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Interaction of α-lactalbumin with Cu^{2+}

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It has been shown by intrinsic fluorescence spectroscopy that α-lactalbumin has several Cu^{2+}-binding sites per molecule. The Ca^{2+}-loaded protein binds two or more Cu^{2+} per molecule with an association constant of about 3×10^3 M^{-1}. Apo-α-lactalbumin binds one Cu^{2+} per molecule with association constant 8×10^4 M^{-1} and from two to three Cu^{2+} with an association constant of about 4×10^3 M^{-1}. The results obtained from spectrofluorometric pH titration of α-lactalbumin in the acidic pH region show the possible involvement of histidine residues in the coordination of Cu^{2+}. The binding of Cu^{2+} to α-lactalbumin lowers significantly its thermostability and stability towards urea denaturation. The stability of Cu^{2+},Ca^{2+}–α-lactalbumin against thermal and urea denaturation is similar to that of the apo protein. The thermal transition in Cu^{2+},Ca^{2+}–α-lactalbumin occurs within the region of physiological temperatures which may suggest the existence of some thermal regulation of its functioning in vivo.

1. Introduction

α-Lactalbumin is one of the two components of the lactose synthase enzyme system, which catalyzes the final step in lactose biosynthesis in the lactating mammary gland [1]. The association of α-lactalbumin with galactosyltransferase imparts a change in specificity of the latter enzyme from terminal *N*-acetylglucosaminyl acceptors to glucose.

α-Lactalbumin is homologous in amino acid sequence to lysozyme. Recent X-ray studies show that its three-dimensional structure is also very similar to that of lysozyme [2]. α-Lactalbumin possesses one Ca^{2+}-binding site ($K_{Ca} \simeq 5\times10^8$ M^{-1}) which is also able to bind Mg^{2+} ($K_{Mg} \simeq 2\times10^3$ M^{-1}). Na^+ ($K_{Na} \simeq 100$ M^{-1}) and K^+ ($K_K \simeq 10$ M^{-1}) [3–5]. In addition, α-lactalbumin has a separate site for binding of Zn^{2+}, Cu^{2+}, Co^{2+} and Al^{3+} [6,7]. The distance between the Ca^{2+}- and Zn^{2+}-binding sites is 11.5 ± 1.5 Å [8]. According to the data of Murakami and Berliner [6], the association constants for Zn^{2+} and Cu^{2+} for Ca^{2+}-loaded α-lactalbumin are 1.5×10^6 and 1.5×10^5 M^{-1}, respectively. Although much is known about Zn^{2+} and Cu^{2+} binding to α-lactalbumin, there are still some problems which should be solved. For example, data on the binding of Cu^{2+} and Zn^{2+} to the apo protein are not available in the literature. The physico-chemical properties displayed by α-lactalbumin when saturated by Zn^{2+} or Cu^{2+} remain unclear thus far. Furthermore, the groups taking part in the coordination of Zn^{2+} and Cu^{2+} in this protein are not known.

In the present work, these problems were studied by means of an intrinsic fluorescence spectroscopic method. It has been revealed that bovine α-lactalbumin possesses more than one Cu^{2+}-binding site. The parameters for Cu^{2+} binding to α-lactalbumin depend upon the Ca^{2+} concentration: the apo-protein has a higher affinity to Cu^{2+} than the Ca^{2+}-loaded form. Some histidine

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residues appear to take part in the coordination of Cu^{2+}. The stability of Ca^{2+},Cu^{2+}-loaded α-lactalbumin is similar to that of the apo-protein.

The problem of Cu^{2+} and Zn^{2+} binding to α-lactalbumin merits further investigation, since the suggestion has been made that the binding of at least Zn^{2+} is of physiological significance for the functioning of this protein [7].

2. Materials and methods

α-Lactalbumin was isolated from bovine milk and purified according to a preparative sequence similar to that described by Kaplanas and Antanavichius [9]. The purity of the preparation was checked electrophoretically. Protein concentrations were evaluated spectrophotometrically using an absorption coefficient of $\epsilon_{280\,nm} = 28\,540$ $M^{-1}\,cm^{-1}$ as reported in the literature [10]. Metal ion-free protein preparations were obtained by means of the method of Blum et al. [11]. Metal ion-free protein preparations contained about 0.1 mol Ca^{2+}/mol protein which was checked spectrofluorometrically [3]. Only deionized water and plastic ware were used in the work with metal-free protein.

Fluorescence spectra were recorded on a laboratory-made spectrofluorimeter described elsewhere [12]. Fluorescence light was collected from the front surface of the cell. All spectra were corrected for the spectral sensitivity of the instrument and in some cases for inner filter effects of screening and reabsorption [13]. 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 rate of cell heating during measurements of the temperature dependences of the fluorescence parameters was about 1 K per min. Fitting of the experimental data to theoretical curves was performed using a non-linear regression scheme (Marquardt's algorithm) [14]. The accuracy in the evaluation of the binding constants by the fitting was about half of an order of magnitude.

3. Results

Fig. 1 demonstrates the results of spectrofluorometric Cu^{2+} titration of Ca^{2+}-loaded bovine α-lactalbumin at pH 8 and 20 °C. The increase in Cu^{2+} concentration causes a shift of the tryptophan fluorescence spectrum towards longer wavelengths by more than 15 nm and a 2-fold decrease in fluorescence quantum yield. It should be noted that the removal of bound Ca^{2+} from α-lactalbumin gives rise to the same spectral shift towards longer wavelengths, however, the fluorescence quantum yield increases in this [3]. The curves of the spectrofluorometric titration in fig. 1 are sigmoidal which reflects the fact that α-lactalbumin binds several Cu^{2+} per molecule and that the binding process seems to be cooperative. The binding parameters were evaluated in this case by the fitting of theoretical curves computed according to the simplest cooperative scheme

$$P + nCu^{2+} \overset{K}{\rightleftharpoons} PCu_n \qquad (1)$$

(P denoting protein) to the experimental points for the relative fluorescence quantum yield. The fitting was carried out by variation of n and K. The best fit was achieved for $K = 3 \times 10^3$ M^{-1} and $n = 2.2 \pm 0.5$

The results of spectrofluorometric Cu^{2+} titration of Ca^{2+}-free α-lactalbumin at pH 8 and 20 °C are depicted in fig. 2. It is clearly seen that

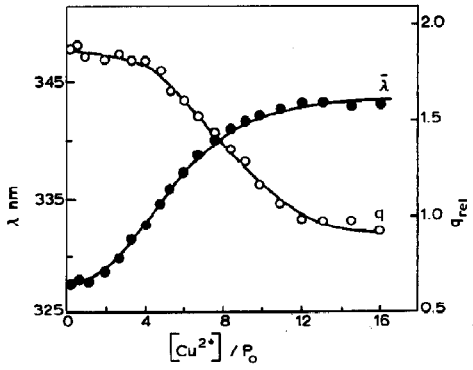

Fig. 1. Spectrofluorometric Cu^{2+} titration ($CuCl_2$) of Ca^{2+}-loaded α-lactalbumin. 10 mM Hepes (pH 8), 1 mM $CaCl_2$, protein concentration $P_0 = 2.3 \times 10^{-5}$ M. $\bar{\lambda}$, spectral position; q, relative fluorescence quantum yield.

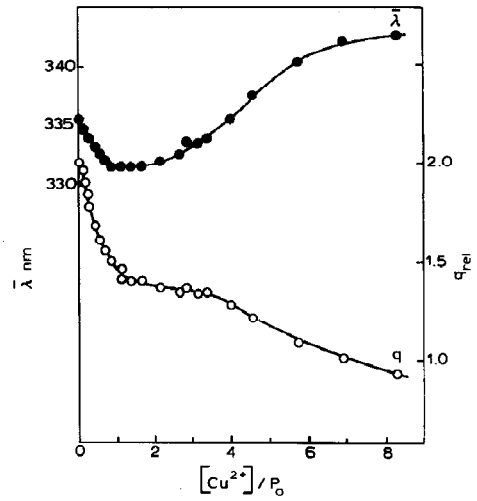

Fig. 2. Spectrofluorometric Cu^{2+} titration ($CuCl_2$) of Ca^{2+}-free α-lactalbumin. 10 mM Hepes (pH 8), protein concentration $P_0 = 2.5 \times 10^{-5}$ M. $\bar{\lambda}$, spectral position; q, relative fluorescence quantum yield.

the spectral changes in this case are different from those for the Ca^{2+}-loaded protein: the increase in Cu^{2+} concentration results in a shift of the fluorescence spectrum towards shorter and then longer wavelengths. In addition, there is a stepped decrease in fluorescence quantum yield. Since the first stage of the spectral changes ends at a Cu^{2+}/protein ratio of about unity, one can assume that these changes are caused by the binding of one Cu^{2+} per protein molecule. Further changes in the fluorescence parameters reflect cooperative binding of Cu^{2+} to another protein site. Therefore, the binding of Cu^{2+} to α-lactalbumin appears to proceed according to the following scheme:

$$\begin{aligned} &P + Cu^{2+} \overset{K_1}{\rightleftharpoons} PCu \\ &PCu + nCu^{2+} \overset{K_2}{\rightleftharpoons} PCu_{n+1} \end{aligned} \qquad (2)$$

Theoretical curves computed according to this scheme were fitted to the experimental data on the relative fluorescence quantum yield. The best fit was achieved for $K_1 = 8 \times 10^4$ M^{-1}, $K_2 = 4 \times 10^3$ M^{-1} and $n = 3.2 \pm 1$. This means that in the absence of Ca^{2+}, α-lactalbumin has a higher affinity to Cu^{2+} (one site with association constant 8×10^4 M^{-1}) than in the presence of saturating concentrations of Ca^{2+}.

Fig. 3 shows the pH dependence of the fluorescence parameters for Ca^{2+}- and Ca^{2+},Cu^{2+}-saturated α-lactalbumin. As shown previously [3,5], the shift of the fluorescence spectrum towards longer wavelengths and the increase in fluorescence yield for Ca^{2+}-loaded α-lactalbumin in the pH range from approx. 4 to approx. 2.5 reflect conformational changes induced by displacement of Ca^{2+} from the binding sites by protons as a result of the competition between Ca^{2+} and H^+

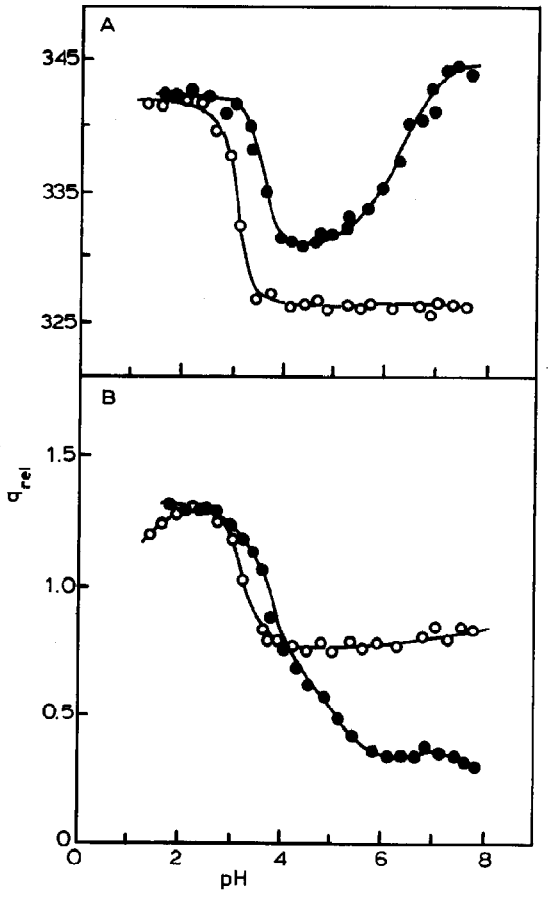

Fig. 3. Spectrofluorometric pH titration of Ca^{2+}-loaded (2.5×10^{-5} M $CaCl_2$) (○) and Ca^{2+},Cu^{2+}-loaded (2.5×10^{-5} M $CaCl_2$ and 3.4×10^{-4} M $CuCl_2$) (●) α-lactalbumin (2.1×10^{-5} M). (A) Spectral position, (B) relative fluorescence quantum yield.

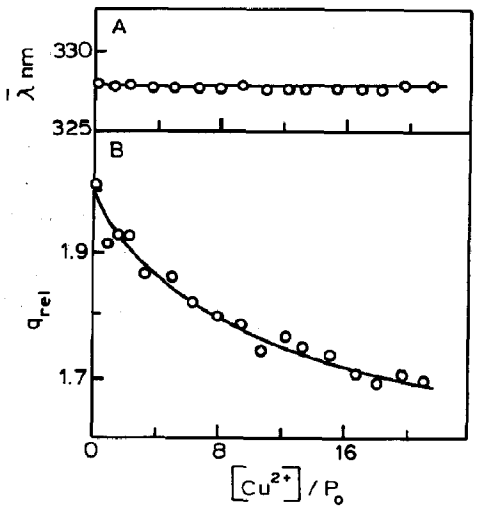

Fig. 4. Spectrofluorometric Cu^{2+} titration ($CuCl_2$) of Ca^{2+}-loaded α-lactalbumin. 10 mM glycine (pH 4.5), 2.5×10^{-5} M $CaCl_2$. Protein concentration $P_0 = 3.4\times10^{-5}$ M. (A) Spectral position, (B) relative fluorescence quantum yield.

for the same carboxylate groups. The Ca^{2+},Cu^{2+}-loaded protein undergoes a similar transition in this pH region, however, the protein in this state displays an additional transition in the pH region from approx. 7.5 to approx. 5, namely, a shift of the fluorescence spectrum towards shorter wavelengths and a rise in the relative fluorescence quantum yield. These changes in fluorescence characteristics are the reverse of those induced by Cu^{2+} binding. The pH region in which these changes occur is characteristic for the titration of side chains of histidine residues (bovine α-lactalbumin contains three histidines, i.e., residues 32, 68 and 107 [15]). These facts lead us to suggest that the fluorescence changes registered for Ca^{2+},Cu^{2+}-loaded α-lactalbumin over the range pH 7.5–5 are caused by conformational changes induced by protonation of the side chains of some histidines, which in turn results in the release of some bound Cu^{2+}. The lowering of the affinity of α-lactalbumin to Cu^{2+} due to this transition is clearly demonstrated by spectrofluorometric Cu^{2+} titration of α-lactalbumin at pH 4.5 (fig. 4). In this case, the increase in Cu^{2+} concentration causes only a non-cooperative decrease in relative fluorescence quantum yield without any spectral shifts.

Evaluation of the effective Cu^{2+}-binding constant using the simplest one-site model results in a value of 5×10^3 M^{-1}, i.e., the affinity to Cu^{2+} of α-lactalbumin in this state is about one order of magnitude lower than that at neutral pH values.

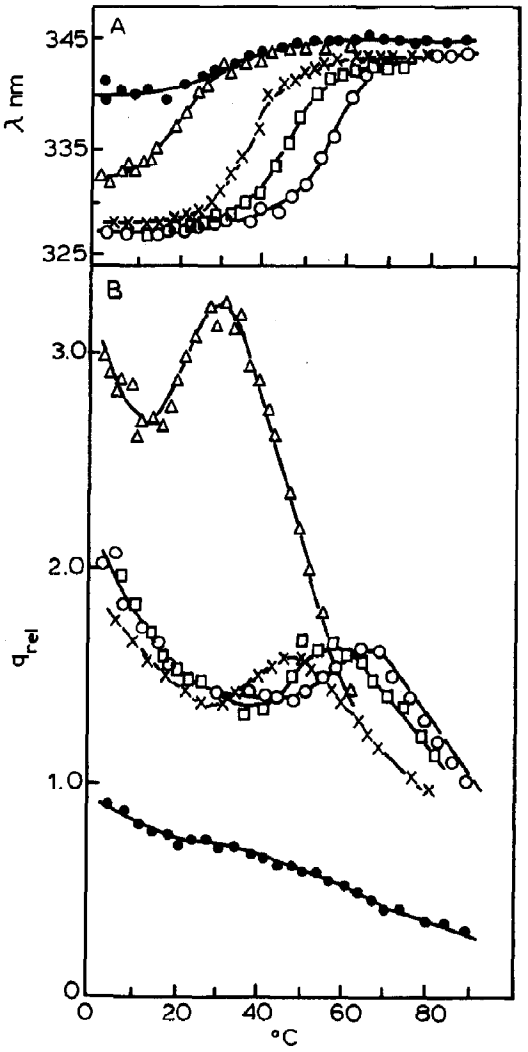

Fig. 5. Thermal denaturation of α-lactalbumin in various states: (○) Ca^{2+}-loaded (2.5×10^{-5} M $CaCl_2$, pH 7.5); (●) Ca^{2+},Cu^{2+}-loaded (2.5×10^{-5} M $CaCl_2$, 3.4×10^{-4} M $CuCl_2$; pH 7.5); (△) apo-protein (0.35 mM EGTA, pH 7.5); (□) Ca^{2+}-loaded (2.5×10^{-5} M $CaCl_2$, pH 4.5); (×) Ca^{2+},Cu^{2+}-loaded (2.5×10^{-5} M $CaCl_2$, pH 4.5). Protein concentration $P_0 = 2.1\times10^{-5}$ M. 10 mM Hepes or glycine. (A) Spectral position, (B) relative fluorescence quantum yield.

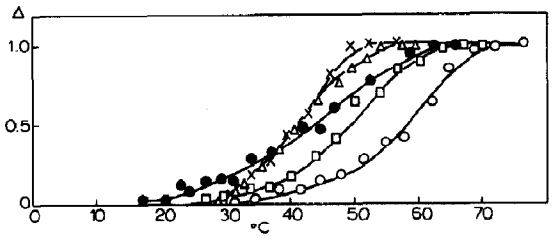

Fig. 6. Temperature dependences of the fraction of conversion from the native to the thermally unfolded state for different forms of α-lactalbumin, computed from the data presented in fig. 5. Designation as in fig. 5.

The mechanism of involvement of histidine residues in the process of Cu^{2+} binding is unclear at present, but one could assume that some histidines participate immediately in the coordination of Cu^{2+} which is corroborated by ESR data [16].

In order to characterize the state of α-lactalbumin in the complexes with Cu^{2+}, we studied the thermal denaturation process for various states of α-lactalbumin at pH 7.5 and 4.5. The results of these measurements are shown in figs. 5 and 6. The thermal denaturation of α-lactalbumin results in a shift of the fluorescence spectrum towards longer wavelengths due to an increase in accessibility of tryptophan residues to the solvent and in a rise of fluorescence yield which is superimposed on the common thermal quenching caused by activation of the thermal motions of some quenching groups in the chromophore's environment (fig. 5B). In order to determine the temperature dependence for the fraction of conversion from the native to thermally denatured state without the thermal quenching effects, we have used a method that we suggested and described previously [17]. This procedure involves the use of $1/q$ vs. T/η plots (q, relative fluorescence quantum yield; T, temperature η, solvent viscosity).

One can observe from fig. 6 that Cu^{2+} binding to Ca^{2+}-loaded α-lactalbumin does not result in stabilization, as might be assumed, but in destabilization of its structure: the midpoint of the thermal transition shifts towards lower temperatures by about 14°C; the transition becomes less cooperative. The transition in Ca^{2+},Cu^{2+}–α-lactalbumin occurs at almost the same temperatures as in the apo-protein. This confirms the conclusion that Ca^{2+},Cu^{2+}–α-lactalbumin is in an apo-like state [6,7]. Lowering of the pH to 4.5 decreases the denaturation temperature of both the Ca^{2+}-loaded and Ca^{2+},Cu^{2+}-loaded proteins, however, the latter still remains less thermostable than the Ca^{2+}-loaded form.

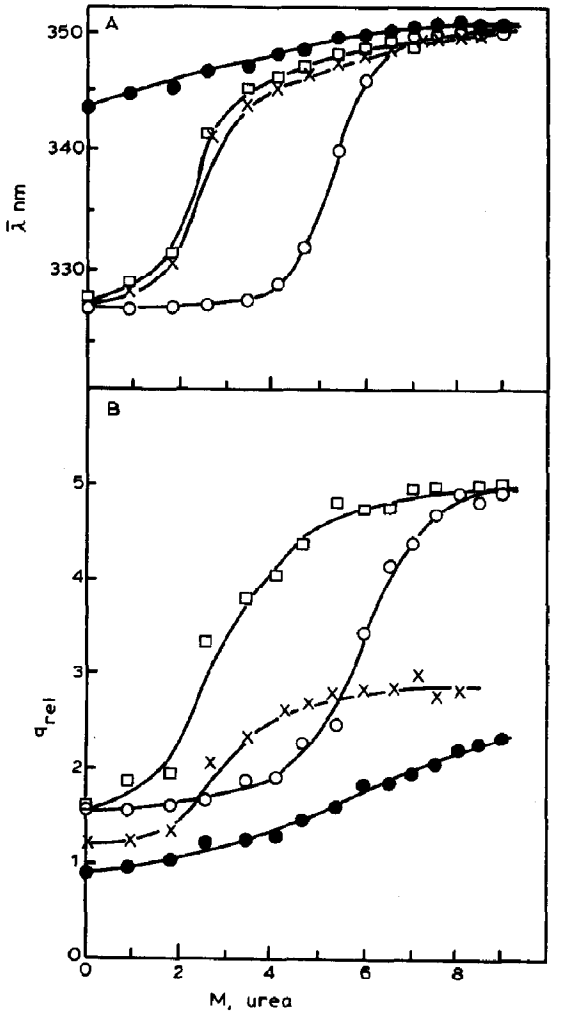

Fig. 7. Urea denaturation of different forms of α-lactalbumin: (○) Ca^{2+}-loaded (2.5×10^{-5} M $CaCl_2$, pH 7.5); (●) Ca^{2+},Cu^{2+}-loaded (2.5×10^{-5} M $CaCl_2$, 3×10^{-4} M $CuCl_2$; pH 7.5); (□) Ca^{2+}-loaded (2.5×10^{-5} M $CaCl_2$, pH 4.5); (×) Ca^{2+},Cu^{2+}-loaded (2.5×10^{-5} M $CaCl_2$, 3×10^{-4} M $CuCl_2$; pH 4.5). Protein concentration 2×10^{-5} M. 10 mM Hepes or glycine. (A) Spectral position, (B) relative fluorescence quantum yield.

Similar conclusions can be drawn from data on the urea-induced denaturation of α-lactalbumin in various states (fig. 7). At pH 7.5, Ca^{2+},Cu^{2+}–α-lactalbumin is much less stable to the action of urea compared to the Ca^{2+}-loaded protein, however, at pH 4.5 the stability of both states is practically equal.

4. Discussion

The experimental data obtained here show that α-lactalbumin possesses several Cu^{2+}-binding sites and that this is not quite in accordance with the data of Berliner et al. [6,7]. Using fluorescence and ESR methods, these authors demonstrated only one Cu^{2+} and Zn^{2+}-binding site in α-lactalbumin. Perhaps they failed to determine the presence of several sites per protein molecule as a result of their spectrofluorometric Zn^{2+}-titration curves not being measured in sufficient detail.

It should also be noted that the Cu^{2+}-binding constants obtained in our work for Ca^{2+}-loaded α-lactalbumin are almost two orders of magnitude lower than those reported by Murakami and Berliner [6]. This could be explained by the fact that we evaluated these parameters from direct spectrofluorometric Cu^{2+} titration of α-lactalbumin whereas they determined the Cu^{2+}- and Zn^{2+}-binding constants from experiments on competition of these cations with Mn^{2+}, however, direct competition by Cu^{2+}, Zn^{2+} and Mn^{2+} for the same binding site has not yet been proven and the observed effects could be due to interactions between separate Cu^{2+}- and Mn^{2+}-binding sites. The experimental spectrofluorometric Zn^{2+} titration curve for α-lactalbumin reported by Murakami and Berliner [6] plateaus at millimolar Zn^{2+} concentrations (protein concentration ~ 3 μM), which suggests that the Zn^{2+}-binding constant for α-lactalbumin is in fact about 10^3 M^{-1}, a value that is in good agreement with our data.

One of the most interesting results of our work is that the Cu^{2+}-binding parameters of α-lactalbumin depend on Ca^{2+} concentration; the affinity of the apo-protein to Cu^{2+} is almost one order of magnitude higher compared to Ca^{2+}-loaded α-lactalbumin. This suggests that interactions take place between the Ca^{2+}- and Cu^{2+}-binding sites in this protein.

Our data on the thermally and urea-induced denaturation of Ca^{2+} and Ca^{2+},Cu^{2+}–α-lactalbumin demonstrate that Cu^{2+} binding gives rise to the apo-like state of the protein. The properties of this state are similar but not identical to those of the apo form. This conclusion is in full agreement with the data of Berliner et al. [6,7]. It should be noted that the thermal transition in Ca^{2+},Cu^{2+}-loaded α-lactalbumin at neutral pH values begins much sooner than that in the Ca^{2+}-loaded protein, therefore, at physiological temperatures around 37°C the Ca^{2+},Cu^{2+}-loaded protein is in equilibrium between the native and thermally denatured forms. This is probably connected with regulation of the temperature for functioning of α-lactalbumin in vivo.

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