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# Calcium-dependent binding between calmodulin and lysozyme

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Calmodulin, an acidic protein that binds calcium with high affinity, has multiple roles in the activation of many enzymes involved in cellular regulation of eukaryotes. In this study we show that calmodulin binding to hen egg-white lysozyme, in a $Ca^{2+}$-dependent way, was observed using electroblots incubated with biotinylated calmodulin and detected with avidin-alkaline phosphatase or for affinity chromatography on a gel calmodulin column. Antimicrobial activity of lysozyme was not modified in the presence of $Ca^{2+}$-calmodulin.

Calmodulin; Lysozyme; $Ca^{2+}$-binding protein; Muramidase

## 1. INTRODUCTION

Calmodulin is a cytoplasmic calcium-binding protein in virtually all eukaryotic cells that regulates multiple calcium-dependent enzymatic activities and cellular processes [1–3]. Among the calmodulin-binding compounds described are several small basic peptides and proteins that bind in a $Ca^{2+}$-dependent manner and have an unknown physiological function [4–8].

Hen egg-white lysozyme is one of the class C lysozymes which include homologous proteins of different animal origin [9]. Lysozyme exerts enzymatic activity by breaking the bond between acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan layer of the bacterial cell wall [10]. Interactions between these proteins, which are well conserved throughout evolution, have not been previously reported. Here, we show that lysozyme (EC 3.2.1.17) is a $Ca^{2+}$-dependent calmodulin-binding protein and that this feature does not modify the lytic enzymatic function.

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## 2. MATERIALS AND METHODS

### 2.1. *Detection of calmodulin-dependent protein*

Analysis of protein on SDS-polyacrylamide gels (12%) was performed as in [11]. After electrophoresis, transfer of hen egg-white lysozyme (Boehringer Mannheim) and standard markers (Biorad) to membranes (Immobilon PVDF, Millipore) was carried out as in [12] for 2 h at 200 mA followed by the detection of calmodulin-dependent protein as described by Billingsley et al. [13]. After blocking of the membrane with 2% bovine serum albumin in blocking solution (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1 mM $CaCl_2$), blots were incubated for 1 h with biotinylated calmodulin (1 $\mu$g/ml). After incubation and washing blots were transferred to an avidin alkaline phosphatase (Sigma) solution for 30 min before the addition of substrates for color development. All incubations and washes were carried out in blocking solution containing either 1 mM $CaCl_2$ or for control studies, 5 mM EGTA or 100 $\mu$M trifluoperazine (Boehringer Mannheim).

### 2.2. *Affinity chromatography on an Affigel-calmodulin column*

The enzyme preparation (500 $\mu$l from a solution of 0.1 mg/ml) was applied to an Affigel-calmodulin (Biorad) column of 1 ml (2.5 × 0.5 cm) which had been equilibrated with solution A (50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$) and washed with 2 vols solution A. After loading, the sample was stopped for 30 min to allow better binding. The column was eluted with solution B (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EGTA) at 1 ml/h and fractions of 300 $\mu$l were collected. Samples of each fraction were taken to measure lysozyme activity.



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2.3. *Enzymatic activity of lysozyme*

The activity of lysozyme was determined as in [14] from the rate of lysis of a fresh suspension of *Micrococcus lysodeikticus* (Sigma) in 0.1 M phosphate buffer, pH 7.0, previously prepared to give an absorbance of 0.8 at 450 nm. The enzyme solution (500 $\mu$l from a solution of 0.1 mg/ml) or 50 $\mu$l eluted lysozyme was added to the suspension (2.9 ml) and the reduction of absorbance was recorded at 25°C. One unit was taken as a decrease in $A_{450}$ of 0.001 per min.

## 3. RESULTS

### 3.1. *Detection of calmodulin binding to lysozyme on transfer membranes*

Samples were run in SDS-PAGE and electroblotted onto transfer membranes and calmodulin-binding proteins were recognized by incubation with biotinylated calmodulin by detection with avidin-alkaline phosphatase chromogens. After blotting membranes were sectioned and the sections were incubated with biotinylated calmodulin in the presence of $Ca^{2+}$, EGTA or trifluoperazine. In several experiments, a prominent band corresponding to lysozyme was observed in the presence of $Ca^{2+}$ after 2 min of color development (fig.1). No band was observed when EGTA or trifluoperazine – an inhibitor of calmodulin binding – were present. The $Ca^{2+}$-dependent calmodulin-binding protein calcineurix was used as control in all experiments. Avidin alkaline phosphatase binding to lysozyme was not observed neither was there unspecific staining of other proteins.

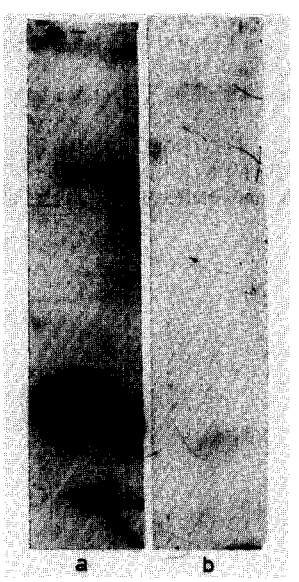


Fig.1. Biotinylated-calmodulin binding to electroblotted lysozyme in the presence of 1 mM $CaCl_2$.

### 3.2. *Binding of lysozyme to an Affigel-calmodulin column*

The ability of lysozyme to bind to calmodulin was tested on an Affigel-calmodulin column. Samples of lysozyme were loaded on the affinity column that was previously equilibrated in buffer A, containing 1 mM $CaCl_2$. The elution with buffer B, containing 5 mM EGTA, showed that lysozyme remained bound to the calmodulin column in the presence of $Ca^{2+}$ and only when EGTA was present in the buffer was the enzyme eluted, enzymatic activity being recovered in the fractions (fig.2).

### 3.3. *Enzymatic activity of lysozyme in the presence of calmodulin*

Since calmodulin had modulatory effects on many enzymes we determined its possible action on

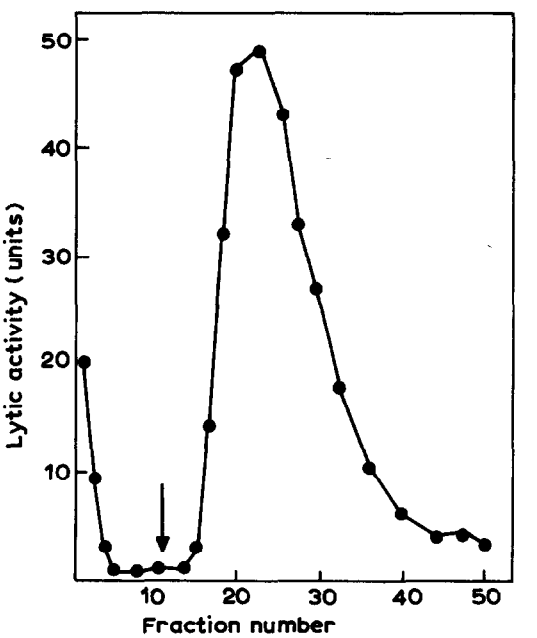


Fig.2. Binding of lysozyme to an Affigel-calmodulin column. A lysozyme preparation (500 $\mu$l from a solution of 0.1 mg/ml) was loaded on a 1 ml Affigel-calmodulin column (2.5 × 0.5 cm). The column was equilibrated in buffer A (with 1 mM $CaCl_2$). Elution was carried out by running buffer B (with 5 mM EGTA). Fractions were taken and the lysozyme activity of the fractions was assayed as described in section 2. Arrow indicates EGTA addition for elution of lysozyme. One unit is taken as a decrease in $A_{450}$ of 0.001 per min.

the lytic activity of lysozyme. Prior enzymatic assays, incubations at 4°C for 30 min of fresh solutions of lysozyme and calmodulin were carried out. The enzymatic activity assays of lysozyme on *M. lysodeikticus* in the presence of different concentrations of calmodulin (2.5, 5, 10 and 50 $\mu$g/ml) and $CaCl_2$ (0.2, 0.5, 1, 2 and 4 mM) were performed at 25°C. No significant alteration of lytic activity was observed (not shown).

## 4. DISCUSSION

The present results show the ability of calmodulin to bind to hen egg-white lysozyme. Calmodulin has many intracellular activities and binding to different proteins has been reported previously. Lysozyme was originally described as an antibacterial lytic enzyme but other possible roles have been suspected [9] and it is known that calcium-binding sites are formed in some types of lysozymes [15]. In this study, binding of biotinylated calmodulin to lysozyme immobilized on synthetic membranes was achieved in the presence of $Ca^{2+}$ but no band was observed when a $Ca^{2+}$-chelating agent such as EGTA was included in the incubations and washes. Affinity chromatography also showed that lysozyme binds to calmodulin in the presence of $Ca^{2+}$ only. These results suggest that the interaction between both proteins is $Ca^{2+}$-dependent. In our case, the antibacterial activity of lysozyme was not modified in the presence of different concentrations of calmodulin and $Ca^{2+}$, and the physiological function of calmodulin binding to lysozyme is unknown. Nevertheless, other functions different from those of lysis have been suggested for lysozyme and recently lysozyme-mediated inhibition of $T_7$ phage transcription has been reported [16]. In this sense, as calmodulin plays different regulatory roles in the cell, the calmodulin-lysozyme binding observed could also suggest other as yet unknown roles for lysozyme; further detailed studies will clarify this assumption and the possibility of using this method to purify calmodulin.

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