stability, structure, and dynamics to probe our system. Numerous studies have found that sugars (e.g., glucose, sucrose, and trehalose) stabilized proteins against denaturation by high temperature, low pH, and denaturants (Lee and Timasheff 1981; Arakawa and Timasheff 1982; Timasheff 1993, 1998; Kita et al. 1994; Hall et al. 1995; Lin and Timasheff 1996; Kendrick et al. 1997, 1998; Xie and Timasheff 1997). The origin of this effect lies predominantly in the higher surface tension of aqueous sugar solutions (Lee and Timasheff 1981; Lin and Timasheff 1996), leading to the inhibition of protein processes (e.g., unfolding) that are accompanied by an increase in surface area (Sinanoglu and Abdulnur 1964, 1965).

Because of the increase in surface tension, we can expect sugars to shift the equilibrium between CS. As mentioned above, CS differ slightly in structure, and in particular, volume (Frauenfelder et al. 1990). According to Richards (1979), the only way to generate volume fluctuations is through the expansion and contraction of interior cavities. It therefore is reasonable that CS with larger volumes also expose larger surface areas; that is, protein expansion as a result of an increase in the number and/or volume of cavities also results in greater surface areas. In this manner, sugars should shift the equilibrium towards the GDH CS with smaller volume and surface area (i.e., H_{CS0}) and thus stabilize against thermoinactivation.

Following the line of reasoning presented above, we examined the effect of trehalose on the thermostability of the WT, D167T, and T138E GDHs. Table 2 gives the half-lives of all three enzymes with and without 0.5 M trehalose. Because of the difference in stability between the GDHs, the

experimental temperatures were selected to obtain similar half-lives for all GDHs in the absence of trehalose. Consistent with our model of inactivation involving shifts between CS that differ in volume as well as surface area (Equation 1), trehalose stabilized all three GDHs. More importantly, the degree of stabilization was different between enzymes and followed the order D167T < WT < T138E — the same order as in the case of pressure-induced stabilization. The correspondence between the order of sucrose- and pressure-induced stabilization is consistent with the proposal that the volume and surface area differences between the CS H_{CS1} and H_{CS0} increase with the magnitude of electrostatic repulsion around positions 138 and 167. Our proposed model of pressure and trehalose stabilization of GDHs also is consistent with the observation by Almagor and coworkers (1998) that various cosolvents (ranging from sucrose to dextran) reduced the specific volume and adiabatic compressibility of myoglobin.

Kendrick et al. (1998) also took advantage of the known effects of sugars to elucidate the mechanism leading to the aggregation of human interferon- γ . As mentioned above, the proposed mechanisms for the aggregation of both human interferon- γ and GDH (Equation 1) are essentially the same; they both invoke the existence of an equilibrium between native conformational species preceding an irreversible step and leading to eventual aggregation. In agreement with our results, sucrose stabilized against aggregation, consistent with a shift of the equilibrium between CS caused by the increase in surface tension upon addition of sucrose (Kendrick et al. 1998).

Finally, the observation that for each GDH, pressure stabilization increases with temperature (Table 1) should be addressed in light of our model of pressure stabilization. Previous studies with the *Pf* GDH also have revealed an increase in the degree of pressure stabilization with temperature (Sun et al. 1999). That observation was rationalized by noting that the compressibility, volume, and volume fluctuations of a protein all increase with temperature (Cooper 1976; Sun et al. 1999), and that the increase is most likely a result of the expansion of cavities and free spaces

between atoms (Richards 1979; Frauenfelder et al. 1987; Young et al. 1994). Therefore, it seemed reasonable that pressure-induced stabilization involving the compression of structure and reduction of volume fluctuations should be amplified at higher temperatures where compressibility and volume fluctuations are greatest (Sun et al. 1999). This explanation can be further elaborated with our present mechanism involving CS. In this case, volume fluctuations are taken into account explicitly by higher-energy CS with larger volumes (i.e., H_{CS1}). As temperature increases, we hypothesize the appearance of new CS of even higher energy and volume beyond that of H_{CS1} . For example, GDH thermoinactivation may proceed through a slightly different mechanism such as

where H_{CS2} is a new, higher-energy and volume CS that is populated to a significant extent only at higher temperatures. For simplicity, we have assumed that the formation of the aggregation-competent species, H_U , now occurs only through H_{CS2} . This equation can be solved, yielding a slightly more complicated expression for the observed rate of GDH thermoinactivation (compare with Equation 3)

$$k_{obs} = \left(\frac{k_i K_1 K_2}{1 + K_1 + K_1 K_2} \right) \quad (6)$$

where $K_2 (= H_{CS2}/H_{CS1})$ is the equilibrium constant for the conversion between H_{CS1} and H_{CS2} . The dependence of K_2 on pressure is governed by the volume difference between H_{CS2} and H_{CS1} , ΔV_2 , and follows an equation analogous to Equation 4. The effect of pressure on the two expressions for k_{obs} (Equation 3 and 6) were analyzed by substituting in some realistic values for ΔV s and K s. As an example, for values of ΔV s from 100–300 Å³ (i.e., <0.1% of the volume of a GDH hexamer) and K values between 0.01–1, the stabilizing effect of pressure was found to be ~2–3 times greater for the mechanism of thermoinactivation hypothesized to occur at higher temperatures. This simple analysis demonstrates that the observed increase in pressure stabilization with temperature (Table 1) can be accounted by a model where pressure shifts the equilibrium between CS.

Materials and methods

GDH Purification

GDH was purified as described previously (Vetriani et al. 1998). Final purified GDHs exhibited single bands when analyzed by SDS-PAGE and stained with Coomassie blue. The concentration of GDH was determined by ultraviolet (UV) absorption at 280 nm of GDH dissolved in 7.84 M guanidine hydrochloride using an extinction coefficient of 82,740 M⁻¹ cm⁻¹ for the unfolded GDH monomer (Edelhoc 1967; Gill and von Hippel 1989).

Activity assays

GDH activity was measured by monitoring the glutamate-dependent reduction of NADP⁺ as described previously (Sun et al. 1999). Assays were performed in an AVIV 14NT-UV-VIS spectrophotometer equipped with a Peltier heater and a magnetic stirrer for accurate temperature control (AVIV Instruments, Inc.). The cell holder was inside a pressure chamber capable of hyperbaric pressurization to 5 atm, allowing assays above 100°C.

Thermoinactivation experiments

Thermoinactivation experiments were performed as described previously in a custom bioreactor kept in a constant-temperature oven (Sun et al. 1999). All thermoinactivation experiments were performed at either 5 or 500 atm of hyperbaric pressure using helium as the pressurizing gas. At 100°C and 500 atm of helium, the liquid mole fraction of helium in water is 0.003094 (Clever 1979); thus, the influence of dissolved helium should be negligible. Temperatures of the thermoinactivation experiments were accurate to within $\pm 0.1^\circ\text{C}$. Unless otherwise indicated, GDH (24–28 µg/mL final concentration) was incubated in 100 mM EPPS (Sigma), at a pH of 7.1–7.2 at the thermoinactivation temperature. For correction of pH as a function of temperature, a $\Delta\text{pH}/\Delta T$ value of -0.0114 was experimentally determined for 100 mM EPPS, with or without 0.5 M trehalose (Sigma). Samples from the bioreactor were withdrawn periodically and immediately assayed for activity at 85°C and 1 atm (Sun et al. 1999).

Analysis of irreversible thermoinactivation

The thermoinactivation trajectories of all three GDHs deviated from a single-exponential decay. As discussed previously, the available evidence suggests that the apparent non-single-exponential thermoinactivation of the GDHs is because of the existence of misfolded and less-stable form(s) of GDH (unpubl.). Specifically, thermoinactivation appears to involve the simultaneous denaturation of less-stable form(s) of GDH and the native, more stable, form of GDH, which inactivates via an irreversible, rate-limiting transition between the native hexamer and an inactivated species. Following this model, the thermoinactivation trajectories were fitted to a double-exponential model with four adjustable parameters:

$$Act(t) = E_1 e^{k_1 t} + E_2 e^{k_2 t} \quad (7)$$

where Act is the remaining activity at time t (i.e., defined as the percentage of the initial activity), E_1 and E_2 are the percentages of the total activity contributed by the less-stable and native forms of GDH, respectively, and k_1 and k_2 are the respective apparent first-order inactivation rate constants. The double-exponential model fitted all thermoinactivation trajectories well. As expected, the fitted parameters E_1 and E_2 summed to nearly 100% in all of the fits. In all samples, the more stable form comprised at least 60% of the total GDH.

The rate constant k_2 corresponding to that of the native GDH, and henceforth identified as k_{obs} , was further analyzed in terms of the activation barrier for thermoinactivation using transition state theory (Eyring 1935):

$$\ln k_{obs} = \ln \frac{\kappa k_b T}{h} - \frac{\Delta G^\ddagger}{RT} \quad (8)$$

where κ is the transmission factor, k_b the Boltzmann constant, T the absolute temperature in Kelvin, h the Planck constant, $\Delta G^\ddagger$ the free energy of activation, and R the gas constant. The difference in

the free energy of activation between 5 and 500 atm ($\Delta\Delta G_p^\ddagger$) were calculated following the analysis of Pappenberger et al. (1997):

$$\Delta\Delta G_p^\ddagger = \Delta G_{500\text{ atm}}^\ddagger - \Delta G_{5\text{ atm}}^\ddagger = -RT \ln \left(\frac{k_{\text{obs}}^{500\text{ atm}}}{k_{\text{obs}}^{5\text{ atm}}} \right) \quad (9)$$

The difference in free energy between conditions of 0 and 0.5 M trehalose, $\Delta\Delta G_{\text{tre}}^\ddagger$, was calculated using the analogous equation and rate constants determined with and without trehalose.

Acknowledgments

This work was supported by the National Science Foundation (BES 9410687), the Schlumberger Fellowship of DSC, and by Kyowa Hakko Kogyo Co. Ltd.

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