High-affinity binding of Ca^{2+} to bovine α -lactalbumin in the absence and presence of EGTA

D. Terence W. BRYANT and Patrick ANDREWS

National Institute for Research in Dairying, Shinfield, Reading, Berkshire RG2 9AT, U.K.

(Received 24 February 1984/Accepted 27 March 1984)

Literature values for the K_d for Ca^{2+} in bovine α -lactalbumin range over 3 orders of magnitude. There is a difference between two results obtained with EGTA as a metal-ion buffer, partly because different values for the K_d of Ca^{2+} –EGTA were used in the calculations, and a much wider difference between results obtained in the presence and absence of EGTA, which has been attributed to an interaction between EGTA and the protein. Titrations in a flow-dialysis cell showed that Mn^{2+} competed with Ca^{2+} for the high-affinity site on the protein, and the results, combined with a K_d for Mn^{2+} of $2.1 \pm 0.1 \mu\text{M}$, which was determined fluorimetrically, gave a K_d for Ca^{2+} of $1.3 \pm 0.1 \text{ nM}$. When α -lactalbumin containing $^{45}\text{Ca}^{2+}$ was titrated with EGTA in a flow-dialysis cell, and widely accepted metal-chelation data for EGTA were used in the calculations, a K_d for Ca^{2+} of $1.10 \pm 0.03 \text{ nM}$ was obtained. The results from the two methods are so similar as to indicate that the affinity for Ca^{2+} was unaffected by the presence of EGTA.

The effect of EGTA on the thermal stability of bovine α -lactalbumin first indicated that the latter was a metalloprotein, and subsequent analyses showed that it contains 1 mol of Ca^{2+} /mol of protein (Hiraoka *et al.*, 1980). Its affinity for Ca^{2+} has been investigated by several authors, but with no agreement as to the result. Fluorimetric titrations with EGTA by Permyakov *et al.* (1981) and Murakami *et al.* (1982) gave K_d values for Ca^{2+} which differed by an order of magnitude (1.5–3 nM and 0.2 nM respectively), and gel-filtration experiments by Kronman *et al.* (1981) gave a value (0.37 μM) which differed again. Kronman & Bratcher (1983) claim that an interaction between α -lactalbumin and EGTA is a source of error in the fluorimetric determinations; on the other hand, it seemed to us that their K_d of 0.37 μM was too high to be consistent with the presence of a stoichiometric amount of Ca^{2+} in purified α -lactalbumin. In order to resolve the problem, therefore, we have determined the K_d by two different methods, one of which used EGTA and one of which avoided its use entirely.

Materials and methods

The buffer used in all experiments was 20 mM-Tris/HCl, pH 7.5, prepared from Aristar Tris (BDH Chemicals, Poole, Dorset, U.K.) and deion-

ized water. It contained $<0.1 \mu\text{M}$ - Ca^{2+} , as determined by atomic-absorption analysis.

Bovine α -lactalbumin was isolated from cow's milk as described by Barman (1970), and its molarity in solution determined spectrophotometrically by using $A_{1\text{cm},280}^{1\%}$ 20 (Kronman & Andreotti, 1964) and an M_r of 14500. The ' Ca^{2+} -free' form was prepared either (a) by treatment with 0.1 M-EDTA/EGTA, followed by gel filtration (Blum *et al.*, 1977) or (b) by gel filtration in 12 mM-HCl (Kronman & Bratcher, 1983), followed by neutralization with Aristar-grade Tris. Measurement of the Ca^{2+} remaining in the molecule, by atomic-absorption analysis, gave values of 0.11 and <0.05 mol/mol for the products from (a) and (b) respectively.

$^{45}\text{CaCl}_2$ was obtained from Amersham International, Amersham, Bucks., U.K., and other chemicals were AnalaR grade.

Flow dialysis

The apparatus and procedures were similar to those described previously (Bryant & Andrews, 1983, 1984). The experiments were performed at room temperature.

Fluorescence titration

Fluorescence measurements were obtained with a Hitachi-Perkin-Elmer model MPF-3L

fluorescence spectrophotometer (Perkin-Elmer, Beaconsfield, Bucks., U.K.). Protein fluorescence was excited at 288nm and measured at 360nm, with 10nm slits for both monochromators. Titration was performed in a 1cm-square cell containing 2ml of protein solution at 25°C, and MnCl_2 solution was added in 5 μl portions. The fluorescence change at saturation was determined from a double-reciprocal plot of the results, and it was assumed that the fluorescence change was proportional to the amount of Mn^{2+} bound at a single site (Murakami *et al.*, 1982).

Results

$K_d^{\text{Ca}^{2+}}$ determined by competition with Mn^{2+}

When Ca^{2+} -free α -lactalbumin was titrated with Ca^{2+} in the flow-dialysis cell in the presence of different concentrations of Mn^{2+} , the results showed that the two cations were bound competitively (Fig. 1a). The K_d for Ca^{2+} was therefore related to the apparent K_d for Ca^{2+} in the presence of Mn^{2+} by

$$\text{apparent } K_d^{\text{Ca}^{2+}} = K_d^{\text{Ca}^{2+}} \left(1 + \frac{[\text{Mn}^{2+}]}{K_d^{\text{Mn}^{2+}}} \right) \quad (1)$$

where $[\text{Mn}^{2+}]$ is the concentration of free Mn^{2+} and $K_d^{\text{Mn}^{2+}}$ is its dissociation constant. The Mn^{2+} concentrations in the experiments were high

enough to use total Mn^{2+} concentration in eqn. (1) instead of free Mn^{2+} concentration without appreciable error, allowing for the fact that α -lactalbumin possesses three weak binding sites for Mn^{2+} as well as one strong one (Murakami *et al.*, 1982). The graph of apparent $K_d^{\text{Ca}^{2+}}$ against Mn^{2+} concentration was linear (Fig. 1b), in agreement with eqn. (1). The results show that the Ca^{2+} -free protein prepared by treatment with either chelating agents or HCl had the same apparent affinities for Ca^{2+} in the presence of Mn^{2+} . The ordinate intercept of the graph ($0.33 \pm 0.27 \mu\text{M}$) was $K_d^{\text{Ca}^{2+}}$, but it was clearly not determined with sufficient accuracy to give a reliable value. The slope of the graph $[(6.25 \pm 0.25) \times 10^{-4}]$ was $K_d^{\text{Ca}^{2+}}/K_d^{\text{Mn}^{2+}}$, and fluorimetric titration of Ca^{2+} -free α -lactalbumin with Mn^{2+} gave a value for $K_d^{\text{Mn}^{2+}}$ of $2.1 \pm 0.1 \mu\text{M}$ (Fig. 2) which, when combined with the slope, gave $K_d^{\text{Ca}^{2+}}$ $1.3 \pm 0.1 \text{ nM}$.

$K_d^{\text{Ca}^{2+}}$ determined by titration with EGTA

Haiech *et al.* (1980) showed that, when a Ca^{2+} -binding protein is saturated with Ca^{2+} containing $^{45}\text{Ca}^{2+}$ and titrated with EGTA in a flow-dialysis cell, the concentration c of $^{45}\text{Ca}^{2+}$ in the buffer flow is given by

$$c = (D_1/f)(L_R/L_T) L_F + (D_2/f)(L_R/L_T)[E_T/(1 + K_d^E/L_F)] \quad (2)$$

Fig. 1. Binding of Ca^{2+} to bovine α -lactalbumin in the presence of Mn^{2+}

(a) The Ca^{2+} -free protein ($21.8 \mu\text{M}$) was titrated with Ca^{2+} in a flow-dialysis cell in the presence of different concentrations of Mn^{2+} . Experimental points, Mn^{2+} concentrations and the values for apparent $K_d^{\text{Ca}^{2+}}$ used to calculate the corresponding curves are respectively: (○), 5 mM, $2.92 \mu\text{M}$; (●), 10 mM, $6.80 \mu\text{M}$; (□), 20 mM, $13.10 \mu\text{M}$. (b) Graph of values for apparent $K_d^{\text{Ca}^{2+}}$ obtained from flow-dialysis titrations against total Mn^{2+} concentration. The experiments were performed with Ca^{2+} -free α -lactalbumin prepared by treatment with (○) EDTA and EGTA or (●) HCl, as described in the text.

in which D_1 and D_2 are the rates at which free Ca^{2+} and the Ca^{2+} -EGTA complex respectively diffuse through the membrane, f is the buffer flow-rate, L_R , L_T and L_F are the concentrations in the upper chamber of free plus bound $^{45}\text{Ca}^{2+}$, free

Fig. 2. Fluorimetric titration of Ca^{2+} -free bovine α -lactalbumin with Mn^{2+}

The protein concentration was $4.2\mu\text{M}$. The open circles are experimental points, and the curve is the calculated result for $K_d^{\text{Mn}^{2+}} = 2.1\mu\text{M}$.

plus bound Ca^{2+} and free Ca^{2+} respectively, E_T is the total EGTA concentration and $K_d^{\text{Ca}^{2+}}$ is the dissociation constant of the Ca^{2+} -EGTA complex. The factors $(D_1/f)L_R$ and $(D_2/f)L_R$ are obtained from the ratio D_1/D_2 and an experiment in which $L_T \gg E_T$, when, from eqn. (2), $c \approx (D_1/f)L_R$. Substitution in eqn. (2) allows the calculation of L_F for each step in the titration by an iterative procedure, L_E can then be obtained, and finally the concentration of protein-bound Ca^{2+} , L_P , from $L_T = L_F + L_E + L_P$.

Bovine α -lactalbumin was prepared for EGTA titration either by adding 1.5 mol of Ca^{2+} containing $5\mu\text{Ci}$ of $^{45}\text{Ca}^{2+}/\text{mol}$ to the Ca^{2+} -free protein, or by adding $5\mu\text{Ci}$ of $^{45}\text{Ca}^{2+}$ to the native protein and allowing it to exchange with the bound Ca^{2+} . A flow-dialysis experiment showed that equilibration was complete in 10 min.

Each titration (Fig. 3a) was preceded by a determination of D_1/D_2 , in the absence of protein, by first adding $^{45}\text{Ca}^{2+}$ to buffer in the cell, followed by an excess of EGTA. Different membrane discs gave values for D_1/D_2 ranging from 4 to 7. The cell was washed clean before the next part of the experiment. When α -lactalbumin labelled with $^{45}\text{Ca}^{2+}$ was titrated, transfer of protein-bound Ca^{2+} to EGTA was shown by increases in the $^{45}\text{Ca}^{2+}$ concentration in the buffer flow. The titration was completed by the addition of a large excess of Ca^{2+} over EGTA ($L_T \gg E_T$).

A value of 19.9 nM , calculated as described by Raaflaub (1956) from the data of Schwarzenbach

Fig. 3. Titration of bovine α -lactalbumin containing $^{45}\text{Ca}^{2+}$ with EGTA in a flow-dialysis cell

(a) The open circles and arrows show a determination of the relative rates of diffusion of Ca^{2+} and the Ca^{2+} -EGTA complex through the membrane (D_1/D_2 ; see the text). $^{45}\text{Ca}^{2+}$ (11 nmol) was added to buffer (2 ml) in the cell at A and EGTA (600 nmol) at B. The closed circles and arrows show a titration of $20.9\mu\text{M}$ -bovine α -lactalbumin containing $^{45}\text{Ca}^{2+}$ with EGTA. Additions of EGTA were as follows: C-F, $5\mu\text{l}$ of 5 mM ; G and H, $5\mu\text{l}$ of 10 mM ; I and J, $5\mu\text{l}$ of 30 mM ; K and L, $10\mu\text{l}$ of 30 mM . At M, $25\mu\text{l}$ of 5 M - CaCl_2 was added in order to measure the $^{45}\text{Ca}^{2+}$ concentration in the buffer flow when essentially all the Ca^{2+} in the cell was in the free form ($L_T \gg E_T$; see the text). (b) Scatchard plot of values for free and bound Ca^{2+} obtained as described in the text from the experiment shown in (a). The K_d for Ca^{2+} is $1.06 \pm 0.03\text{ nM}$.

et al. (1957), was used for K_d^E in the calculations, and volume changes in the protein solution during the titration were allowed for. A Scatchard plot with values for L_F and L_P obtained in one experiment is shown in Fig. 3(b). Three EGTA titrations, two in which α -lactalbumin was labelled with $^{45}\text{Ca}^{2+}$ by exchange and one in which $^{45}\text{Ca}^{2+}$ was added to the Ca^{2+} -free protein, gave $K_d^{\text{Ca}^{2+}} 1.10 \pm 0.03 \text{ nM}$.

Discussion

Since the K_d values reported for Ca^{2+} - α -lactalbumin range from 0.2 nM to 0.37 μM , some of them must be misleading. Most of them are based on K_d values for other binding equilibria which were used in the calculations. In the competitive method with Mn^{2+} the result depended on the K_d of Mn^{2+} - α -lactalbumin, but this was uncertain to the extent that a fluorimetric titration by Kronman *et al.* (1981) gave a value of 2.9 μM , whereas titrations by Murakami *et al.* (1981) gave 1–2 μM with reagent-grade MnCl_2 and 31 μM with ultra-pure salt. Since we used AnalaR MnCl_2 in the competition experiments, we used the same material in the fluorimetric titration, and obtained a value of $2.1 \pm 0.1 \mu\text{M}$. When EGTA is used as a Ca^{2+} buffer, K_d values for Ca^{2+} -EGTA for different experimental conditions are usually calculated from the metal-chelation data of Schwarzenbach *et al.* (1957). According to Kim & Padilla (1978), however, these K_d values may not be correct. Murakami *et al.* (1982) used a K_d for Ca^{2+} -EGTA suggested by Kim & Padilla (1978), and obtained the lowest of the K_d values reported for Ca^{2+} - α -lactalbumin. The competitive method with Mn^{2+} did not confirm this low value, but gave a K_d which agreed well with our result from EGTA titration, obtained when the calculations for EGTA were based on data from Schwarzenbach *et al.* (1957). Both results were near the range of K_d values reported by Permyakov *et al.* (1981), who used the same data for EGTA.

According to Kronman & Bratcher (1983), interaction between α -lactalbumin and chelator as the reason why the affinity between Ca^{2+} and α -lactalbumin determined by fluorimetric titration in

the presence of EGTA was much greater than that determined by gel filtration in its absence. However, we obtained essentially the same low K_d in both the presence and the absence of EGTA, and interaction between α -lactalbumin and EGTA was not detected. The results of the EGTA titrations gave linear Scatchard plots, whereas, if the concentration of EGTA free to diffuse through the membrane had been appreciably decreased by interaction with the protein, we would have expected non-linear plots (Haiech *et al.*, 1980). The direct determination of very low K_d values for Ca^{2+} is hindered by contamination with Ca^{2+} , and a possible reason for the discrepancy between the gel-filtration result and those of the other methods is that, in gel filtration, the accurate control of free Ca^{2+} concentrations in the nanomolar region is especially difficult in the absence of a metal-ion buffer.

References

- Barman, T. E. (1970) *Biochim. Biophys. Acta* **214**, 242–244
- Blum, H. E., Lehky, P., Kohler, L., Stein, E. A. & Fisher, E. H. (1977) *J. Biol. Chem.* **252**, 2834–2838
- Bryant, D. T. W. & Andrews, P. (1983) *Biochem. J.* **211**, 709–716
- Bryant, D. T. W. & Andrews, P. (1984) *Biochem. J.* **219**, 287–292
- Haiech, J., Vallet, B., Aquaron, R. & Demaille, J. G. (1980) *Anal. Biochem.* **105**, 18–23
- Hiraoka, Y., Segawa, T., Kuwajima, K., Sugai, S. & Murai, N. (1980) *Biochem. Biophys. Res. Commun.* **95**, 1098–1104
- Kim, Y. S. & Padilla, G. M. (1980) *Anal. Biochem.* **89**, 521–528
- Kronman, M. J. & Andreotti, R. E. (1964) *Biochemistry* **3**, 1145–1151
- Kronman, M. J. & Bratcher, S. C. (1983) *J. Biol. Chem.* **258**, 5707–5709
- Kronman, M. J., Sinha, S. K. & Brew, K. (1981) *J. Biol. Chem.* **256**, 8582–8587
- Murakami, K., Andree, P. J. & Berliner, L. J. (1982) *Biochemistry* **21**, 5488–5494
- Permyakov, E. A., Yarmolenko, V. V., Kalinichenko, L. P., Morozova, L. A. & Burstein, E. A. (1981) *Biochem. Biophys. Res. Commun.* **100**, 191–197
- Raaflaub, J. (1956) *Methods Biochem. Anal.* **3**, 301–325
- Schwarzenbach, G., Senn, H. & Anderegg, G. (1957) *Helv. Chim. Acta* **40**, 1886–1900