Effects of pH, Temperature and Ca^{2+} Content on the Conformation of α-lactalbumin in a Medium Modelling Physiological Conditions

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Abstract. Data obtained by the intrinsic protein fluorescence technique showed that, in addition to Ca^{2+} and Mg^{2+} ions, bovine α-lactalbumin also binds physiologically significant Na^+ and K^+ ions, the nucleotides ATP, ADP, UTP, UDP and UDP-galactose. The release of the bound Ca^{2+} ions from the protein in a medium modelling physiological conditions (containing Mg^{2+}, Na^+, K^+, ATP and ADP in physiological concentrations) induced a transition of the protein from the native state of the Ca^{2+}-loaded form to a state which is a mixture of native and thermally changed states of the apo- and metal bound forms. Any variations in temperature result in changes in the populations of these states. This may by associated with some Ca^{2+} and temperature dependent regulation of the protein function. Variations of pH within the physiological limits had little influence on the conformation of both Ca^{2+}-loaded and Ca^{2+}-free α-lactalbumin.

Key words: α-lactalbumin — Cation binding — Thermal transition — Fluorescence

Introduction

Alpha-lactalbumin is the noncatalytic regulatory subunit of the lactose synthase enzyme system which catalyzes the final step in lactose biosynthesis in the lactating mammary gland (Brew et al. 1968):

UDP-galactose + glucose → lactose + UDP

The catalytic subunit of the system is galactosyltransferase. α-lactalbumin promotes binding of glucose to galactosyltransferase through an association with the enzyme to form a 1:1 complex. α-lactalbumin is homologous in amino acid sequence and probably in conformation to lysozyme (Brew et al. 1970; Brown et

Abbreviation: EGTA — Ethylene glycol bis(2-aminoethylether)N,N'-tetraacetic acid

al. 1969; Warme et al. 1974). Hiraoka et al. showed in 1980 that α-lactalbumin is a calcium metalloprotein. Permyakov et al. (1981b) and then Kronman et al. (1981); Murakami et al. (1982) and Segawa and Sugai (1983) demonstrated that the binding of one Ca^{2+} ion to bovine α-lactalbumin molecule results in a conformational change reflected in very pronounced changes of the tryptophan residues environment. The apparent calcium binding constant evaluated from fluorometric EGTA-titration data at 20 °C (Permyakov et al. 1981b; Segawa and Sugai 1983) was determined to be approx. 3×10^8 l/mol. This strong site also binds Mn^{2+}, Cd^{2+}, Mg^{2+} albeit with a weaker affinity (Kronman et al. 1981; Permyakov et al. 1981a; Murakami et al. 1982). Apart from this site, α-lactalbumin has a distinct zinc binding site which also associates with Al^{3+}, Co^{2+} and Cu^{2+} (Murakami and Berliner 1983).

The binding of divalent cations to α-lactalbumin results in an increase in the protein resistance to thermal, pH and guanidine-HCl denaturation (Hiraoka et al. 1980; Permyakov et al. 1981b; Segawa and Sugai 1983). The position of the transitions of α-lactalbumin from the native to partially unfolded states on temperature, pH or denaturants concentration scales strongly depends upon Ca^{2+} concentration. Since in the absence of metal ions the thermal transition of α-lactalbumin occurs within a temperature range from approx. 20 to approx. 50 °C, i.e. near the physiological temperatures, it may be assumed that parameters such as temperature, pH and Ca^{2+} concentration can control the conformation of α-lactalbumin in vivo.

In view of this the aim of the present work was to study effects of "physiological" temperatures, pH and Ca^{2+} concentration on the conformation of α-lactalbumin. Changes in the protein conformation were monitored by the intrinsic tryptophan fluorescence of α-lactalbumin. Since all the living cells contain rather high concentrations of Na^+ and K^+ ions, it is important to investigate possible interactions of α-lactalbumin with these ions. In addition to Ca^{2+}, Mg^{2+}, Na^+ and K^+ ions, α-lactalbumin could also bind nucleotides ATP, ADP, UPT and UDP and UDP-galactose. For this reason the experiments on the thermal denaturation of the protein were carried out in a medium modelling physiological conditions, i.e. containing "physiological" concentrations of Mg^{2+}, Na^+, K^+, ATP and ADP.

Materials and Methods

Bovine α-lactalbumin prepared as described by Kaplanas and Antanavichius (1975) was kindly supplied to us by Dr. V. V. Yarmolenko (Kaunas Medical Institute, Kaunas, USSR). Protein concentrations were evaluated spectrophotometrically using $E_{1\,cm,\,1\,\%} = 20.1$ at 280 nm (Kuwajima and Sugai 1978).

All solutions were made with deionized water. Only plastic ware was used in this work.

EGTA, HEPES (from Sigma), ATP and ADP (from Reanal) were used without further purification.

Fluorescence measurements were performed with a laboratory-made spectrofluorimeter described earlier (Permyakov et al. 1977). The fluorescence was collected from the front surface of the cell. The emission spectra were corrected for the instrumental spectral sensitivity. Corrections for the effects of screening and reabsorbing inner filter were made using the derived correction factor (Burstein 1968) for each fluorescence wavelength λ:

$$w(\lambda)=\frac{1-T_p}{1-T_pT_eT_r}\cdot\frac{D_p+D_e+D_r}{D_p}$$

where T and D are transparency and absorbance ($T=10^{-D}$), respectively. Subscripts p and e refer to protein and screening agents (nucleotides) at the excitation wavelength (296.7 nm), respectively; r refers to reabsorbing agents (nucleotides as well) at the fluorescence wavelength λ. The protein fluorescence quantum yield, q, was evaluated by comparing areas under emission spectra of a protein sample with that of aqueous tryptophan solution (quantum yield 0.23 at 20 °C) (Teale and Weber 1957) with the same absorbance at the excitation wavelength. The position of the middle of a chord drawn at the 80 % level of the maximal intensity ($\bar{\lambda}$) was taken as the position of the spectrum.

The temperature in the thermostatted cell of the instrument was measured by means of a copper-constantan thermocouple with an accuracy of approx. 1°. The heating rate was approx. 1 K . min^{-1}.

Ultraviolet absorption spectra were recorded with a Specord UV-VIS spectrophotometer (Carl Zeiss, Jena).

Fitting of the experimental data with theoretical curves was carried out with an M-4030 computer using a nonlinear regression scheme (Marquardt's algorithm) (Reich et al. 1972).

Results

1. *Binding of divalent and monovalent cations to α-lactalbumin*

The apparent Ca^{2+} and Mg^{2+} binding constants of α-lactalbumin measured at 20 °C and 37 °C using metal ion-induced changes in tryptophan fluorescence of the protein are shown in Table 1. Fig. 1 shows the relationship between the fluorescence parameters of α-lactalbumin and the concentration of NaCl and KCl at 20 °C.

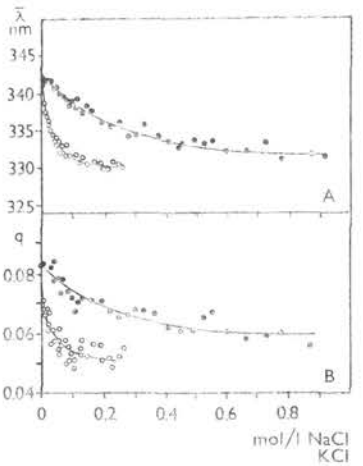
Table 1. Apparent equilibrium constants for the binding of Ca^{2+}, Mg^{2+}, Na^+ and K^+ ions to bovine α-lactalbumin at 20 °C and 37 °C. 50 mmol/l HEPES, pH 8.0.

Cation	Association constant, K_{app}, l/mol	
	37 °C	20 °C
Ca^{2+}	$\log K_{app}=7.3\pm0.5$	$\log K_{app}=8.6\pm0.5$
Mg^{2+}	210 ± 20, 46 ± 10	2000 ± 100, 200 ± 20
Na^+	36 ± 10	100 ± 10
K^+	6 ± 3	8 ± 3

Similar experiments were also carried out at 37 °C (the results are not shown). To prevent the metal-free protein from being contaminated by calcium ions the measurements were performed in the presence of 0.2 mmol/l EGTA. Titration of

the protein with Na^+ and K^+ ions resulted in spectral changes which were qualitatively similar but quantitatively smaller than those induced by Ca^{2+} or Mg^{2+} binding. These ions induced a shift in the fluorescence spectrum towards shorter wavelenghts by 8—10 nm and a decrease in the fluorescence yield. The Na^+- and K^+-induced spectral changes at 37 °C were less pronounced. The spectral changes seem to reflect some changes in the environment of tryptophan residues in α-lactalbumin due to a conformational rearrangement induced by ion binding. It would be reasonable to suppose that the spectral changes induced by Na^+ and K^+ ions are in fact due to the effect of the ionic strength, i.e. collapse of the protein structure as repulsive electric charges are screened by the electrolyte, however, this is not the case since NaCl and KCl cause quantitatively different changes.

The results shown in Fig. 1 do not permit stoichiometry determination of the Na^+ and K^+ binding; however some conclusions can be drawn from the fluorescence phase plots (Burstein 1977) which are shown in Fig. 2. The fluorescence phase plot, i.e. the relationship between fluorescence intensity at a fixed emission wavelength and the intensity at any other fixed wavelength, has to be a segment of a stright line (as in the case of K^+-titration) for a transition between two states. In a more complex case, where the transition passes through an intermediate, the phase plot has a more or less pronounced bend or break just as in the case of Na^+-titration. It is reasonable to assume that the two stright-linear perts in the phase plot for Na^+-titration correspond to the binding of at least two Na^+ ions to α-lactalbumin.



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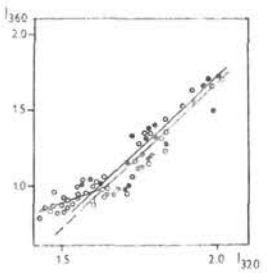

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Fig. 1. Na^+- and K^+-titration of α-lactalbumin. *A* — fluorescence spectrum position; *B* — fluorescence quantum yield. 50 mmol/l HEPES, pH 8.0; 0.2 mmol/l EGTA; 20 °C. Protein concentration 0.015 mmol/l. ○ — NaCl; ● — KCl.

Fig. 2. Fluorescence phase plots corresponding to Na^+- and K^+-titration of α-lactalbumin. For conditions, see Fig. 1. ○ — NaCl; ● — KCl.

The apparent Na^+ and K^+ binding constants were evaluated from the fitting of q versus NaCl or KCl concentration plots by theoretical curves, computed on the assumption of the existence of only one binding site for the monovalent cations on the protein molecule. The fit was carried out by varying the apparent binding constant (Reich et al. 1972). It was not reasonable to fit the data for Na^+-titration by the two-site scheme since the fluorescence change corresponding to the binding of the second Na^+ ion is very small. The values of the apparent association constants obtained for Na^+ and K^+ ions at 20 °C and 37 °C are summarized in Table 1. As for divalent cation binding, the constants for the monovalent cations measured at 37 °C are lower than those determined at 20 °C.

2. *Binding of nucleotides and UDP-galactose to α-lactalbumin*

The concentrations of ATP and ADP in living cells are rather high. It could be therefore important to investigate possible interactions of α-lactalbumin with these nucleotides. Fig. 3 shows results of titration of Ca^{2+}-free and Ca^{2+}-loaded α-lactalbumin with ATP and ADP at 37 °C. The gradual addition of ATP and ADP resulted in changes in both fluorescence yield and fluorescence spectrum position.

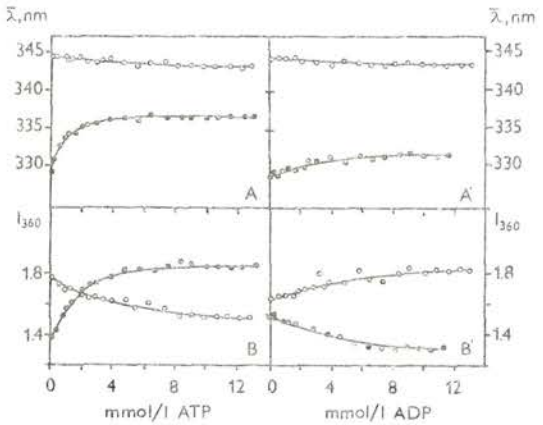


Fig. 3. ATP- (*A*, *B*) and ADP- (*A'*, *B'*) titrations of Ca^{2+}-loaded (●) (0.040 mmol/l Ca^{2+}) and Ca^{2+}-free (○) (0.25 mmol/l EGTA) α-lactalbumin. 50 mmol/l HEPES, pH 8.0; 37 °C. Protein concentration 0.020 mmol/l. *A*, *A'* — fluorescence spectrum position; *B*, *B'* — fluorescence intensity at 360 nm.

The spectrum shifted to longer wavelengths for the Ca^{2+}-loaded, and to shorter wavelengths for the Ca^{2+}-free protein. Nucleotide titration resulted in an increase in the fluorescence yield (and in intensity at a fixed wavelength) on ATP-titration of Ca^{2+}-loaded α-lactalbumin and on ADP-titration of the Ca^{2+}-free protein.

ATP-titration of Ca^{2+}-free α-lactalbumin and ADP-titration of the Ca^{2+}-loaded protein resulted in quenching of the protein fluorescence. The curves reached plateau at high nucleotide concentrations. The spectral changes observed seem to be due to binding of the nucleotides to α-lactalbumin. It may be suggested that the spectral changes induced by ATP-titration of the Ca^{2+}-loaded protein reflected an effect of the nucleotide as a Ca^{2+}-chelator. However this is not the case since α-lactalbumin binds Ca^{2+} ions 10^4 times stronger than does the nucleotide, and the nucleotide concentration used was only about 10^2—10^3 times higher than that of the protein. Moreover, the position of the fluorescence spectrum for apo-α-lactalbumin at 37 °C is approx. 343—344 nm, while the nucleotide shifts it only to 336—337 nm. (Fig. 3 *A*, *A'*).

Similar spectral changes were observed with titration of Ca^{2+}-loaded and Ca^{2+}-free α-lactalbumin with UTP and UDP.

Fig. 4 shows results of titration of Ca^{2+}-loaded and Ca^{2+}-free α-lactalbumin with UDP-galactose, the substrate of the lactose synthase reaction. It is clearly seen that the nature of the UDP-galactose binding to the Ca^{2+}-loaded protein is different from that to the Ca^{2+}-free protein.

We evaluated the apparent equilibrium constants for the nucleotide and UDP-galactose binding to different forms of α-lactalbumin. ATP- and ADP-titrations of α-lactalbumin were approximated by the simplest scheme assuming a binding stoichiometry of 1:1. In the case of the two-step curve for UDP-galactose titration the following successive binding scheme was assumed:

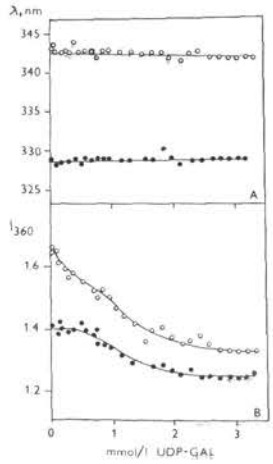
$$P + N \overset{K_1}{\rightleftharpoons} PN$$
$$PN + mN \overset{K_2}{\rightleftharpoons} PN_{m+1}$$

(P and N are the protein and UDP-galactose, respectively). Theoretical curves for I_{360}, computed according to the schemes, were fitted to the experimental points by varying the apparent binding constant (Reich et al. 1972). Values of the binding constants which give the best fits are shown in Table 2. It can be seen that the

Table 2. Apparent equilibrium constants for the binding of ATP, ADP, UTP, UDP and UDP-galactose to α-lactalbumin. 50 mmol/l HEPES, pH 8.0, 37 °C.

Compound	$+Ca^{2+}$ K_{app} (l/mol)	$-Ca^{2+}$ K_{app} (l/mol)
ATP	1050 ± 100	130 ± 50
ADP	130 ± 50	180 ± 50
UDP	2500 ± 500	510 ± 50
UTP	1500 ± 100	1100 ± 100
UDP-Gal	1000 ± 100 $m = 3.8$	8600 ± 500 770 ± 100 $m = 3.2$

apparent equilibrium constants for ATP, UDP and UDP-galactose binding to α-lactalbumin depend upon the state of the protein. ATP and UDP have higher affinities to the Ca^{2+}-loaded than to the Ca^{2+}-free protein. By contrast, UDP-galactose has a higher affinity to the Ca^{2+}-free than to the Ca^{2+}-loaded protein. The binding of ADP and UTP to the Ca^{2+}-loaded and Ca^{2+}-free protein is practically the same.



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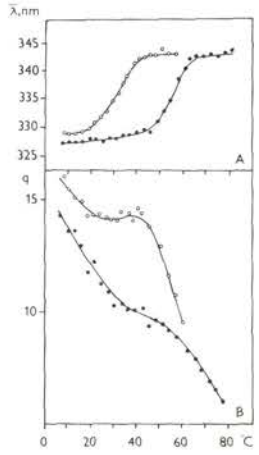

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Fig. 4. UDP-galactose-titration of Ca^{2+}-loaded (●) and Ca^{2+}-free (○) (0.35 mmol/l EGTA) α-lactalbumin. Protein concentration 0.020 mmol/l, 50 mmol/l HEPES, pH 8.0; 37 °C. *A* — fluorescence spectrum position; *B* — fluorescence quantum yield.

Fig. 5. Temperature dependences of the fluorescence spectrum position (*A*) and the relative quantum yield (*B*) for Ca^{2+}-loaded (0.8 mmol/l Ca^{2+}) (●) and Ca^{2+}-free (1 mmol/l EGTA) (○) α-lactalbumin. 50 mmol/l HEPES, pH 7.3; 150 mmol/l KCl, 13 mmol/l $MgCl_2$, 10 mmol/l Na_2ATP, 1 mmol/l Na_2ADP.

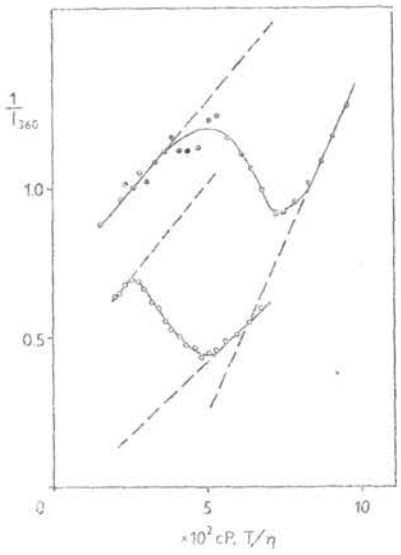
3. *Effects of pH and Ca^{2+} content on the thermal transitions in α-lactalbumin*

In view of the fact that α-lactalbumin is not only able to interact with Ca^{2+} ions, but also with Mg^{2+}, Na^+, K^+, ATP and ADP, further experiments were performed in a medium modelling physiological conditions, i.e. in 50 mmol/l HEPES, containing 13 mmol/l $MgCl_2$, 150 mmol/l KCl, 10 mmol/l Na_2 ATP, 1 mmol/l Na_2ADP (Prosser 1977). In order to free the protein from Ca^{2+} ions the metal chelator EGTA was used.

Heating of α-lactalbumin solution from 5 °C up to 80 °C resulted in a shift in its fluorescence spectrum maximum from 327—329 nm to 344—345 nm (Fig. 5*A*). The shift dependent strongly upon the Ca^{2+} content in the protein sample. These spectral changes reflect a transfer of some tryptophan residues from

the protein interior to the surface of the protein globule in contact with bound water molecules (Burstein 1977). The transfer is due to a thermal rearrangement of the protein structure. The fluorescence data are in a good agreement with the CD (Hiraoka et al. 1980) and microcalorimetry (Dolgikh et al. 1981) results obtained in a pure buffer solution. It should be emphasized that thermally induced changes in α-lactalbumin involve mainly its tertiary structure and not the secondary one. The thermally changed protein is nearly as compact as the native one.

The progress of the thermally induced transition is also well seen in plots of the fluorescence yield or intensity at a fixed wavelength, versus temperature (Fig. 4*B*); the changes in these parameters however develop on the background of a common thermal quenching of the fluorescence which reflects thermal activation of intramolecular collisions between the excited chromophores and neighbouring quenching groups (Burstein 1977). In order to eliminate the effects of the trivial thermal quenching an earlier described method (Permyakov and Burstein 1984) was used. It is based on the observation by Bushueva et al. (1978) that the temperature dependence of the fluorescence quantum yield q (or intensity at a fixed wavelength I) for native proteins containing a single fluorescent



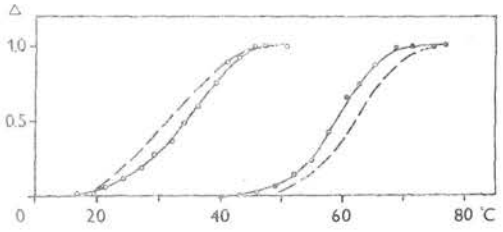


Fig. 6. The dependences of reciprocal fluorescence intensity at 360 nm on T/η for Ca^{2+}-loaded (●) and Ca^{2+}-free (○) α-lactalbumin. For conditions, see Fig. 4.

Fig. 7. Temaperature dependences of conversion from native to thermally changed state for Ca^{2+}-loaded (1) and Ca^{2+}-free (2) α-lactalbumin. For conditions, see Fig. 4. Broken lines (3 and 4) represent similar curves measured in pure buffer solution without any additions.

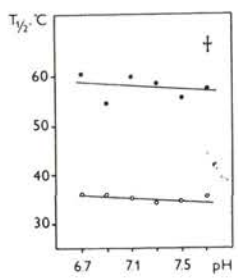
chromophore within the nondenaturing temperature range can be described by the equation

$$1/I = a + b \,.\, T/\eta$$

where a and b are the temperature-independent constants, T is the temperature and η is the solvent viscosity. Fig. 6 shows $1/I_{360}$ versus T/η plots for α-lactalbumin in the presence and absence of Ca^{2+} ions at pH 7.3. Although α-lactalbumin contains four tryptophan residues per molecule, the plots in the temperature regions below and above the thermally induced transition are stright lines. The fluorescence intensity at a fixed wavelength for a given temperature T is

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

where the subscripts N and H refer to the native and "high" temperature conformers; Δ is the fractional conversion of the N to the H form, and $(I_\lambda)_{N,T}$ and $(I_\lambda)_{H,T}$ represent the fluorescence intensities of the two conformers at the temperature T. $(I_\lambda)_{N,T}$ and $(I_\lambda)_{H,T}$ can be determined from an extrapolation of the linear parts of $1/I_{360}$ versus T/η plots to the thermal transition regions. From $(I_\lambda)_{N,T}$ and $(I)_{H,T}$, Δ can be obtained. Fig. 7 shows the thermally induced transition curves (Δ versus temperature) for α-lactalbumin in the presence and absence of Ca^{2+} ions. Broken curves are similar data obtained in 50 mmol/l HEPES without any salts and nucleotides.



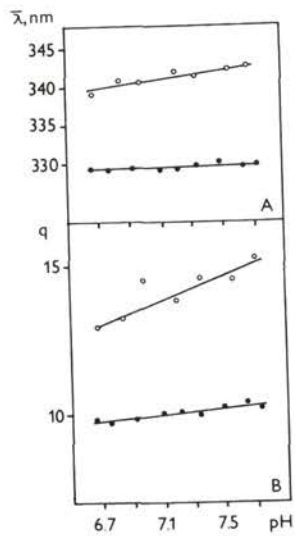


Fig. 8. pH dependences of the half transition temperatures for thermally induced changes in Ca^{2+}-loaded(●) and Ca^{2+}-free (○) α-lactalbumin. For conditions, see Fig. 4.

Fig. 9. pH-dependences of the fluorescence spectrum position (*A*) and the relative quantum yield (*B*) for Ca^{2+}-loaded (●) and Ca^{2+}-free (○) α-lactalbumin. Temperature 37 °C. For conditions, see Fig. 4.

The thermal transition curves for α-lactalbumin in the presence and absence of Ca^{2+} were measured within a pH range between 6.7 to 7.7. The half-transition temperature, $t_{1/2}$ for α-lactalbumin in the presence and absence of Ca^{2+} ions is almost independent on pH in this region (35 °C and 58 °C in the absence and in the presence of Ca^{2+} ions, respectively) (Fig. 8). Fig. 9 shows pH dependences of the fluorescence parameters (spectrum position and relative fluorescence quantum yield) for α-lactalbumin in the absence and presence of Ca^{2+} ions. An acidification of the solution resulted in a small shortwavelength shift of the fluorescence spectrum and in a slight decrease in the fluorescence yield. It should be noted that these spectral changes occur only in the presence of the nucleotides.

Discussion

The results of the present work show that α-lactalbumin interacts with the main monovalent and divalent cations of a cell. The protein has the strongest affinity to Ca^{2+} ions. The binding of Ca^{2+}, Mg^{2+}, $Na^{+}$and K^{+} ions to α-lactalbumin at 37 °C results in changes in its tertiary structure, resulting in turn in a transfer of at least one exposed tryptophan residue from the protein surface to the hydrophobic interior of the protein globule. Since this induces a decrease in the fluorescence yield, it is reasonable to assume that the environment of the transferable tryptophan residues in the protein interior contains some quenching groups, most likely disulfids. Ca^{2+} ions induce the most pronounced conformational change. It should be noted once more that the changes mainly involve the tertiary structure. The value of the apparent Ca^{2+} binding constant for α-lactalbumin is within the same range as those for the high affinity sites in Ca^{2+}-binding proteins such as parvalbumin and troponin C. However, the values of the apparent Mg^{2+} binding constants for α-lactalbumin are much lower than those for the sites in parvalbumin or troponin C (Permyakov et al. 1983). The affinity of α-lactalbumin to Na^{+} and K^{+} ions is almost that of parvalbumins (Permyakov et al. 1983). Like parvalbumins, α-lactalbumin binds Na^{+} ions better than it does K^{+} ions. The apparent association constants of Na^{+} and K^{+} ions are low; however, taking into consideration their high concentrations in any cell it can be assumed that at physiological concentrations these cations can successfuly compete with Mg^{2+} ions for the same binding sites. It is of interest that α-lactalbumin has at least two binding sites for both Mg^{2+} and Na^{+} ions.

The affinity of α-lactalbumin to ATP, UTP, UDP and especially to ADP is also low, but the high concentrations of the nucleotides ATP and ADP in a cell lead us to take into account their interactions with the protein. Since the apparent equilibrium constant for ATP binding to Ca^{2+}-loaded protein is approx. 10^3 l/mol

and the ATP concentration in a cell is several mmol/l, it can be suggested that in vivo most of the Ca^{2+}-loaded protein is in the complex with ATP.

It is of interest to note here that the binding of UDP-galactose does not induce any spectral shifts, while the nucleotides induce rather significant shifts in the fluorescence spectrum. This seems to suggest that UDP-galactose and the nucleotides ATP, ADP, UTP and UDP bind to different sites in α-lactalbumin.

Thus, bovine α-lactalbumin binds UDP-galactose and nucleotides UDP, UTP, ATP and ADP, and the binding parameters depend upon the protein state. It is difficult to say now whether this binding plays any functional role, since in vivo, α-lactalbumin functions in a complex with galactosyltransferase which can influence the binding of nucleotides. Further investigations are required to get a clear picture.

Fig. 7 shows that at physiological temperatures Ca^{2+}-loaded α-lactalbumin is in its native state in both pure buffer solution and solution modelling physiological conditions. The release of the protein from Ca^{2+} ions results in a transition of the protein from this state to a state which is a mixture of different protein states. α-lactalbumin in the pure buffer solution is a mixture of native and thermally changed states of the apo-protein, and in the presence of Mg^{2+}, Na^+, K^+, ATP and ADP a mixture of native and thermally changed states of the apo- and metal bound forms. The equilibrium scheme of the binding of one metal ion (Me) to α-lactalbumin molecule, taking into consideration an equilibrium between native (P, PMe) and thermally changed (P*, P*Me) states of the protein, is:

$$\begin{array}{ccc} P + Me & \overset{K_{Me}}{\rightleftharpoons} & PMe \\ \alpha \downarrow\uparrow & & \uparrow\downarrow \beta \\ P^* + Me & \overset{K^*_{Me}}{\rightleftharpoons} & P^*ME \end{array}$$

where K_{Me} and K^*_{Me} are intrinsic metal ion binding constants for the native and the thermally changed protein, respectively, and α and β are equilibrium constants of the thermal denaturation of the protein in its apo- and metal ion-lodaded forms, respectively. The scheme gets more complex when more than one ion becomes bound (as in the case of Mg^{2+} or Na^+ ions). In the absence of Ca^{2+} ions the solution at physiological temperatures contains all the states of the complexes of α-lactalbumin with Mg^{2+}, Na^+ and K^+, since in α-lactalbumin with bound Mg^{2+}, Na^+ or K^+ ions thermal transitions occur at much lower temperatures than in the Ca^{2+}-loaded protein, but at higher temperatures than in the apo-protein. The binding of nucleotides makes the situation still more complex.

Any variations in temperature must result in changes in the populations of the states. It would be rather attractive to suppose that this is related to some temperature regulation of the α-lactalbumin function; at the present this remains

a mere assumption since we still do not know what is the physiological function of metal binding to α-lactalbumin.

Variations in pH within the physiological region induce little changes in the position of the thermal transition in α-lactalbumin in both the absence and presence of Ca^{2+} ions, though the fluorescence parameters of the Ca^{2+}-loaded and Ca^{2+}-free α-lactalbumin in the presence of the nucleotides at 37 °C show slight pH dependence (Fig. 8). The spectral changes seem to reflect the pH-dependence of protonation of ATP and ADP, the pK_a values of which are within the region of 6.6—6.8. It is reasonable to assume that the parameters of the interaction of α-lactalbumin with the protonated forms of the nucleotides are different from those of the interaction with deprotonated forms.

Thus, the conformation of α-lactalbumin in a medium modelling physiological conditions can be controlled by varying temperature and Ca^{2+} concentrations within the physiological limits. This may be associated with the existence of a Ca^{2+}- or temperature-dependent regulation of the α-lactalbumin function in vivo.

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Received December 4, 1984/Accepted September 16, 1985