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An Experimental Artifact in the Use of Chelating Metal Ion Buffers

BINDING OF CHELATORS TO BOVINE α-LACTALBUMIN*

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The binding of EDTA and EGTA to bovine α-lactalbumin was shown to stabilize the native conformation relative to that characteristic of the metal-free protein (A conformer). Fluorescence titration of the metal-free protein with Ca^{2+} in the presence of EDTA was markedly influenced by micromolar concentrations of EDTA such that the apparent association constant of calcium binding would be significantly larger than obtained in the absence of chelator. These observations explain the discrepancy in values of K_a reported by different investigators for binding near neutral pH: 2.75×10^6 M^{-1} (Kronman, M. J., Sinha, S. K., and Brew, K. (1981) *J. Biol. Chem.* 256, 8582–8586); 6.3×10^8 M^{-1} (Permyakov, E. A., Yarmolenko, V. V., Kalinichemko, L. P., Morozova, L. A., and Burstein, E. A. (1981) *Biochem. Biophys. Res. Commun.* 100, 191–197); and 4×10^9 M^{-1} (Murakami, K., Andree, P., and Berliner, L. J. (1982) *Biochemistry* 21, 5488–5494). The last two high values were obtained by fluorescence titration in the presence of EGTA, while the former lower one was determined by a gel filtration technique in the absence of chelators. The anomolous association constants for calcium binding and the alteration of a conformational equilibrium observed in the presence of chelators demonstrate the need for great care in the use of chelating metal ion buffers in the studying of metalloproteins.

Native α-lactalbumin as isolated from milk contains a single strongly bound calcium ion (1). Removal of the calcium has a marked effect on the molecular conformation of the protein. This transformation from the N to the A conformational state is accompanied by a long wavelength shift of the tryptophan emission spectrum and an increase in the quantum yield (2, 3). On further examination of this conformational change, as part of a program of study on the role of metal ions in lactose synthetase action, we considered the use of chelating metal ion buffers. Such EDTA or EGTA[1] chelating buffers have been widely used in studying metalloproteins where accurately known metal ion concentrations sufficiently low to measure high affinity constants are required. In large part, uncertainty in metal ion concentration has been due to contamination of reagents, glassware, etc. Although the use of chelating buffers has been widely accepted in such applications, there appear to have been few instances where investigators have demonstrated their "innocuous character," *i.e.* the absence of chelator binding with concomitant alteration of protein properties.

In our attempts to use EDTA or EGTA buffers, we found that these chelators bind to α-lactalbumin and appear to modify the equilibrium between N and A conformers. This study will present evidence for this conclusion and consider the influence of chelator binding on the measurement of Ca^{2+} binding and the apparent thermal stability of the protein.

EXPERIMENTAL PROCEDURES

Materials—BLA was prepared by a modification (4) of the method of Aschaffenburg and Drewry (5) and stored as a frozen lyphilized powder. Metal free buffers and water were prepared as described earlier (3). Other chemicals such as HCl, which could not be purified by treatment with Chelex 100 (Bio-Rad), were of MCB Manufacturing Chemists, Inc. "SUPRAPUR" grade. ^{14}C-labeled EDTA was obtained from Amersham Corp.

Preparation of Apo-BLA—Our earlier procedure for preparing apo-BLA called for 24 h of dialysis of the protein solution against 100 mM EDTA to remove the calcium, followed by 48 h of dialysis *versus* a minimum of 10 changes of buffer to remove chelator. In view of our concerns about the effect of EDTA binding to the protein, we have carried out this preparation in the presence of ^{14}C-labeled EDTA. Preparations of apo-BLA were found typically to contain no more than 0.02 to 0.03 mol of EDTA/mol of protein. Nonetheless, in order to circumvent the potential problem of binding of the chelator, we have developed the following procedure which eliminates the use of EDTA. This procedure is based on our observation that apo-BLA does not bind Ca^{2+} at pH 1.73.

Approximately 120 mg of BLA was dissolved in 3 ml of 2 mM Tris buffer, pH 7.6. The pH was then adjusted to 1.7 with 10 M metal-free HCl using *very efficient* stirring, the solution was applied to a column (2.5 × 95 cm) of Sephadex G-15 which had been pre-equilibrated with metal free 0.012 M HCl, and then eluted with the same solvent. In a separate experiment, $^{45}Ca^{2+}$ was eluted through the same column in the absence of protein; the position of this peak of radioactivity relative to the protein peak indicates complete separation of the apoprotein and calcium. The apoprotein so obtained was stored in the 0.012 M HCl solvent in the frozen state. In most cases, however, the high concentration of H^+ interferes with subsequent measurements. In such cases, the bulk of the HCl is removed by lyophilization, and the solid protein is dissolved in 1 mM Tris buffer, pH 7.5, prior to storage in the frozen state. Apoprotein prepared by this procedure is indistinguishable (fluorescence, binding affinity for calcium, and the ability to catalyze the lactose synthetase reaction in the presence of metal ion) from that prepared (see above) by the EDTA method.

Methods—While our primary method for determining if calcium has been removed from the protein has been atomic absorption (3), typical values for apo-BLA being about 0.05 G atom of Ca^{2+}/mol of protein, our *routine* test for its removal makes use of the observed tryptophan fluorescence. For preparations of apoprotein essentially free of Ca^{2+} (atomic absorption), addition of this ion to a final concentration of 10 mM gives rise to a 3-fold decrease in fluorescence intensity at 370 nm. Values of the fluorescence ratio at 370 nm significantly lower than 3 are indicative of incomplete removal of calcium or, as we shall see below, incomplete removal of chelator if

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[1] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; BLA, bovine α-lactalbumin; apo-BLA, metal-free bovine α-lactalbumin.

the EDTA procedure has been used to prepare apoprotein. The binding of Ca^{2+} will also shift the tryptophan maximum emission from 340 to 329 nm, but this is a less sensitive measure of calcium contamination.

Experiments were carried out under stringent metal-free conditions; procedures for cleaning glassware and plasticware have been described, as have methods for carrying out fluorescence measurements (3). Some of the latter were made using an SLM 8000 spectrofluorometer, rather than the laboratory built instrument employed earlier. Emission spectra obtained in this study were corrected for detector response.

RESULTS AND DISCUSSION

Removal of Ca^{2+} from BLA—If chelators such as EDTA and EGTA are innocuous and only abstract Ca^{2+} from BLA, the ratio of the fluorescence at 370 nm in the presence and absence of chelator should be close to 3, the inverse of the value obtained on saturation of *apo*-BLA with calcium. Shown in Fig. 1 is the concentration dependence of $(F_{370})_{rel}$ for both EGTA and EDTA. At very high concentrations of chelator, we have the paradoxical situation where the apparent "efficiency" of the chelator in abstracting calcium from the protein is *inversely* proportional to its concentration. $(F_{370})_{rel}$ goes through a maximum with decreasing concentration. In the case of EDTA, the maximum ratio is considerably greater than 3 (*dotted horizontal line*), the value cited above for complete removal of Ca^{2+}, while for EGTA the maximum value is significantly lower than 3. We have shown in ancillary experiments (data not shown) that neither EDTA nor EGTA have any influence on the fluorescence of free tryptophan. The complex character of the curves of Fig. 1 indicate that binding of the chelator, or perhaps chelator·Ca^{2+} complex, to protein occurs (see below).

Binding of Chelators to Apo-BLA—Titration of apo-BLA with EDTA or with EGTA (Fig. 2) reduces the fluorescence intensity and produces a short wavelength shift of the emission spectrum. Shown also is a fluorescence titration curve for Ca^{2+}-apo-BLA obtained in the absence of chelator. The change in fluorescence on saturation with Ca^{2+} is about 3-fold, as indicated above, while for EDTA and EGTA the change is approximately 2.2 and 1.7, respectively. It is interesting that the emission maximum obtained on titration with EDTA or EGTA approaches the value found on binding of calcium ion, suggesting that binding of chelators may stabilize the N conformational state relative to the A state even when Ca^{2+} is absent.

Inspection of Fig. 2 indicates that binding of chelator to apo-BLA is not significant below a concentration of 0.1 mM. Thus, if binding of EDTA or EGTA to apoprotein were the *sole* cause of the anomalous results presented in Fig. 1, we would expect that plots of (F_{370})rel *versus* log (chelator) would exhibit plateaus at $(F_{370})_{rel} = 3$ for chelator concentrations below 0.1 mM. Since this is not the case, it seems probable that binding of a calcium complex of chelator to α-lactalbumin may also occur even at very low concentrations of chelator.

Titration of Apo-BLA with Calcium in the Presence of EDTA—Comparison of fluorescence titration curves for Ca^{2+}-apo-BLA obtained in the presence and absence of EDTA (Fig. 3) reveal the effect of binding of chelator. It is convenient to represent such titration data as plots of log K_{app} *versus* log (Ca^{2+}). We refer to "apparent" values of K since the fluorescence change which occurs in the presence of chelator may reflect spectral changes other than those associated with the following conformational changes (2, 3).

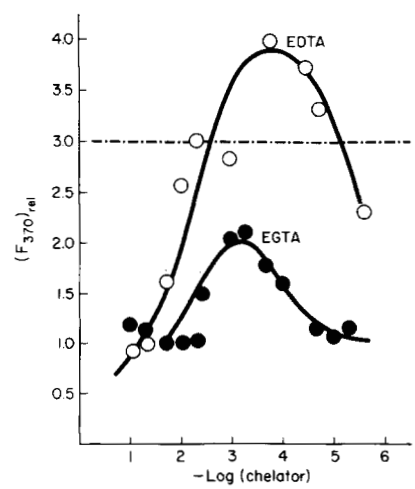

FIG. 1. **Effect of EDTA and EGTA on the fluorescence of BLA.** $(F_{370})_{rel}$ is the fluorescence intensity at 370 nm (excitation, 295 nm) for BLA in the presence of chelator relative to that found in its absence. Measurements were made in 0.02 M Tris buffer, pH 7.5, 30 min after preparation of the solution. Protein concentration, 3.5 μM. —·—·— indicates the maximum value predicted, assuming that the change is due solely to the removal of Ca^{2+}.

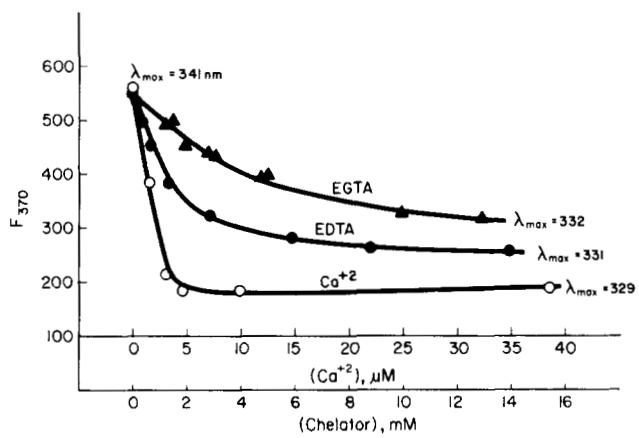

FIG. 2. **Fluorescence titration of apo-BLA with EDTA, EGTA, or Ca^{2+}.** 0.02 M Tris buffer, pH 7.5; protein concentration, 12 μM; emission wavelength, 370 nm; excitation wavelength, 295 nm. Titrations were carried out by addition of aliquots of concentrated EDTA, EGTA, or Ca^{2+} stock solutions to the fluorescence cell containing *3.00 ml* of the protein solution. The fluorescence intensity was corrected for dilution. Emission maxima (corrected spectra) were obtained for the *final points* on the *titration curves*.

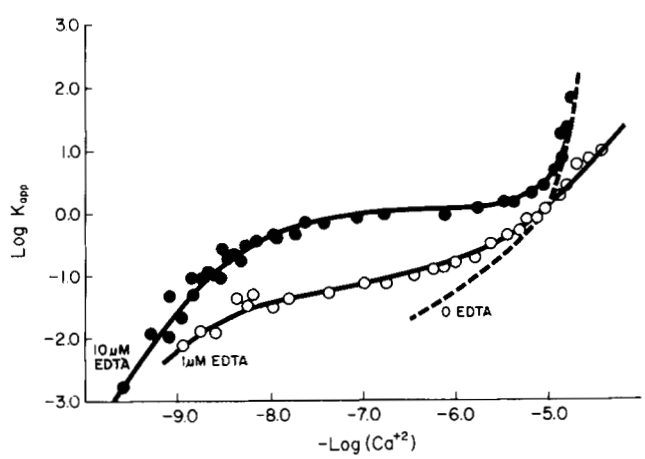

FIG. 3. **Log K_{app} *versus* log (Ca^{2+}) obtained from fluorescence titration of apo-BLA with Ca^{2+} at 370 nm in the presence and absence of EDTA (see text).** Excitation wavelength, 295 nm; temperature, 25.0 °C; 0.02 M Tris buffer, pH 7.5; protein concentration, 16.1 μM.

$$(BLA)_A + Ca^{2+} \underset{}{\overset{}{\rightleftharpoons}} (BLA)_N \quad (1)$$

$$K_{app} = \frac{\Delta F_{370\ nm}}{(\Delta F_{max} - \Delta F)_{370\ nm}} = \frac{(BLA)_N}{(BLA)_A} \quad (2)$$

The calcium ion concentration (Fig. 3) in the absence of EDTA is the total concentration, while in the presence of EDTA the calcium ion concentration is the *free* concentration computed from the EDTA concentration and the total calcium concentration using the EDTA association constants given by Portzehl *et al.* (6).

The equilibrium between N and A conformers lies far toward A in the absence of EDTA below $p_{Ca^{2+}}$ equal to 6.5, while in the presence of EDTA significant amounts of N appear to be present at $p_{Ca^{2+}}$ values as low as 8 to 8.5. This difference in the Ca^{2+} titration curves obtained in the presence and absence of chelator suggests that its binding is still significant at micromolar concentrations.

The short wavelength shift which is observed on binding of chelator (see above) is consistent with its preferential binding to the N conformer, thereby stabilizing it relative to the A form of the protein. Likewise, although Kronman *et al.* (3) showed with fluorescence measurements that chelator-free apo-BLA is in the A conformation even at room temperature (no cooperative melting), Hiroaka *et al.* (1) have observed cooperative melting (circular dichroism measurements) from the N to A state for BLA in the presence of 1 mM EDTA. Hiroaka *et al.* (1) concluded that EDTA only *destabilizes* the protein with respect to thermal melting. Their observation of the N state at room temperature is almost certainly due to the artifact of EDTA stabilizing this conformation even when Ca^{2+} might have been removed from the protein.

Binding of Ca^{2+} to Apo-BLA—The fluorescence titrations curves of Fig. 3 could be used to determine the binding affinities of Ca^{2+} and apo-BLA assuming that a single calcium ion is bound to the N conformer. Based on the data of Fig. 3, the effect of chelator would be to increase the apparent affinity of apo-BLA for calcium.

In our original publication (3) describing the preparation and characteristics of apo-BLA, we used $^{45}Ca^{2+}$ and the Hummel-Dryer (see Ref. 7) gel filtration technique to determine binding of Ca^{2+} in the absence of chelators and obtained a K_a of 2.75×10^6 M^{-1} at pH 7.4. Since then, Permyakov *et al.* (8) and Murakami *et al.* (9), using tryptophan fluorescence titrations in the presence of EGTA to characterize binding of Ca^{2+} to apo-BLA in the pH range of 6.5 to 9, find values of 6.3×10^8 and 4×10^9 M^{-1}, respectively, for K_a. No explanation is given in either paper as to the reason for the 200 to 1500-fold difference in association constants from the value we reported earlier. Since our original report, we have carried out extensive measurements of the pH and ionic strength dependence of binding of Ca^{2+} to apo-BLA using the gel filtration technique cited above.[2] The values of K_a that we observe between pH 5.9 and 8 are in the range of 1.6 to 2.2×10^6 M^{-1} in good agreement with our earlier reported value, but totally inconsistent with those found by Permyakov *et al.* (8) and Murakami *et al.* (9). In view of the observations reported in this study, it seems likely that the association constants obtained by fluorescence titration in the presence of EGTA are anomolously high due to binding of the chelator to the protein. These observations suggest that great care must be exercised in the use of chelating metal ion buffers. Demonstration that such chelators have no effect on the protein other than abstracting metal ions would seem to be essential.

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