Kinetic Study on the Irreversible Thermal Denaturation of Yeast Phosphoglycerate Kinase†

Maria L. Galisteo, Pedro L. Mateo, and Jose M. Sanchez-Ruiz*

Departamento de Quimica Fisica (Facultad de Ciencias) e Instituto de Biotecnologia, Universidad de Granada, 18071 Granada, Spain

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ABSTRACT: Differential scanning calorimetry transitions for the irreversible thermal denaturation of yeast phosphoglycerate kinase at pH 7.0 are strongly scanning-rate dependent, suggesting that the denaturation is, at least in part, under kinetic control. To test this possibility, we have carried out a kinetic study on the thermal inactivation of the enzyme. The inactivation kinetics are comparatively fast within the temperature range of the calorimetric transitions and can be described phenomenologically by the equation $dC/dt = -\alpha C^2/(\beta + C)$, where C is the concentration of active enzyme at a given time, t, and α and β are rate coefficients that depend on temperature. This equation, together with the values of α and β (within the temperature range 50–59 °C) have allowed us to calculate the fraction of irreversibly denatured protein versus temperature profiles corresponding to the calorimetric experiments. We have found that (a) irreversible denaturation takes place during the time the protein spends in the transition region and (b) there is an excellent correlation between the temperatures of the maximum of the calorimetric transitions (T_m) and the temperatures (T_h) at which half of the protein is irreversibly denatured. These results show that the differential scanning calorimetry transitions for the denaturation of phosphoglycerate kinase are highly distorted by the rate-limited irreversible process. Finally, some comments are made as to the use of equilibrium thermodynamics in the analysis of irreversible protein denaturation.

High-sensitivity differential scanning microcalorimetry (DSC)[1] has been extensively used in the last 15 years to study protein thermal denaturation [for a recent review, see Privalov (1989)]. Under certain conditions (pH, ionic strength, ...) the thermal denaturation of many small globular proteins (Privalov, 1979) and some complex proteins (Privalov, 1982) is reversible; that is, the protein recovers its native structure upon cooling. The reversibility of the denaturation is easily checked in DSC studies by comparing the first scan of the sample with a second one carried out after cooling to room temperature; in the case of reversible thermal denaturation, both thermograms show identical (or almost identical) transitions. Reversible thermal protein denaturation involves the unfolding of the native structure. Unfolding–refolding processes are generally assumed to be fast enough, within the transition region, to allow chemical equilibrium to be attained at denaturational temperatures [see, however, Lopez-Mayorga and Freire (1987)]; therefore, the corresponding DSC transitions are interpreted on the basis of equilibrium thermodynamics.

Analysis of the DSC transitions according to equilibrium thermodynamics provides detailed information about the energetics and mechanism of reversible thermal denaturation (unfolding) of proteins [for reviews, see Privalov (1979, 1982, 1989)]. It must be recognized, however, that the thermal denaturation of many proteins is irreversible, as shown by the lack of thermal effect in the DSC thermogram corresponding to the second heating of the sample. Irreversible protein denaturation is thought to involve, at least, two steps: (a) reversible unfolding of the native protein (N) and (b) irreversible alteration of the unfolded protein (U) to yield a final state (F) that is unable to fold back to the native structure. This two-step nature of irreversible denaturation is depicted in the following simplified scheme:

$$N \rightleftharpoons U \rightarrow F$$

which is usually referred to as the Lumry and Eyring model [see Lumry and Eyring (1954)]. [For a recent review on the several processes responsible for the irreversible step, see Klibanov and Ahern (1987).]

It is important to note that the step U → F, whatever its nature, is a time-dependent process and must be described by a rate equation (and *not* by a temperature-dependent equilibrium constant). Therefore, it is not a priori clear whether or not the DSC transitions corresponding to irreversible protein denaturation can be analyzed according to equilibrium thermodynamics. In fact, recent work (Sanchez-Ruiz et al., 1988a,b; Guzman-Casado et al., 1990) has shown that the DSC transitions for the irreversible thermal denaturation of several proteins could be quantitatively described by a two-state irreversible model, N → F. This model represents an extreme case: only the native and final states are significantly populated (the amount of unfolded state is very small), the heat absorption is entirely determined by the kinetics of the irreversible process, and no thermodynamic information can be derived from the transitions (other than the total enthalpy change for the process N → F). The opposite case may be exemplified by the thermal denaturation of the B subunit of cholera toxin; Goins and Freire (1988) have shown that, in this case, the irreversible process takes place with little thermal effect at temperatures somewhat higher than those corresponding to the DSC transition; this strongly suggests that the calorimetric transition of the toxin is due, in fact, to the reversible unfolding and is, therefore, amenable to equilibrium thermodynamics analysis. Of course, intermediate situations may also be expected to occur.

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* Author to whom correspondence should be addressed.

[1] Abbreviations: DSC, differential scanning calorimetry; PGK, yeast phosphoglycerate kinase; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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Analysis of irreversible DSC transitions appears to be a somewhat controversial issue in recent literature; thus, some authors [see, for instance, Brandts et al. (1989) and Bertazzon et al. (1990)] assume that equilibrium thermodynamic analysis of the transitions is permissible, while others [see, for instance, Lepock et al. (1990) and Morin et al. (1990)] take into account the possible kinetically controlled character of the transitions and analyze the calorimetric data on the basis of the two-state irreversible model. It appears desirable, therefore, to develop experimental approaches that allow the researcher to determine to what extent a given calorimetric transition is affected by the occurrence of irreversible processes and what kind of information (thermodynamic, kinetic, or both) can be derived from it. The approach proposed in this work is based on the analysis of the scanning rate effect on the calorimetric transitions and the kinetics of thermal denaturation of the protein.

Yeast phosphoglycerate kinase (PGK) is a single polypeptide chain protein (420 amino acid residues) with a molecular weight of 45 000. Hu and Sturtevant (1987, 1989) have recently studied its thermal denaturation by differential scanning calorimetry. They found the denaturation of PGK to be irreversible; however, the effect of several ligands on the calorimetric transitions appeared to follow the dictates of the van't Hoff equation. This result was considered as evidence supporting the applicability of equilibrium thermodynamics to the thermal denaturation of PGK. On the other hand, as the authors recognized (Hu & Sturtevant, 1987), no reasonable equilibrium model was able to fit the shape of the DSC transitions. It was suggested (Hu & Sturtevant, 1987) that the high-temperature side of the DSC transitions was affected (sharpened) by the rate-limited irreversible process, corresponding the rest of the transitions to the reversible unfolding. If this hypothesis is correct, the irreversible thermal denaturation of PGK should make an excellent model system to test the approach we propose in this work.

Experimental Procedures

Yeast phosphoglycerate kinase was purchased from Sigma Chemical Co. as an ammonium sulfate precipitate and was used without further purification. Enzyme activity was assayed in the back reaction (3-phosphoglycerate to 1,3-diphosphoglycerate) by using the coupled assay with glyceraldehyde-3-phosphate dehydrogenase as described by Scopes (1975). It was ascertained that the measured activity was proportional to the amount of enzyme present in the assay, at least within the range 0–0.013 μg/mL (all activity assays were carried out with enzyme concentrations within this range). The specific activity of the PGK used in this work was found to be about 1000 units/mg (close to that stated by Sigma). Protein solutions were prepared by exhaustive dialysis against 50 mM PIPES buffer, pH 7.0, or by passage through a Sephadex G-25 column previously equilibrated with that buffer (the way the protein solutions were prepared had no effect on the calorimetric results). Prior to use, protein solutions were centrifuged to eliminate any solid residue. Protein concentrations were calculated spectrophotometrically by using an E_{280nm} for a 1 mg/mL solution of 0.57 (Blake et al., 1972).

The disodium salt of ATP, the disodium salt of D-(−)-3-phosphoglyceric acid and the glyceraldehyde-3-phosphate dehydrogenase were purchased from Sigma. Other chemicals were of reagent grade. Distilled, deionized water was used throughout.

Calorimetric experiments were carried out in a differential scanning calorimeter DASM-4, described by Privalov (1980), with 0.47-mL cells. Different scanning rates within the range 0.11–2 K/min were employed. Degassing during the calorimetric experiment was prevented by an additional constant pressure of 2.5 atm over the liquids in the cells. The reversibility of the DSC transitions was checked by reheating the solution in the calorimetric cell after cooling from the first run. In all cases, the thermal denaturation of PGK was found to be irreversible; therefore, the thermogram corresponding to the reheating run was used as instrumental base line. The transitions were corrected for the difference in heat capacity between the initial and final states by using a sigmoidal, chemical base line, as described by Takahashi and Sturtevant (1981).

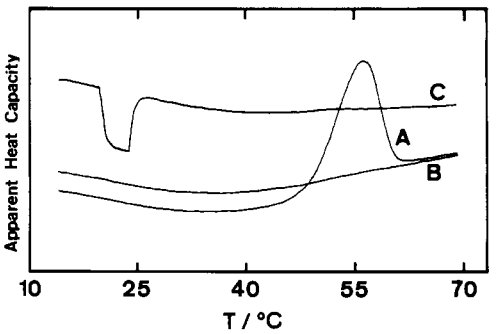

FIGURE 1: (A) Original recording of heat absorption of PGK solution at 2.02 K/min and pH 7.0, protein concentration 1.16 mg/mL. (B) Reheating run. (C) Buffer–buffer base line showing a 25-μW calibration mark (power was communicated to the sample cell, and therefore, the calibration mark appears as an exotherm).

Using the procedure of Lopez-Mayorga and Freire (1987), we found that the instrument response could be adequately described by a single response time of 15 s, which did not depend on the scanning rate; DSC transitions were corrected for the effect of this response time, as described by these authors. It is important to note that the effect of this correction is much smaller than the total scanning rate effect on the transitions (see Figure 2); in fact, we found it impossible to bring to coincidence the transitions obtained at different scanning rates, not even by using very large (and grossly unrealistic) values for the response time.

The kinetics of thermal inactivation of PGK was studied by using the following experimental procedure: microliter aliquots of a PGK solution were introduced into capillary tubes (diameter 0.5 mm), and the tubes were sealed and immersed in a water bath at the desired temperature. At given times, tubes were extracted from the high-temperature bath and immediately immersed in a water–ice mixture to stop the inactivation process. Subsequently, the enzyme activity was measured as described above (no recovery of activity with time was detected after the thermal inactivation process had been stopped). The time required for the capillary tubes to reach the temperature of the bath appears to be very short and, in fact, no lag was observed in any of the inactivation profiles (not even in those obtained at the higher temperatures, which show fast kinetics; see Figure 3).

Results and Discussion

Scanning Rate Effect on the DSC Thermograms for the Thermal Denaturation of PGK. We have carried out DSC experiments on the thermal denaturation of PGK in 50 mM PIPES buffer, pH 7.0. The DSC transitions were calorimetrically irreversible (see Figure 1 for an original DSC recording) and the protein samples extracted from the calorimetric cell showed strong aggregation. The shape of the transitions, as well as the calorimetric enthalpies (855 ± 36 kJ/mol) and denaturation temperatures (Figure 6) were in good agreement with the results reported by Hu and Sturtevant

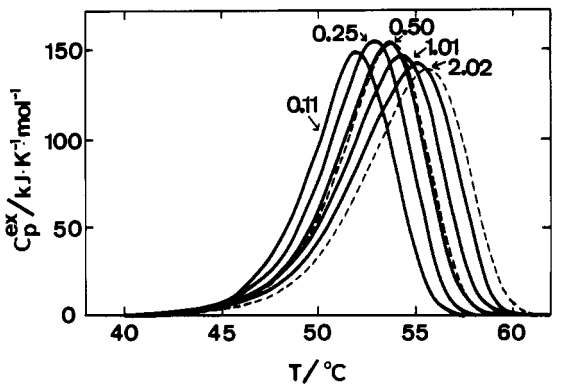

FIGURE 2: Differential scanning calorimetry transitions for the thermal denaturation of PGK in 50 mM PIPES buffer, pH 7.0. In all cases, the total protein concentration is 3.72 mg/mL. The numbers alongside the transitions stand for the scanning rate in K/min. (—) DSC transitions corrected for the instrumental base line, the chemical base line, and the slow time response of the calorimeter (see text for details). (---) DSC transitions (corresponding to the scanning rates 2.02 and 0.50 K/min) corrected for the instrumental base line and the chemical base line.

(1987) under the same ionic and scanning rate conditions (these authors employed a scanning rate of 1 K/min, exclusively). We found, however, that (a) the DSC transitions are strongly scanning-rate dependent, even after correcting for the effect of the instrument response time (Figure 2), and (b) there is a small, although significant, protein concentration effect on the temperatures, T_m, corresponding to the maximum excess heat capacity (Figure 6).[2]

It is clear that the thermal denaturation of PGK is, at least in part, under kinetic control (as indicated by the strong scanning-rate effect); in this case, however, the two-state irreversible model [N → F; see Sanchez-Ruiz et al. (1988a)] does not provide an adequate representation of the DSC data. Thus, for instance, no acceptable agreement was found between the "rate constants" calculated (according to the model) from DSC transitions obtained at different scanning rates (results not shown).

Kinetics of Thermal Inactivation of PGK. The kinetics of the thermal inactivation of PGK in 50 mM PIPES buffer, pH 7.0, was investigated by following the time dependence of the enzyme activity (see Experimental Procedures) at given temperatures within the range 50–59 °C; at each temperature, several inactivation experiments were carried out at different total protein concentrations within the range 0.2–3.7 mg/mL. Figure 3 shows some examples of the inactivation profiles obtained.

Aggregation is probably one of the main causes of irreversibility in the thermal denaturation of PGK. Therefore, the inactivation kinetics might be expected to have a higher than one reaction order (Jaenicke, 1987). On the other hand, first order kinetics cannot be ruled out a priori, given that, if aggregation is very fast, the rate-limiting step might be a previous first-order step in the unfolding pathway. In addition, the kinetics might be very complex if, for instance, changes in rate-determining step (with temperature or protein concentration) take place. The purpose of the following analysis, therefore, is to find the simplest, phenomenological rate equation that actually describes the experimental inactivation data.

[2] The shape of the transitions also appears to depend somewhat on the protein concentration. Thus, the DSC transitions obtained at the lower concentrations gave some indication of a shoulder on the low-temperature side.

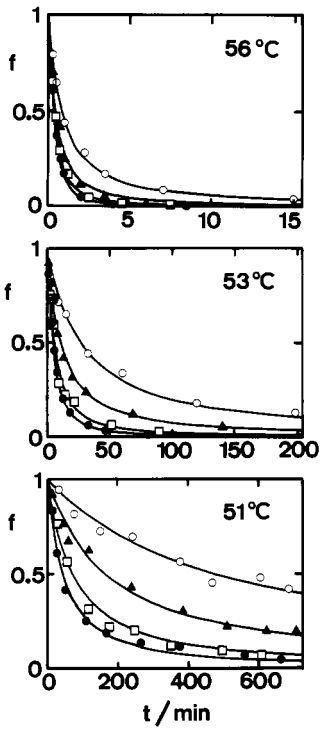

FIGURE 3: Kinetics of thermal inactivation of PGK in 50 mM PIPES buffer, pH 7.0, at the indicated temperatures. f stands for the fraction of active enzyme at a given time t (f is calculated as the ratio A/A_0, where A is the enzyme activity measured at a time t and A_0 is the activity for $t = 0$; see text for details). The inactivation kinetics correspond to the following total protein concentrations (in mg/mL): at 51 °C, 0.21 (○), 0.65 (▲), 1.80 (□), and 3.58 (●); at 53 °C, 0.21 (○), 0.64 (▲), 1.96 (□), and 3.70 (●); at 56 °C, 0.21 (○), 0.66 (▲), 1.80 (□), and 3.58 (●). (—) Curves predicted by eq 2 with the optimum values of the parameters α and β (the four curves drawn at each temperature are computed from a single set of α and β values).

Preliminary data analysis showed that the inactivation profiles obtained at high temperature and high concentration (within the ranges studied) could be approximately described by exponentials, while those corresponding to low concentration and low temperature gave reasonable fits to the integrated second-order rate equation. Accordingly, we tried to simultaneously describe all the inactivation profiles obtained at a given temperature by a single rate equation:

$$\frac{dC}{dt} = -\frac{\alpha C^2}{\beta + C} \quad (1)$$

where α and β are constants (for a given temperature) and C stands for the concentration of "active" enzyme at a time t; here, by active enzyme, we mean not only the native enzyme but also any forms of the enzyme (unfolded or partially unfolded) that are able to fold back to the native state upon cooling (see Experimental Procedures). Accordingly, we consider $C_t - C$ (where C_t is the total protein concentration) as the concentration of irreversibly denatured protein (protein unable to refold to the native state).

It must be noted that eq 1 reduces to the first-order rate equation if $C \gg \beta$ and to the second-order one if $C \ll \beta$, thus providing the desired change in reaction order with protein concentration.

Separation of variables in (1) followed by integration from $t = 0$ (active protein concentration = C_t) to t (active protein concentration = C) yields

$$(\beta/C_t)[(f - 1)/f] + \ln f = -\alpha t \quad (2)$$

where f is the fraction of active enzyme (C/C_t) at a time t. Note that f is experimentally available as the ratio A/A_0,

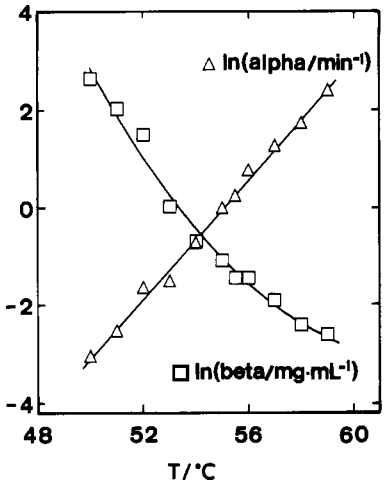

FIGURE 4: Temperature dependence of the rate coefficients α (△) and β (□) in eq 1. (—) Curves predicted by eq 3.

where A is the enzyme activity measured at a time t in a given inactivation kinetics and A_0 is the activity at time zero.

Equation 2 was employed to simultaneously fit all the inactivation kinetics obtained at a given temperature. The optimum values of α and β were obtained by nonlinear minimization (using the simplex algorithm) of $\sum (f_{exp} - f_{pre})^2$, where f_{exp} is the experimental value of f and f_{pre} is the value predicted by eq 2 (obviously, this procedure involved numerical solving of eq 2 for given values of α, β, and t). We found that, in all cases, a single set of α and β values was able to acceptably fit all the inactivation kinetics obtained at a given temperature (see, for instance, Figure 3). α and β, however, do change with temperature (Figure 4); these dependences can be adequately described by the following empirical relationships:

$$\ln (\alpha/\text{min}^{-1}) = -33.78 + 0.613T$$

$$\ln (\beta/\text{mg}\cdot\text{mL}^{-1}) = 153.61 - 5.058T + (4.084 \times 10^{-2})T^2 \quad (3)$$

where T is the temperature in degrees Celsius.

We must clearly state that we have not proposed any specific kinetic mechanism for the thermal inactivation of PGK; we consider eqs 1–3 as a convenient, phenomenological description of the thermal inactivation kinetics, valid within the studied concentration and temperature ranges.

Comparison between the DSC Transitions and the Thermal Inactivation Kinetics. Equation 1, together with eq 3, gives the rate of inactivation of PGK at a given temperature and active protein concentration (C). Equation 1 was integrated for constant temperature to yield 2; in a DSC experiment, however, temperature changes with time according to a constant (to a good degree of approximation) scanning rate. The fraction of irreversibly denaturated protein versus temperature profile for a given DSC experiment can be easily obtained by using the following procedure.

Taking into account that the scanning rate is given by $v = dT/dt$, eq 1 can be rewritten as

$$\frac{dC}{dT} = -\frac{1}{v}\frac{\alpha(T)C^2}{\beta(T) + C} \quad (4)$$

where $\alpha(T)$ and $\beta(T)$ are the temperature-dependent values of α and β, given by eq 3. Equation 4 was then integrated numerically (by using the fourth-order Runge–Kutta algorithm with an integration interval of 0.02 deg) from a temperature T_0, at which the inactivation rate is negligible (active protein concentration = C_t), to a temperature T (active protein concentration = C):

$$C(T) = -\frac{1}{v}\int_{T_0}^{T}\frac{\alpha(T)C^2}{\beta(T) + C}\,dT \quad (5)$$

The integration was carried out for the scanning rate (v) and total protein concentration (C_t) values corresponding to the DSC experiments. The results have been plotted as $(C_t - C)/C_t$ (fraction of irreversibly denatured protein) versus temperature profiles (see, for instance, Figure 5A).

It must be noted that integration of eq 4 involves the use of the values of α and β given by eq 3 in a temperature range that is, in general, wider than the one (50–59 °C) employed in the thermal inactivation experiments. This does not introduce any significant errors as the values of α and β given by eq 3 (together with eq 1) predict that the inactivation rate is negligible at temperatures below 50 °C, which is, in fact, the experimental result we obtained; in addition, the values of α and β computed for $T > 59$ °C do not affect at all the results of the integration, as, in all cases, essentially all the protein is irreversibly denatured [$(C_t - C)/C_t \simeq 1$] at $T > 59$ °C (see, for instance, Figure 5A).

It is also important to note that profiles of fraction of irreversibly denatured protein versus temperature (such as the one shown in Figure 5A) can also be obtained from scanning inactivation experiments in which a protein solution is heated at a constant rate and aliqouts are extracted at given temperatures and assayed for enzyme activity (after stopping the inactivation process, usually by cooling). We favor, however, an indirect way (thermal inactivation experiments at constant temperature and integration of eq 4) to obtain the abovementioned profiles for the following two reasons:

First, kinetic analysis of scanning inactivation experiments is rather complicated, as temperature is changed with time and, therefore, rate coefficients (such as α and β in eq 1) behave as time-dependent parameters.

Second, and most important, in some cases, interpretation of scanning inactivation experiments may be ambiguous. For instance, it might be argued that the denaturation process is a reversible unfolding of the protein and that upon sudden cooling (to stop the denaturation process) the equilibrium between the native and unfolded states of the protein is "frozen" due to the slowness of the refolding kinetics at low

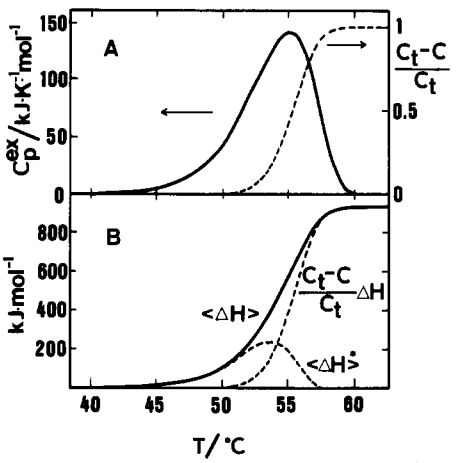

FIGURE 5: (A) DSC transition and fraction of irreversibly denatured protein versus temperature profile for the thermal denaturation of PGK in 50 mM PIPES buffer, pH 7.0; total protein concentration 3.72 mg/mL, scanning rate 2.02 K/min. (B) Deconvolution of the apparent excess enthalpy versus temperature profile corresponding to the DSC transition in (A), according to eq 7.

temperature; it could also be assumed that the irreversible alteration of the unfolded state (aggregation for instance) takes place, in fact, at low temperature. Under these assumptions, the enzyme activity versus temperature profile would monitor the equilibrium unfolding process; in fact, such profiles have been interpreted, in some cases, according to the van't Hoff equation [see, for instance, Fukada et al. (1985)]. This kind of interpretation, however, cannot be applied to the profiles obtained by integration of eq 4, given that the inactivation experiments, from which the data required to integrate eq 4 were obtained, were carried out at constant temperature [whatever criticisms are made to the procedure used to stop the inactivation process, it is clear that the fraction of active enzyme versus time profiles (such as those shown in Figure 3) correspond to a time-dependent process, described by a rate equation and *not* by equilibrium thermodynamics].

Comparison between the $(C_t - C)/C_t$ versus temperature profiles and the DSC thermograms (see, for instance, Figure 5A) indicates that the irreversible denaturation takes place within the temperature range of the calorimetric transitions. It is clear, therefore, that the DSC transitions for the thermal denaturation of PGK are distorted by the occurrence of the irreversible process. This last statement can be illustrated by the following analysis, which is based on an additional and, we believe, reasonable assumption.

The apparent excess enthalpy, $\langle \Delta H \rangle$ (obtained by integration of the C_p^{ex} versus temperature data) is an average over all the protein states

$$\langle \Delta H \rangle = \sum_i x_i \Delta H_i \qquad (6)$$

where x_i is the fraction of protein molecules present in a given state and ΔH_i stands for the enthalpy difference between that state and the reference state (the native state). The contribution of the irreversibly denatured state, F, to the average in eq 6 is given by $x_F \Delta H_F$, where x_F is equal to $(C_t - C)/C_t$. We will *assume* that the enthalpy difference for the final state, ΔH_F, is equal to the total enthalpy of the transition, ΔH, as eventually all the protein molecules will be found in the state F. Accordingly, eq 6 can be rewritten as

$$\langle \Delta H \rangle = \langle \Delta H \rangle^* + [(C_t - C)/C_t]\Delta H \qquad (7)$$

where $[(C_t - C)/C_t]\Delta H$ is the contribution of the final state and $\langle \Delta H \rangle^*$ is the contribution of all other states [which is calculated as $\langle \Delta H \rangle - [(C_t - C)/C_t]\Delta H$]. Figure 5B shows the $\langle \Delta H \rangle$, $\langle \Delta H \rangle^*$, and $[(C_t - C)/C_t]\Delta H$ versus temperature profiles[3] corresponding to the DSC transition obtained at a scanning rate of 2 K/min and a total protein concentration of 3.72 mg/mL (similar results are obtained for DSC transitions corresponding to other values of v and C_t). According to the analysis shown in Figure 5B, at temperatures close to the T_m value, most of the excess enthalpy (heat absorbed) is associated with the irreversible formation of the final state.

Finally, in Figure 6 we compare the temperatures corresponding to the maximum heat capacity, T_m, with the temperatures, T_h, at which half of the protein is irreversibly denatured [that is, for $T = T_h$, $(C_t - C)/C_t = 0.5$]; as was to be expected from the above analysis, both temperatures do not differ in more than a few tenths of a degree and change with the scanning rate (Figure 6A) and the total protein concentration (Figure 6B) in a very similar fashion. The excellent correlation found between the T_m and T_h values shows clearly that the scanning rate and protein concentration effects on the T_m values are to be attributed to the kinetics of irreversible denaturation of the enzyme, as described by eqs 1 and 3.

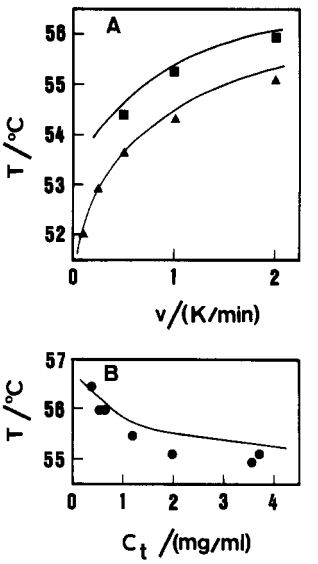

FIGURE 6: Correlation between the T_m values of the DSC transitions and the temperatures, T_h, at which half of the protein is irreversibly denatured. (A) Scanning rate effect on the T_m and T_h values; the T_m values correspond to total protein concentrations of 3.72 mg/mL (■) and 0.54 mg/mL (▲). The curves represent the T_h values calculated from the numerical integration of eq 4 (the lower curve corresponds to C_t = 3.72 mg/mL and the upper one to C_t = 0.54 mg/mL). (B) Total protein concentration effect on the T_m (●) and T_h (—) values (both corresponding to scanning rate 2.02 K/min).

Concluding Remarks

The structure of yeast phosphoglycerate kinase (Banks et al., 1979) is composed of two globular lobes of approximately equal size that interact through a narrow hinge region. Differential scanning calorimetry thermograms for the thermal denaturation of PGK, however, show a single peak (Hu & Sturtevant, 1987; this work). Hu and Sturtevant (1987) found that, under most ionic conditions, the van't Hoff enthalpies were roughly equal to the calorimetric enthalpies. They concluded that the interaction between the lobes of the protein is strong enough to cause an approximately two-state unfolding process for the molecule.

Recently, Brandts et al. (1989) have developed a new model to interpret differential scanning calorimetry data on proteins that have interacting domains. This model was applied to the thermal denaturation of wild-type and several mutant forms of phosphoglycerate kinase; in fact, Brandts et al. deconvoluted the DSC peak for the wild-type enzyme (0.02 M triethanolamine buffer pH 7.5) into two overlapping transitions that were attributed to the unfolding of the two lobes (or domains) of the protein.

The analysis of the DSC transitions carried out by Brandts et al. (1989) and Hu and Sturtevant (1987) were based on equilibrium thermodynamics; however, the thermal denaturation of yeast phosphoglycerate kinase is calorimetrically irreversible. It is often argued that irreversible denaturation does not take place significantly during the short time the protein spends in the transition region; the irreversible calorimetric transition is then assumed to correspond to the reversible unfolding and is analyzed according to equilibrium thermodynamics. We have shown, however, that, at least under the ionic conditions employed in this work, irreversible

[3] The profiles in Figure 5B have been calculated assuming that ΔH is constant while, in fact, it changes with temperature according to Kirchoff's equation and the denaturational heat capacity change. However, a more rigorous calculation (using $\Delta C_p = 20 \pm 3$ kJ K^{-1} mol^{-1}, obtained from the DSC transitions) yields essentially identical results, as was to be expected from the narrow temperature span of the profiles.

denaturation of PGK takes place, in fact, during the time the protein spends in the transition region (Figure 5) and that, at temperatures near the T_m values, the DSC transitions are highly distorted by the occurrence of the irreversible process (as indicated by the excellent correlation found between the T_m values and the temperatures, T_h, at which half of the protein is irreversibly denatured; see Figure 6). The DSC transitions for the irreversible denaturation of PGK reported in this work might perhaps contain some thermodynamic information (associated with the $\langle \Delta H \rangle^*$ term in eq 7); it is clear, nevertheless, that analysis of these transitions according to equilibrium models that do not take into account the kinetics of the irreversible formation of the final state is not permissible.

This work illustrates several important points with regard to the general problem of the analysis of irreversible DSC transitions:

(A) The results obtained in this work, together with those previously reported on other protein systems (Sanchez-Ruiz et al., 1988a,b; Guzman-Casado et al., 1990) show that, very often, DSC transitions corresponding to irreversible protein denaturation are highly distorted by the kinetics of the irreversible formation of the final state. Therefore, direct analysis of irreversible DSC transitions according to equilibrium thermodynamic models is, at least, risky. Prior to any kind of analysis of irreversible DSC transitions, it appears essential to determine to what extent the transitions are distorted by the rate-limited process.

(B) Studies on the scanning rate effect on the DSC transitions and the kinetics of thermal denaturation of the protein may be used to determine to what extent the transitions are affected by the occurrence of the irreversible process.

(C) The main evidence supporting equilibrium thermodynamic analysis of the DSC transitions for the irreversible thermal denaturation of PGK (Hu & Sturtevant, 1987, 1989) and other proteins (Manly et al., 1985; Edge et al., 1985) comes from the fact that ligand and protein concentration effects on the T_m values appear to conform the dictates of the van't Hoff equation.[4] Clearly, the results reported in this work cast serious doubts on the reliability of these effects as equilibrium criteria for DSC of proteins.

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[4] We have found that the DSC transitions for the thermal denaturation of PGK in the presence of the ligands employed by Hu and Sturtevant (1987) are also scanning-rate dependent (results not shown).

Registry No. PGK, 9001-83-6.

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