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CALCIUM BINDING TO α-LACTALBUMIN: STRUCTURAL REARRANGEMENT AND ASSOCIATION CONSTANT EVALUATION BY MEANS OF INTRINSIC PROTEIN FLUORESCENCE CHANGES.

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The binding of one Ca^{2+} ion to bovine α-lactalbumin molecule causes a conformational change reflected in a decrease of the tryptophan fluorescence quantum yield and very pronounced spectral shift towards shorter wavelengths (by ca. 15 nm). The calcium binding constant evaluated from the fluorimetric EGTA- and pH-titrations is $(3-6)\times10^8$ M^{-1}. The results obtained allow to suggest that the well known acid conformational change in α-lactalbumin is due in fact to a competetive replacement of the bound calcium ion by three H^+ ions (pK=5.0±0.1) in the calcium binding site.

INTRODUCTION: Recently it has been shown that bovine α-lactalbumin is able to bind Ca^{2+} ions (1). Metal analysis and the studies of the effects of EDTA on unfolding reactions have led to a suggestion that the protein has at least two kinds of Ca^{2+} binding sites. The success achieved in evaluation of the Ca^{2+} binding properties of parvalbumin monitoring the changes of intrinsic protein fluorescence during metal or chelator titrations (2) has led us to an idea to investigate the fluorescence properties of α-lactalbumin complexed with different amounts of Ca^{2+} and to try to obtain some information concerning the calcium binding to the protein. 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 $E_{1cm,1\%}=20.1$ at 280.4 nm (4).

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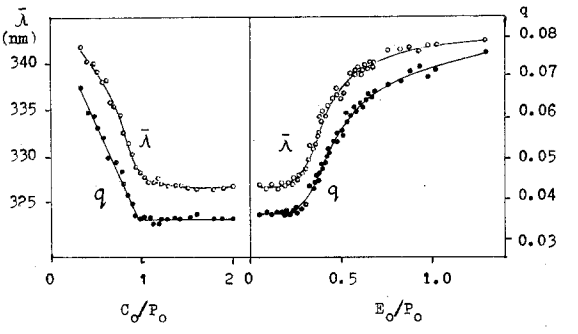


Fig.1. Left part: Titration of Ca^{2+}-free bovine α-lactalbumin with Ca^{2+}. 0.005 M Tris buffer, pH 8.04; 20°C. C_o - total calcium concentration; protein concentration P_o=21.3 μM. $\bar{\lambda}$- spectrum position (position of the middle of a chord drawn at the 80% level of the maximal intensity); q - fluorescence quantum yield. Fluorescence was excited at 280.4 nm. Right part: Titration of Ca^{2+}-loaded α-lactalbumin with calcium chelator EGTA. 0.005 M Tris buffer, pH 8.04; 20°C, P_o=20.3 μM; C_o=46.7 μM; E_o - EGTA concentration. $\bar{\lambda}$ - spectrum position; q - fluorescence quantum yield (points are experimental data, curve is a theoretical best fit computed according to scheme (i)).

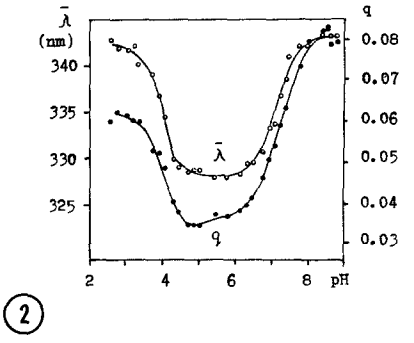
Calcium-freed preparations of α-lactalbumin were obtained by method of Blum et al. (5). All solutions were made using deionized water distilled in all-quartz apparatus. Only plastic ware was used in this work. Total calcium content in preparations was estimated by atomic absorption spectrophotometry. 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 free tryptophan solutions (quantum yield 0.23 at 20°C (7)) with the same absorbance at the excitation wavelength.
Fitting of the experimental data with theoretical ones was carried out with the computer M-4030 using a known optimization program (8). Schwarzenbach's set of EGTA-Ca^{2+} binding constants (9) was used in calculations.

RESULTS: The left part of Fig.1 shows that the gradual titration of Ca^{2+}-freed bovine α-lactalbumin with $CaCl_2$ at pH 8.04 causes an almost two-fold decrease in the fluorescence quantum yield value, q, and a ca. 20 nm shift of the fluorescence spectrum towards shorter wavelengths which seems to reflect some conformational changes in the protein caused by

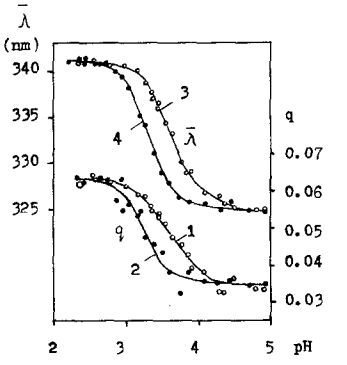
the Ca^{2+} binding. The curves approach a plateau at total calcium to protein molar ratio (C_o/P_o) ca. 1 which suggests that the intrinsic protein fluorescence is sensitive to the binding of only one Ca^{2+} ion to the protein molecule. The plot of q vs. C_o/P_o consists of two stright-linear parts intersecting at $C_o/P_o \sim 1.0$. It suggests a very high Ca^{2+} binding constant but does not allow to estimate its value. However, it can be evaluated from the experiment on the titration of Ca^{2+}-loaded α-lactalbumin with a strong Ca^{2+}-chelator, EGTA (right part of Fig.1). As expected, EGTA induces a full reversal of the fluorescence parameters of α-lactalbumin changed by the Ca^{2+}-titration. Since the fluorescence quantum yield is a linear measure of a conversion extent (10), the plot of q vs. E_o/P_o (E_o is total EGTA concentration) was taken for an evaluation of the Ca^{2+} binding constant of α-lactalbumin, K. The curve for q vs. E_o/P_o in Fig.1 is theoretical one computed according to the following equilibrium scheme:

$$\begin{aligned} \text{Protein} + Ca^{2+} &\overset{K}{\rightleftharpoons} \text{Protein.Ca} \\ \text{EGTA} + Ca^{2+} &\overset{K_E}{\rightleftharpoons} \text{EGTA.Ca} \end{aligned} \qquad (1)$$

and fitted to the experimental points as described earlier (2). The best fit was achieved at $K=2.9\times10^{8}\ M^{-1}$ ($\log K=8.5\pm0.5$). Since Ca^{2+} ions are coordinated in proteins mainly by carboxylate and carbonyl oxigens (11) one can assume that Ca^{2+} binding properties of α-lactalbumin are independent on pH from 6.5 to 9. On the other hand, the Ca^{2+}-EGTA association constant varies from ca. $10^{4}\ M^{-1}$ to ca. $10^{10}\ M^{-1}$ in this pH range (9). A significant redistribution of Ca^{2+} between EGTA and the protein during the pH-titration of the system contai-



(2)



(3)

Fig.2. pH-titration of α-lactalbumin. P_0=17.4 μM; C_0=39.9 μM; E_0=703 μM. 0.005 M Tris; 20°C. $\bar{\lambda}$ - spectrum position; q - fluorescence quantum yield (points are experimental data, curve in the pH region from 5.5 to 9 is a theoretical best fit computed according to scheme (i)). Fluorescence was excited at 280.4 nm.

Fig.3. pH-titration of α-lactalbumin. •-P_0=26 μM, C_0=1.32 mM; o-P_0=24 μM, C_0=24 μM. 0.005 M Tris, 20°C. $\bar{\lambda}$ (curves 3 and 4) - spectrum position; q (curves 1 and 2) - fluorescence quantum yield (points are experimental data, curves are theoretical best fits computed according to scheme (ii)).

ning α-lactalbumin, Ca^{2+} and EGTA can be easily monitored by tryptophan fluorescence (Fig.2). The experimental points in the q vs. pH plot in pH range from 6 to 9 were fitted by theoretical curve computed according to the scheme (1). The best fit was achived for $K=6.5\times10^{8}$ M^{-1} ($\log K=8.8\pm0.5$). This value practically coincides with that obtained from the EGTA-titration experiment at pH 8.04.

The spectral changes at acidic pH values (pH 5) in Fig.2 are very similar to those caused by the Ca^{2+} binding. It leads to an assumption that the low-pH conformational change, which has been studied by many authors with various techniques (see e.g. (12,13)), could be caused in fact by a competition of protons and Ca^{2+} ions for the same site. Fig.3 shows a pronounced shift to lower pH values of the fluorimetric pH-

titration curves for α-lactalbumin with an increase of the total Ca^{2+} concentration. The curves q vs. pH in Fig.3 are theoretical ones computed according to the following competition scheme:

$$\begin{aligned} \text{Protein} + Ca^{2+} &\overset{K}{\rightleftharpoons} \text{Protein.Ca} \\ \text{Protein} + nH^{+} &\overset{pK}{\rightleftharpoons} \text{Protein.H}_n \end{aligned} \qquad \text{(ii)}$$

The fitting to the experimental points was performed by variation of n and pK values. The best fit is achieved when n=2.9 and pK=4.9 for $C_o=P_o$ (curve 1) and when n=3.2 and pK=5.1 for $C_o=55xP_o$ (curve 2). This result may suggest the existence of three carboxyl groups with pK ca. 5.0 in the Ca^{2+}-binding site of α-lactalbumin.

Tb^{3+} ions, commonly used as an useful tool in the study of protein Ca^{2+} binding sites (14), cause the changes in the tryptophan fluorescence of Ca^{2+}-freed α-lactalbumin which are very similar to those induced by Ca^{2+} ions. However we have failed to detect the intrinsic Tb^{3+} luminescence excited by means of the excitation energy transfer from aromatic protein chromophores to the bound Tb^{3+} ions. It may be a result of a rather long distance of the metal binding site in α-lactalbumin from any of tryptophan and tyrosine residues.

DISCUSSION: An analysis of the Ca^{2+}-induced changes in the α-lactalbumin fluorescence spectra shows that thay seem to be caused by a conformational change in the protein molecule which results in a transfer of some exposed tryptophan residues from the protein surface to a rigid nonpolar interior of the protein globule (10). The results of our study clearly indicate that tryptophan fluorescence of α-lactalbumin is

very sensitive to the binding of one Ca^{2+} ion to the protein molecule. Worth to note that it may be the case that in fact more than one Ca^{2+} ion bind to α-lactalbumin molecule but the intrinsic protein fluorescence is not sensitive to such a binding. The value of the Ca^{2+} binding constant for α-lactalbumin ($(3-6)\times10^{8}$ M^{-1}) is in the same range as those for high affinity sites in such typical Ca^{2+}-binding proteins as parvalbumin (2), troponin C (15) and calmodulin (16). It has been suggested by Kretsinger (17) that any protein which contains an "EF-hand" (region of amino acid sequence homologous to the EF region of carp parvalbumin) is calcium modulated. A consideration of the amino acid sequence of bovine α-lactalbumin (18) shows the existence of some, though non-ideal "EF-hand"-like region between Trp 26 and Gln 54, which includes three carboxyl groups of Asp 37. Glu 39 and Glu 43 that could take part in the coordination of bound Ca^{2+}. The region suggested by Hiraoka et al. (1) to be the Ca^{2+} binding site (from Thr 33 to Cys 61) is less like "EF-hand".

Although due to the high binding constant the usual preparations of α-lactalbumin have to be saturated with bound Ca^{2+}, the fact of significant changes in the protein structure induced by the cation binding puts the problem of a reconsideration of many data on the physico-chemical and structural properties of this protein. Particularly it concerns the acid pH conformational change which may be due in fact to a competition of Ca^{2+} and $3H^{+}$ ions for the same site.

REFERENCES

1. Hiraoka,Y., Segawa,T., Kuwajima,K., Sugai,S., and Murai,N. (1980) Biochem. Biophys. Res. Commu. 95, 1098-1104.

2. Permyakov,E.A., Yarmolenko,V.V., Emelyanenko,V.I., Burstein,E.A., Closset,J., and Gerday,Ch. (1980) Eur. J.Biochem. 109, 307-315.
3. Kaplanas,R.I., and Antanavichus,A.I. (1975) Biokhimia (Moscow) 40, 584-587.
4. Kuwajima,K., and Sugai,S. (1978) Biophysical Chem. 8, 247-254.
5. Blum,H.E., Lehky, P., Kohler,L., Stein,E.A., and Fisher, E.H. (1977) J.Biol. Chem. 252, 2834-2838.
6. Burstein,E.A., Permyakov,E.A., Yashin,V.A., Burkhanov,S.A., and Finazzi-Agro,A. (1977) Biochim.Biophys.Acta, 491, 155-159.
7. Teale,F.W.J., and Weber,G. (1957) Biochem.J. 65, 476-482.
8. Reich,J.G., Wangermann,G., Falk,M., and Rohde,K. (1972) Eur. J. Biochem. 26, 368-379.
9. Schwarzenbach,G., and Flaschka,H. (1965) Die Komplexometrische Titration. Ferdinand Enke Verlag, Stuttgart.
10. Burstein,E.A. (1977) Science and Engeneering Results: Biophysics, vol.7, VINITI, Moscow.
11. Kretsinger,R.H. (1976) in Ann. Rev. Biochem. (Snell,E.E., ed.) vol. 45, pp.239-266, Palo Alto, California.
12. Sommers,P.B., and Kronman,M.J. (1980) Biophysical Chem. 11, 217-232.
13. Kuwajima,K., Nitta,K., and Sugai,S. (1980) Biochim. Biophys. Acta, 623, 389-401.
14. Brittain,H.G.,Richardson,F.S., and Martin,R.B. (1976) J. Am. Chem. Soc. 98, 8255-8260.
15. Potter,J.D., and Gergely,J. (1975) J. Biol. Chem. 250, 4628-4633.
16. Potter,J.D., Johnson,J.D., Dedman,J.R., Schreiber,W.E., Mandel,F., Jackson,R.L., and Means,A.R. (1977) in Calcium Binding Proteins and Calcium Function (Wasserman, R.H., et al., eds.), pp.239-250, North-Holland, New York.
17. Kretsinger,R.H. (1975) in Calcium Transport in Contraction and Secretion (Carafoli,E., ed.), pp.469-478, North-Holland Publishing Company.
18. Brew,K., Vanaman,T.C., and Hill,R.L. (1968) Proc. Natl. Acad. Sci. USA, 59, 491-497.