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α-LACTALBUMIN BINDS MAGNESIUM IONS: STUDY BY MEANS OF INTRINSIC FLUORESCENCE TECHNIQUE

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The titration of metal-freed bovine α-lactalbumin with Mg^{2+} ions causes a two-stepped decrease in the tryptophan fluorescence quantum yield and a pronounced spectral shift towards shorter wavelengths, which seems to be a result of the binding of two magnesium ions to the protein molecule. The magnesium binding constants evaluated from the fluorimetric Mg^{2+}-titration are $2 \cdot 10^3$ and $2 \cdot 10^2$ M^{-1}. Mg^{2+} ions in millimolar concentrations almost do not influence the binding of Ca^{2+} ions to the protein.

INTRODUCTION: Recently it has been shown that α-lactalbumin is a calcium metalloprotein (1). Our previous study (2) has shown that the binding of one Ca^{2+} ion to bovine α-lactalbumin molecule causes a conformational change reflected in very pronounced changes of the tryptophan fluorescence of the protein. The calcium binding constant evaluated from fluorimetric EGTA- and pH-titration data has been shown to be $(3-6) \cdot 10^8 M^{-1}$.

Magnesium ions are surely present in many biological systems, including milk and mammary gland cells, in millimolar concentrations. It was therefore reasonable to study an ability of α-lactalbumin to bind Mg^{2+} ions. Here we report some results of this study.

MATERIALS AND METHODS: Bovine α-lactalbumin was prepared as described in (3). The protein concentrations were evaluated spectrophotometrically, using $E_{1\ cm,1\%} = 20.1$ at 280 nm (4). Metal freed preparations of α-lactalbumin were obtained by

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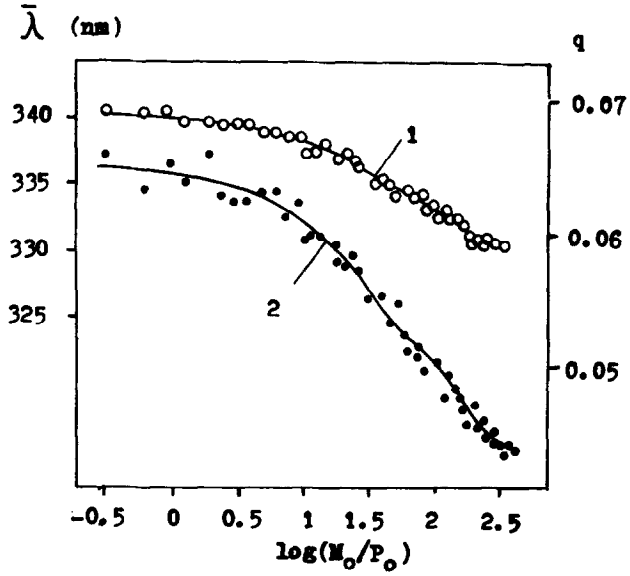


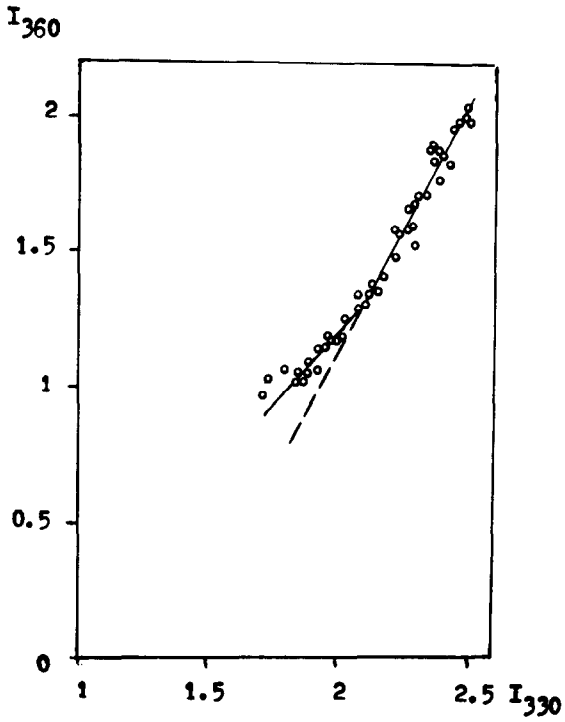
Fig.1. Titration of metal-freed α-lactalbumin with Mg^{2+} ions. 0.005 M Tris buffer, pH 8,04; 20°C. M_0-total Mg^{2+} concentration; protein concentration P_0=28.7 μM; total Ca^{2+} concentration $C_0=0.4 \cdot P_0 \cdot \bar{\lambda}$ (1) - spectrum position (position of the middle of a chord drawn at the 80% level of the maximal intensity); q (2) - fluorescence quantum yield.

the method of Blum et al. (5). All solutions were made using deionized water distilled in all-quartz apparatus. Only plastic ware was used in this work. Total calcium content in magnesium preparations was estimated by atomic absorption spectrophotometry.

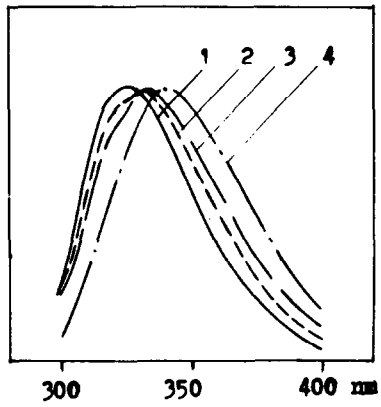
Fluorescence measurements were performed with a lab-made spectrofluorimeter described earlier (6). All spectra were corrected for the instrumental spectral sensitivity. Fluorescence quantum yield was evaluated by comparing the areas under fluorescence spectra of protein preparations with those of aqueous tryptophan solutions (quantum yield 0.20 at 25°C (7) with the same absorbance at the excitation wavelength (280.4nm).

Fitting of the experimental data with theoretical ones was carried out with the computer M-4030 using a standard optimization program (8). Schwarzenbach's of EGTA-Ca^{2+} and EGTA-Mg^{2+} binding constants (9) was used in the calculations.

RESULTS AND DISCUSSION: Fig.1 shows that a gradual adding of $MgCl_2$ to the metal-freed α-lactalbumin at pH 8.04 results in a decrease of the fluorescence quantum yield value, q, and a ca. 10 nm shift of the fluorescence spectrum towards shorter wavelengths which seems to reflect some conformational changes in the protein structure induced by the Mg^{2+} binding. The cur-



(2)



(3)

Fig.2. Fluorescence phase plot corresponding to the Mg^{2+}-titration of α-lactalbumin. Conditions as in Fig.1. Fluorescence intensities at 330 and 360 nm are expressed in relative units.

Fig.3. Normalized fluorescence spectra of α-lactalbumin in different metal-states. 1 - Ca^{2+}-state, P_o=21.3 μM, C_o/P_o=2.5; 2 -2Mg^{2+}-state, P_o=28.7 μM, C_o/P_o = 0.4, M_o/P_o=354; 3 - Mg^{2+}-state, P_o=28.7 μ M, C_o/P_o=0.4, M_o/P_o=75; 4 - apo-state, in the presence of a high EGTA concentration E_o=40·P_o, P_o=20.3 μM. 0.005 M Tris buffer, pH 8.04; 20°C.

ves seem to approach a plateau at very high Mg^{2+} concentrations ($>$ 9 mM) which suggests a rather weak affinity of α-lactalbumin to Mg^{2+} ions. The curves in Fig.1 may be assumed to be two-stepped ones. However the two-stepped character of the plots becomes obvious from the phase representation (10) (Fig.2) where the fluorescence intensity at any amission wavelength is plotted versus the fluorescence intensity at another wavelength. The fluorescence phase plot has to be a segment of

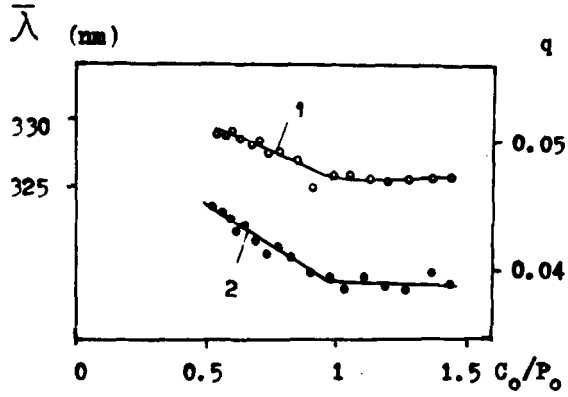


Fig.4. Titration of α-lactalbumin with Ca^{2+} ions in the presence of 8.7 mM Mg^{2+}. P_o=24.7 μM; 0.005 M Tris buffer, pH 8.04; 20°C. $\bar{\lambda}$ (1) - spectrum position, q (2) - fluorescence quantum yield.

straight line for a transition between two states (10). 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 our case (Fig.2). It is reasonable to assume that the two-stright-linear parts in the phase plot correspond to the successive binding of two Mg^{2+} ions to α-lactalbumin.

Fig.3 shows fluorescence spectra of α-lactalbumin in the states corresponding to different metal contents in the protein complex. One can see that the spectra of the protein in the metal-free-, mono-Ca^{2+}-, mono-Mg^{2+}- and di-Mg^{2+}- states have different positions and shapes which seems to suggest different conformations of these protein states.

Since the fluorescence quantum yield is a linear measure of a conversion extent (10), the plot of the fluorescence yield vs. Mg^{2+} concentration was taken for an evaluation of the protein-Mg^{2+} association constants. The constants of the successive binding of two Mg^{2+} ions evaluated from the middle points

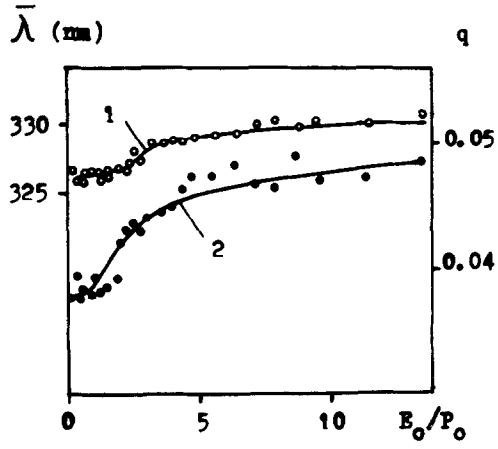


Fig.5. Titration of α-lactalbumin with EGTA in the presence of 35.2 M Ca^{2+} and 8.5 mM Mg^{2+}. P_o=24.1 μM; 0.005 M Tris buffer, pH 8.04; 20°C. $\bar{\lambda}$ (1) - spectrum position; q (2) - fluorescence quantum yield (points are experimental data, curve is a theoretical best fit computed according to scheme (i)).

of the q vs. total Mg^{2+} concentration plot (Fig.1) are ca. $2 \cdot 10^3$ and $2 \cdot 10^2$ M^{-1}, respectively.

Fig.4 shows that a titration of α-lactalbumin with Ca^{2+} in the presence of millimolar concentrations of Mg^{2+} causes an additional decrease in the fluorescence quantum yield and a further spectral shift towards shorter wavelengths, which seems to correspond to the binding of Ca^{2+} to the protein. It is important that the curves reach a plateau at the total Ca^{2+} to protein molar ratio (C_o/P_o) about 1 and the plot of q vs. C_o/P_o consists of two stright-linear parts. It suggests a very high ($\gg 1/P_o$) value of the Ca^{2+} binding constant, which can be evaluated from the experiment on the titration of Ca^{2+}-loaded α-lactalbumin in the presence of Mg^{2+} ions with a strong Ca^{2+}-chelator EGTA. EGTA has a rather high Ca^{2+} selectivity: the association constants ratio $K_{Ca}:K_{Mg}$ equals ca. 10^6 (9). The fluorescence changes induced by EGTA in Fig.4 seems to correspond to a release of Ca^{2+} from the protein. Points in Fig.5 are

experimental ones and the curve for q is theoretical one computed according to an aquilibrium scheme:

$$\begin{aligned} \text{Protein} + Ca^{2+} &\overset{K_P}{\rightleftharpoons} \text{Protein}\cdot Ca \\ \text{EGTA} + Ca^{2+} &\overset{K_{Ca}}{\rightleftharpoons} \text{EGTA}\cdot Ca \\ \text{EGTA} + Mg^{2+} &\overset{K_{Mg}}{\rightleftharpoons} \text{EGTA}\cdot Mg \end{aligned} \qquad (1)$$

The curve was fitted to the experimental points by variation of K_p value as described earlier (2,11). The best fit was achived at $K_P=1\cdot10^8 M^{-1}$. Worth to note that the Ca^{2+} association constant for α-lactalbumin in the absence of Mg^{2+} ions is $(3-6)\cdot10^8 M^{-1}$(2), i.e. the binding of Mg^{2+} ions almost does not change the calcium binding constant.It seems to mean that Ca^{2+} and Mg^{2+} ions bind to different sites of α-lactalbumin, which is corraborated by the fact that the titration of Ca^{2+}-loaded α-lactalbumin with Mg^{2+} ions (up to 0.45 M Mg^{2+}) does not result in an appearance of the spectrum of the Mg^{2+}-state of the protein.

Our preliminary measurements have shown that Mn^{2+} ions also bind to α-lactalbumin. The binding of Mn^{2+} ions may have a physiological importance since α-lactalbumin is a component of lactososintetase complex including also galactosyltransferase which needs Mn^{2+} ions for its activity (12).

Thus α-lactalbumin binds not only Ca^{2+} ions but also Mg^{2+}, Mn^{2+} and perheps some other ions. As it has been shown in this work the sites for Ca^{2+} and Mg^{2+} binding may be located in different parts of the protein molecule. The binding of cations to α-lactalbumin may modulate its function.

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