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Probing the nucleotide-binding site of sarcoplasmic reticulum (Ca^{2+}-Mg^{2+})-ATPase with anti-fluorescein antibodies

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Antibodies raised against fluorescein were unable to bind to the fluorophore when bound at the nucleotide-binding site of native (Ca^{2+}-Mg^{2+})-ATPase, as judged by fluorescence quenching assays or competitive ELISAs, but were able to bind when the ATPase was denatured. Indirect ELISAs, in which native and denatured FITC-ATPase were used to coat ELISA plates, were unable to detect the difference in accessibility of the fluorescein bound to the native and denatured ATPase. These results indicate that the nucleotide-binding site is relatively inaccessible in the native structure, even though fluorescence energy transfer studies [(1987) Biochim. Biophys. Acta 897, 207–216] indicate that this site must be close to the surface of the ATPase. In addition the results suggest that the indirect ELISA method may be of limited value in probing the accessibility of epitopes using antibodies.

Nucleotide-binding site; ATPase, (Ca^{2+}+Mg^{2+})-; Fluorescein antibody; Fluorescence quenching; ELISA

1. INTRODUCTION

(Ca^{2+}-Mg^{2+})-ATPase, the calcium pump of sarcoplasmic reticulum, belongs to a class of cation ATPases which share sequence homology [1]. All members of this class are believed to pump cations by a similar mechanism involving two conformational extremes of the enzyme, designated E1 and E2. In the case of (Ca^{2+}-Mg^{2+})-ATPase it is the phosphorylation of the E1 form of the ATPase (with its 2 high affinity outward facing calcium-binding sites) by ATP to give Ca_2E1-P and the conformational change to give Ca_2E2-P (with its 2 low affinity outward facing calcium-binding sites) which constitutes the transport event [2–4].

On the basis of the sequence and labelling studies, a schematic structural model has been proposed for the ATPase [1]. However the complete 3-dimensional structure of this protein remains to be elucidated. One probe which has been used extensively in studies of the ATPase is fluorescein isothiocyanate (FITC) which covalently labels the ATPase at or close to the nucleotide-binding site [5]. This probe has been used in energy transfer studies to locate the position of sites on the ATPase relative to the nucleotide-binding site [6,7]. In this study we have examined the binding of anti-fluorescein antibodies to fluorescein attached to the nucleotide-binding site of the ATPase to gain information about the accessibility and therefore the possible location of this site.

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Abbreviations: BSA, bovine serum albumin; ELISA, enzyme linked immunosorbent assay; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline

2. MATERIALS AND METHODS

Sarcoplasmic reticulum (Ca^{2+}-Mg^{2+})-ATPase was prepared as outlined previously [8]. Fluorescein-labelled ATPase (FITC-ATPase) was prepared by incubating 9.0 nmol of ATPase for 1 h in 50 μl buffer (0.3 M sucrose, 1 mM Hepes, pH 7.5) with 4.5 nmol FITC (added as a 1.0 $mg \cdot ml^{-1}$ solution in dimethylformamide). Any unreacted FITC was removed from the sample by passing the incubate through a Sephadex G50 column equilibrated with PBS (0.137 mM NaCl, 2.7 mM KCl,

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8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4). The amount of FITC bound to the ATPase was estimated by spectrophotometry as outlined previously [8] and was typically 0.3 mol FITC/mol ATPase. Denatured FITC-ATPase was prepared as above except that the incubation was terminated by the addition of 5 μl of a 10% sodium dodecyl sulphate solution before passing down the Sephadex column. FITC-labelled BSA was prepared by adding 5 mg FITC in 50 μl dimethyl formamide to 10 mg BSA in 1 ml of 0.1 M $NaHCO_3$ buffer, pH 9.0. Unreacted FITC was removed by passing the incubate through a Sephadex G50 column and collecting the FITC-BSA in the void volume.

Anti-FITC antibodies were prepared by injecting a New Zealand White rabbit intramuscularly with 100 μg of FITC-BSA in Freund's complete adjuvant followed by an identical booster injection 8 weeks later. Serum was collected 7 days after the boost.

Fluorescence studies were carried out using a Perkin Elmer fluorescence spectrometer model LS-3B. The excitation and emission wavelengths were 495 nm and 525 nm, respectively.

ELISAs were performed using the method outlined by Hudson and Hay [9] with ELISA plates coated with 1 μg of the appropriate antigen in PBS. The competitive ELISA was performed with native and denatured ATPase. A range of concentrations of the native and denatured ATPase (0–20 μg) were incubated overnight with a 1:50000 dilution of the anti-fluorescein antiserum in PBS plus 0.05% Tween. The incubates were then transferred to an ELISA plate coated with native FITC-ATPase and the unbound anti-fluorescein estimated by the ELISA method outlined above.

3. RESULTS AND DISCUSSION

Fig.1 shows the effect of anti-fluorescein antibodies on fluorescein fluorescence. As observed previously [10] addition of the anti-fluorescein antibodies markedly reduces the fluorescence of free fluorescein with a maximum quenching of 60% being achieved in this study on addition of 20 μl of antiserum. Addition of the antiserum to the denatured fluorescein-labelled ATPase resulted in a maximum fluorescence quenching of 40%. However, only a 6% reduction in fluorescence was achieved when 20 μl of the anti-fluorescein antiserum was added to the native form of the fluorescein-labelled ATPase. Half-maximal quenching was achieved with ~1.4 and 1.7 μl of antiserum for free fluorescein and fluorescein bound to the denatured ATPase, respectively. Preimmune serum was without effect on fluorescein fluorescence.

These results indicate that fluorescein bound to the native form of the ATPase is located in a site inaccessible to anti-fluorescein antibodies and supports the view that the fluorescein is occupying an

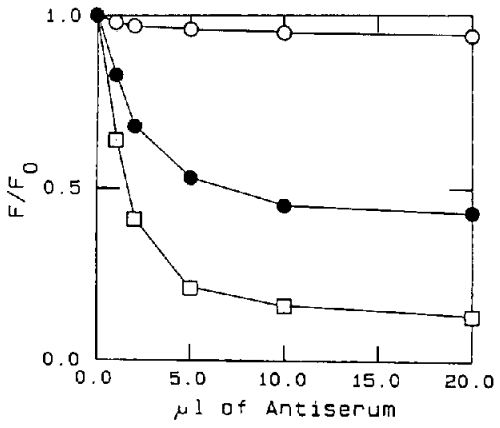

Fig.1. The effect of anti-fluorescein antibodies on the fluorescence emission of fluorescein. Native FITC-ATPase (○), denatured ATPase (●) or free fluorescein (□) (equivalent to 0.06 nmol of fluorescein) were dispersed in 3 ml PBS. Anti-fluorescein antibodies were added sequentially and the fluorescence intensity of the fluorescein was measured.

environment partially protected from the aqueous phase which makes up part of the nucleotide-binding site [11]. Fluorescence energy transfer studies indicate that the nucleotide-binding site is approx. 80 Å from the plane of the bilayer [7] which, from the current dimensional information [12], places the nucleotide-binding site on the ex-

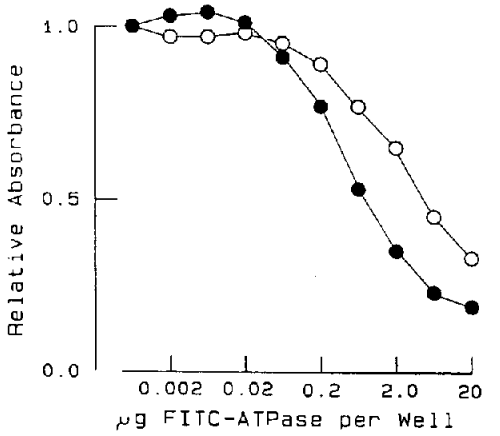

Fig.2. Determination of the accessibility of the FITC-binding site by competitive ELISA. Native (○) or denatured (●) FITC-ATPase was incubated overnight in the presence of anti-fluorescein antiserum (1:50000 dilution). The incubation was transferred to ELISA plates coated with 1 μg of native ATPase and the amount of free anti-fluorescein antibody estimated by ELISA.

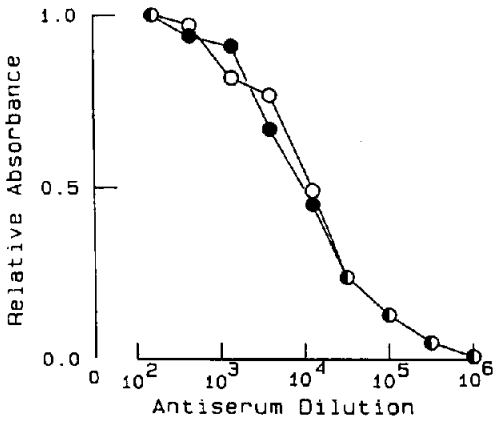

Fig.3. An examination of the ability of anti-fluorescein antibodies to bind to native and denatured ATPase adsorbed to ELISA plates. ELISA plates were coated with either native (○) or denatured (●) ATPase and incubated for 1 h with a range of antiserum concentrations. The amount of bound antibody was estimated by ELISA.

treme cytoplasmic surface of the ATPase. The nucleotide-binding domain cannot, however, be surface exposed.

Fig.2 shows a typical competitive ELIS profile showing the difference in binding of the antifluorescein antibodies to the native and denatured forms of the fluorescein-labelled (Ca^{2+}-Mg^{2+})-ATPase. The native form of the fluorescein-labelled ATPase is less efficient at binding the anti-fluorescein antibodies than is the denatured form, confirming the conclusion that fluorescein is inaccessible to antibodies in the native enzyme. Typically 6 times more of the native ATPase (5.4 μg) was required to reduce the ELISA reading by 50% compared with the denatured fluorescein-labelled ATPase (0.92 μg), indicating that 1 in 6 of the bound fluorescein moieties was available for binding in the native ATPase. This may be due to the presence of some denatured ATPase in the 'native' preparation.

When the native and denatured FITC-ATPase samples were used to coat ELISA plates and the binding of the anti-FITC antibodies was examined, the antibodies showed a similar dilution profile with a 50% reduction in binding being achieved at an antibody dilution of 1:10000 (fig.3). This is surprising in view of the finding that the competitive ELISA was able to discriminate between the native and denatured ATPase (fig.2). It may be that the adsorption of the ATPase to the polystyrene ELISA plates results in conformational changes to the native FITC-ATPase which increase the accessibility of the fluorescein moiety. Whatever the mechanism, there are obviously potential pitfalls in the use of the indirect ELISA assay for detecting changes in protein conformation.

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