

# Electrostatic repulsion between molecules of like charge can be misinterpreted as binding

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Spectroscopic methods have shown that  $\text{Ca}^{2+}$  chelators interact with  $\text{Ca}^{2+}$ -binding proteins. These spectral alterations have been interpreted as evidence for the binding of chelator by the proteins. We show by direct examination of EDTA interaction with calmodulin and  $\alpha$ -lactalbumin that these proteins repel EDTA rather than bind it. The repulsion is reduced by increased salt concentration but is unaffected by  $\text{Ca}^{2+}$  binding to the proteins. The acidic protein,  $\alpha$ -lactalbumin, repels the negatively charged EDTA and inorganic phosphate whereas the basic protein, lysozyme, repels the positively charged spermine. Thus, spectroscopic changes induced by negatively charged  $\text{Ca}^{2+}$  chelators on negatively charged  $\text{Ca}^{2+}$ -binding proteins are due to electrostatic repulsion, and not to binding. These observations underscore the possible pitfalls of using spectroscopic methods alone to analyze protein-ligand interactions.

EDTA binding; Electrostatic repulsion; Calmodulin;  $\alpha$ -Lactalbumin; Chelator

## 1. INTRODUCTION

Spectroscopic methods have demonstrated an interaction of the chelators EDTA and EGTA with many  $\text{Ca}^{2+}$ -binding proteins [1-3]. These spectral changes have been interpreted as evidence for binding of the chelators to the proteins [1-3]. Spectral changes are also induced in these proteins by the  $\text{Ca}^{2+}$  indicator dyes, BAPTA and Fura-2 [4,5]. Since  $\text{Ca}^{2+}$  chelators are often used during the isolation of  $\text{Ca}^{2+}$ -binding proteins, in measurements of their  $\text{Ca}^{2+}$  binding properties and in assays of the  $\text{Ca}^{2+}$ -dependent activation of  $\text{Ca}^{2+}$ -regulated enzymes, it is important to determine whether these chelators actually bind to  $\text{Ca}^{2+}$  regulatory proteins.

Because spectroscopic data cannot be interpreted unambiguously in the absence of direct binding experiments, there is still some doubt as to whether  $\text{Ca}^{2+}$  binding proteins really bind  $\text{Ca}^{2+}$  chelators [6-9]. Nuclear magnetic resonance studies indicate that although  $\alpha$ -lactalbumin interacts with EGTA, the observed effects are inconsistent with binding [7]. It has been suggested that the effect of the chelators on protein is mediated via the  $\text{Na}^+$  or  $\text{K}^+$  added as the counterion with the chelator [9]. Because of the like charges of these proteins and their putative ligands, one would expect that they should not bind to each other. We therefore decided to directly reanalyze the interaction between EDTA, and two  $\text{Ca}^{2+}$ -binding proteins, calmodulin and  $\alpha$ -lactalbumin, using the Hummel-Dreyer gel filtration technique [10].

## 2. MATERIALS AND METHODS

Calmodulin was isolated according to Newton et al. [11] and decalcified as previously described [12]. DEAE cellulose-purified  $\alpha$ -lactalbumin was a gift from Dr W. Klee (NIMH, Bethesda MD). Lysozyme was a product of Worthington Biochemical Co. Protein concentrations were determined spectrophotometrically using the following extinction coefficients:  $\alpha$ -lactalbumin,  $E_{280\text{nm}} = 28\,500$  [13]; lysozyme,  $E_{281\text{nm}} = 38\,900$  [13]; and calmodulin,  $E_{277\text{nm}} = 3300$ . All proteins migrated as single bands when subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. [ $^{14}\text{C}$ ]EDTA (48 mCi/mmol), [ $^{14}\text{C}$ ]spermine (*N,N'*-bis-(3-aminopropyl)-1,4- $^{14}\text{C}$ -tetramethylene-1,4-diamine) (111 mCi/mmol),  $^{45}\text{Ca}$  (0.75 mCi/mmol), and [ $^{32}\text{P}$ ]orthophosphate (200 mCi/mmol) were from Amersham.

Gel filtration experiments were performed using 3.7 ( $0.6 \times 13$  cm) or 2.1 ( $0.6 \times 7.5$  cm) ml columns packed with Sephadex G-25 fine as previously described [10]. Except as indicated, columns were equilibrated with binding buffer, 20 mM Tris-Cl, pH 8.0, containing 3.0 mM of either [ $^{14}\text{C}$ ]EDTA, [ $^{14}\text{C}$ ]spermine, or [ $^{32}\text{P}$ ]orthophosphate, and the concentration of KCl indicated in figure legends. Lyophilized proteins were dissolved in 30 mM Tris-Cl buffer, pH 8.0, and gel filtered on columns equilibrated with 30 mM Tris-Cl, pH 8.0. These concentrated stock protein solutions were appropriately diluted with solutions of KCl and radiolabeled ligands. The final concentrations of Tris, KCl and labeled ligands in the samples loaded on to the columns were identical to those of the binding buffers used for the column equilibrations. Aliquots of 250 or 125  $\mu\text{l}$  were applied to the 3.7 or 2.1 ml columns, respectively, and the respective fraction sizes were 125 and 75  $\mu\text{l}$ . Elution of proteins and radioactive ligands was monitored by absorbance at 280 nm and duplicate radioactivity determinations. All experiments were performed at room temperature.

## 3. RESULTS AND DISCUSSION

Two small acidic  $\text{Ca}^{2+}$ -binding proteins, calmodulin and  $\alpha$ -lactalbumin, which bind  $\text{Ca}^{2+}$  with high affinity and have been reported to bind EDTA on the basis of spectral data, were selected for our binding studies. We used for this purpose the gel filtration method of Hum-

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mel and Dreyer in which a protein is subjected to gel filtration in the presence of a constant amount of ligand in the column buffer. The elution patterns of calmodulin and of EDTA from a gel filtration column equilibrated with 2 mM [ $^{14}\text{C}$ ]EDTA are shown in Fig. 1. Instead of the expected peak of bound ligand followed by a trough, we observed a trough, indicating a depletion of [ $^{14}\text{C}$ ]EDTA at the position of calmodulin, followed by a peak of EDTA. The same pattern was observed with  $\alpha$ -lactalbumin (Fig. 3A). The decrease in EDTA concentration in fractions containing calmodulin or  $\alpha$ -lactalbumin and the corresponding increase in late fractions indicate that both proteins are actually repelling instead of binding EDTA.

The repulsion observed with negatively charged EDTA and the negatively charged proteins, calmodulin and  $\alpha$ -lactalbumin, also occurs between positively charged proteins and effectors. Hummel-Dreyer experiments performed using the acidic protein  $\alpha$ -lactalbumin ( $pI=4.5$ ) and the basic protein lysozyme ( $pI=10.5-11$ ) are illustrated in Fig. 2. Each of these proteins is also known to bind  $\text{Ca}^{2+}$  [14]. In addition to [ $^{14}\text{C}$ ]EDTA, [ $^{32}\text{P}$ ]orthophosphate was used as a negatively charged agent and [ $^{14}\text{C}$ ]spermine was the positively charged effector. As shown in Fig. 2A and B, the two negatively charged ligands, EDTA and  $\text{P}_i$ , were repelled by  $\alpha$ -lactalbumin and bound to lysozyme. Conversely, the positively charged spermine was bound to  $\alpha$ -lactalbumin but repelled by lysozyme (Fig. 2C).

The interaction of  $\alpha$ -lactalbumin and EDTA was studied at several levels of ionic strength. In the absence of added salt, a strong repulsion between EDTA and  $\alpha$ -lactalbumin was evident (Fig. 3A) in the large trough coincident with the protein-containing fractions followed by a correspondingly large peak in the elution pattern of [ $^{14}\text{C}$ ]EDTA. At 75 (Fig. 3B) or 300 mM KCl (Fig. 3C) a concentration-dependent decrease in the

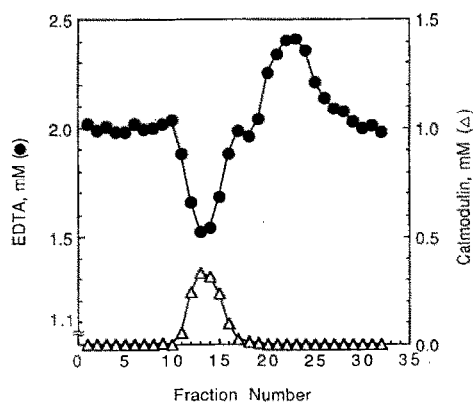


Fig. 1. Interaction of EDTA and calmodulin. Decalcified calmodulin was equilibrated with the buffer used for the binding experiment by gel filtration and appropriately diluted with a solution of radiolabeled ligand. The 3.7 ml column was equilibrated with 20 mM Tris-Cl buffer, pH 8.0, containing 2.0 mM [ $^{14}\text{C}$ ]EDTA (spec. act. 53 000 cpm/ $\mu\text{mol}$ ).

sizes of the protein-related troughs was observed. In the absence of KCl, EDTA concentration in the protein-containing fractions was reduced by 15%. In the presence of 75 or 300 mM KCl, EDTA concentration in the same fractions was reduced 9 and 7%, respectively. In these experiments, performed with  $\alpha$ -lactalbumin solutions which had not been decalcified, a second trough, particularly noticeable in the presence of KCl, is present between the protein-associated trough and the included radioactive peak (Fig. 3B,C). The presence of a double trough was also observed with non-decalcified calmodulin. The second trough was eliminated either by decalcifying the protein or by adding  $\text{Ca}^{2+}$  to the EDTA buffer (data not shown). As is evident from the data shown in Fig. 4, this second trough in EDTA concentration is due only to the interaction of  $\text{Ca}^{2+}$  and EDTA. Because the Sephadex matrix carries a negative charge, it repels free EDTA more so than the Ca-EDTA complex and thus accounts for the pattern. Regardless of the presence or absence of  $\text{Ca}^{2+}$  in the protein solutions or the binding buffers the size of the

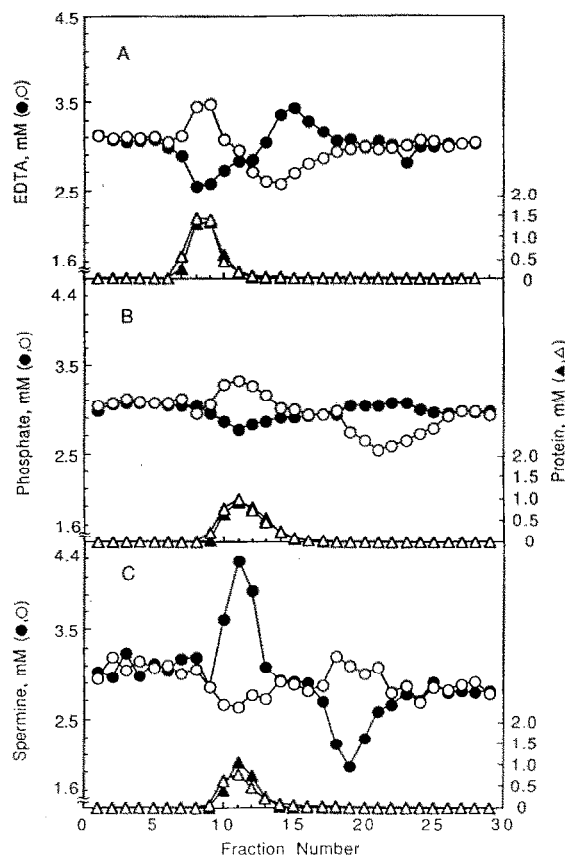


Fig. 2. Interactions of  $\alpha$ -lactalbumin (filled symbols) and lysozyme (open symbols) with EDTA (A), orthophosphate (B) and spermine (C). The binding buffer contained: (A) 25 mM KCl and 3 mM [ $^{14}\text{C}$ ]EDTA (spec. act. 78 000 cpm/ $\mu\text{mol}$ ); or (B) and (C) 50 mM KCl and 3 mM [ $^{14}\text{C}$ ]spermine (spec. act. 89 000 cpm/ $\mu\text{mol}$ ) or [ $^{32}\text{P}$ ]orthophosphate (spec. act. 103 000 cpm/ $\mu\text{mol}$ ). Column size was 2.1 ml.

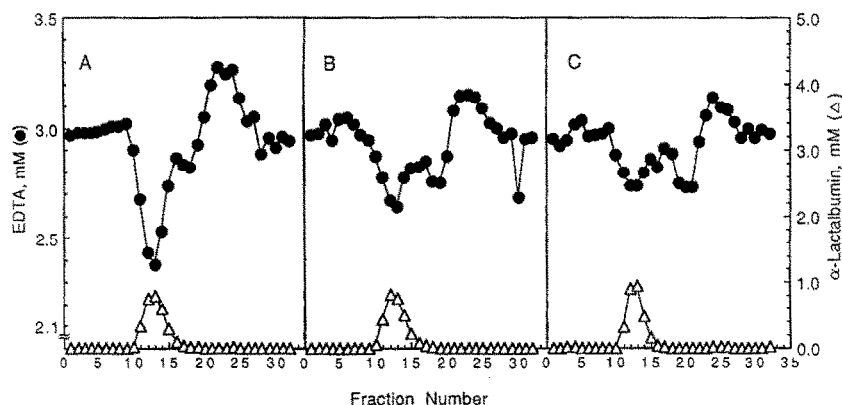


Fig. 3. Effect of KCl on the interaction of EDTA and  $\alpha$ -lactalbumin.  $\alpha$ -Lactalbumin was equilibrated in binding buffer as described in legend to Fig. 1 without prior decalcification. This stock solution was appropriately diluted with solutions of KCl and [ $^{14}$ C]EDTA to bring the final concentration of Tris (20 mM), EDTA (3 mM) and KCl identical to those buffers used for equilibration and elution of the columns. Three successive runs were performed using 2.1 ml column, in the absence (A), or presence of 75 mM (B) and 300 mM (C) KCl.

first trough was unchanged showing that the chelating property of EDTA was not involved in the repulsion process.

The study of the interaction of EDTA with calmodulin and  $\alpha$ -lactalbumin using the Hummel-Dreyer gel filtration technique demonstrates that neither of these proteins bind EDTA and that both repel it. The absence of binding is consistent with the sharpening of proton magnetic resonance of the acetate groups of EDTA and EGTA found in lieu of the line broadening expected were EDTA and EGTA bound to  $\alpha$ -lactalbumin [7]. The repulsion is related not to the chelating properties of EDTA but to its negative charge. The experiments reported here use concentrations of EDTA that are similar to those used in the spectral experiments reported previously and both the repulsion observed here and the spectral changes reported by others are reduced at moderate ionic strength [4]. Thus, the interaction of  $\text{Ca}^{2+}$  chelators with  $\text{Ca}^{2+}$ -binding

proteins first revealed by spectroscopy is to be attributed to a change in conformation of the protein in the environment of a negatively charged ion and not, as previously proposed, to the binding of the chelator [1–5] or to the binding of  $\text{Na}^+$  or  $\text{K}^+$ , added as EDTA counterions, to the protein [7]. On the basis of similar  $\text{Ca}^{2+}$ - and EDTA-induced spectral changes of  $\alpha$ -lactalbumin, it had been proposed that binding of EDTA promotes a conformation similar to that of the  $\text{Ca}^{2+}$  saturated protein [2]. It is more likely that in the presence of high concentrations of EDTA,  $\alpha$ -lactalbumin exists as a distinct conformer. This form of the protein binds to hydrophobic supports whereas  $\text{Ca}^{2+}$ -loaded  $\alpha$ -lactalbumin does not [15]. These observations emphasize the need for direct binding measurements to unequivocally interpret spectroscopic experiments. Clearly, repulsive electrostatic forces can cause spectral changes when samples contain high concentrations of charged species. At the same time caution should be taken in the use of chelating agents to measure  $\text{Ca}^{2+}$  binding or  $\text{Ca}^{2+}$ -dependent activation of enzymes since these chelators may not only buffer  $\text{Ca}^{2+}$  but also modify the structure of proteins.

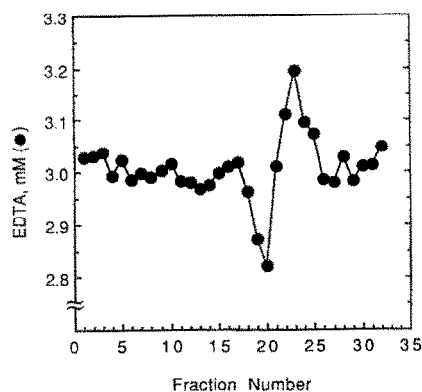


Fig. 4. EDTA elution from Sephadex is retarded by  $\text{Ca}^{2+}$ . A 3.7 ml column of Sephadex G-25 fine was equilibrated with binding buffer containing 200 mM KCl and 3.0 mM [ $^{14}$ C]EDTA (20 000 cpm/ $\mu$ mol). A 250 ml aliquot of this buffer was made 1.5 mM in  $\text{Ca}^{2+}$  and loaded on the column.

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