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Microcalorimetric studies of conformational transitions of ferricytochrome *c* in acidic solution

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The conformational transitions of ferricytochrome *c* in acidic solutions with different NaCl concentrations have been studied by scanning and isothermal microcalorimetry. It is shown that ferricytochrome *c* adopts three different forms which are realized under the considered conditions: native, denatured (unfolded) and a compact native-like form with unique tertiary structure. The thermodynamic parameters of the corresponding transitions have been measured and the changes in the number of bound ligands (H^+ and Cl^-), accompanying these transitions, have been determined by analyzing the temperature, pH and ionic strength dependence of these parameters.

1. Introduction

The crystallographic model of ferricytochrome *c* shows that in this molecule the heme moiety is located in a crevice of the native conformation, bound by two thioether linkages to Cys 14 and Cys 17, and that the heme iron is coordinated by His 18 and Met 80 [1]. Therefore, one can expect that heme-polypeptide interactions in cytochrome *c* must be essential for stabilization of the globular conformation of the protein. Indeed, it has been shown that there is no detectable ordered structure in apo-cytochrome *c* and that the stability of the globular structure of cytochrome *c* depends largely on the state of the heme: it is markedly different for the ferri and ferro forms [1–3]. Acidification of ferricytochrome *c* solution causes the displacement of the two strong field ligands by two weak field ligands, most likely supplied by the solvent. An increase in ionic strength of acidic solutions leads to a change in the interaction of the heme iron with protein ligands which results in an alternative structure for the molecule. The state of ferricytochrome *c* observed under these conditions was classified by Ohgushi and Wada [4] as a 'molten-globule' state.

This new type of structural state was initially proposed as being a liquid-like phase of globular proteins [5]. In this case, the polypeptide chain is supposed to be packed compactly, nearly as occurs in the native state, while the intramolecular motions of atoms are extensively increased.

The possibility of the existence of this new structural state and the lack of thermodynamic data on the conformational transitions of ferricytochrome *c* in acidic solution prompted us to perform the present investigation.

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2. Materials and methods

Horse heart cytochrome *c* was prepared as described previously [6]. The protein in the ferri form was further purified, using a Sephadex G-75 column (3.5 × 100 cm) equilibrated with 20 mM sodium phosphate buffer (pH 8.0). Cytochrome *c* was oxidized by adding 1 mM $K_3Fe(CN)_6$.

The covalently bound heme moiety in cytochrome *c* was cleaved from the polypeptide chain by reaction with silver sulfate in acidic solution. The apo-protein was purified by gel-filtration chromatography on a Sephadex G-75 column, equilibrated with 50 mM ammonium acetate (pH 5.0) [2]. The protein solution showed no measurable absorbance at 410 nm.

The purity of preparations was checked by electrophoresis in 10% polyacrylamide gels in the presence of SDS according to the method of Weber and Osborn [7].

The protein concentrations were determined from the light absorption of solutions at the standard pH value. The solutions were prepared by diluting samples 2-fold or more with 0.2 M glycine-HCl buffer solution (pH 6.5). The following values of the extinction coefficients were used for ferricytochrome *c* and apo-protein, respectively: $A_{530\ nm} = 9.36\ cm^2/mg$ and $A_{280\ nm} = 1.01\ cm^2/mg$. The coefficients were obtained by measuring the light absorption and nitrogen content of the stock solution. Nitrogen content was determined according to the procedure in ref. 8.

Calorimetric measurements were performed on a DASM-1M scanning microcalorimeter [9] at a heating rate of 2 K/min and with protein concentrations varying from 2 to 4 mg/ml. The general accuracy of heat capacity measurements is about 0.3 mJ/K per ml. The partial specific heat capacity of the protein was determined from calorimetric recordings as described earlier [10], assuming that the partial specific volume of cytochrome *c* amounts to 0.75 cm^3/g. The total calorimetric enthalpy of transitions was determined from the partial specific heat capacity curves, as described in the same paper.

Isothermal calorimetric experiments were carried out in an LKB batch calorimeter which was calibrated electrically. The heat of mixing of the solution of ferricytochrome *c* in 1 mM glycine-HCl (pH 2.6) with NaCl solutions of the required concentrations was measured. Initial concentrations of ferricytochrome *c* ranging from 4 to 10 mg/ml were used in such experiments. The enthalpy of transfer of ferricytochrome *c* to NaCl solutions was calculated as the difference between the integral heat effect and the enthalpy of dilution of the corresponding NaCl solution. The dilution enthalpy of the latter was measured in additional experiments [11].

Immediately before use, samples for calorimetric studies were incubated in a solution of neutral pH containing 1 mM $K_3Fe(CH)_6$, and then were adjusted to the required conditions by passage through the Sephadex G-25 column. It was proposed that the activity of Cl^{-1} is equal to its concentration in the buffer solution. The changes in Cl^- concentration as a result of conformational transitions of the protein were not taken into account.

3. Results

3.1. Conformational transitions of ferricytochrome c in solutions of low Cl^- concentration

Fig. 1 shows the partial heat capacity of ferricytochrome *c* at low ionic strength and different pH values of solutions. The reversibility of the

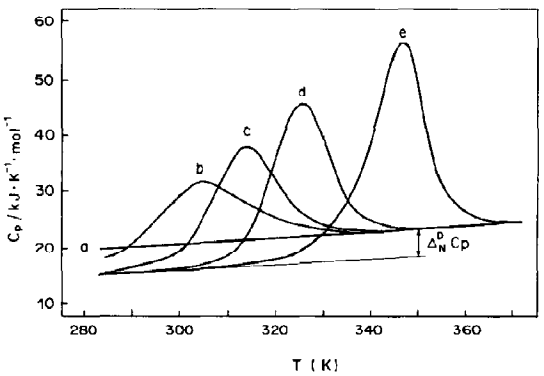

Fig. 1. Dependence of the partial molar heat capacity of apocytochrome *c* (a) and ferricytochrome *c* (b–e) on temperature. 25 mM glycine-HCl: pH 2.6 (a), pH 2.2–3.2 (b–e).

system under study upon reheating was 80% or more. The observed melting curve was independent of heating rate between 0.25 and 2 K/min. Thus, the melting process can be considered as being at equilibrium and therefore can be analyzed thermodynamically.

The partial heat capacities of ferricytochrome *c* and apo-cytochrome are equal at higher temperatures. On the other hand, it has been shown that apo-cytochrome has no detectable ordered structure [2,3]. Therefore, a conformational transition of ferricytochrome *c* under these conditions leads to the complete denaturation of the protein as judged from heat capacity measurements.

Taking the partial heat capacity of apo-cytochrome *c* to be an approximation of C_p for denatured ferricytochrome *c*, one can calculate the populations of all intermediate states for the latter at any temperature. The results of such a calculation show that at pH $\geq$ 2.2 the population of the intermediate states in all cases is below 20%. In other words, the denaturation of ferricytochrome *c* under these conditions can be considered as a transition between two states-the native and the denatured ones, since there is no appreciable concentration of thermodynamically stable intermediate forms.

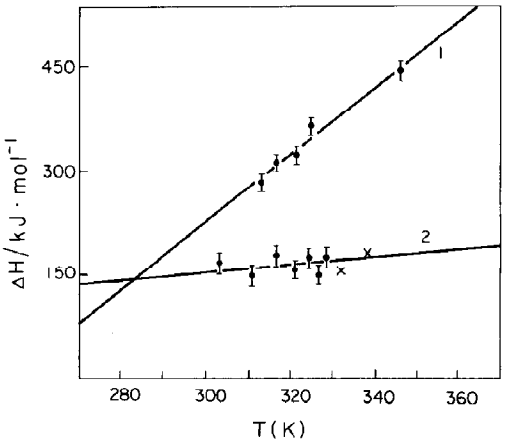

Fig. 2. Dependence of the enthalpy of the observed transitions of ferricytochrome *c* on temperature. (1) 25 mM glycine-HCl, pH 2.3–3.2. (2) (ł) 0.05–0.5 M NaOH + HCl, pH 2.1, (×) 0.5 M NaOH + HCl, pH 2.6 and 2.9.

Fig. 2 shows plots of the specific denaturation enthalpies of ferricytochrome *c* in solutions at different pH values vs. the corresponding temperature of denaturation. The slope of this function is numerically equal to the observed denaturational heat capacity change of the protein (figs. 1 and 2; table 1). In other words, the value of the total derivative of enthalpy with respect to temperature coincides with that of the partial derivative:

$$\left(\mathrm{d}\Delta_N^D H\right)/\left(\mathrm{d}T_d\right) = \left(\partial\Delta_N^D H\right)/\left(\partial T\right) + \left(\partial\Delta_N^D H\right)/\left(\partial \mathrm{pH}\right) \cdot \left(\partial \mathrm{pH}\right)/\left(\partial T_d\right) \cong \Delta_N^D C_p$$

Since $(\partial\Delta_N^D H)/(\partial T) = \Delta_N^D C_p$ and $(\partial pH)/(\partial T_d)$ is not sufficiently small, the partial derivative of enthalpy with respect to pH must be small. Thus the denaturation enthalpy of ferricytochrome *c* does not depend on the pH, but is a direct linear function of temperature, i.e.

$$\Delta_N^D H = a_N^D + \Delta_N^D C_p T \tag{1}$$

This is not a new result obtained in this paper. Side chain ionization at acidic pH values does not significantly contribute to the enthalpy change [14]. However, this connection provides additional evidence that the transitions detected are the same (between the same states of the protein) at any pH.

It is well known that the thermal denaturation of a protein is ordinarily accompanied by net uptake or release of protons, caused by changes in the environment of ionizable residues. The existence of the pH dependence for the stability of a protein structure is usually explained by this fact and can be interpreted in corresponding terms [10,13]. A general scheme of denaturation in this case can be presented as

$$\mathrm{N} + m^{\mathrm{l}}_{\mathrm{H}^+}\mathrm{H}^+ \rightleftharpoons \mathrm{DH}^{\mathrm{l}}_{m^{\mathrm{l}}_{\mathrm{H}^+}} \tag{2}$$

The equilibrium constant of the reaction can be expressed as

$$K = \left[\mathrm{DH}^+_{m^{\mathrm{l}}_{\mathrm{H}^+}}\right]/[\mathrm{N}][a_{\mathrm{H}^+}]^{m^{\mathrm{l}}_{\mathrm{H}^+}} \tag{3}$$

where $[\mathrm{DH}^+_{m^{\mathrm{l}}_{\mathrm{H}^+}}]$ and [N] denote the concentrations of ferricytochrome *c* in the native and denatured

states, respectively; $[a_{H^+}]$ is the activity of protons in solution, and $m^1_{H^+}$ the number of protons bound upon denaturation per protein molecule.

Of course, in general the value of m depends on the pH of the solution and is formed by a certain distribution of pK values of abnormally titrated groups. However, there is a certain region of pH where [m] can be supposed to remain constant.

If there is only one mechanism of variation in protein stability (which is directly described by eq. 2), the expression, connecting pH of solution and temperature denaturation of proteins, can be obtained. Since ΔC_p is independent of pH and temperature of solution and

$$(\partial \Delta S)/(\partial T) = (1/T)[(\partial \Delta H)/(\partial T)] = \Delta C_p/T$$

the standard entropy of transition can be presented as

$$\Delta S = \Delta C_p \ln(T/T_0) \qquad (4)$$

where T_0 is the temperature at which the entropy change for the transition between the native and denatured states is zero. T_0 and ΔS may only be weak functions of pH because the dependence of protein stability on the latter was taken into consideration as explicit functions. The numerical value of T_0 can be evaluated as follows. Since at the point of denaturation the concentrations of the native and denatured forms are equal, we obtain from eq. 3

$$-\Delta G/RT_d + m \ln[a_{H^+}] = 0 \qquad (5)$$

where ΔG designates the standard Gibbs' energy for this reaction. Substituting the expression for enthalpy (eq. 1) and entropy (eq. 4) into eq. 5, we obtain for $T = T_d$ the relation between the pH value of solutions and denaturation temperature of protein

$$2.303\mathrm{pH} = -(1/Rm^1_{H^+})\left[\left(a^D_N/T_d\right) - \left(\Delta^D_N C_p \ln T_d\right)\right] - \left[\left(\Delta^D_N C_p\right)/Rm^1_{H^+}\right](1 + \ln T_0)$$

Hence, there should be a linear relationship between the pH of the solution and the parameter $(a^D_N/T_d) - \Delta^D_N C_p \ln T_d$, if our presumption is valid. The slope of this function must be equal to $(-1/2.303\ Rm^1_{H^+})$ and intersect the pH axis at a point numerically equal to $[(\Delta^D_N C_p)/(2.303\ Rm^1_{H^+})](1 + \ln T_0)$ (fig. 3). This permits calcula-

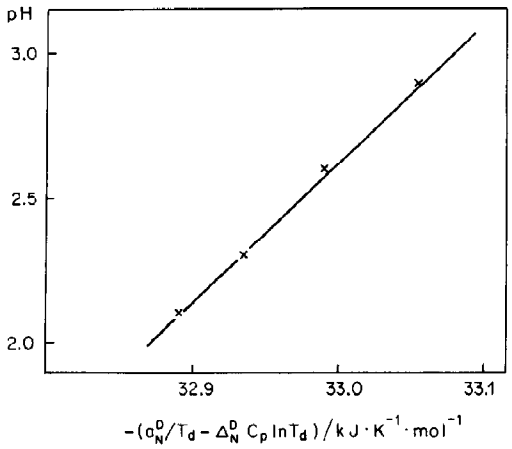

Fig. 3. Dependence of the pH value of the medium at which the transition occurs as a function of the thermodynamic parameters for the transition $(a^D_N/T_d - \Delta^D_N C_p \ln T_d)$. Results obtained in 25 mM glycine-HCl solution.

Table 1

Thermodynamic parameters of transitions

m_{H^+} and m_{Cl^-} denote the respective numbers of H^+ and Cl^- bound upon transition, a and ΔC_p parameters characterizing the dependence of transition enthalpy on temperature ($\Delta H = a + \Delta C_p T$) and T_0 the temperature at which the transition entropy falls to zero (assuming ΔC_p to be constant).

Transition	m_{H^+}	m_{Cl^-}	a (kJ/mol)	ΔC_p (kJ/K per mol)	T_0 (K)
$N \rightleftharpoons N^*$	4.4	1.7	−1257.1	4.44	268
$N^* \rightleftharpoons D$	1.2	−1.7	−15.4	0.56	125
$N \rightleftharpoons D$	5.6	–	1272.5	5.00	246

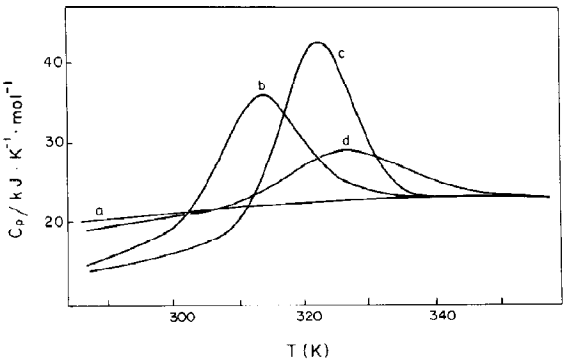

Fig. 4. Dependence of the partial molar heat capacity of apocytochrome *c* (a) and ferricytochrome *c* (b–d) on temperature. 25 mM glycine-HCl, pH 2.55 (b), pH 2.8 (c); 0.5 M NaOH-HCl, pH 2.1 (a, d).

tion of the T_0 and $m^1_{H^+}$ values. The results obtained from calculation carried out with the use of a least-squares method are summarized in table 1.

3.2. Conformational transitions of ferricytochrome c in solutions of high Cl^- concentration

To determine the thermodynamic parameters for the state of ferricytochrome *c* which is observed at sufficiently low pH and ionic strength, we constructed melting curves for varying pH values over the range pH 2.1–2.9 and different NaCl concentrations within the range 0.1–0.5 M. As in the case of low ionic strength, the maximum value of the protein fraction in all states, excluding the initial and final ones, does not exceed 20% in the temperature ranges of the transitions. The final states of ferricytochrome *c* in this case do not differ from the apo-protein state. This follows from the lack of any difference in the heat capacities of protein in these forms (fig. 4). As has been shown earlier [14], the heat capacity function is sensitive to the number of buried hydrophobic groups, and one may conclude that, in both cases, the proteins assume a conformation in which the side chain groups are accessible to water.

Despite appreciable variation in the conditions (pH and ionic strength), only small changes in the transition enthalpy are observed which depend on the temperature at which they are measured (fig. 2). The possibility that this results from compensation is improbable. A more real assumption is that we observe denaturation of protein under these conditions from the same state and that its enthalpy is a function only of the temperature. Due to the difference in temperature dependence of the enthalpy, the initial state of the protein is distinguishable from that at a low ionic strength. The values of a and ΔC_p for this transition can be obtained from the temperature dependence of the denaturation enthalpy. In this case, direct measurement of ΔC_p is somewhat hindered due to possible conformational transitions between the low and high salt forms of the protein at low temperatures (see section 4). The stability of this new state depends on both the pH and the NaCl concentration in solution. In accordance with the considerations presented in section 1, we suggest the following scheme of reaction:

$$N^*Cl^-_{m_{Cl}} + m^2_{H^+}H^+ \overset{K_2}{\rightleftharpoons} DH^+_{m^2_{H^+}} + m_{Cl^-}Cl^- \qquad (6)$$

where the equilibrium constant of the process, K_2, is

$$K_2 = \left[DH^+_{m^2_{H^+}}\right]\left[a_{Cl^-}\right]^{m_{Cl^-}} / \left[N^*Cl^-_{m_{Cl^-}}\right]\left[a_{H^+}\right]^{m^2_{H^+}}$$

As in the case considered previously, the expression connecting the pH and pCl of the solution and the denaturation temperature of the protein can be presented as

$$2.303\left(m^2_{H^+}\mathrm{pH} - m_{Cl^-}\,\mathrm{p}Cl\right) = -(1/R)\left(a^D_{N^*}/T_d - \Delta^D_{N^*}C_p \ln T_d\right) - \left(\Delta^D_{N^*}C_p/R\right)(1 + \ln T_0) \qquad (7)$$

The number of bound H^+ and Cl^- and the temperature, T_0, at which the value of the entropy of the initial and final states is equal under standard conditions, can be calculated as in the previous case using the results from two sets of experiments: (1) at pH 2.1 and NaCl concentration varying from 0.1 to 0.5 M; (2) at 0.5 M NaCl and pH varying from pH 2.1 to 2.9. One of the curves is shown in fig. 5. The thermodynamic parameters for transition calculated according to eq. 7 are listed in table 1.

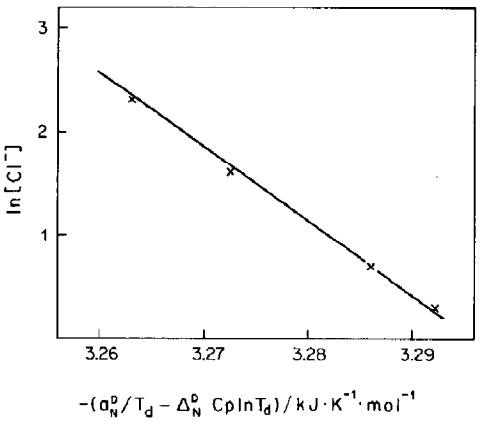

Fig. 5. Dependence of the medium p*Cl* value at which the transition takes place as a function of the thermodynamic parameters of the transition ($a^{D}_{N*}/T_d - \Delta^{D}_{N*}C_p \ln T_d$).

3.3. General scheme of conformational transitions of ferricytochrome c in solution at acidic pH values

As follows from the above, there are three different states of ferricytochrome *c* in solution at pH $\leqslant$ 3.2 and at different concentrations of NaCl and temperatures: native, native-like with bound Cl^- and denatured. The general scheme for the conformational transitions taking place under the studied conditions can be represented as follows:

$$N + 5.6H^+ + 1.7Cl^- \overset{K_1}{\rightleftharpoons} N^*H^+_{4.4}Cl^-_{1.7} + 1.2H^+$$

$$\overset{K_2}{\rightleftharpoons} DH^+_{5.6} + 1.7Cl^- \qquad (8)$$

There are two reasons for the appearance of fractional stoichiometric coefficients in this reaction scheme: (i) substitution of the real situation (when the protein in all of the states can bind ions, but the binding constants differ significantly) by models and (ii) possible experimental errors.

In fact, in scanning calorimetry experiments we are able to detect the transition which corresponds to the second step of the scheme, namely N* $\rightarrow$ D, or if the NaCl concentration is extremely low and state N* is not observed, the direct transition between the native and denatured states (N $\rightarrow$ D). The equilibrium constant for the latter transition is given by $K = K_1K_2$ (see above). Using our results, we can calculate the thermodynamic parameters for the first step of our formal scheme (eq. 8), which is accompanied by the binding of 1.7 mol Cl^- and 4.4 mol H^+ as the difference between the corresponding parameters of the two transitions. The results of this calculation are given in table 1. As one may observe, the value of the enthalpy for this process is so small (see also fig. 2) that the transition is not detectable by scanning calorimetry under the given conditions. Indeed, the two transitions should be separated with respect to temperature for a reliable recording of the first one. For this reason, the first transition can be recorded by scanning calorimetry only at $T \leqslant$ 300 K. In this case, the enthalpy of the second transition is about 3.5-times greater than that of the first. As the maximum amplitude of the heat absorption peak is proportional to the enthalpy squared [10], for the second transition it must be approx. 12-times greater as compared to the first. If one takes into account that, in this instance, the half-width of the first transition is 3.5-times smaller and that the heat absorption curves are obtainable only from 5 °C upward, then there is obviously no possibility of obtaining a reliable recording of the first transition by scanning calorimetry.

In order to demonstrate the occurrence of the first transition, we have carried out isothermal titration of ferricytochrome *c* with NaCl solutions at pH 2.6 [11]. Fig. 6 depicts the variation in enthalpy of transfer of ferricytochrome *c* from a solution containing 1 mM glycine-HCl (pH 2.6) to one containing NaCl at final concentrations that result in an equal value of the pH. Real errors due to the necessity for extrapolating the heat absorption curve corresponding to the initial and final states of the protein render a detailed analysis of the shape of the curve impossible. Nevertheless, it can be concluded that the values of the transition enthalpy obtained from this curve and from scanning calorimetry data are equal within the accuracy of measurements.

4. Discussion

It was noted by several authors that ferricytochrome *c* adopts at least three different confor-

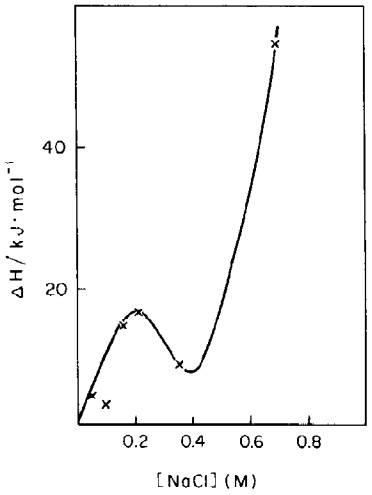

Fig. 6. Dependence of the enthalpy of transition of ferricytochrome *c* from 1 mM glycine-HCl solution (pH 2.6) to a salt solution of corresponding concentration of identical pH value.

mations at acidic pH values [1,4,15,16]. The resistance of a particular state to the action of pH and temperature depends essentially on the concentration of monovalent salts. Good correlation between the sodium salt effect and the partial molar entropy of dissolution for the corresponding anions has been reported [17]. This fact and the results obtained in a study of ion binding to cytochrome *c* by means of the nuclear magnetic quadruple relaxation method [18] allowed us to confirm that it is solely the anions that bind to the protein.The number of bound anions and protons changes when the molecules undergo transition from one form to another. Information on the number of bound protons and anions was derived from data on equilibrium [15,16,19] and kinetic [16] titration of the protein using different models. However, the thermodynamic parameters for stabilization of the structure of cytochrome *c* at acidic pH were not determined. In this work the absorption of heat that accompanies the transition of molecules between three states, observed under these conditions, was measured directly. This, in turn, permitted calculation of the changes in number of bound protons and anions accompanying the transition and, thus, of the fraction of protein in any state in solution for arbitrary (within the investigated region) values of pH, p*Cl* and temperature. It is interesting to consider some of the immediate consequences of the resulting model. The stability of both the first and second transitions should increase with rise in pH. This dependence is greater for the first transition. Hence, at any concentration of salt, a pH range must exist in which the state N* is not observed (e.g., low salt, pH > 2.1).

The second transition is stabilized and the first one destabilized with increasing salt concentration. Hence, at any pH (within the limits of the model) one should be able to determine a salt concentration at which one cooperative transition becomes separated into two different components. In fact, in scanning calorimetry experiments, only the second transition is reliably detectable due to the low value of the enthalpy of the first one. Nevertheless, all possible states are realized in this case. For this reason, direct determination of ΔC_p is impossible at intermediate values of the ionic strength. The principal result obtained in this study on the determination of protein fractions in any state is the intersection of the plots for the two temperature dependences of the transition enthalpy. It is clear that in this case there should exist not only cold denaturation of the protein (low salt concentration), but also the cold-induced transition from N to N* (intermediate salt concentration). With increasing salt concentration, the temperature at which N disappears last (the temperature of maximum stability for the native state) must be near 283 K.

There is some divergence between our scheme and that suggested by Ohgushi and Wada [4]. For example, according to these authors, the temperature for the N → D transition reaches 293 K at pH ≅ 2.5, while from our data it occurs at pH ≅ 2.1. The suggested diagram corresponds to ours, but was obtained at a higher temperature (difference of about 10–15 °C). This effect may be connected with the greater sensitivity of NMR to increasing fluctuations in structure when the temperature reaches that of the transition. However, beyond any doubt, the form which they classified as the 'molten-globule' state corresponds to our state N*.

The transition to state N* results in a significant decrease in number of abnormally titrated groups (table 1), signifying that a change occurs in the environment of ionizable groups. In particular, the aspartic and glutamic acid residues titrated under these conditions become more accessible to water molecules; the pK for a certain number of groups has a value close to that of the denatured state. Moreover, the accessibility of hydrophobic groups of ferricytochrome c also increases, which can be concluded from the change ΔC_p (as has been shown, the value of the heat capacity change correlates with the number of contacts of hydrophobic groups [14]. Nevertheless, in no way at all does this mean that this state can be regarded as a molten-globule state [4]. The main argument against this is provided by the fact that a phase transition occurs between this state and the denatured form of the protein. The existence of molten globular states was assumed by Shakhnovich and Finkelstein [5] to explain the two-state mechanism of protein denaturation and there is no possibility of observation for this mechanism when the protein undergoes transition from this state to the coil-like one [20–22].

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