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The existence of a hexameric intermediate with molten-globule-like properties in the thermal denaturation of bovine-liver glutamate dehydrogenase

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Abstract

We have studied the thermal denaturation of hexameric beef-liver glutamate dehydrogenase by itself and in the presence of ADP and guanidine–HCl by a variety of techniques. In differential scanning calorimetry studies, the observed melting temperature and total enthalpy of denaturation show no dependence on protein concentration, but do show a significant dependence on the scan rate. This suggests that the overall denaturation process is irreversible and kinetically controlled. Isothermal unfolding kinetics from spectrophotometry confirm this result. The size of the protein, as shown by quasi-elastic light scattering measurements, does not change during the denaturation process. We interpret these results in terms of the following model:

$$N_6 \rightleftharpoons N_6' \rightarrow 6U(\rightarrow F)$$

where N_6 and N_6' are, respectively, the native hexamer and a hexameric, highly folded high-enthalpy species, U is the unfolded monomer and F is some final aggregated state. The kinetic intermediate, N_6', possesses the properties of one definition of a molten globule, having a very high enthalpy and a hexameric compact structured form. This "molten globule" is an obligatory intermediate in the unfolding pathway of the protein. The stabilization of the protein by ADP is due to the modulation of the high-enthalpy two-state predenaturational $E \rightleftharpoons E'$ transition, resulting in the lowering of the energy of the native state of the protein.

Keywords: Unfolding; Predenaturational transition; Ligand effects; Inactivation

1. Introduction

In recent years, differential scanning calorimetry (DSC) has established itself as the prime technique for the study of the thermal stability of proteins, especially since the availability of ultrasensitive microcalorimeters and convenient deconvolution algorithms. Most reversibly unfolding protein systems obey the two-state van't Hoff model and, hence, can be described completely in terms of the thermodynamic properties of the folded and unfolded forms [1,2]. In the case of irreversibly denaturing proteins, the process is often kinetically controlled, restricting

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any formal thermodynamic analysis. The overall irreversibility of a denaturation process can be established by the reduction in size of the endothermic peak in a rescan of a rapidly chilled denatured sample. Kinetic control of the process is customarily inferred from the scan rate dependence of the observed melting temperature [3–5]. In certain special cases the DSC scans can be fitted with a van't Hoff model, in spite of the overall irreversibility, and the thermodynamic melting temperature and van't Hoff enthalpy of denaturation can still be obtained reliably since the effect of the kinetics on the irreversible step on melting is small [6,7]. This is "situation A" as defined by Sanchez-Ruiz [7]. However, in most cases where the rate of the irreversible step is significant during melting, the endotherms are highly distorted from the van't Hoff shapes and, hence, provide very little thermodynamic information. This is situation C, case B being the intermediate situation. Lumry–Eyring kinetic models [8] have been employed in such cases to define and evaluate the process of irreversible denaturation. In the extreme case, this model $N \rightleftharpoons U \rightarrow F$ (where N is the folded native protein, U is the reversibly unfolded molecule and F is some irreversibly unfolded state) reduces to $N \rightarrow F$ with negligible accumulation of U at any temperature. In such a system, the scan rate dependence of the observed melting temperature can be analyzed using one-step kinetics involving the temperature dependence of the reaction rate constant, yielding the activation energy [3]. Additionally, the total enthalpy of denaturation can be obtained by integration of the endothermic transition. Protein concentration and ligand binding effects in irreversible denaturation have also been interpreted in terms of modified Lumry–Eyring models [7]. In these models, a change in the state of association of the protein on denaturation results in protein concentration dependence of the observed melting temperature, whereas the preferential binding of a ligand to the folded form of the protein only leads to ligand concentration dependence of the melting temperature.

The folding–unfolding pathways in reversible [9,10] as well as irreversible denaturation [11] have been shown to include folding intermediates frequently referred to as "molten globules". Some studies indicate that these "hot" intermediates have a compact structured form, retaining most of the secondary structure of the native protein [9,11,12], whereas other studies suggest that molten globules have a compact unstructured form resulting from nonspecific hydrophobic collapse [13]. In either case, these molten-globule species play an important role in the thermal denaturation processes.

Beef-liver L-glutamate dehydrogenase (GDH) is a hexameric protein with a single subunit of molecular mass 56 100. At concentrations above 10 μM, it exists in the form of labile associated chains of the hexamer [14], but the catalytic properties are independent of the degree of association [15]. The coenzyme, NADPH, is known to reduce the thermal stability of this enzyme [16], whereas the presence of trace amounts of phosphate is necessary for the survival of the enzyme even at low temperatures [17]. We have established that the cooperative binding of ligands such as NADPH and ADP [18], and anions such as phosphate and acetate [18,19], all of which affect the thermal stability of the enzyme, also induce at least one high-enthalpy, proton-ionization linked two-state transition in the enzyme. This predenaturational transition, $E \rightleftharpoons E'$, is characterized in each complex by a temperature, T°, at which the equilibrium constant is unity and the two forms must be equal in concentration. The coenzyme, NADPH, lowers the T° of the enzyme from 43 to -15°C, whereas ADP raises it to 56°C. The purpose of this study is to determine the unfolding mechanism of the protein, and to provide a rational interpretation of the ligand-induced changes in the thermal stability of the enzyme through a combination of differential scanning calorimetric and isothermal denaturation kinetic approaches.

2. Materials and methods

2.1. Materials

Beef-liver L-glutamate dehydrogenase (EC.1.4.1.3), purchased from Boehringer Mannheim as an ammonium sulfate suspension, was purified as described previously [20]. The stock enzyme solution was dialyzed in 0.1 M phosphate buffer at pH 7.60 with at least four changes, and the concentration was determined spectrophotometrically using $\epsilon = 0.97$

mg^{-1} ml cm^{-1} at 280 nm for a subunit of M_r = 56 100. Guanidine–HCl, ADP and the buffer constituents were of high purity and were used without any further purification. All solutions were prepared in 0.1 M phosphate buffer, pH 7.60, at 25°C. ADP concentration was measured spectrophotometrically using $\epsilon = 15.4$ mM^{-1} cm^{-1} at 259 nm.

2.2. Differential scanning calorimetry

DSC studies were conducted on an ultrasensitive MC-2D microcalorimeter (MicroCal Inc., Northampton, MA). Protein samples, after the adjustment of concentration and pH to 7.60 at 25°C, were degassed with constant stirring at room temperature for 15–20 min. The sample cell was filled with the protein solution and the reference cell with a solution containing all the constituents of the sample solution except the protein. While filling the cells, care was taken to avoid air bubbles. After applying an air pressure of 2 atm over the cells, the instrument was turned on its side to obviate the occurrence of artifactual endotherms due to aggregation of denatured protein. DSC scans were performed in the protein concentration range of 10–100 μM and scan rate range of 10–90 K h^{-1}. Appropriate solvent–solvent base lines were collected in each case at the same scan rate as for the protein sample. Base line subtraction, concentration normalization and integration of the endothermic transitions were performed using the Origin software (MicroCal Inc., Northampton, MA). The deconvolution of the DSC scans by this software is based on the algorithms developed by Freire and Biltonen [21,22]. The scan rate dependence of denaturation was studied at a fixed enzyme concentration of 20 μM, whereas the protein concentration dependence was obtained at a fixed scan rate of 90 K h^{-1}.

2.3. Isothermal denaturation kinetics

The denaturation kinetic experiments were carried out on a HP-8450A diode array spectrophotometer equipped with a HP-89100A temperature controller. This controller can maintain a temperature accuracy of ±0.1°C when a fluid temperature probe is placed in the sample cuvette. In all the runs, the enzyme in a buffer containing 0.1 M phosphate at pH 7.60 was mixed with stock guanidine–HCl solution to obtain a final enzyme concentration of 10 μM in 2 M guanidine–HCl. These studies could only be performed in the presence of guanidine–HCl, since otherwise the samples aggregated as the denaturation progressed. The reference cell was always maintained at 5°C and the sample cell at the temperature of the run. After filling both the cells with the enzyme solution and allowing them to equilibrate, a balance spectrum was obtained. The denaturation was started by adding calculated volumes of pre-incubated guanidine–HCl solution to the reference and sample cells. Immediately upon adding the denaturant solution to the sample cell with a stirrer-adder, absorption spectra in the range of 250–500 nm were collected at regular time intervals. The data were transferred directly to a microcomputer through a modem for analysis.

2.4. Inactivation kinetics

Activity-loss time courses were measured using the apparatus described above, in 0.1 M phosphate buffer, pH 7.6. Stock enzyme solution was added to pre-incubated phosphate buffer or 2 M guanidine–HCl solution to begin the denaturation. At regular time intervals, aliquots were drawn into chilled test tubes kept in ice. The partially denatured enzyme samples were assayed for enzymatic activity on a Gilford Response II spectrophotometer equipped with the rapid sampling accessory using the standard assay described earlier [20]. The assay solution contained 0.5 mg ml^{-1} bovine serum albumin, 100 μM EDTA, 50 mM ammonium sulfate, 2 mM α-ketoglutarate and 100 μM NAD. Various kinetic models were fitted to the time-dependent residual activity.

2.5. Particle diameter by quasi-elastic light scattering (QELS)

The diameter of protein samples during the process of denaturation was determined on a Nicomp submicron particle sizer model 370 (Particle Sizing Systems, Inc., Santa Barbara, CA) equipped with a 5 mW 632.8 nm He–Ne laser. Aliquots were drawn from enzyme samples denatured by 2 M guanidine–HCl, as described in Section 2.4, and measured at 23°C. Autocorrelation functions collected after integration over several minutes were analyzed by the

methods of cumulants in order to obtain a number distribution of particle diameters [23].

3. Results and discussion

3.1. Thermal denaturation of GDH and its complexes studied by DSC

We studied the thermal denaturation of glutamate dehydrogenase by DSC under a variety of experimental conditions of scan rate, protein concentration, and the presence of ADP and guanidine–HCl. Typical DSC scans after subtraction of appropriate base lines and computer smoothing are shown in Fig. 1. In no case did a rescan of a rapidly chilled denatured sample produce any endothermic peak. Thus, the overall process of denaturation is irreversible and must, therefore, contain at least one irreversible step. The peak temperature of the transition (henceforth called "the observed melting temperature, T_m") was determined graphically in each case, and the corresponding enthalpy of denaturation, ΔH_d, was obtained by integration of the transition peak. It can be seen from Fig. 2 that both of these parameters are strongly dependent on the scan rate in the available range of 10–90 K h^{-1}. It can also be seen from Fig. 2 that the half-band width of the peak decreases markedly as the scan rate increases. A simple two-state equilibrium transition should provide a symmetrical DSC peak whose integrated ΔH, T_m, and half-band width are invariant with increasing observed rates, as required by the van't Hoff equation [21]. The deviation of the DSC scans shown in Fig. 2 from all of these criteria indicates that the process of denaturation as studied by this technique is under kinetic control, and that the heat absorbing process is the rate-limiting step.

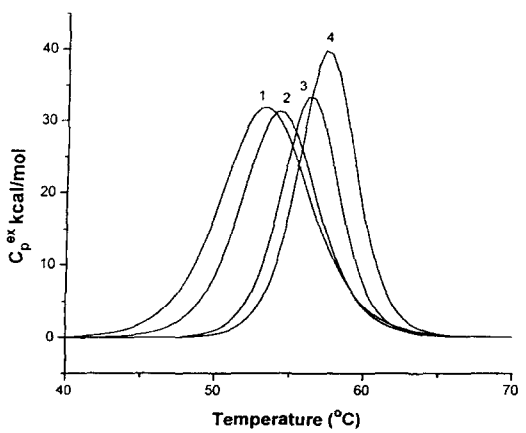

Fig. 1. Typical DSC scans, after base line subtraction and smoothing, as a function of scan rate. The scans were performed on 20 μM GDH in 0.1 M phosphate buffer at pH 7.6, with the buffer in the reference cell, at scan rates of 10, 20, 45 and 90 K h^{-1}, labeled as 1, 2, 3 and 4 respectively.

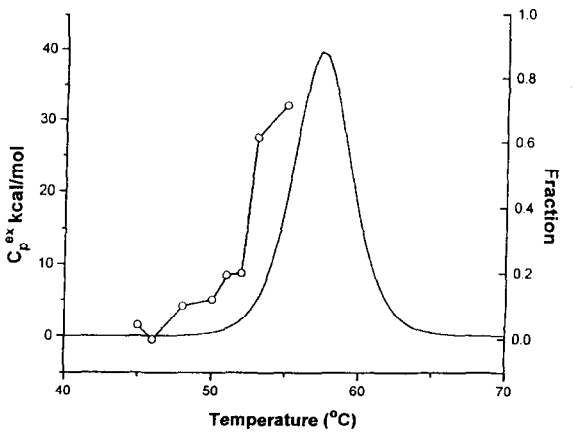

Fig. 2. Temperature dependence of the heat capacity (————) and the fraction of irreversibly denatured protein (– ∘ –) for the denaturation of 20 μM GDH at the scan rate of 90 K h^{-1}, the other conditions being the same as in Fig. 1. The calculation of the fraction of denatured protein is described in the text.

To determine the extent of irreversibility of denaturation, the enzyme samples were scanned in each case, first to a specific temperature below the melting transition and then, after rapid cooling, to a temperature well past the melting transition. Any irreversibly denatured protein will not contribute to heat absorbed under the DSC peak in this second scan. Thus, the ratio of the integrated enthalpy of denaturation from the peak in the second scan to the total enthalpy of denaturation of the native enzyme gives the fraction of the native enzyme (including the reversibly denaturing enzyme) remaining at the final temperature of the first scan. The temperature dependence of this fraction shows that the irreversible denaturation begins at temperatures below the onset of the DSC melting transition (Fig. 2). Clearly, our system is in situation C [7], where the

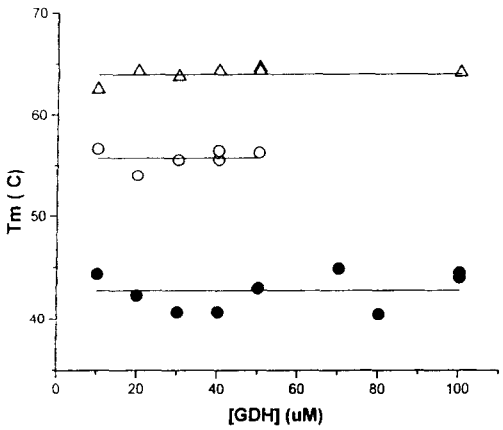

Fig. 3. Protein concentration dependence of the observed melting temperature for the denaturation of GDH (○), GDH–ADP complex (△), and GDH in 2 M guanidine–HCl (●).

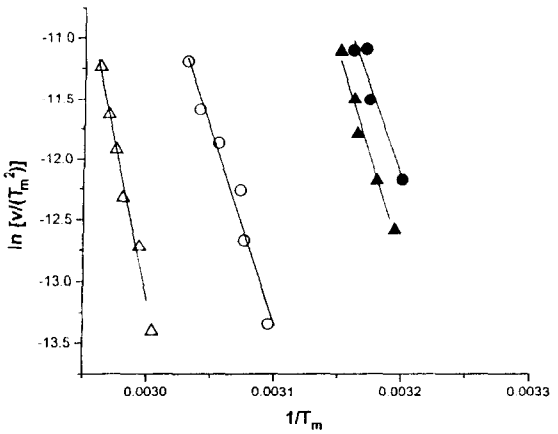

Fig. 4. Plots of $\ln(v/T_m^2)$ vs. $1/T_m$ for the denaturation of GDH (○), GDH–ADP complex (△), GDH in 2 M guanidine–HCl (●) and GDH–ADP complex in 2 M guanidine–HCl (▲). The concentrations are [GDH] = 20 μM and [ADP] = 1 mM. T_m is the absolute temperature at the peak maximum. v is the scanning rate in deg h^{-1}.

temperature is far below the observed melting temperature at which the rate constant for denaturation is 1 min^{-1}. In such a situation, there is no appreciable accumulation of the reversibly unfolded intermediate, U, in the Lumry–Eyring scheme at any given temperature. However, the data indicated by the open circles in Fig. 2 demonstrate that a substantial fraction of the denatured protein contains a *reversibly* denatured form at temperatures below T_m. This fraction, on cooling and rescanning, is observed to absorb an additional amount of heat for complete denaturation. While we have not actually observed this reversible entity at higher temperatures, there is no reason to believe that it is not an obligatory intermediate; it is reasonable to expect that at higher temperatures, the subsequent entropy-driven irreversible step proceeds much faster, and that substantial amounts of the reversible entity may not accumulate.

The thermal denaturation of free GDH and GDH–ADP complex, as well as of free GDH in 2 M guanidine–HCl, is independent of the total protein concentration as shown in Fig. 3. Therefore, for the denaturation of a hexameric protein such as GDH, the dissociation of the folded hexamer to either a folded or an unfolded monomer must occur during or after the endothermic rate-limiting step. This finding implies that this early occurring reversibly denatured intermediate must still retain its hexameric nature.

The GDH–ADP complex melts at temperatures about 6–8°C higher than the free enzyme at any given scan rate. Guanidine–HCl lowers the melting temperature of the enzyme, the shift depending on the concentration of the denaturant.

Table 1
Activation energies of GDH and its complexes calculated from the scan rate dependence of the melting temperature

System	Activation energy (kcal mol^{-1}) (from Fujita model)	Activation energy (kcal mol^{-1}) (from Lumry–Eyring model)
GDH	65.1	66
GDH in 2 M guanidine–HCl	53.2	56.4
(GDH–ADP) complex	100.7	106
(GDH–ADP) in guanidine–HCl	66.6	65

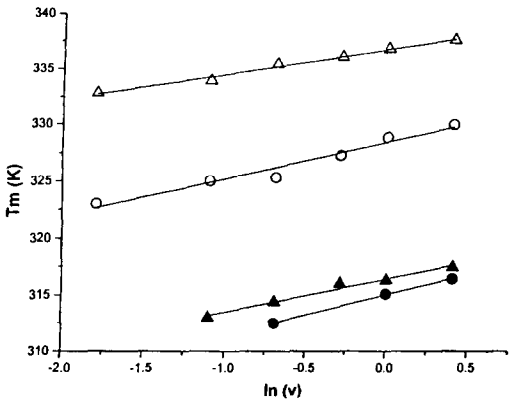

Fig. 5. Plots of T_m vs. $\ln v$ for the denaturation of GDH (○), GDH–ADP complex (△), GDH in 2 M guanidine–HCl (●) and GDH–ADP complex in 2 M guanidine–HCl (▲). The experimental data are the same as in Fig. 4.

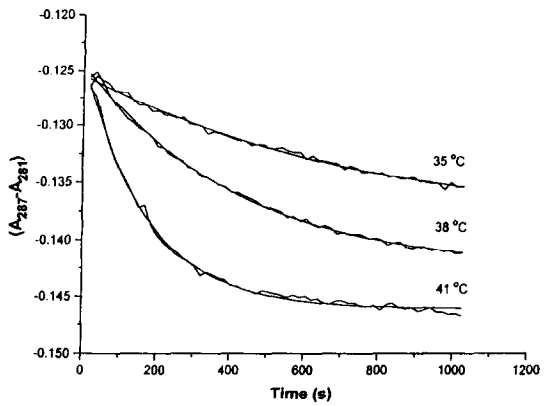

Fig. 6. Isothermal unfolding kinetics using tyrosine spectral changes as the signal. 10 μM GDH in 2 M guanidine–HCl denatured at the indicated temperatures. The solid lines are one-exponential fits to the data.

3.2. Activation energy of denaturation

The activation energies for the irreversible kinetic denaturation of GDH under various conditions were calculated (according to the Lumry–Eyring model [21]) from the slopes of the linear plots of $\ln(v/T_m^2)$ vs. $1/T_m$, as shown in Fig. 4 and Table 1. Similar values were obtained from linear plots of T_m vs. $\ln v$ (using the Fugita model [23]) (Fig. 5). The linearity of the slopes suggests the presence of a single rate-limiting step in the denaturation process.

3.3. Isothermal denaturation kinetics by difference spectroscopy

We studied the denaturation kinetics of GDH in the presence of 2 M guanidine–HCl as a function of temperature by difference spectroscopy, monitoring the changes in the tyrosine band. Under these conditions, the solution remained free of any aggregation throughout the denaturation process. The results, as shown in Fig. 6, indicate only a single-exponential kinetic process. An Arrhenius plot of the temperature dependence of the observed rate constant (graph not shown) gives an activation energy of 53 kcal mol^{-1} which is comparable to the value obtained from the DSC studies (56 kcal mol^{-1}). This implies that in these measurements we are directly studying the kinetics of the same rate-limiting step as that which controls the denaturation in the DSC scans.

3.4. Inactivation kinetics

The inactivation kinetics of GDH in the presence of guanidine–HCl are shown in Fig. 7. The activation energies of this process cannot be calculated from these data with any useful degree of accuracy, but that of the rate constant associated with the faster

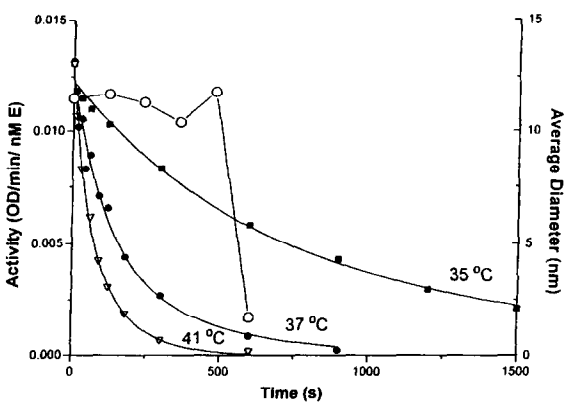

Fig. 7. Temperature dependence of the catalytic inactivation kinetics of 10 μM GDH in 0.1 M phosphate buffer at pH 7.60; the incubation temperatures are as indicated. The solid lines are two-exponential fits to the kinetic data for the 37°C and 41°C reactions, and a single-exponential fit was used for the 35°C reaction. Also shown is the time dependence of the number-average particle diameter (– ∘ –) measured by QELS. 100 μM GDH in 2 M guanidine–HCl was incubated at 35°C for specific time intervals and measured after cooling to 23°C.

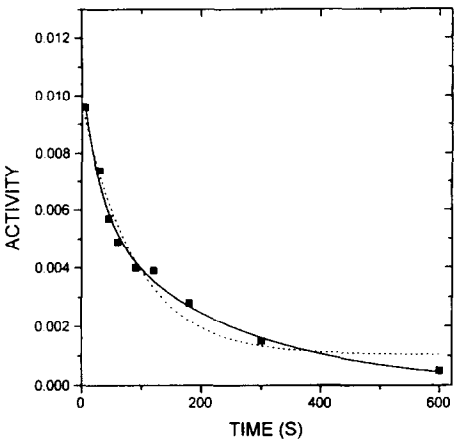

Fig. 8. Demonstration of the two-exponential character of the 37°C curve from Fig. 7. The solid line represents the best fit for a two-exponential curve with $k_1 = 0.0038\ s^{-1}$ and $k_2 = 0.029$. The dotted line represents the best fit for a single-exponential curve with $k = 0.00866\ s^{-1}$.

step is similar in magnitude to that obtained from DSC or isothermal kinetics. [1] The 35°C data have been fitted with a single-exponential function, since a two-exponential function provides a nearly identical curve with two nearly identical rate constants. The 37°C and 41°C curves, however, are fitted more closely by two-exponential functions (as shown in Fig. 8). The results depicted in Fig. 2 indicate that a substantial fraction of the denatured protein consists of a reversibly denatured form at temperatures below the melting temperature. The presence of a multi-exponential inactivation process in the same temperature range confirms the existence of an intermediate in the reaction pathway. It may be presumed that this kinetically implicated intermediate corresponds to the early reversibly denatured entity whose existence was deduced from the thermal data shown in Fig. 2.

[1] The failure of the spectrophotometric kinetic measurements shown in Fig. 6 to detect the intermediate revealed by the inactivation kinetics shown in Fig. 7 may be explained on the basis of differing contributions to the observed signal by the two intermediates. The spectral signal appears to result from the first step of the reaction $N_6 \rightleftharpoons N_6'$, in which some disruption of the tertiary structure results in the exposure of one or more buried tyrosine residues, concomitant with the major portion of the total increase in enthalpy that characterizes the overall reaction. Evidently, the relative contributions of tyrosine exposure and loss of activity must differ between the two consecutive steps.

3.5. Change in particle size during denaturation by QELS

The particle size of the protein during the guanidine–HCl-induced denaturation was measured as a function of time by QELS. The results in Fig. 7 show that in the initial phase of the process, where substantial denaturation has occurred as indicated by inactivation kinetics, there is no significant change in the particle size of the hexamer. At longer times, a rapid drop in size is observed, presumably corresponding to the dissociation of hexamer to monomer. This confirms our earlier hypothesis (based on the lack of protein concentration dependence of the melting transition) that the dissociation step follows the highly endothermic rate-limiting step. Thus, we conclude that neither dissociation of the hexamer nor any major unfolding of the protein occurs in the rate-limiting step.

3.6. Unfolding model

We have already established that the process of thermal denaturation of glutamate dehydrogenase is irreversible overall and kinetically controlled by a single rate-limiting step, in which no dissociation of the hexamer occurs. The dissociation of the hexameric enzyme must then occur in a subsequent step. Based on these conclusions, we propose the following model:

$$N_6 \rightleftharpoons N_6' \rightarrow 6U(\rightarrow F)$$

where N_6 and N_6' are, respectively, the native hexamer and the high-energy hexameric intermediate, U is the unfolded monomer and F is some final state of the aggregated enzyme. In this scheme, the first step is rate limiting and involves only partial loss of structure, but is accompanied by the absorption of most of the total heat of denaturation. Since there is no significant change in particle size in this step, presumably only a small change in the tertiary structure takes place without any appreciable change in the secondary structure. The rapid second step results in the dissociation of the hexameric intermediate and a complete loss of structure without any significant absorption of heat.

The two-exponential process of catalytic inactivation on heating suggests that, if N_6 and N_6' are,

respectively, the active and partially active forms of the enzyme, then they must be linked through a reversible step. This step must be followed by a rapid, presumably irreversible, step. A kinetic scheme with two consecutive unimolecular irreversible steps would lead to single-exponential behavior of the first reactant, i.e. N_6, and thus does not agree with our observations.

3.7. The molten-globule intermediate

The first step in the denaturation process, in which most of the heat is absorbed, is not accompanied by any substantial change in the hydrodynamic radius as shown by the QELS studies. This indicates that the high-energy protein molecule is still hexameric and has not lost much of its structure. These properties fit those conventionally ascribed to a molten globule. In the literature [24–26], there are two somewhat different definitions of a molten globule, one in which the molecule is in a compact structured form [25] and the other in which it is in a collapsed but largely unfolded form [26]. In multimeric proteins, the subunits can associate only if they are essentially folded; unfolded chains can form random aggregates but cannot form stoichiometric multimers. Hence, the glutamate dehydrogenase molten globule must be of the first kind, namely, the structured form. Studies on bovine α-lactalbumin and human α-lactalbumin [10,27] have shown that the cooperative melting transition is absent in the case of the molten-globule state. Since most of the secondary structure is preserved in this state with some loss of the tertiary structure, these authors conclude that the process of heat absorption and cooperative melting is associated with the transition from the native state to the molten-globule state. Any subsequent loss of secondary structure on going from the molten-globule state to the unfolded state must, therefore, be an enthalpyless entropy-driven process. In the case of thermal denaturation of GDH and its complexes, heat is absorbed in the conversion of the native state to the molten-globule state only. Therefore, the subsequent process of dissociation and unfolding must involve only entropy changes.

Baker et al. [29] recently published the three-dimensional crystal structure of the NAD^+-linked glutamate dehydrogenase from *Clostridium symbiosum* (CS). This CS enzyme is hexameric, possesses substantial homology to the beef-liver enzyme and has an overall structure closely resembling the electron microscopic structure of the beef-liver enzyme [31]. Thus, it is pertinent to discuss the thermal denaturation of beef-liver enzyme on the basis of the reported crystal structure. The hexameric CS enzyme possesses 32-fold symmetry, with each subunit organized into two domains separated by a deep cleft in which the coenzyme and substrate molecules bind. Domain I is involved in the dimer interface, trimer interface and additional contact surface in the formation of the hexamer. The smaller domain II contains the regulatory site to which ADP binds and regulates the enzymatic activity in the beef-liver GDH [28]. This domain is not involved in the inter-subunit contacts of the hexamer. Thus, it is very likely that the thermal denaturation of GDH proceeds through the following steps: (1) the partial unfolding of domain II or the ADP binding regulatory site, (2) the simultaneous dissociation and unfolding of the rest of the subunits, and (3) the final aggregation of the unfolded chains. This sequence of steps also explains the lack of any protein concentration dependence and the ADP-induced stabilization as discussed below.

3.8. Ligand effects

We have shown that the activation energy for the thermal denaturation of the GDH–ADP complex is substantially higher (about 40 kcal mol^{-1}) than that of the native enzyme. On an a priori basis, this difference could be attributed either to an increase in the activation energy of the ligand complex or, equally well, to a decrease in the energy of its ground state. We prefer the latter assignment in this case, however, based on our earlier demonstration of the existance of a predenaturational highly enthalpic $E \rightleftharpoons E'$ transition and its modulation by ADP [30,32]. The enthalpy of this transition is 20 kcal mol^{-1} and the free energy is 2.2 kcal mol^{-1}. ADP raises the transition temperature of this predenaturational equilibrium from 43 to about 56°C. Thus, in the presence of ADP, the enzyme is mainly in the E form with a lower free energy, but higher melting temperature. In contrast, other ligands, such as NADPH, favor the lower enthalpy "E" form of the enzyme, raising its ΔG° by as much as 5–7 kcal mol^{-1} from that of the

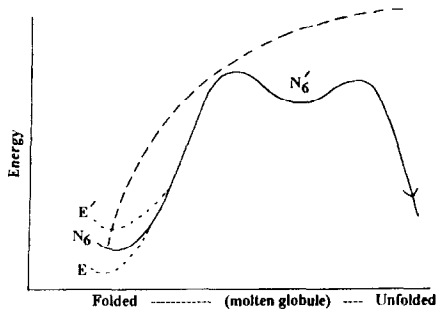

Fig. 9. Free energy (————) and enthalpy (– – –) changes are qualitatively represented for the thermal unfolding process via the molten-globule intermediate. E and E′ denote, respectively, the ligand-induced low-energy and high-energy forms of the ground state, N_6.

unliganded enzyme. The GDH–NADPH complex is markedly more thermolabile than either the free enzyme or its ADP complex. Based on this apparent coupling between the predenaturational equilibrium and the thermal denaturation process described here, we have chosen to present the energy profile of the denaturation process by assuming a common transition state for both forms of the enzyme and a ligand-modulated ground state, as shown in Fig. 9. However, the denaturants are presumed to effect the thermal stability of the protein by stabilizing either the molten-globule state or the unfolded state relative to the ground state. In the case described here, guanidine–HCl presumably binds to the N_6' form, facilitating the denaturation of the native enzyme.

The thermal denaturation of hexameric beef-liver glutamate dehydrogenase is unusual in the sense that the denaturation process is independent of the subunit dissociation effects. The endothermic rate-limiting step is decoupled from the dissociation step. We conclude that the kinetic intermediate, N_6', is a compact denatured state with properties resembling those of a molten globule, and that such an intermediate is a viable entity in the denaturation process.

Acknowledgements

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