On Experimental Artifacts in the Use of Metal Ion Chelators for the Determination of the Cation Binding Constants of α-Lactalbumin

A REPLY* (Received for publication, September 19, 1986)

Eugene A. Permyakov‡, Kentaro Murakami¶, and Lawrence J. Berliner¶||
From the Institute of Biological Physics, Union of Soviet Socialist Republics Academy of Sciences, Pushchino, Moscow Region, 142292, Union of Soviet Socialist Republics and ‡Department of Chemistry, The Ohio State University, Columbus, Ohio 43210

The binding constant of Ca\(^{2+}\) to the strong cation site of bovine \(\alpha\)-lactalbumin has been measured directly by monitoring the free calcium concentration by Quin 2 fluorescence. A dissociation constant of 1–4 nM was calculated, which confirms the strong calcium binding properties of this protein. In order to examine whether the metal ion chelators EDTA or EGTA affect the cation binding equilibria by binding to bovine \(\alpha\)-lactalbumin, calcium binding equilibria were carefully measured under highly stabilized pH and temperature conditions. Within the concentration ranges required for competitive binding by these ligands (EDTA or EGTA) (<1–3 mM) these chelators produced no artifacts, in contradiction to the data of Kronman and Bratcher (Kronman, M. J., and Bratcher, S. C. (1983) J. Biol. Chem. 258, 5707–5709).

In 1980 Hiraoka et al. (1) revealed that typical common preparations of bovine \(\alpha\)-lactalbumin \((\alpha\text{-LA})\) contained Ca\(^{2+}\) ions. Since then, several groups of researchers determined equilibrium parameters for the interaction of \(\alpha\text{-LA}\) with Ca\(^{2+}\) ions. Most of them used either a competitive EGTA backtitration of the Ca\(^{2+}\)-loaded protein or Ca\(^{2+}\)-EGTA buffers (4–7). The Ca\(^{2+}\) dissociation constant for the single strong binding site of \(\alpha\text{-LA}\) obtained in the presence of the chelator was in the 0.2–3 nM range. Kronman et al. (8), using the Hummel-Dreyer gel-filtration method in the absence of any chelators, obtained a value almost 2–3 orders of magnitude larger \((2.7 \times 10^8 \text{ M}^{-1})\). In a later report, Kronman and Bratcher (9) explained this discrepancy as due to the binding of EGTA or EDTA to \(\alpha\text{-LA}\), even at the very low concentrations employed for the determination of Ca\(^{2+}\) binding constants in the presence of the chelators. While they never directly measured \(\alpha\text{-LA}\)-chelator binding, their main evidence for the existence of an EGTA (or EDTA)–\(\alpha\text{-LA}\) complex at low concentrations was the fact that fluorometric EDTA (EGTA) titration curves went through different maxima.

In the present work we have measured the Ca\(^{2+}\)–\(\alpha\text{-LA}\) dissociation constant by quantitating free Ca\(^{2+}\) directly. Furthermore, we have thoroughly remeasured the fluorometric EGTA and EDTA titration curves for bovine \(\alpha\text{-LA}\) under highly stabilized pH and temperature conditions. It was found that these curves (which differed in chelator concentrations due to their different affinities for Ca\(^{2+}\)) go through the same maxima and coincide with one other. Thus, the suggestion of the existence of EGTA or EDTA binding to \(\alpha\text{-LA}\) at low chelator concentrations (<1–3 mM) is unfounded (9).

MATERIALS AND METHODS

Bovine \(\alpha\text{-LA}\) was from the Kaunas Medical Institute (U. S. S. R.) (10) or Sigma (Lot No. 52P-8075-1). Protein concentrations were evaluated spectrophotometrically using \(E_{\text{280nm}}^{1000} = 20.1\) at 280 nm (11). Metal-free \(\alpha\text{-LA}\) was prepared either according to the method of Blum et al. (12) or Murakami et al. (3). The "metal-free" protein preparations contained no detectable chelator. All solutions were prepared using deionized water and were treated with Chelex 100 prior to use. Plasticware was used exclusively. Calcium content was determined by atomic absorption.

The fluorescence emission of Quin 2 (50 mM Tris-HCl, pH 7.4, 26 °C) was monitored at 490 nm \((\lambda_{\text{ex}} = 340 \text{ nm})\) on an SLM 4800S spectrophotometer. Calibration curves were prepared by titrating 1.3 μM Quin 2 with a standard Ca\(^{2+}\) solution. The curve was linear up to 1000 nM Ca\(^{2+}\). A calcium \(\alpha\text{-LA}\) (1:1) solution (274 μM) was placed inside a carefully prewashed dialysis sack (Spectrapor, M<sub>W</sub> 1200 cutoff) and equilibrated versus Chelex-treated dialysis buffer (50 mM Tris-HCl, pH 7.4). An aliquot of the dialysate was removed over time and measured versus the same Quin 2 calibration standard until the measured calcium concentration leveled off. The data were corrected for trace calcium levels in the buffer alone (~5–10 nM), which was 20% above background intensity (at 490 nm) on the SLM instrument. The equilibrium data were treated in a manner similar to that used by Bertho et al. (13).

Intrinsic fluorescence measurements were carried out as described earlier (3, 14). Protein fluorescence quantum yield was evaluated by comparing the areas under the spectra with that of free aqueous tryptophan (fluorescence yield 0.23 at 20 °C (15)) with the same absorbance at the excitation wavelength (280.4 nm). The center position of a chord drawn at 80% intensity was used to determine λ, the spectral maximum. Cell temperature was measured with a polyethylene-covered copper-constantan thermocouple.

RESULTS AND DISCUSSION

In an attempt to measure directly the \(\alpha\text{-LA}\) Ca\(^{2+}\) affinity constant, we quantitated free unbound Ca\(^{2+}\) concentration by equilibrium dialysis at pH 7.4 (50 mM Tris-HCl, 26 °C). The dialysate was then quantitated for free Ca\(^{2+}\) by Quin 2 fluorescence at 490 nm until the measured free Ca\(^{2+}\) levels were constant. A \(K_{\text{dis}}\) value of 1.2 ± 0.4 nM was determined by calculating \([\alpha\text{-LA}]_{\text{free}}\) based on the free [Ca\(^{2+}\)]. If, on the other hand, we estimated [\(\alpha\text{-LA}\)] from the protein intrinsic fluorescence R value of Murakami et al. (3) then the \(K_{\text{dis}}\) value...
increased to 4.3 ± 1.5 nm; i.e. the $K_{\text{diss}}$, must be in the 1-4 nm range, in total agreement with other methods (2-7).

The main argument of Kronman and Bratcher (9) in favor of the existence of EGTA and EDTA binding to $\alpha$-LA at low chelator concentrations was based on their spectrofluorometric titration curves that went through different maximal levels which were also different from those for the apoprotein in the absence of chelators. According to their data, the ratio of the fluorescence intensities at 370 nm in the absence and presence of calcium ions ($F_{370\text{nm}}$) was equal to 3.0 in the absence of chelators. In the presence of EGTA, $F_{370\text{nm}}$ did not reach 3.0, but in the presence of EDTA it exceeded 3.0. They explained this discrepancy as being due to different binding characteristics of each chelator (i.e. EGTA or EDTA) to $\alpha$-LA.

Fig. 1 shows the results of EGTA and EDTA titrations of $1.5 \times 10^{-3} \text{m} \text{Ca}^{2+}$-loaded bovine $\alpha$-LA carried out in 50 mM Hepes, pH 8.1, 25 °C. The only difference from the experiments of Kronman and Bratcher (9) was that the buffer (Hepes versus Tris-HCl) concentration was 2.5-fold higher in order to stabilize the pH at high chelator concentrations. The EGTA (EDTA) titrations caused a shift of the tryptophan fluorescence to longer wavelengths with a concomitant rise in fluorescence quantum yield (Fig. 1, A and B). Fig. 1C depicts the protein fluorescence intensity changes, $F_{370\text{nm}}$, as defined by Kronman and Bratcher (9). Since EGTA binds $\text{Ca}^{2+}$ more tightly than EDTA at pH 8.1 (16), the EGTA titration curve plateaus at lower chelator concentration. However, note that both titration curves (EGTA and EDTA) reach exactly the same level (i.e. ~3.0). According to Kronman and Bratcher (9) an $F_{370\text{nm}}$ value of 3.0 is inherent to the apoprotein in the absence of the chelators. Note that this fluorescence parameter plateaus and remains constant up to a concentration of ~1 mM chelator and then begins to change, but to a much lesser extent than in the data of Kronman and Bratcher (9). Even at chelator concentrations up to 10 mM, the fluorescence intensity at 370 nm is quenched by only ~20%, and the emission maximum of $\alpha$-LA is only 2 nm shorter than that in the plateau region. It is possible that these $\alpha$-LA fluorescence changes in the several millimolar chelator concentration range may reflect real binding of EGTA (or EDTA), but their association constants would be rather low ($<10^3 \text{ M}^{-1}$). Some weak binding of chelators to $\alpha$-LA would not be unexpected, since it is well known that other $\text{Ca}^{2+}$-binding proteins (e.g. parvalbumin, calmodulin) also bind EGTA and EDTA weakly (17, 18). Most important, however, is that such binding would have a negligible influence on the results of an EGTA (or EDTA) titration of low chelator concentrations.

Another piece of evidence that negates the effect of any influence of EGTA or EDTA on $\alpha$-LA calcium affinity is the fact that cation binding constants determined by ESR (by $\text{Ca}^{2+}$-Mn$^{2+}$ competition (3) in the absence of chelators) were in good agreement with those obtained in the competitive titration fluorescence experiments.

It is also true that binding of $\text{Ca}^{2+}$ to EGTA or EDTA at neutral pH results in a release of protons. If buffer capacity were insufficient, i.e. 20 mM Tris-HCl at high chelator concentration (9), the resultant acidification would be in concert with an apparent decrease in $\text{Ca}^{2+}$ affinity. Furthermore, it is also possible that under the high EGTA/EDTA concentrations employed by Kronman and Bratcher (9), sufficiently high ionic strengths were present in their solutions to alter the activity coefficient of $\text{Ca}^{2+}$. For example, Mitani et al. (5) have shown that the "apparent" $K_{\text{diss}}$ for $\text{Ca}^{2+}$ decreases by factors of $10^5$–$10^6$ for Na$^+$ concentrations of 10–100 mM, respectively. Another contribution to altered $\text{Ca}^{2+}$ binding...
may arise from temperature effects on pH. Specifically, Tris buffers have a substantial negative $\Delta pK_a/\Delta T$ coefficient around neutral pH which is, of course, reflected in the affinity of Ca$^{2+}$ for EGTA/EDTA (19). For example, a 2–3 °C temperature fluctuation results in an ~1.5-fold change in the Ca$^{2+}$-EDTA equilibrium constant (19). There are also thermal denaturation effects on apo-α-LA, which are reflected dramatically in its intrinsic fluorescence emission (20). The consequences of slight (~2–3 °C) temperature fluctuations in the range employed by Kronman and Bratcher (9) for apo-α-LA are substantial variations in the observed fluorescence parameters (20). While it is impossible to assess which of those contributions above accounted for the irreproducible results of Ref. 9, it is not difficult to imagine that a combination of the above was responsible.

Other more quantitative errors appear to exist in the work of Kronman and Bratcher (9). They computed free calcium concentration in the presence of EDTA assuming their Ca$^{2+}$-α-LA binding constant of $2.75 \times 10^6$ M$^{-1}$, but did not take into consideration the competition for Ca$^{2+}$ ions between EDTA and the protein (16). Since it is certainly clear that the Ca$^{2+}$-α-LA dissociation constant lies in the nanomolar region (2-7), such corrections would shift the Kronman and Bratcher curves in the presence of α-LA (16.1–5). Kronman and Bratcher (9) for apo-α-LA, they never succeeded in detecting the Ca$^{2+}$ affinity constant for α-LA is 2.75 $\times 10^6$ M$^{-1}$, and EDTA, pH 7.5, (1 or 10 μM) to higher, not lower, Ca$^{2+}$ concentrations. There are, in fact, several other contradictions in their results. In spite of their assumption that there is a strong binding site for chelators on α-LA, they never succeeded in detecting radioactive EGTA binding to α-LA. In addition, their spectrophotometric Ca$^{2+}$ titration curves showed saturation at 5 or 10 μM Ca$^{2+}$, whereas the initial protein concentrations were reported to be 12 or 16 μM, respectively. This is impossible for an allegedly Ca$^{2+}$-free protein!

CONCLUSIONS

It is clear from the results of this study, as well as previously reported results from at least five independent research groups around the world, that the Ca$^{2+}$ affinity constant for α-LA is in the nanomolar range and is not a function of the presence of chelators such as EDTA or EGTA. Of course, errors and discrepancies occur in the literature from time to time due to experimental oversights which are frequently understandable. What was very unfortunate in the paper of Kronman and Bratcher (9) was the misrepresentation of the title to apparently erroneous data that did not actually support its implications.

Acknowledgments—We are extremely grateful to Dr. Keiko Koga for advice and discussion. Some of the bovine α-LA was a gift from Dr. V.V. Yarmolenko, Kaunas Medical Institute, U. S. R.

REFERENCES