Effect of Zn^{2+} on the Thermal Denaturation of Carboxypeptidase B†

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Received June 11, 1990; Revised Manuscript Received November 14, 1990

ABSTRACT: A differential scanning calorimetry study on the thermal denaturation of porcine pancreas carboxypeptidase B (in 20 mM pyrophosphate buffer, pH 9.0) has been carried out. The calorimetric transitions have been found to be calorimetrically irreversible and to depend on the Zn^{2+} concentration in the buffer. The effect of the Zn^{2+} concentration on the temperatures corresponding to maximum heat capacity appears to conform the dictates of the van't Hoff equation. In spite of this, analysis of the scanning rate effect on the transitions, together with studies on the thermal inactivation kinetics, show that the heat absorption is entirely determined by the rate of formation of the final (irreversibly denatured) state of the protein; therefore, analysis of the calorimetric transitions according to equilibrium thermodynamics models is not permissible. The effect of Zn^{2+} on the calorimetric transitions can be explained on the basis of a simple kinetic model that does not assume chemical equilibrium to be established between the significantly populated states of the protein.

Pancreatic carboxypeptidase B (CPB)[1] is a zinc exopeptidase that preferentially catalyzes the hydrolysis of the C-terminal peptide bonds of peptide substrates that bear a basic carboxyl-terminal amino acid residue (Folk, 1971; Blackburn, 1976). It is composed of a single polypeptide chain (molecular weight 34 500) containing 1 mol of Zn/mol of protein (Folk et al., 1960), and it has a tertiary structure (Schmid & Herriott, 1976) and a kinetic mechanism (Alter et al., 1977) homologous to those of carboxypeptidase A (Auld & Vallee, 1987). We have found that, under certain ionic conditions (20 mM pyrophosphate buffer, pH 9.0) the DSC transitions corresponding to the thermal denaturation of porcine pancreatic CPB depend on the Zn^{2+} concentration in the buffer, in a way that appears to conform the dictates of the van't Hoff equation; the transitions, however, were found to be calorimetrically irreversible. Following the approach previously proposed (Galisteo et al., 1991), we have carried out a study on the scanning rate effect on the DSC transitions and the kinetics of thermal denaturation of the protein, in order to determine to what extent the heat absorption is determined by the rate of formation of the final (irreversibly denatured) state of the protein. We have found that the thermal denaturation of CPB follows quantitatively the two-state irreversible model (Sanchez-Ruiz et al., 1988), and therefore, analysis of the DSC transitions according to equilibrium thermodynamics models is not permissible. It is shown that the effect of Zn^{2+} on the thermal denaturation of CPB can be explained on the basis of that simple kinetic model.

Experimental Procedures

Procarboxypeptidase B was prepared from porcine pancreas following the method described by Vilanova et al. (1985). CPB was obtained by tryptic digestion of native procarboxypeptidase B (Wintersberger et al., 1962) followed by application to a DEAE-Sepharose column in 40 mM Tris buffer, pH 8.0, and elution with a linear NaCl gradient (0–0.4 M) to separate CPB from the activation fragment. The enzyme was found to be electrophoretically pure and showed no detectable trypsin activity. It was stored frozen in a buffer of 40 mM Tris, pH 8.0, and 100 mM NaCl.

Enzyme activity was assayed spectrophotometrically by following the hydrolysis of hippuryl-L-arginine, as described by Folk et al. (1960). The specific activity of the enzyme was 170–180 units/mg. Protein solutions were prepared by exhaustive dialysis against 20 mM pyrophosphate buffer, pH 9.0, with the desired concentration of $ZnCl_2$ or 1,10-phenanthroline. Protein concentrations were calculated spectrophotometrically by using an E_{280nm} for a 1 mg/mL solution of 2.0. Zn concentrations were determined by atomic absorption spectroscopy. Hippuryl-L-arginine was purchased from Sigma. Other chemicals were of reagent grade. Distilled, deionized water was used throughout.

DSC experiments were performed in a differential scanning calorimeter DASM-4, described by Privalov (1980), with 0.47-mL cells (see Figure 1 for an original DSC thermogram). Different scanning rates within the range 0.5–2 K/min were employed. The protein concentrations of the solutions employed in the calorimetric experiments were in the range 1–1.5 mg/mL. The DSC transitions were corrected for the instrumental base line, the chemical base line, and the effect of the instrument response time, as previously described (Galisteo et al., 1991).

The kinetics of thermal inactivation of CPB in the presence of Zn^{2+} was studied by following the time dependence of the enzyme activity, as previously described (Galisteo et al., 1991). The kinetics of thermal inactivation of CPB in the presence of 1,10-phenanthroline was studied in the same way, except that, prior to the activity assays, Zn^{2+} (in 5-fold excess over phenanthroline) was added to the protein samples. Most inactivation experiments were carried out with protein concentrations about 1.2 mg/mL; additional experiments with lower protein concentrations (about 0.1 mg/mL) showed that the kinetics of thermal inactivation of CPB does not change

† This research was supported by Grants PB87-0871 from the DGICYT and 0460.E. (JR) from the Biotechnology Action Programme of the European Communities.

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[1] Abbreviations: DSC, differential scanning calorimetry; CPB, carboxypeptidase B; Tris, tris(hydroxymethyl)aminomethane.

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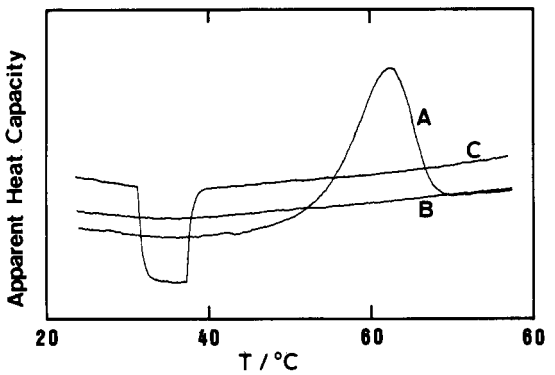


FIGURE 1: (A) Original recording of heat absorption of CPB solution at 2.02 K/min and pH 9.0; protein concentration 1.13 mg/mL; Zn^{2+} concentration 0.37 mM. (B) Reheating run. (C) Buffer − buffer base line showing a 25-μW calibration mark (power was communicated to the sample cell, and therefore, the calibration mark appears as an exotherm).

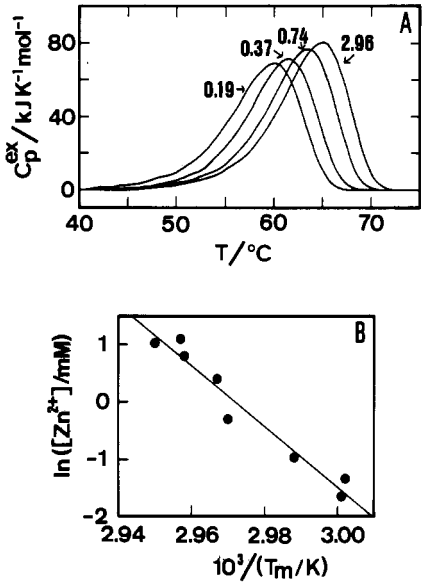


FIGURE 2: (A) Effect of Zn^{2+} concentration on the DSC transitions corresponding to the thermal denaturation of CPB in 20 mM pyrophosphate buffer, pH 9.0; the numbers alongside the transitions stand for the Zn^{2+} concentration in millimolar. In all cases, the scanning rate is 2.02 K/min. (B) Plot of ln $[Zn^{2+}]$ versus $1/T_m$, according to eq 1; all the data correspond to a scanning rate of 2.02 K/min.

significantly with protein concentration.

Results and Discussion

Effect of Zn^{2+} on the DSC Transitions for the Thermal Denaturation of CPB. Figure 2A shows DSC transitions corresponding to the thermal denaturation of CPB in 20 mM pyrophosphate buffer, pH 9.0, and in the presence of increasing concentrations of Zn^{2+}. These transitions were obtained at a scanning rate of 2.02 K/min and have been corrected for the instrumental base line, the chemical base line, and the effect of the slow time response of the calorimeter. The transitions in Figure 2 (as well as all the calorimetric transitions reported in this work) were found to be irreversible. It is apparent that the temperature, T_m, corresponding to the maximum heat capacity increases with Zn^{2+} concentration (there also appears to be an increase in the total enthalpy change of the transition, which might be attributed to a ΔC_p effect; see Figure 9).

Equilibrium thermodynamics predicts that, for a monomeric protein that undergoes two-state reversible unfolding with simultaneous ligand loss, the T_m values of the DSC transitions

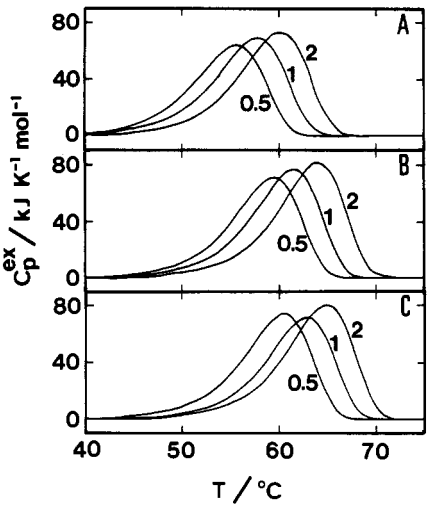


FIGURE 3: Scanning rate effect on the DSC transitions for the thermal denaturation of CPB in the presence of several Zn^{2+} concentrations: (A) 0.26 mM, (B) 1.48 mM, and (C) 2.96 mM. The numbers alongside the transitions stand for the scanning rate in kelvins per minute.

change with ligand concentration according to (Fukada et al., 1983)

$$\Delta H^{vH}/RT_m + \nu \ln L_0 = \text{constant} \quad (1)$$

where ΔH^{vH} is the apparent, or van't Hoff, enthalpy, L_0 is the ligand concentration, and ν is the number of ligand molecules bound to a native protein molecule. Note that eq 1 assumes that the ligand concentration is much larger than the total protein concentration (as is the case for the calorimetric experiments in Figure 2A) and that no ligand is bound to the unfolded protein.

For CPB, and as a first assumption, $\nu = 1$. Therefore, a plot of ln $[Zn^{2+}]$ versus $1/T_m$ should be a straight line with a slope equal to $-\Delta H^{vH}/R$. This plot is shown in Figure 2B; the plot is linear (within the scatter of the experimental data) and the ΔH^{vH} value derived from the slope (445 ± 38 kJ/mol) agrees acceptably with the value obtained for this parameter from the shape of the transitions (414 ± 15 kJ/mol) by using the well-known equation

$$\Delta H^{vH} = 4RT_m^2 C_{p,m}^{ex}/\Delta H \quad (2)$$

where $C_{p,m}^{ex}$ is the excess heat capacity at the maximum and ΔH is the calorimetric enthalpy.

Thus, the effect of Zn^{2+} on the DSC transitions apparently follows equilibrium thermodynamics. We will show here, however, that the DSC transitions for the irreversible denaturation of CPB in the presence of Zn^{2+} are strongly rate-limited and conform, in fact, to the two-state irreversible model (Sanchez-Ruiz et al., 1988).

Scanning Rate Effect on the DSC Transitions for the Thermal Denaturation of CPB. Figure 3 shows the scanning rate effect on DSC transitions corresponding to the thermal denaturation of CPB in the presence of several Zn^{2+} concentrations. These transitions have been corrected for the effect of the slow time response of the calorimeter. From the strong scanning rate effect observed it can be deduced that the thermal denaturation of CPB is, at least in part, under kinetic control.

The simplest theoretical scheme that takes into account the possible kinetically controlled character of irreversible DSC transitions is the two-state irreversible model (Sanchez-Ruiz et al., 1988), which assumes that only the native and final (irreversibly denatured) states of the protein are significantly populated and that the rate of denaturation is determined by

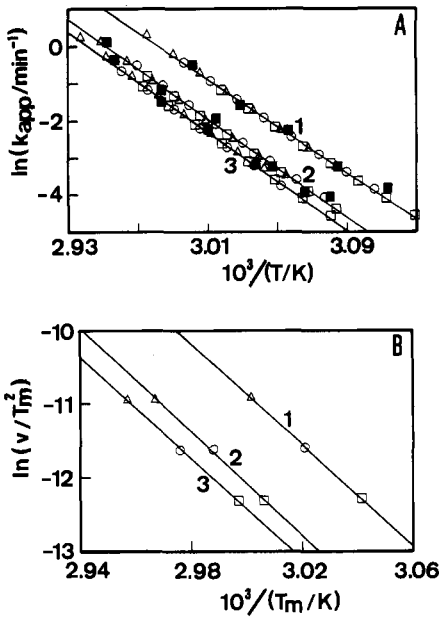


FIGURE 4: (A) Arrhenius plots for the apparent first-order rate constant for CPB thermal denaturation. □ (0.5 K/min), ○ (1.01 K/min), and △ (2.02 K/min): k_{app} values obtained from the DSC transitions by using eq 4. ■: k_{app} values obtained from thermal inactivation experiments. (B) Plots of ln (v/T_m^2) versus $1/T_m$, according to eq 6. In both (A) and (B), the Zn^{2+} concentrations are (1) 0.26 mM, (2) 1.48 mM, and (3) 2.96 mM.

a first-order rate constant that changes with temperature according to the Arrhenius equation:

$$k_{app} = \exp(E_{app}/RT^*) \exp(-E_{app}/RT) \quad (3)$$

where k_{app} is the rate constant, E_{app} is the energy of activation, and T^* is the temperature at which $k_{app} = 1\ min^{-1}$. Values of k_{app} can be obtained from the DSC transitions by using (Sanchez-Ruiz et al., 1988)

$$k_{app} = \frac{vC_p^{ex}}{\Delta H - \langle \Delta H \rangle} \quad (4)$$

where v stands for the scanning rate, ΔH for the total enthalpy of the transition, and C_p^{ex} and $\langle \Delta H \rangle$ for the apparent excess heat capacity and the apparent excess enthalpy at the temperature at which k_{app} is calculated. Once the values of k_{app} have been calculated, the values of E_{app} and T^* can be obtained from Arrhenius plots of ln k_{app} versus $1/T$.

If the thermal denaturation of CPB follows the two-state irreversible model, there must be agreement between the k_{app} values computed from transitions obtained at different scanning rates. The agreement is, in fact, excellent, as shown by the Arrhenius plots of Figure 4A; the values of E_{app} and T^* derived from these plots are given in Table I.

The energy of activation can also be obtained from the parameters of the maximum of the transitions by using (Sanchez-Ruiz et al., 1988)[2]

$$E_{app} = eRT_m^2 C_{p,m}^{ex}/\Delta H \quad (5)$$

or from the slope of a plot of ln (v/T_m^2) versus $1/T_m$ (see Figure 4B), as indicated by (Sanchez-Ruiz et al., 1988)

$$\ln (v/T_m^2) = \text{constant} - E_{app}/RT_m \quad (6)$$

The E_{app} values obtained by using these two methods are in good agreement with those calculated from the Arrhenius plots (Table I).

[2] It must be noted that, as the thermal denaturation of CPB follows the two-state irreversible model, eq 2 does not, in fact, yield the van't Hoff enthalpy but simply $(4/e)E_{app}$ (compare eqs 2 and 5).

Table I: Kinetic Parameters Corresponding to the Thermal Denaturation of CPB[a]

	$[Zn^{2+}]$ (mM)			
	0.26	1.48	2.78	2.96
E_{app}[b] (kJ/mol)	270 ± 5	284 ± 5	289 ± 13	284 ± 5
E_{app}[c] (kJ/mol)	286 ± 4	289 ± 13	269 ± 8	284 ± 8
E_{app}[d] (kJ/mol)	267 ± 5	284 ± 4	286 ± 11	284 ± 6
E_{app}[e] (kJ/mol)	262 ± 8 [266]	278 ± 5 [282]	282 ± 13 [289]	278 ± 7 [289]
T^*[e] (°C)	62.2 ± 0.3 [62.5]	65.6 ± 0.2 [65.9]	67.5 ± 0.2 [66.5]	66.8 ± 0.2 [66.6]

[a] Thermal denaturation data were determined from DSC experiments carried out in 20 mM pyrophosphate buffer, pH 9.0, at different scanning rates and in the presence of several $[Zn^{2+}]$ concentrations. [b] From the parameters of the maximum of the DSC transitions by using eq 5. [c] From the scanning rate effect on the T_m values, according to eq 6. [d] From the nonlinear least-squares fitting of eq 7 to the C_p^{ex} versus temperature profiles. [e] From the fitting of eq 3 to the k_{app} values calculated from the DSC transitions by using eq 4. The numbers in brackets were obtained from the fitting of eq 3 to the k_{app} values predicted by eqs 12–14.

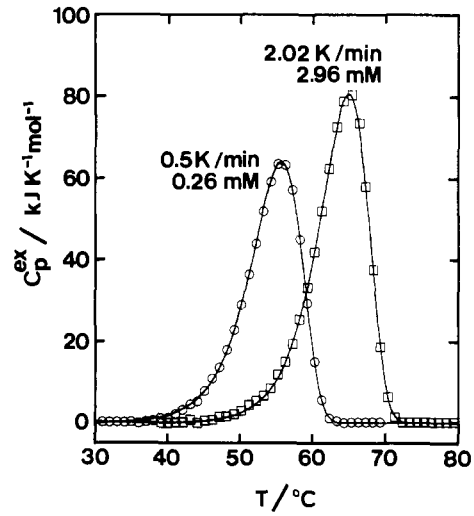


FIGURE 5: DSC transitions for the thermal denaturation of CPB at the indicated scanning rates and Zn^{2+} concentrations. (□, ○) Experimental excess heat capacity data. (—) Best fits of the theoretical curve given by eq 7.

Finally, the shape of DSC transitions that follow the two-state irreversible model with first-order kinetics is given by

$$C_p^{ex} = \frac{\Delta H E_{app}}{RT_m^2} \exp\left(\frac{E_{app}\Delta T}{RT_m^2}\right) \exp\left[-\exp\left(\frac{E_{app}\Delta T}{RT_m^2}\right)\right] \quad (7)$$

where C_p^{ex} is the apparent excess heat capacity at a temperature T and $\Delta T = T - T_m$ [eq 7 can be easily obtained by differentiation from eq A16 in Sanchez-Ruiz et al. (1988)].

The DSC transitions for the thermal denaturation of CPB in the presence of Zn^{2+} (Figures 2A and 3) are quantitatively described by eq 7, as shown in Figure 5. The E_{app} values derived from the nonlinear least-squares fitting of eq 7 to the experimental C_p^{ex} data are also in good agreement with those obtained by using the other methods described above (see Table I).

Kinetics of Thermal Inactivation of CPB. The kinetics of thermal inactivation of CPB in 20 mM pyrophosphate buffer, pH 9.0, in the presence of Zn^{2+} was investigated by following the time dependence of the enzyme activity (see Experimental Procedures) at given temperatures within the ranges of the corresponding DSC transitions.

As was to be expected from the fact that the DSC transitions follow the two-state irreversible model with first-order kinetics,

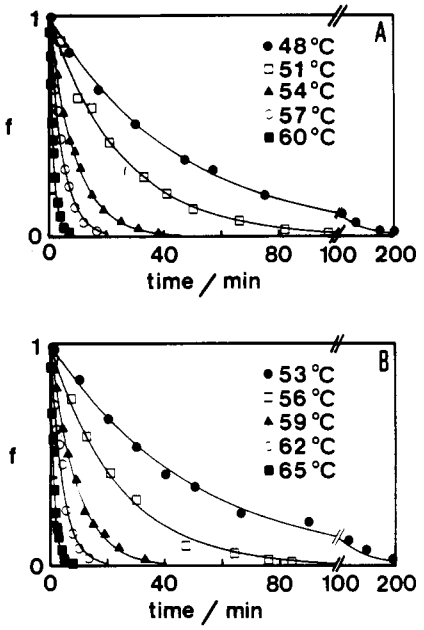


FIGURE 6: Thermal inactivation kinetics of CPB in 20 mM pyrophosphate buffer, pH 9.0, at the indicated temperatures. (A) $[Zn^{2+}]$ = 0.26 mM. (B) $[Zn^{2+}]$ = 2.96 mM. f stands for the fraction of active enzyme at a time t (f is calculated as the ratio A/A_0, where A is the enzyme activity measured at a time t and A_0 is the activity for t = 0). (—) Curves predicted by eq 8 with the optimum values of k_{app}.

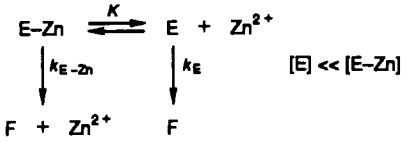
the activity versus time profiles did not depend on the total protein concentration (within the range 0.2–2 mg/mL) and gave excellent fits (Figure 6) to the integrated first-order rate equation

$$f = A/A_0 = \exp(-k_{app}t) \tag{8}$$

where A is the enzyme activity at a time t, A_0 is the activity at time zero, and f is the fraction of irreversibly denatured enzyme. In addition, the values determined for the rate constant k_{app} were in good agreement with those obtained from the DSC transitions by using eq 4 (see Figure 4A).

A Simple Kinetic Model To Explain the Effect of Zn^{2+} on the Thermal Denaturation of CPB. The study we have carried out on the scanning rate effect on the DSC transitions and the thermal inactivation kinetics of CPB shows conclusively that the thermal denaturation of this enzyme (in 20 mM pyrophosphate buffer, pH 9.0, and in the presence on Zn^{2+} concentrations larger than the total protein concentration), follows the two-state irreversible model with first-order kinetics. This indicates that (a) only the native and final (irreversibly denatured) states of the protein are significantly populated during denaturation, (b) chemical equilibrium between these two states is not established, and therefore, the DSC transitions cannot be analyzed on the basis of equilibrium thermodynamics models, and (c) the conversion from the native to the final state is determined by a first-order rate constant (k_{app}).

It is clear, therefore, that the effect of Zn^{2+} on the DSC transitions (Figure 2) must be attributed to the fact that the apparent first-order rate constant depends on Zn^{2+} concentration (see Figure 4A). We have found that this dependence can be explained on the basis of a simple kinetic model:



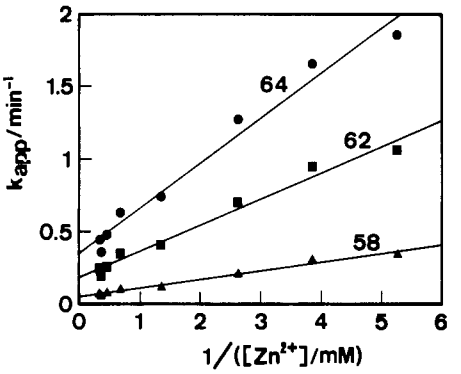


FIGURE 7: Effect of the Zn^{2+} concentration on the apparent first-order rate constant for CPB thermal denaturation (see eq 12 in the text). The numbers alongside the lines stand for the temperature in degrees Celsius.

We will assume that the native state of CPB is the folded protein with Zn^{2+} bound (E–Zn), although a small amount of protein without bound Zn^{2+} (E) is present in equilibrium with E–Zn. Both E–Zn and E can react irreversibly to yield the final state (F) according to first-order rate constants k_{E-Zn} and k_E, respectively. Finally, K is the equilibrium constant for Zn^{2+} dissociation:

$$K = \frac{[E][Zn^{2+}]}{[E\text{–}Zn]} \tag{9}$$

It is important to note that we assume [E] ≪ [E–Zn], and therefore, only two states (E–Zn and F) are significantly populated during denaturation.

The rate of formation of the final state is given by

$$d[F]/dt = k_{E-Zn}[E\text{–}Zn] + k_E[E] \tag{10}$$

Taking into account eq 9 and the fact that $d[F]/dt \cong -d[E\text{–}Zn]/dt$ (given that the concentration of E is assumed to be very low), eq 10 can be written as

$$\frac{d[E\text{–}Zn]}{dt} = -\left(k_{E-Zn} + \frac{k_E K}{[Zn^{2+}]}\right)[E\text{–}Zn] \tag{11}$$

The Zn^{2+} concentrations in the calorimetric experiments of Figures 2 and 3 were much larger than the total protein concentration; therefore, $[Zn^{2+}]$ can be taken as a constant (and equal to the total zinc concentration) and eq 11 indicates that the denaturation kinetics is first order with an apparent rate constant given by

$$k_{app} = k_{E-Zn} + k_E K/[Zn^{2+}] \tag{12}$$

Plots of k_{app} versus $1/[Zn^{2+}]$ are linear (within the scatter of the data) as predicted by eq 12 (see Figure 7).[3] The values of $k_E K$ and k_{E-Zn}, obtained from the slope and intercept of these plots, show Arrhenius-like dependences with temperature, which can be described by the following equations and parameters:

$$\ln [k_{E-Zn}/\text{min}^{-1}] = \frac{E_{E-Zn}}{R}\left(\frac{1}{T_{E-Zn}^*} - \frac{1}{T}\right)$$

$$E_{E-Zn} = 300 \text{ kJ/mol} \qquad T_{E-Zn}^* = 67.3\ ^\circ\text{C} \tag{13}$$

[3] One of the reviewers of this paper has correctly noted that eq 12 predicts $k_{app} = \infty$ for $[Zn^{2+}] = 0$, whereas the correct result is $k_{app} = k_E$. This is a consequence of the fact that eq 12 is valid under the assumption [E] ≪ [E–Zn]. Obviously, this assumption does not hold in the limit $Zn^{2+} \rightarrow 0$.

$$\ln [k_E K/(\text{mM}\cdot\text{min}^{-1})] = \frac{E_{kK}}{R}\left(\frac{1}{T_{kK}^*} - \frac{1}{T}\right)$$

$$E_{kK} = 259 \text{ kJ/mol} \qquad T_{kK}^* = 68.3 \text{ °C} \qquad (14)$$

where E_{E-Zn} and E_{kK} are the energies of activation and T_{E-Zn}^* and T_{kK}^* are the temperatures at which $k_{E-Zn} = 1$ min^{-1} and $k_E K = 1$ mM·min^{-1}, respectively. We should note that the term $k_E K$ is the product of a rate constant and an equilibrium constant; the fact that $k_E K$ follows an Arrhenius-like dependence with temperature can be explained if we assume that, within the studied temperature range, k_E follows the Arrhenius equation and K the van't Hoff equation with a constant enthalpy change. Clearly, the apparent energy of activation for the term $k_E K$ is equal to $\Delta H_{Zn} + E_E$, where ΔH_{Zn} is the enthalpy change for Zn^{2+} dissociation (E–Zn ↔ E + Zn^{2+}) and E_E is the energy of activation corresponding to the irreversible step E → F.

Given that k_{E-Zn} and $k_E K$ change with $1/T$ in an exponential fashion (eqs 13 and 14), some curvature could be expected in the plots of ln k_{app} versus $1/T$, for a given Zn^{2+} concentration (see eq 12); numerical simulation (using eqs 12–14) of the temperature dependence of k_{app} shows, however, that the curvature is too small to be detected within the narrow temperature range of the calorimetric transitions, which explains the fact that the experimental k_{app} values follow the Arrhenius equation (Figure 4A). It must be noted that the E_{app} and T^* values (eq 3) derived from the k_{app} values calculated from eqs 12–14 are in good agreement with those obtained from the calorimetric transitions (see Table I).

Equation 11 gives the time dependence of the concentration of native enzyme (E–Zn) for a kinetic experiment carried out at constant temperature. For a DSC experiment, in which temperature and time change simultaneously according to a constant scanning rate, the relevant differential equation is

$$\frac{d[E-Zn]}{dT} = -\frac{k_{app}}{v}[E-Zn] \qquad (15)$$

At $T = T_m$, $d^2[E-Zn]/dT^2 = 0$, and therefore, the condition of the maximum of the DSC transitions can be written as

$$k_{app,m} = v\left(\frac{d \ln k_{app}}{dT}\right)_m \qquad (16)$$

where $k_{app,m}$ and $(d \ln k_{app}/dT)_m$ are the values of k_{app} and $(d \ln k_{app}/dT)$ at the temperature T_m. Equations 12–14 (together with the previously determined values of E_{E-Zn}, T_{E-Zn}^*, E_{kK}, and T_{kK}^*) give the temperature dependence of k_{app}; therefore, eq 16 can be numerically solved to obtain the T_m value corresponding to given values of the scanning rate and the Zn^{2+} concentration. These calculated T_m values are compared with the experimental ones (obtained from the DSC transitions) in Figure 8. Clearly, the proposed kinetic model explains both the Zn^{2+} concentration effect and the scanning rate effect on the T_m values (it may be worth pointing out here that no equilibrium thermodynamics model can explain a scanning rate effect on DSC transitions).

Thermal Denaturation of CPB in the Presence of 1,10-Phenanthroline. DSC transitions for the thermal denaturation of CPB in 20 mM pyrophosphate buffer, pH 9.0, and in the presence of 5 mM 1,10-phenanthroline were also found to be strongly scanning-rate dependent (T_m = 48.1 °C at v = 2.02 K/min and T_m = 41.0 °C at v = 0.5 K/min). The transitions could be adequately described by the two-state irreversible model (eqs 4–7) and the rate constants calculated by using eq 4 were in good agreement with those obtained from thermal inactivation experiments (results not shown). Given that 1,10-phenanthroline is an inhibitor of CPB that acts as a Zn^{2+}-chelating agent (Folk et al., 1960), it appears plausible to assume that the DSC transitions obtained in the presence of this inhibitor correspond to the denaturation of the protein without bound Zn^{2+}; accordingly, the rate constants calculated from the transitions by using eq 4 should be equal to the constant k_E:

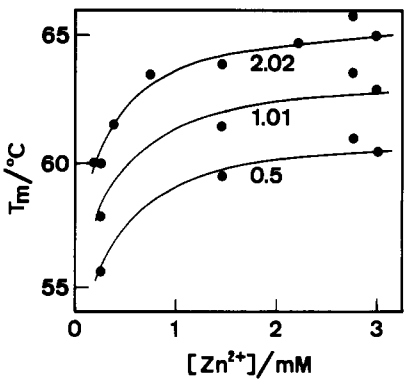
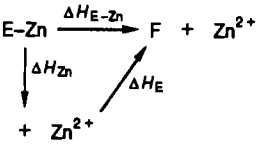


FIGURE 8: Effect of Zn^{2+} concentration on the temperatures of the maximum of the DSC transitions. (●) Experimental T_m values. (—) Theoretical curves predicted by eqs 12 and 16, together with the values of k_{E-Zn} and $k_E K$ given by eqs 13 and 14. The numbers alongside the curves stand for the scanning rate in kelvins per minute.

$$E \xrightarrow{k_E} F$$

The temperature dependence of the calculated k_E values can be described by the Arrhenius equation with an energy of activation of 177 ± 10 kJ/mol and a temperature, T^*, at which $k_E = 1$ min^{-1} equal to 52.1 ± 0.3 °C. These values of the rate constant k_E, together with those previously determined for the term $k_E K$, allow us to calculate the equilibrium constant for Zn^{2+} dissociation, K. This calculation of K involves extrapolation of the Arrhenius dependences of k_E and $k_E K$ outside the temperature ranges in which they were determined (given that these ranges did not overlap); therefore, the values obtained for the equilibrium constant K are to be considered as estimates. It is interesting, nevertheless, that the calculated K values (for instance, K = 0.0009 mM at 27 °C, K = 0.01 mM at 53 °C, and K = 0.06 mM at 72 °C) indicate that, in the presence of the Zn^{2+} concentrations employed in the calorimetric experiments of Figures 1 and 2 (0.19–3.0 mM), the concentration of E is, in fact, very low.

The enthalpy change for Zn^{2+} dissociation (ΔH_{Zn}) derived from the temperature dependence of the equilibrium constant K is 82 kJ/mol. According to the thermodynamic cycle[4]



the following relationship holds between the three enthalpy changes:

$$\Delta H_{Ezn} = \Delta H_E + \Delta H_{Zn} \qquad (17)$$

where the ΔH_{E-Zn} values are determined from the DSC transitions obtained in the presence of Zn^{2+} concentrations

[4] It may be worth pointing out that, according to the first law, the total heat absorbed (obtained by integration of the apparent excess heat capacity function) equals the total enthalpy change, even if the denaturation process is irreversible. The total entropy change (and, hence, the Gibbs energy change) cannot be determined from the transitions, given that ΔS calculations from experimental C_p data are based upon the Clausius equality, which does not hold for an irreversible, rate-limited process.

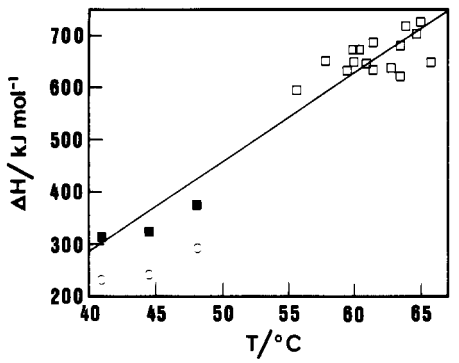


FIGURE 9: Plot of calorimetric enthalpy change versus temperature for the thermal denaturation of CPB. The enthalpies have been ascribed to the corresponding T_m values. (□) ΔH_{E-Zn} values calculated from the DSC transitions obtained in the presence of Zn^{2+} concentrations much larger than the protein concentration. (○) ΔH_E values calculated from DSC transitions obtained in the presence of 1,10-phenanthroline. (■) ΔH_{E-Zn} values obtained by application of eq 17. The line is a linear least-squares fit to all the ΔH_{E-Zn} values.

much larger than the protein concentration, and the ΔH_E values from the DSC transitions obtained in the presence of 1,10-phenanthroline. Note that the values of ΔH_{E-Zn} and ΔH_E are to be ascribed to different temperatures (in Figure 9, each enthalpy change has been assigned to the corresponding T_m value). ΔH_{E-Zn} values at the T_m's of the DSC transitions obtained in the presence of 1,10-phenanthroline were calculated from eq 17 and the previous estimate of ΔH_{Zn} (82 kJ/mol). A least-squares linear fit to all the ΔH_{E-Zn} values (see Figure 9) gives a denaturation heat capacity change of 16 kJ K^{-1} mol^{-1}, in reasonable agreement with the values determined from the individual DSC transitions (8–12 kJ K^{-1} mol^{-1}). Clearly, the analysis shown in Figure 9 is only approximate, as it is based on an estimated value of ΔH_{Zn}, which has been assumed to be temperature-independent (in addition, we have assumed that the irreversibly denatured protein, F, is a single macroscopic state); the analysis is simply intended to show that the estimated ΔH_{Zn} value (82 kJ/mol) does not appear to be inconsistent with the calorimetric enthalpies determined from the DSC transitions.

Concluding Remarks

The DSC transitions for the thermal denaturation of CPB in 20 mM pyrophosphate buffer, pH 9.0, and in the presence of Zn^{2+} concentrations much larger than the total protein concentration are calorimetrically irreversible. In addition, they are quantitatively described by the two-state irreversible model with first-order kinetics (Sanchez-Ruiz et al., 1988). The heat absorption, therefore, is entirely determined by the rate of formation of the final (irreversibly denatured) state and no thermodynamic information (other than the total enthalpy change) can be derived from the transitions.

The effect of Zn^{2+} concentration on the DSC transitions has been explained in terms of a very simple kinetic model. We believe that the proposed kinetic model is, in fact, the simplest one able to explain the effect of Zn^{2+} on the thermal denaturation of CPB; we must clearly state, however, that this model (as any other kinetic model) might be modified (or even rejected) on the light of new experimental information. The results reported in this work show, nevertheless, several important points that are independent of the specific assumptions of our kinetic model:

(a) Contrary to the points of view expressed by several authors in recent literature (Manly et al., 1985; Edge et al., 1985; Hu & Sturtevant, 1987; Sturtevant, 1987) the fact that ligand effects on the T_m values appear to conform to the dictates of the van't Hoff equation does *not* constitute evidence supporting the equilibrium thermodynamics analysis of irreversible DSC transitions. Thus, the "van't Hoff plot" of ln $[Zn^{2+}]$ versus $1/T_m$ for the thermal denaturation of CPB (Figure 2B) is linear (within the scatter of the experimental data) and yields a van't Hoff enthalpy value (eq 1) which agrees with that obtained from the shape of the transitions (eq 2). In spite of this, equilibrium thermodynamics analysis of the DSC transitions is not permissible, as the denaturation was found to obey the two-state irreversible model and chemical equilibrium between the significantly populated states (native and final) is not established.

(b) Ligand effects on irreversible DSC transitions can be explained on the basis of kinetic models that include ligand dissociation as a preliminary equilibrium step.

(c) Whatever kinetic model is proposed, the immediate reason for the Zn^{2+} effect on the thermal denaturation of CPB is the fact that, for a given temperature, increasing the Zn^{2+} concentration causes a decrease in the rate of irreversible denaturation of the protein.

There have been in recent literature several attempts to obtain mutant proteins with enhanced thermal stability; the basic approach consists of introducing mutations that are thought to increase the Gibbs energy difference between the unfolded and native states of the protein. In some of these studies, DSC was used to characterize the thermal stability of the wild-type and mutant forms of the protein. Given that protein thermal denaturation is often found to be irreversible, we would like to suggest that the possibility that the effect of a mutation might arise from its influence on the rate of irreversible denaturation should be taken into account.

Acknowledgments

F.C.-L. acknowledges a predoctoral fellowship from the Direccion General de Investigacion Cientifica y Tecnica.

Registry No. CPB, 9025-24-5; Zn^{2+}, 7440-66-6.

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