Vol. 95, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1980 Pages 1098-1104

α-LACTALBUMIN: A CALCIUM METALLOPROTEIN

Yoshiki Hiraoka[1], Tatsuhisa Segawa[1], Kunihiro Kuwajima[1], Shintaro Sugai[1] and Norio Murai[2]

[1]Department of Polymer Science, Faculty of Science, Hokkaido University, Kita-Ku, Sapporo 060, Japan

[2]Yagai-Kagaku Co., Higashi-Naebo-Chō, Higashi-Ku, Sapporo 065, Japan

Received July 1,1980

Metal analyses and the studies of the effects of EDTA on unfolding reactions have shown that α-lactalbumin is a calcium metalloprotein. The role of the calcium binding in its biological activity is considered. A plausible site of binding is presented on the basis of the metal-binding site of lysozyme and of the structural models of the protein based on the lysozyme structure.

INTRODUCTION: α-Lactalbumin is a globular protein contained in mammalian-milk whey. Much interest in this protein has been excited by the following discoveries; (i) it is the B component of the lactose synthetase enzyme system (EC 2.4.1.22) (1) and acts as a specifier or modifier of the catalytic activity of galactosyltransferase (2), (ii) its primary sequence is highly homologous to vertebrate lysozyme (3) and hence both the proteins may also exhibit great similarity in their stereoregular structures (4,5). Many reports have appeared along with the above aspects (reviewed in (6,7)).

We wish to report here new observations which undoubtedly demonstrate that α-lactalbumin is a Ca^{2+}-binding metalloprotein.

Our previous studies on the Ca^{2+}-containing α-lactalbumin have shown that the GuHCl†-induced unfolding is represented as a three-state transition involving a stable intermediate which has an appreciable amount of backbone secondary structure (8-10). Kinetics of the transition between the native and the intermediate states has also been investigated (10,11). The present study indicates that the removal of Ca^{2+} by chelation with EDTA shifts this transition to a lower concentration of GuHCl with a remarkable loss of the

† Abbreviations used: GuHCl, guanidine hydrochloride; CD, circular dichroism; NMR, nuclear magnetic resonance; ESR, electron spin resonance.

0006-291X/80/101098-07$01.00/0

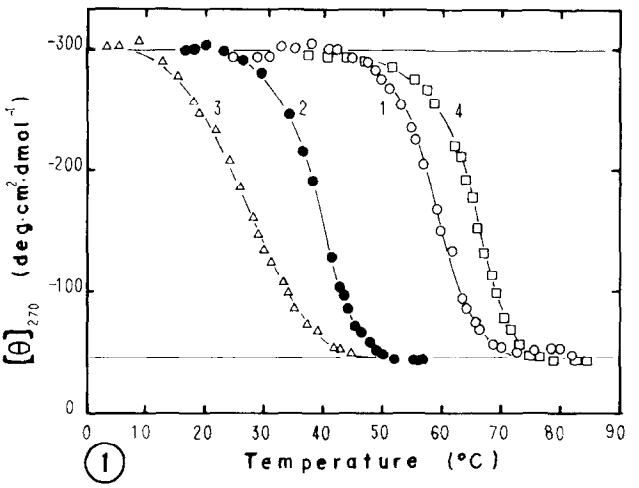
stability of the native structure, although the overall denaturation can also be expressed as a three-state mechanism.

MATERIALS AND METHODS: Bovine, human and goat α-lactalbumins were prepared in the same manner as reported previously (8,10,12). At the final step of the purification, the protein solutions were dialyzed exhaustively against distilled water, and then lyophilized. Specially prepared reagent grade GuHCl was purchased from Nakarai Chemicals Ltd., Kyoto. Other chemicals were analytical reagent grade. Concentrations of the proteins and GuHCl were determined as reported previously (8,10,12).

The stability of the proteins against the thermal and GuHCl-induced unfolding reactions was studied in the presence and absence of EDTA (disodium salt). The effect of $CaCl_2$ on the stability in the absence of EDTA was also examined. The unfolding transition curves were obtained by means of CD measurements done in a Jasco J-20 or a Union CD-1000 recording spectroplarimeter (8). The protein solutions containing EDTA were allowed to stand at 4°C or at a room temperature for more than 10 hours before the measurements.

Metal analyses were performed for bovine α-lactalbumin by flame spectrophotometry (for Ca and Mg) or by atomic absorption spectrophotometry (for Mn and Zn) in a Shimadzu AA-610S Atomic Absorption/Flame Spectrophotometer (13). The lyophilized powder of the protein was dissolved in deionized distilled water, which contained 10 mM EDTA, known to be effective to suppress interference (13), and also contained 15 mM Tris (pH 8.2). The protein solution was directly sprayed into an air-acetylene flame.

RESULTS: The effect of EDTA on the thermal stability of bovine α-lactalbumin is shown in Fig. 1. The transition curves are expressed as temperature dependence of the mean residue ellipticity at 270 nm, $[\theta]_{270}$. The transition curve in the absence of EDTA at neutral pH (curve 1), having a transition temperature of about 58°C, is in good agreement with our previous observation of the transition measured by aromatic absorption at 292 nm (14), and also coincides well with the transition curves measured by other investigators by a variety of techniques; e.g., the temperature dependence of a fluorescence property (transition temperature of 55°C at pH 6.0) (15), aromatic absorption (53~55°C at pH 5.2~6.8) (16,17), and optical rotation (58°C at pH 8.1) (18). Addition of EDTA (1 mM) drastically decreases the stability of the protein, and the transition temperature falls by 20°~32°C (curves 2 and 3 in Fig. 1), although the shape of the CD spectrum of the EDTA-treated protein at a low temperature before the unfolding was found to be essentially the same as that of the original protein. The similar effect of EDTA was also observed for human and goat α-lactalbumins.



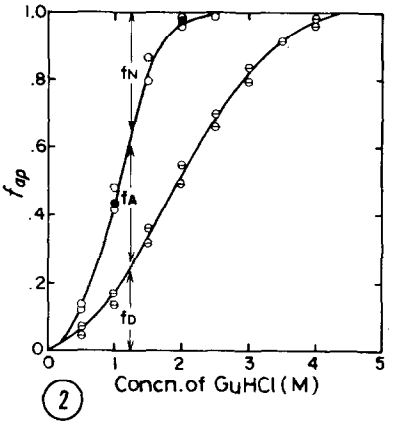


Figure 1: Thermal transition curves of bovine α-lactalbumin: (1) in the absence of EDTA at pH 7.5 (20 mM Tris-HCl); (2) in the presence of 1 mM EDTA at pH 7.0 (0.2 M KCl, 0.05 M phosphate); (3) in the presence of 1 mM EDTA at pH 7.5 (20 mM Tris-HCl); and (4) in the presence of 1 mM $CaCl_2$ at pH 7.5 (20 mM Tris-HCl). Protein concentration was 0.04~0.05%.

Figure 2: The apparent transition curves of the bovine protein for the GuHCl-induced unfolding in the presence of EDTA at 25°C and pH 6.9 (0.2 M KCl, 0.05 M phosphate). The apparent fractional extents of unfolding, $\underline{f}_{ap}$, are plotted against GuHCl concentration: the data from $[\underline{\theta}]_{270}$, 1 mM (○) and 10 mM EDTA (●); and the data from $[\underline{\theta}]_{222}$, 1 mM EDTA (⊖). The fractional extents of the three states are also shown ($\underline{f}_N$, $\underline{f}_A$, and $\underline{f}_D$). Protein concentration was 0.05%.

Figure 2 shows the apparent transition curves of the bovine protein obtained from the variations of $[\underline{\theta}]_{270}$ and $[\underline{\theta}]_{222}$ with the concentration of GuHCl in the presence of 1 or 10 mM EDTA. As shown in the previous study for the unfolding in the absence of chelating agents (8), the transition curves monitored by the two different probes are not coincident with each other; some tertiary structure, measured by aromatic CD, unfolds in a first transition at a low GuHCl concentration and then secondary structure, measured by far ultraviolet CD, is disrupted at a higher GuHCl concentration. The apparent transition curves, however, shift to lower GuHCl concentrations as compared with those in the absence of EDTA (8). If we assume a three-state mechanism, in which only one stable intermediate exists during the unfolding, we can estimate the effects of EDTA on the transitions from the native (N) to

Table I

Metal Analyses of Bovine α-Lactalbumin

	Metal content (mg/liter)	Limits of detection (mg/liter)	Protein concentration (M)	Number of metal atoms per a molecule of the protein
Ca 1	2.78	~0.05	6.23×10^{-5}	1.11
2	1.47	~0.05	$3.11<10^{-5}$	1.18
Mg	*	~1	3.68×10^{-4}	<~0.11
Mn	*	~0.02	1.82×10^{-4}	<~0.002
Zn	0.152	~0.07	3.68×10^{-4}	0.006

*: not detected.

the intermediate (A) and from the A to the fully unfolded (D) state by using the same analytical method as used previously (8). The N⇄A transition is affected remarkably and the midpoint of the transition shifts from 2.3 M to 1.2 M GuHCl on treatment with EDTA, while the A⇄D transition shifts only by 0.3 M in the denaturant concentration. Thus, the mode of action of EDTA is to labilize native α-lactalbumin, and after the disruption of the tertiary structure EDTA does not significantly affect a further conformational transition.

The metal analyses of bovine α-lactalbumin are shown in Table I. The protein contains about one atom of Ca per a molecule of the protein, and the concentration of other metals examined is insignificant.

All of the above results demonstrate that native α-lactalbumin binds at least one Ca^{2+} ion and that the removal of Ca^{2+} by chelation with EDTA reduces the stability of the native tertiary structure.

Addition of $CaCl_2$ to the protein solutions in the absence of EDTA further stabilize the native structure. The result for the bovine protein is also shown in Fig. 1 (curve 4). A similar stabilizing effect was also observed in the GuHCl-induced unfolding and also for the goat and human proteins. Thus, there are at least two kinds of binding sites for Ca^{2+} in the protein. The first which binds one Ca^{2+} has such a high affinity that the Ca^{2+} cannot be removed from the protein by the purification procedure. The second sites

have relatively low affinities, but the binding of Ca^{2+} to these sites brings about the further stabilization of the native structure.

DISCUSSION: The criteria for the identification of metalloprotein have been delineated (19), and the present data establish that α-lactalbumin is a calcium metalloprotein. We can safely conclude that the highly purified α-lactalbumin prepared by the currently used techniques contains a stoichiometric quantity of Ca^{2+}, namely, one Ca^{2+} per a molecule of the protein.

Whether or not α-lactalbumin requires Ca^{2+} for its biological activity, remains to be proven. However, in view of a high content of Ca^{2+} in mammalian milk (35~130 mg per 100 ml of milk) (20), it is reasonably expected that the Ca^{2+} binding might serve as a key role for the action of the protein or the biosynthesis of lactose in mammary glands. Most of the extracellular enzymes that bind Ca^{2+} are not only stabilized but also partially activated by Ca^{2+} at the concentration as is normally found in the extracellular environment (21). With free Ca^{2+} concentrations in the cytosol (~10^{-7} M), these enzymes may not be activated prior to their secretion into a secretory granule (21). There is a paradox of the secretion into milk of large quantities of α-lactalbumin with its important intracellular function during lactation. It has been suggested that not only the presence of α-lactalbumin in the secretory cells of the mammary glands but also its movement through the cisternae of the Golgi menbrane, which binds galactosyltransferase, during lactation is required for maximal synthesis of lactose (6,22).

It has been shown that hen egg-white lysozyme can also bind polyvalent metal cations and may be classfied into the group of Ca^{2+}-binding proteins (21,23). X-Ray crystallographic, NMR, ESR and spectroscopic studies on the lysozyme-metal complexes have been carried out extensively (23-31). This protein in solution forms a 1:1 stoichiometric complex with each metal cation, and the binding site is located between the two carboxylate groups of Glu-35 and Asp-52 in the active site cleft. Model building studies of α-lactalbumin

(4,5) suggest that a cleft region also appears in the region corresponding to the metal-binding site of lysozyme. Although the occupation of Tyr-103 in the cleft of α-lactalbumin blocks the binding with lysozyme substrate (4,5), the Ca^{2+} binding with α-lactalbumin is expected to occur in this cleft region in view of relatively small size of the Ca^{2+} ion. Alignment of the known sequences of six α-lactalbumins (bovine, human, guinea pig, goat, rabbit, and rat) indicates that one of the two functional carboxyl residues in lysozyme is conserved throughout in the α-lactalbumins (Asp-49 or Glu-49) (3,7,32-34). The residue corresponding to Glu-35 is replaced by Thr-33 except for the replacements in the guinea pig and kangaroo proteins (Ile-33) (7). It is also noteworthy that the residues neighboring with Asp-(or Glu-)49 (e.g., Ser-47, Thr-48, Tyr-50, Gly-51) are also conserved in the α-lactalbumin-lysozyme group of proteins. Our previous studies have suggested that one of these conserved residues, Tyr-50, has an abnormally high p$\underline{K}$ value in the N state and cooperates with some carboxyl group(s) to stabilize the native structure (35,36).

ACKNOWLEDGMENTS:

The authors wish to thank the Ministry of Education of Japan for supporting them with Grants in Aid. They are also grateful to Prof. Michio Yoneyama and Dr. Katsutoshi Nitta for their helpful discussions and advice.

REFERENCES

1. Brodbeck, U., Denton, W.L., Tanahashi, N., and Ebner, K.E. (1967) J. Biol. Chem. 242, 1391-1397.
2. Brew, K., Vanaman, T.C., and Hill, R.L. (1968) Proc. Natl. Acad. Sci. USA, 59, 491-497
3. Brew, K., Castellino, F.J., Vanaman, T.C., and Hill, R.L. (1970) J. Biol. Chem. 245, 4570-4582.
4. Browne, W.J., North, A.C.T., Phillips, D.C., Brew, K., Vanaman, T.C., and Hill, R.L. (1969) J. Mol. Biol. 42, 65-86.
5. Warme, P.K., Momany, F.A., Rumball, S.V., Tuttle, R.W., and Scheraga, H.A. (1974) Biochemistry, 13, 768-782.
6. Hill, R.L., and Brew, K. (1975) Adv. Enzymol. Relat. Areas Mol. Biol. 43, 411-490
7. Hill, R.L., Steinman, H.M., and Brew, K. (1974) in Lysozyme (Osserman, E.F., Canfield, R.E., and Beychok, S., eds.), pp. 55-62, Academic Press, New York.
8. Kuwajima, K., Nitta, K., Yoneyama, M., and Sugai, S. (1976) J. Mol. Biol. 106, 359-373.
9. Kuwajima, K. (1977) J. Mol. Biol. 114, 241-258.

10. Nozaka, M., Kuwajima, K., Nitta, K., and Sugai, S. (1978) Biochemistry, 17, 3753-3758.
11. Nitta, K., Kita, N., Kuwajima, K., and Sugai, S. (1977) Biochim. Biophys. Acta, 490, 200-208.
12. Kuwajima, K., Nitta, K., and Sugai, S. (1980) Biochim. Biophys. Acta, in press.
13. Willis, J.B. (1963) Methods of Biochem. Analysis, 11, 1-67.
14. Kuwajima, K., and Sugai, S. (1978) Biophysical Chem. 8, 247-254.
15. Sommers, P.B., and Kronman, M.J. (1980) Biophysical Chem. 11, 217-232.
16. Takase, K., Niki, R., and Arima, S. (1976) Agr. Biol. Chem. (Tokyo), 40, 1273-1277.
17. Takesada, H., Nakanishi, M., and Tsuboi, M. (1973) J. Mol. Biol. 77, 605-614.
18. Barel, A.O., Prieels, J.P., Maes, E., Looze, Y., and Léonis, J. (1972) Biochim. Biophys. Acta, 257, 288-296.
19. Vallee, B.L., and Wacker, W.E.C. (1970) The Proteins 2nd Ed. 5, 1-170.
20. Evans, M.T.A., and Gordon, J.F. (1980) in Applied Protein Chem. (Grant, R.A., ed.), pp. 31-67, Applied Sci. Publishers, Ltd., London.
21. Kretsinger, R.H. (1976) Internatl. Rev. Cytology, 46, 323-393.
22. Brew, K. (1969) Nature, 222, 671-672.
23. Levine, B.A., Williams, R.J.P., Fullmer, C.S., and Wasserman, R.H. (1977) in Calcium-Binding Proteins and Calcium Function (Wasserman, R.H., Corradino, R.A., Carafoli, E., Kretsinger, R.H., MacLennan, D.H., and Siegel, F.L., eds.), pp. 29-37, Elsevier North-Holland, Inc., New York & Amsterdam.
24. McDonald, C.C., and Phillips, W.D. (1969) Biochem. Biophys. Res. Commu. 35, 43-51.
25. Gallo, A.A., Swift, T.J., and Sable, H.Z. (1971) Biochem. Biophys. Res. Commu. 43, 1232-1238.
26. Jori, G., Gennari, G., Galiazzo, G., and Scoffone, E. (1971) Biochim. Biophys. Acta, 236, 749-766.
27. Ikeda, K., and Hamaguchi, K. (1973) J. Biochem. (Tokyo), 73, 307-322.
28. Teichberg, V., Sharon, N., Moult, J., Smilansky, A., and Yonath, A.(1974) J. Mol. Biol. 87, 357-368.
29. Secemski, I.I., and Lienhard, G.E. (1974) J. Biol. Chem. 249, 2932-2938.
30. Jones, R., Dwek, R.A., and Forsén, S. (1974) Eur. J. Biochem. 47, 271-283.
31. Perkins, S.J., Johnson, L.N., Machin, P.A., and Phillips, D.C. (1979) Biochem. J. 181. 21-36.
32. MacGillivray, R.T.A., Brew, K., and Barnes, K. (1979) Arch. Biochem. Biophys. 197, 404-414.
33. Hopp, T.P., and Woods, K.R. (1979) Biochemistry, 18, 5182-5191.
34. Prasad, R., Hudson, B.G., Butkowski, R., Hamilton, J.W., and Ebner, K.E. (1979) J. Biol. Chem. 254, 10607-10614.
35. Kuwajima, K., Ogawa, Y., and Sugai, S. (1979) Biochemistry, 18, 878-882.
36. Kuwajima, K., Ogawa, Y., and Sugai, S. (1980) submitted.