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CATION BINDING EFFECTS ON THE pH, THERMAL AND UREA DENATURATION TRANSITIONS IN α-LACTALBUMIN

Eugene A. PERMYAKOV, Ludmila A. MOROZOVA and Edward A. BURSTEIN

Institute of Biological Physics, U.S.S.R. Academy of Sciences, Pushchino, 142292 Moscow Region, U.S.S.R.

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The binding of monovalent (Na^+, K^+) and divalent (Ca^{2+}, Mg^{2+}) cations to bovine α-lactalbumin at 20 and 37°C has been studied by means of instrinsic protein fluorescence. The values of apparent binding constants for these ions obtained at 37°C are about one order of magnitude lower than those measured at 20°C. Urea and alkali (pH > 10) induce unfolding transitions which involve stable partially unfolded intermediates for all metal ion-bound forms of α-lactalbumin. Heating induces similar partially unfolded states. Nevertheless, the partially unfolded states induced by heating, urea, alkaline or acidic treatments are somewhat different in their tryptophan residue environment properties. The results have been interpreted in terms of a simple scheme of equilibria between metal-free and metal-bound forms in their native, partially unfolded and unfolded states. The scheme provides an approach to the quantitative interpretation of any transition equlibrium shift induced by a low molecular mass species able to be bound by a protein.

1. Introduction

α-Lactalbumin is one of the two components of the lactose synthetase system which catalyzes the final step in lactose biosynthesis in the lactating mammary gland [1]. Molecular conformations of α-lactalbumin have been extensively studied. In numerous papers the existence of stable intermediates in the folding and unfolding transitions of bovine and human α-lactalbumins has been shown [2–9]. For example, guanidine hydrochloride and urea induce an unfolding transition which involves a stable intermediate. This intermediate is very similar to the partially unfolded states produced by acid and alkaline transitions in α-lactalbumin. A similar partially unfolded conformer is formed above 60°C [10].

Recently, it has been shown that α-lactalbumin is a calcium metalloprotein [11]. Our previous studies [12,13] and those of Kronman et al. [14]. Murakami et al. [15] and Segawa and Sugai [16] have shown that α-lactalbumin posesses a high-affinity binding site for divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}) and that the binding causes a conformational change which results in a transfer of some exposed tryptophan residues from the protein surface to a rigid nonpolar interior of the protein macromolecule. This change closely resembles those that occur during the acid transition of the native protein. Our earlier results [12] allowed us to suggest that the well known acid transition in α-lactalbumin is in fact due to a competitive cooperative replacement of the bound Ca^{2+} by three H^+ ($pK = 5.0 \pm 0.1$) at the Ca^{2+}-binding site: the fluorometric pH-titration curve for α-lactalbumin shifts to lower pH values with increase of total Ca^{2+} concentration. Hiraoka et al. [11] have demonstrated the effect of Ca^{2+} binding on the resistance of bivine α-lactalbumin to thermal and guanidine hydrochloride denaturation. Thus, the position of the transitions of α-lactalbumin from the native state to partially unfolded and unfolded

states on temperature, pH or denaturant concentration scales strongly depends upon Ca^{2+} concentration. These new data forced us to revise some conclusions concerning the nature of the conformational transitions in α-lactalbumin taking into consideration the effects of cation binding.

Here we present some results of a fluorometric study of the binding of divalent (Ca^{2+}, Mg^{2+}) and monovalent (Na^+, K^+) cations to bovine α-lactalbumin at different temperatures. The apparent binding constants for these ions have been evaluated and the stability of the metal-free and metal-bound states of α-lactalbumin during temperature, urea, acid and alkali treatment has been studied. Urea and alkali induce unfolding transitions which involve stable intermediates for all metal forms of the protein. Temperature induces similar stable partially unfolded states. The results of these experiments can be interpreted in terms of a simple scheme of equilibria between metal-free and metal-bound native, partially unfolded and unfolded states of the protein.

2. Materials and methods

Bovine α-lactalbumin, prepared as described by Kaplanas and Antanavicius [17], was kindly supplied by Dr. V.V. Yarmolenko (Kaunas Medical Institute, Kaunas, U.S.S.R.). The protein concentrations were evaluated spectrophotometrically using $E_{1\,cm,1\%} = 20.1$ at 280 nm [18]. The metal-free α-lactalbumin was prepared according to Blum et al. [19]. All solutions were made using deionized water. Only plastic ware was used in this work. Total calcium content in buffers and salt preparations was estimated by atomic absorption spectrophotometry.

Fluorescence measurements were performed with a laboratory-made spectrofluorimeter described earlier [20]. All fluorescence spectra were corrected for the spectral sensitivity of the instrument. Intensities in the corrected spectra are proportional to the number of photons emitted per unit wavelength interval.

Protein fluorescence quantum yield was evaluated by comparing areas under corrected fluorescence spectra of a protein sample with that of aqueous tryptophan (quantum yield 0.23 at 20°C [21]) with the same absorbance at the excitation wavelength (280.4 nm). The position of the middle of a chord drawn at the 80% level of the maximal intensity ($\bar{\lambda}$) was taken as a measure of the spectral position.

The temperature dependences of the fluorescence parameters were measured using thermostatically controlled water circulating in the hollow brass cell-holder. The temperature in the cell was measured with a copper-constantan thermocouple covered by polyethylene film. The rate of the cell heating was about 1°C per min.

Fitting of the experimental data with theoretical curves was done using a non-linear regression scheme (Marquardt's algorithm) [22] on an M-4030 computer. The set of EGTA-Ca^{2+} and EGTA-Mg^{2+} binding constants of Schwarzenbach and Flaschka [23] was used in our calculations.

3. Results

3.1. The binding of cations to α-lactalbumin at 20 and 37°C

3.1.1. Ca^{2+} and Mg^{2+}

In our previous work [12,13] we evaluated the apparent Ca^{2+} and Mg^{2+} binding constants of α-lactalbumin at 20°C and pH 8.0 using the cation-induced changes of tryptophan fluorescence of the protein. The results are presented in table 1. This table also contains the apparent Ca^{2+} and Mg^{2+} binding constants of α-lactalbumin measured by the same method at 37°C.

3.1.2. Na^+ and K^+

Fig. 1 shows the dependence of the protein spectrum position, λ, quantum yield, q, and fluorescence intensity at 360 nm upon NaCl and KCl concentrations at 37°C. The titration with Na^+ or K^+ results in a shift of the fluorescence spectrum towards shorter wavelengths by 2–3 nm and a slight decrease in the fluorescence yield. The Na^+ and K^+-induced spectral changes at 20°C are more pronounced. The spectral changes seem to reflect some changes in the environment of tryptophan residues in α-lactalbumin caused by rather

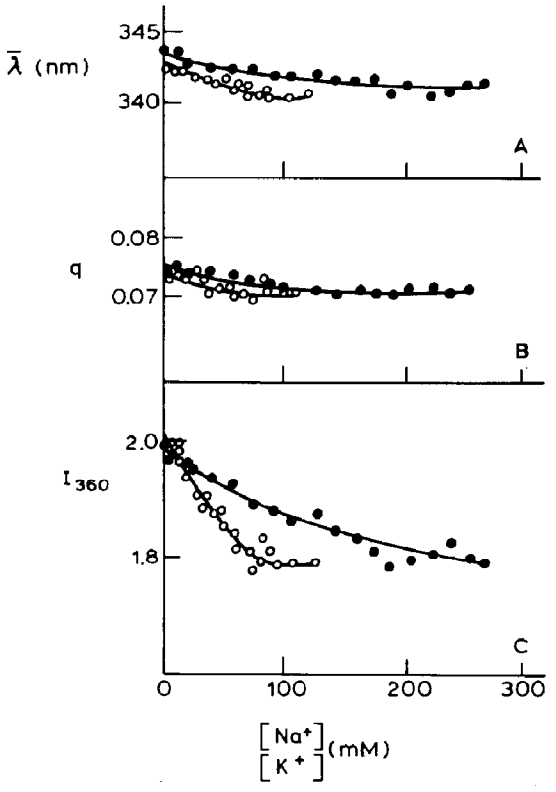

Fig. 1. Titration of bovine α-lactalbumin with Na^+ (○) and K^+ (●) at 37°C. Protein concentration 15 μM; 1 mM EGTA; 50 mM Hepes, pH 8.0. (A) Spectrum position, (B) fluorescence yield, (C) fluorescence intensity at 360 nm.

subtle conformational rearrangement induced by the ion binding. The apparent Na^+ and K^+ binding constant were evaluated from the I_{360} vs. NaCl or KCl concentration plots assuming that only one binding site exists for the monovalent cations on the protein molecule. The values of the constants obtained at 20 and 37°C are summarized in table 1. As for Ca^{2+} and Mg^{2+} binding the constants for the monovalent cations measured at 37°C are lower than those determined at 20°C.

Table 1

The apparent equilibrium constants for the binding of Ca^{2+}, Mg^{2+}, Na^+ and K^+ to bovine α-lactalbumin at 20 and 37°C (50 mM Hepes, pH 8.0)

Cation	Association constant, K_{app} (M^{-1})	
	37°C	20°C
Ca^{2+}	$\log K_{app} = 7.3 \pm 0.5$	$\log K_{app} = 8.6 \pm 0.5$
Mg^{2+}	211 ± 20; 46 ± 10	2000 ± 100; 200 ± 20
Na^+	36 ± 10	100 ± 10
K^+	6 ± 3	8 ± 3

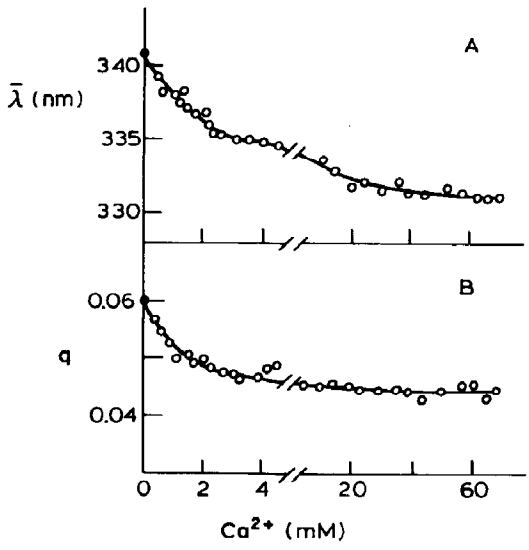

Fig. 2. Titration of bovine α-lactalbumin with Ca^{2+} at pH 2.9. Protein concentration 20 μM; 50 mM glycine; 20°C. (A) Spectrum position, (B) fluorescence quantum yield.

3.1.3. *The binding of Ca^{2+} at pH 2.9*

In our earlier study [12] we assumed that the acid conformational change in α-lactalbumin which has been studied by many authors with various techniques (see, e.g., refs. 7 and 10) could in fact be caused by a competitive removal of the bound Ca^{2+} by protons:

$$\text{Protein} + Ca^{2+} \overset{K_{Ca}}{\rightleftharpoons} \text{protein} \cdot Ca$$

$$\text{Protein} + nH^+ \overset{pK}{\rightleftharpoons} \text{protein} \cdot H_n \qquad (1)$$

An increase in the total Ca^{2+} concentration shifts the pH-titration curve for α-lactalbumin to lower pH values. Fig. 2 shows that the titration of α-lactalbumin with Ca^{2+} at pH 2.9 (20°C), where the protein is in its so-called acid form, causes the same spectral changes as those induced by the Ca^{2+} binding at pH 8.0, but at much higher Ca^{2+}

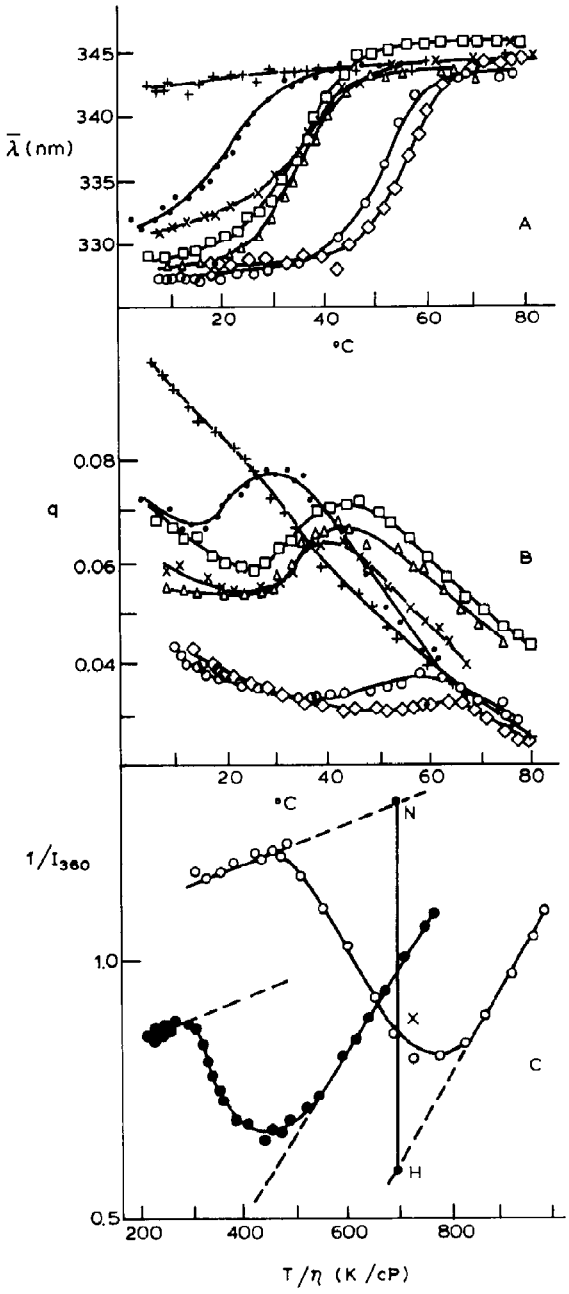

Fig. 3. Temperature dependence of spectrum position (A) and fluorescence quantum yield (B) for different metal ion forms of bovine α-lactalbumin. Protein concentration $P_0 = 15$–25 μM; 50 mM Hepes, pH 8.0. (○) Ca^{2+} form (Ca^{2+} concentration $C_0 = 1.5P_0$); (◇) Ca^{2+} form ($C_0 = 10P_0$); (×) Mg^{2+} form (12 mM $MgCl_2$, 1 mM EGTA); (△) Na^+ form (210 mM NaCl, 1 mM EGTA); (□) K^+ form (970 mM KCl, 1 mM EGTA); (●) apo form (0.35 mM EGTA); (+) acid form (pH 2.5. 50 mM glycine). (C) The plots of reciprocal fluorescence intensity at 360 nm vs. T/η (T, temperature; η, solvent viscosity) for two forms of α-lactalbumin. Protein concentration $P_0 = 20$ μM; 50 mM Hepes, pH 8.0. (○) Ca^{2+} form ($C_0 = 1.5P_0$); (●) apo form (0.35 mM EGTA).

concentrations. The apparent Ca^{2+} binding constant at pH 2.9 is about 1×10^3 M^{-1}. The data for fluorescence quantum yield in fig. 2 can also be fitted by the theoretical curve computed according to scheme 1. The best fit is achieved when $n = 2.9$ and $pK = 5.0$ which is in good agreement with our previous results [12].

3.2. Thermal stability of different metal ion forms of α-lactalbumin

The heating of α-lactalbumin from 5 to 75°C results in a shift of its fluorescence spectrum maximum from 327–331 to 344–346 nm. However, the position of the shift depends strongly upon the ionic form of the protein (fig. 3A). The progress of the thermally induced transition is also well seen in plots of either fluorescence quantum yield or intensity vs. temperature (fig. 3B), but the changes of these parameters are developed on the background of a common thermal quenching of the fluorescence which reflects the activation of intramolecular collisions between excited chromophores and neighbouring quenching groups [24,25].

The fluorescence intensity at a constant wavelength λ for a given temperature T is

$$F_\lambda = (1 - \Delta)(F_\lambda)_{N,T} + \Delta(F_\lambda)_{H,T} \qquad (2)$$

where the subscripts N and H refer to the native and 'high' temperature conformers (according to the terminology of Sommers and Kronman [10]), Δ is the fractional conversion of the N to the H form and $(F_\lambda)_{N,T}$ and $(F_\lambda)_{H,T}$ represent the fluorescence intensities of the two conformers at the temperature T of measurement.

For elimination of the thermal quenching effects we have used the finding of Bushueva et al. [25,26] that the temperature dependence of the fluorescence quantum yield q (or intensity, I) for native proteins containing a single fluorescent chromophore within the non-denaturing tempera-

Table 2

The apparent parameters of the thermal transitions in the different metal ion forms of α-lactalbumin evaluated according to the Van't Hoff treatment of the curves shown in fig. 4 and the positions of their fluorescence spectra before (λ_N) and after (λ_H) the thermal transitions. Conditions are as indicated in fig. 3.

Initial form of α-lactalbumin	T_m (°C)	ΔH (kcal/mol)	ΔS (cal/mol per K)	λ_N (nm) (±0.5 nm)	λ_H (nm) (±0.5 nm)
Ca^{2+}-loaded, $C_0 = 1.5P_0$	58±1	48± 5	146±10	328	344
Ca^{2+} – loaded, $C_0 = 10P_0$	62±1	57± 5	174±10	328	344
Mg^{2+}-loaded	45±1	40± 5	126±10	331	345
Na^+-loaded	40±1	38± 5	124±10	328	344
K^+-loaded	41±1	40± 5	128±10	329	346
Apo form	31±1	37± 5	124±10	332	343
Acid form (pH 2.5)	32±2	31±10	102±20	342	344

ture range can be described by the equation

$$1/q = a + bT/\eta \qquad (3)$$

where a and b are temperature-independent constants, T the temperature (K) and η the solvent viscosity (cP). Fig. 3C presents the $1/I_{360}$ vs. T/η plots for two ionic forms of α-lactalbumin. Despite the fact that α-lactalbumin contains four tryptophan residue per molecule, the plots in the temperature regions below and above the thermally indiced transition are straight lines. Thus, $(F_\lambda)_{N,T}$ and $(F_\lambda)_{H,T}$ can be determined from an extrapolation of the linear parts of the $1/I_{360}$ vs. T/η plots to the thermal transition region ($\Delta(T_X) = [NX][H]/[NH][X]$). Fig. 4 shows the thermally induced transition curves (Δ vs. temperature) for α-lactalbumin containing either Ca^{2+}, Mg^{2+}, Na^+, K^+ or no metal (in the presence of EGTA). Van't Hoff plots obtained for these data were linear. The values of the apparent transition en-

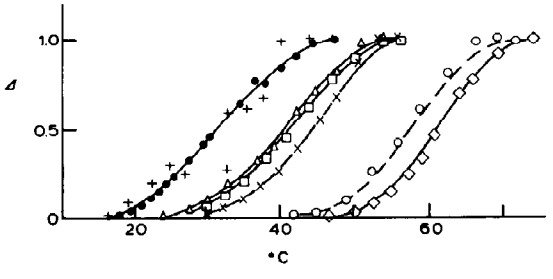

Fig. 4. The temperature dependence of the fraction of conversion from native to thermally unfolded state for different forms of α-lactalbumin. Designation as in fig. 3.

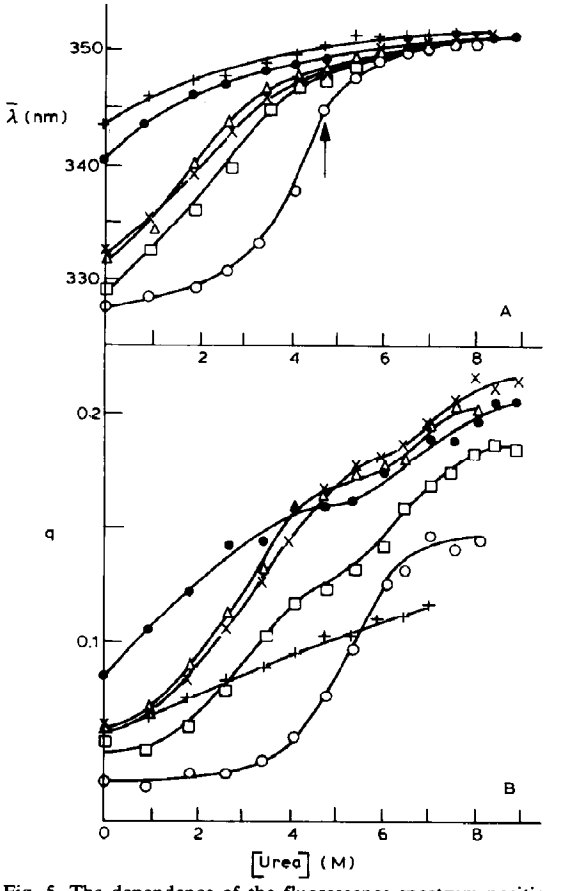

Fig. 5. The dependence of the fluorescence spectrum position (A) and quantum yield (B) of different α-lactalbumin forms upon urea concentration. Protein concentration $P_0 = 15$–$20\ \mu M$. 50 mM Hepes, pH 8.0; 20°C. (○) Ca^{2+} form ($C_0 = 1.5P_0$); (×) Mg^{2+} form (10 mM $MgCl_2$, 1 mM EGTA); (△) Na^+ form (184 mM NaCl, 1 mM EGTA); (□) K^+ form (1 M KCl, 1 mM EGTA); (●) apo form (0.35 mM EGTA); (+) acid form (pH 2.9).

thalpy (ΔH), entropy (ΔS) and midpoint temperature (T_m) are collected in table 2.

3.3. Denaturation of bovine α-lactalbumin by urea

Fig. 5 shows the dependences of the fluorescence spectrum position and quantum yield of different metal ion forms of α-lactalbumin upon urea concentration at 20°C. The increase of urea concentration results in a long wavelength shift of the protein fluorescence spectrum to 351 nm (i.e., almost to the position intrinsic to free aqueous tryptophan) and in a pronounced increase of its fluorescence quantum yield. The two-stepped quantum yield increase (fig. 5B) indicates the existence of intermediate states of the protein. The intermediate is not evident for the Ca^{2+}-loaded protein; however, the corresponding fluorescence phase-like plot [24] suggests its existence in this case as well. In all cases studied the maximal population of the intermediate exists at approx. 4–5 M urea.

Fig. 6 shows the results of the calcium titration of α-lactalbumin in the presence of 8.9 M urea. The gradual increase in calcium concentration pro-

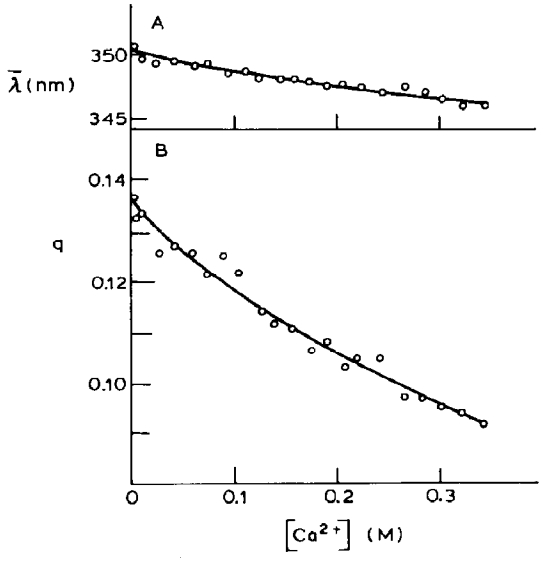

Fig. 6. Titration of α-lactalbumin with Ca^{2+} in the presence of 8.9 M urea. Protein concentration $P_0 = 19$ μM. (A) Spectrum position, (B) fluorescence quantum yield.

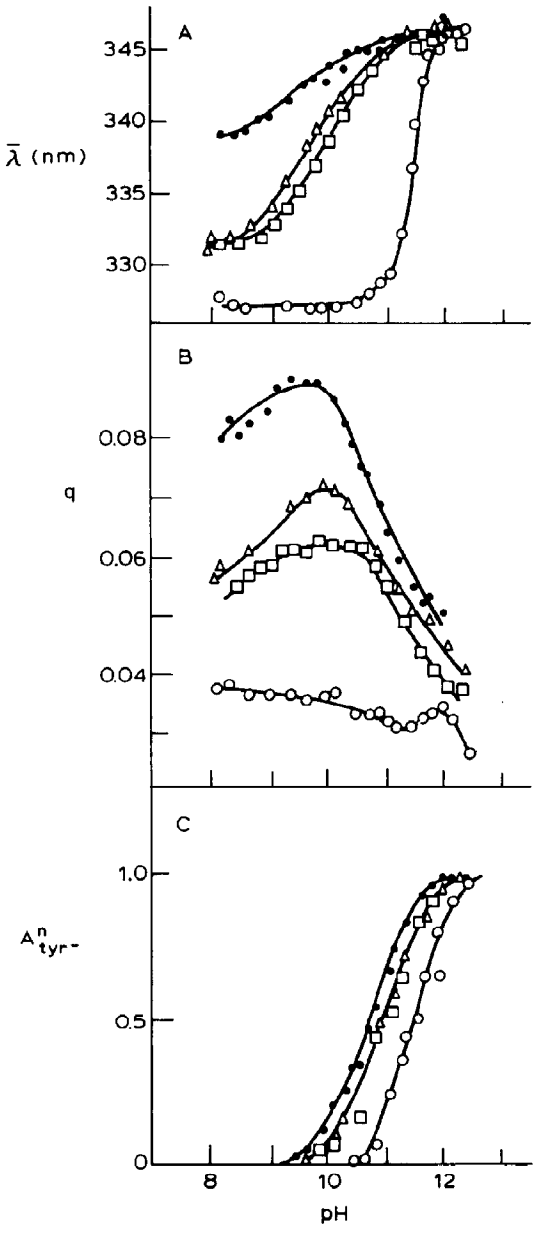

Fig. 7. pH dependences of the fluorescence spectrum position (A), quantum yield (B) and normalized absorption at 300 nm (tyrosinate) (C) for different metal ion forms of α-lactalbumin. Protein concentration 17–23 μM. (○) Ca^{2+}-loaded protein ($C_0 = 1.5P_0$); (△) Na^+ form (373 mM NaCl, 1 mM EGTA); (□) K^+ form (990 mM KCl, 1 mM EGTA); (●) apoprotein (1 mM EGTA).

duces changes in the fluorescence parameters which are opposite to those induced by an increase in urea concentration. However, the effective Ca^{2+} binding constant in this case is very low and we could not approach the characteristics of the spectrum inherent to the calcium-loaded state of α-lactalbumin in the absence of urea even at very high calcium concentrations (up to 0.35 M). Nevertheless, the data obtained mean that the effective parameters of the urea denaturation of α-lactalbumin are strongly dependent upon the total metal ion concentration in the solution.

3.4. Alkaline denaturation of α-lactalbumin

Fig. 7 depicts the pH dependence of the fluorescence spectrum characteristics of different metal ion forms of α-lactalbumin at 20°C. The shift of the fluorescence spectrum to longer wavelengths could be due to alkaline denaturation of the protein (fig. 7A). The fluorescence quantum yield vs. pH plots (fig. 7B) suggest the existence of intermediate states in the course of the alkali-induced denaturation. This is clearly corroborated by the fluorescence phase-like plots (fig. 8). The figures in fig. 8 denote pH values at which the maximal populations of the intermediates are observed. The straight line extrapolated to the origin corresponds to fluorescence quenching by OH^- at higher pH values without any conformational change and two other parts of the plots are due to two successive structural changes in α-lactalbumin which seem to be initiated mostly by a deprotonation of lysyl and tyrosyl side chains.

Deprotonation of tyrosyl side chains correlates with the second pH-induced conformational change. The appearance of the tyrosinate is well seen in the protein absorption at 300 nm (fig. 7c).

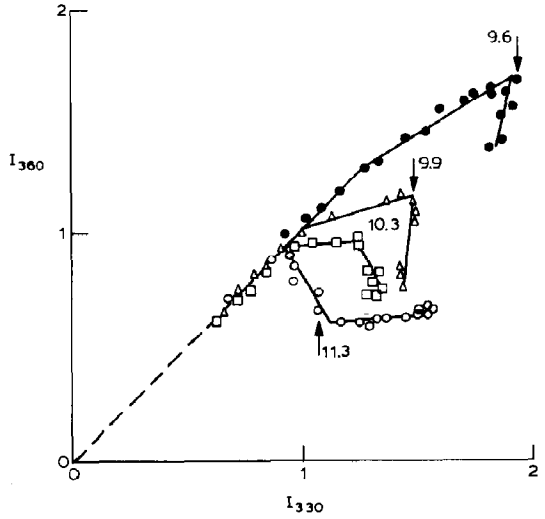

Fig. 8. Fluorescence phase-like plots corresponding to the pH dependences of the fluorescence parameters for α-lactalbumin in different metal ion forms represented in fig. 7. Designations as in fig. 7.

4. Discussion

An analysis of the metal ion-induced changes in the bovine α-lactalbumin fluorescence spectra shows that they are caused by a conformational change in the protein molecule, which results in a transfer of at least one exposed tryptophan residue from the protein surface to a more hydrophobic interior of the protein globule [12,13,24]. Ca^{2+} induces the most significant conformation change. At 20°C the spectral effects are more pronounced than those at 37°C. The value of the apparent Ca^{2+}-binding constant for α-lactalbumin is in the same range as those for high-affinity sites in such typical Ca^{2+}-binding proteins as parvalbumin [27], troponin C [28,29] and calmodulin [30,31]. However the values of the apparent Mg^{2+}-binding constants for α-lactalbumin are much lower than those for parvalbumin, troponin C and calmodulin [28,30]. Na^+ and K^+ associate with α-lactalbumin still more weakly but, taking into consideration their high concentrations in any cell, one can assume that Na^+ and K^+ at 'physiological' concentrations can compete successfuly with Mg^{2+} for the same binding sites. Our experiments on the effects of Na^+ and K^+ on the binding of Ca^{2+} to α-lactalbumin show that these monovalent cations, even at 'physiological' concentrations, are not able to compete successfully with Ca^{2+} for the same site because of the very great difference in their affinities.

Fig. 5 shows that the metal ion titration of apoprotein at 20°C results in a transition from the

native state of the apoform to the native state of the metal ion-loaded protein while the metal ion titration of apoprotein at 37°C induces a transition between the states which are mixtures of the native and thermally denatured states of apo and metal ion-bound forms of the protein in the cases of Mg^{2+}, K^+ and Na^+ and a transition into the native metal ion-loaded state in the case of Ca^{2+}. Therefore, the metal ion binding constants measured in this work and presented in table 1 are apparent constants.

The equilibrium scheme of the binding of one metal ion (Me) to the α-lactalbumin molecule taking into consideration an equilibrium between native (P, PMe) and thermally changed (P', P'Me) states of the protein is:

$$\begin{array}{ccc} P + Me & \overset{K_{Me}}{\rightleftharpoons} & PMe \\ \alpha \downarrow \uparrow & & \downarrow \uparrow \beta \\ P' + Me & \overset{K'_{Me}}{\rightleftharpoons} & P'Me \end{array} \qquad (4)$$

where K_{Me} and K'_{Me} are intrinsic metal ion binding constants for the native and thermally denatured protein, respectively, and α and β are equilibrium constants of the thermal denaturation of the protein in its apo and metal ion-loaded forms, respectively.

$$K_{Me} = \exp\left[-(\Delta H_{Me} - T\Delta S_{Me})/RT\right] \qquad (5)$$

$$K'_{Me} = \exp\left[-(\Delta H'_{Me} - T\Delta S'_{Me})/RT\right] \qquad (6)$$

$$\alpha = \exp\left[-(\Delta H_{\alpha} - T\Delta S_{\alpha})/RT\right] \qquad (7)$$

$$\beta = \exp\left[-(\Delta H_{\beta} - T\Delta S_{\beta})/RT\right] \qquad (8)$$

ΔH_{Me}, $\Delta H'_{Me}$ and ΔS_{Me}, $\Delta S'_{Me}$ are enthalpy and entropy changes for the metal ion binding to the native and thermally denatured protein. ΔH_{α}, ΔH_{β} and ΔS_{α}, ΔS_{β} are enthalpy and entropy changes for the thermal transitions in the apo and metal ion-loaded protein. ΔH_{α} and ΔS_{α} were still determined from the experiment on the thermal denaturation of the apoprotein, while ΔH_{β} and ΔS_{β} were determined from the thermal denaturation curve for the metal ion-loaded protein (table 2).

The apparent metal ion binding constant determined from the fluorescence experiment (table 1) is

$$K_{app} = \frac{[PMe] + [P'Me]}{([P] + [P'])[Me]} = K_{Me} \cdot \frac{1 + \beta}{1 + \alpha} \qquad (9)$$

while

$$K_{Me}/K'_{Me} = \alpha/\beta \qquad (10)$$

The values of K_{Me} and K'_{Me} at 20 and 37°C for Ca^{2+}, Mg^{2+} Na^+ and K^+ were estimated from eqs. 9 and 10. Using eqs. 5 and 6, ΔH_{Me}, $\Delta H'_{Me}$ and ΔS_{Me}, $\Delta S'_{Me}$ were evaluated (table 3). Knowledge of ΔH_{Me}, $\Delta H'_{Me}$, ΔS_{Me}, $\Delta S'_{Me}$ and ΔH_{α}, ΔH_{β}, ΔS_{α}, ΔS_{β} for Ca^{2+}, Mg^{2+}, Na^+ and K^+ allows us to calculate the thermal denaturation curve of α-lactalbumin in the presence of any given concentrations of the metal ion. The dotted curve in fig. 5 is a theoretical one computed for the calcium concentration $C_0 = 1.5P_0$ (P_0 protein concentration). Points are experimental ones measured at this calcium concentration. One can see very good agreement between the theoretical and experimental data.

Table 3

Thermodynamic parameters of metal ion binding to α-lactalbumin

ΔH_{Me}, $\Delta H'_{Me}$, ΔS_{Me}, $\Delta S'_{Me}$ and $\Delta G_{37°C}$, $\Delta G'_{37°C}$: are enthalpy, entropy and free energy changes for the metal ion binding to the native and thermally denatured protein, respectively.

Cation	Native protein			Thermally denatured protein		
	ΔH_{Me} (kcal/mol)	ΔS_{Me} (cal/mol per K)	$\Delta G_{37°C}$ (kcal/mol)	$\Delta H'_{Me}$ (kcal/mol)	$\Delta S'_{Me}$ (cal/mol per K)	$\Delta G'_{37°C}$ (kcal/mol)
Ca^{2+}	−17.5	−20.3	−11.2	2.8	29.1	−6.2
Mg^{2+}	−11.4	−23.8	−4.0	−8.9	−21.0	−2.4
Na^+	1.0	5.9	−0.8	0.2	6.2	−1.7
K^+	7.5	29.9	−1.8	10.0	34.3	−0.6

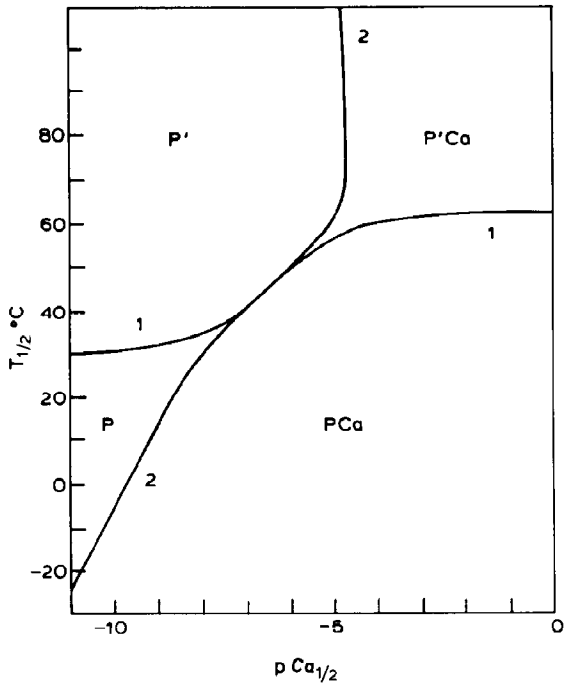

Fig. 9. Phase state diagram for bovine α-lactalbumin computed according to the thermodynamic parameters determined. Protein concentration 20 μM. (Curve 1) Dependence of the thermal transition midpoint (T_m) upon the logarithm of free Ca^{2+} concentration; (curve 2) temperature dependence of the logarithm of free Ca^{2+} concentration of the midpoint of the Ca^{2+}-induced transition ($pCa_{1/2}$).

Fig. 9 shows a state diagram of α-lactalbumin computed on the base of the knowledge of the thermodynamic parameters for the thermal and Ca^{2+}-induced transitions in it. One curve is the dependence of the thermal transition midpoint (T_m) upon the logarithm of free Ca^{2+} concentration, the other being the temperature dependence of the logarithm of the free calcium concentration at the midpoint of the Ca^{2+}-induced transition ($pCa_{1/2}$). One can see the regions of predominance of the different states of the protein.

Very recently, Segawa and Sugai [16] reported practically the same equilibrium scheme for bovine, human and goat α-lactalbumins, but they did not succeed in obtaining thermodynamic parameters. They evaluated the constants K_{Me} and α at 25°C (Me = Ca) for the three α-lactalbumins. There is a little disagreement of their data with our estimations. According to Segawa and Sugai, $K_{Ca} = (2.9 \pm 1.0) \times 10^9$ M^{-1} and $\alpha = 11.5 \pm 4.0$ at 25°C, while according to our data $K_{Ca} = 2.8 \times 10^8$ M^{-1} and $\alpha = 2.7$ for bovine α-lactalbumin, but taking into account the rather poor accuracy of such estimations one can conclude that this disagreement is not serious.

The fluorescence parameters of the thermally denatured states of α-lactalbumin (spectrum positions 343–346 nm) suggest their partially unfolded conformations. There are some tryptophan residues in these states which remain in the interior of the protein globule. Sommers and Kronman [10] came to the same conclusion for Ca^{2+}-loaded bovine, human and goat α-lactalbumins. The value of ΔH for the thermal transition in Ca^{2+}-loaded bovine α-lactalbumin obtained in our work (57 ± 5 kcal/mol) is comparable with values of 59 ± 4 and 50 kcal/mol obtained by Sommers and Kronman [10] and Barel et al. [32], respectively.

In our previous study [12] we assumed that the low pH conformational change in α-lactalbumin arises from a competition between protons and Ca^{2+} for the same binding site (scheme 1). The results of the present study support this assumption. The titration of the protein in acid form (pH 2.9) with Ca^{2+} shifts the competition equilibrium in favour of the Ca^{2+}-associated forms of the protein. The acid form of α-lactalbumin resembles the apo form of this protein. They have similar circular dichroism spectra in the far- and near-ultraviolet regions [33], and their temperature-induced transitions occur within the same temperature region and have similar thermodynamic parametes (fig. 4, table 2). However, these forms of α-lactalbumin show some differences. The fluorescence spectrum positions for the native states (at approx. 5°C) of the acid and apo forms of bovine α-lactalbumin are different. The spectrum of the native acid form has a more long-wavelength position (approx. 342 nm) than has the native apo form (approx. 332 nm) (fig. 3). This suggests that tryptophan residues in the native acid form are more exposed to water than those in the native apo form [24]. The temperature-induced spectral changes for the acid form (spectral shift approx. 2 nm) are much smaller than those for the apo form (spectral shift approx. 12 nm). The denaturation of

the acid form by urea is a two-state transition while the urea-induced unfolding transition in the apo form of α-lactalbumin involves a stable intermediate (fig. 5). Such findings led us to conclude that in spite of some similarities between the acid and apo forms of bovine α-lactalbumin, there are some significant differences between their structures which seem to be caused by the protonation of acid form carboxylates, some of which are located at the metal ion binding site.

Figs. 4–8 show that the denaturation processes in various ionic forms of bovine α-lactalbumin induced by urea and alkali pass through stable intermediates. Therefore, the equilibrium scheme accounting for the metal ion binding for such an unfolding process at low temperatures (below the temperature-induced transition) is

$$\begin{array}{ccc} P + Me & \overset{K_{Me}}{\rightleftharpoons} & PMe \\ \alpha_1 \downarrow\uparrow & & \downarrow\uparrow\ \beta_1 \\ P' + Me & \overset{K'_{Me}}{\rightleftharpoons} & P'Me \\ \alpha_2 \downarrow\uparrow & & \downarrow\uparrow\ \beta_2 \\ P'' + Me & \overset{K''_{Me}}{\rightleftharpoons} & P''Me \end{array} \qquad (11)$$

where P′ and P′Me are apo and metal ion-loaded intermediates; P″ and P″Me are apo and metal ion-bound more unfolded states of the protein. α_1, α_2 and β_1, β_2 are constants of equilibrium between native, intermediate and denaturated states of apo and metal ion-loaded forms of the protein at a given urea concentration (or at given pH value).

This scheme (eq. 11) is not complete, since each state in it is in fact in equilibrium with the corresponding high temperature conformer.

Urea and alkaline pH shift the equilibria towards the unfolded conformers of α-lactalbumin while metal ions shift the equilibrium towards the folded conformers of the protein. This is clearly seen from the experiment on the Ca^{2+} titration of the protein in 8.9 M urea.

The intermediates arising in urea and alkaline denaturations seem to have different structures. The longer wavelength position of the fluorescence spectra for the urea-induced intermediates suggests that their tryptophan residues are more exposed to solvent than the chromophores in the alkali-induced intermediates.

The results of the present work clearly show that the simple scheme of denaturation of α-lactalbumin by urea, guanidine hydrochloride, alkali and other denaturants used by many authors N ⇌ I ⇌ D (N, native molecule; D, completely disordered conformation; I, partially denatured conformation) is not valid in some cases, especially when the pH or thermal transitions are investigated in the presence of a denaturant [5,8], since in such systems all sets of the apo, and metal ion-loaded states of the scheme (eq. 11) are present. This scheme is reduced to the three-state one only for the apoprotein or for the metal ion-loaded protein in the presence of a great molar excess of the metal ions. The simultaneous action of two or more denaturing factors complicates the situation, since the partially unfolded conformations of α-lactalbumin induced by different denaturing factors are somewhat different.

It should be noted that the approach presented here based on a consideration of the complex equilibria seems to be suitable not only for the metal ion-binding proteins, but also for the quantitative interpretation of any transition equilibrium shifts induced by a low molecular mass species able to be bound by a protein.

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