Identification of a labelled peptide after stoicheiometric reaction of fluorescein isothiocyanate with the Ca^{2+}-dependent adenosine triphosphatase of sarcoplasmic reticulum

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Incorporation of 4.5 nmol fluorescein isothiocyanate/mg rabbit sarcoplasmic reticulum, or of 7.4 nmol/mg purified ATPase, was sufficient to inhibit the activity completely. These results are not consistent with the suggestion (Pick, U. and Karlish, S.J.D. (1980) Biochim. Biophys. Acta 626, 255–261) that 2 mol ATPase were inhibited by each mole of reagent incorporated. A single labelled peptide was purified from the inhibited ATPase and it was shown that Lys 3/190, 10 residues from the N-terminus of tryptic fragment B, was the reactive lysine residue. This site is close to a potential nucleotide-binding fold in the ATPase sequence. A similar peptide showing only 2 conservative replacements was isolated from the sarcoplasmic reticulum of the lobster.

$(Ca^{2+} + Mg^{2+})$ATPase inhibition *Reactive lysine residue* *Nucleotide-binding fold*
Fluorescein isothiocyanate *Integral membrane protein* *(Sarcoplasmic reticulum)*

1. INTRODUCTION

The Ca^{2+}-transporting ATPase of sarcoplasmic reticulum is an integral membrane protein containing a single polypeptide chain of 115 000 M_r [1,2]. It is still an open question whether the functional unit contains one or more chains. Several experiments [3,4] have suggested the formation of oligomeric units in the membrane, although the conclusions are open to criticism [5]. However, there is good evidence that the monomeric unit possesses full ATPase activity, albeit with slightly changed kinetics [6].

Experiments which determine the ratio of catalytic sites to polypeptide chains provide the most direct evidence on the problem. While there has been general agreement that there are twice as many Ca^{2+}-binding sites as there are phosphorylation sites or high affinity ATPase-binding sites (4 nmol/mg SR protein), the estimated number of peptide chains is ~6 nmol/mg [7,8], an intermediate figure, from which it is difficult to draw clear conclusions.

In [9–12] a new approach to this problem was described based on observations of a stoicheiometric inhibition of the Ca^{2+}-ATPase by fluorescein isothiocyanate (4.2 nmol/mg protein), from which it was concluded that 1 mol FITC was sufficient to inhibit 2 mol ATPase. Here we reinvestigate this question and identify the site which is labelled by the FITC.

Abbreviations: bicine, *N,N*-bis-(2-hydroxyethyl)-glycine; Ca^{2+}-ATPase, calcium- and magnesium-dependent adenosine triphosphatase (EC 3.6.1.3); $C_{12}E_9$, nona-ethyleneglycol dodecyl ether; FITC, Fluorescein 5 isothiocyanate (isomer I); FTC, fluorescein thiocarbamyl; SR, sarcoplasmic reticulum

2. MATERIALS AND METHODS

The reagents for sequencing [13] and enzyme assay [14] were as described. The detergent $C_{12}E_9$ was from Sigma (polidocanol). Fluorescein isothiocyanate was obtained from Molecular Probes (24750 Lawrence Road, Junction City, OR 97448) and assayed by titration against mercaptoethanol [15]. Different preparations contained 70–85% of active material.

Sarcoplasmic reticulum vesicles were prepared from rabbit muscle [16] or from the abdominal

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muscle of lobster [17]. ATPase-enriched vesicles were obtained from the rabbit SR by deoxycholate extraction, using method 2 from [18]. Detergent-purified ATPase [6] was obtained from rabbit SR by dissolving SR vesicles (30 mg/ml) in 3 vol. extraction buffer (10% $C_{12}E_9$, 20% glycerol, 4 mM $CaCl_2$, 50 mM Tris–HCl (pH 8.25)). After 15 min at 4°C any insoluble material was spun down (180 000 × *g* for 20 min) and the supernatant applied to a column (1 cm × 20 cm) of DEAE–Sephacel pre-equilibrated in 0.2% $C_{12}E_9$, 20% glycerol, 1 mM $CaCl_2$, 50 mM Tris–HCl (pH 8.25). After washing with 10 ml of this buffer the delipidated ATPase was eluted at 0.2 M NaCl by a gradient (0–0.5 M) of NaCl in the elution buffer (as above except for a lower concentration (0.02%) of $C_{12}E_9$). The ATPase (1–2 mg/ml) was used on the day of preparation.

Reactions with FITC were carried out at pH 7.0 or pH 8.8 in 50 mM Tris–HCl or bicine, respectively, in the presence of $CaCl_2$ (0.1 mM or 0.5 mM). The reaction was complete within 20 min at pH 8.8 or within 3 h at pH 7.0. The lobster ATPase reacted more slowly than that from rabbit and the labelled protein was obtained by incubation of 80 mg of the SR with 0.9 μmol FITC at pH 8.8 for 105 min (90% inhibition). Unbound fluorescein compounds were removed by gentle shaking with Dowex II (20–50 mesh, 0.1 g/ml) for 15 min. The bound fluorescein was calculated from A_{500} in 1% SDS, 0.1 M NaOH (to ensure complete ionization of the fluorescein in SDS). The extinction coefficient of our FITC was 76 000 [15] and it was changed by < 3% by reaction with ATPase. However, in view of the impurities in the FITC we have used a figure of 80 000, widely used in other laboratories.

Rabbits were immunized by subdermal injection of 5 mg bovine γ-globulin labelled with fluorescein (20 mol/mol) and dispersed in Freund's complete adjuvant. They were boosted with a similar injection after 5 weeks and bled at weekly intervals after a further 3 weeks. The antibody was isolated from the serum on a column of FITC–BSA coupled to Sepharose 4B, from which it was eluted with acetic acid (0.2 M). It was detected and estimated by titration with fluorescein, the fluorescence of which was quenched [21]. The purified antibody (200 mg) was precipitated with ammonium sulphate (2 M), dialysed and coupled to Sepharose 4B (100 ml) which had been activated with CNBr [22]. The immunoadsorbent bound to 20 nmol fluorescein/ml column vol.

2.1. *Isolation of FITC-labelled peptides*

In a typical experiment 30 mg purified ATPase (3 mg/ml) in either 50 mM Tris–glycine (pH 7.0) or 50 mM bicine (pH 8.8) were inhibited with equimolar amounts of active FITC. Unreacted FITC was removed by three 15 min treatments with Dowex II (0.1 g/ml). Membrane vesicles were spun down at 180 000 × *g* for 20 min and the pellet resuspended (10 mg protein/ml in 10 mM Tris–HCl, 1 mM $CaCl_2$ (pH 7.5)) before digestion with trypsin (1:30, w/w) for 60 min at 37°C. Digestion was halted by the addition of soybean trypsin inhibitor (1:15, w/w) and the digest spun at 180 000 × *g* for 120 min.

The supernatant was fractionated on a column (1.6 × 90 cm) of Sephadex G-50 in 50 mM ammonium bicarbonate, containing thiodiglycol (1 mg/ml). The fluorescent fractions were pooled, freeze-dried, dissolved in ammonium acetate (pH 7.7) adsorbed on a column of immunoadsorbent (4 ml) and eluted with 0.1 M triethylamine. The freeze dried samples were further purified by TLC on 0.25 mm silica layers in *n*-butanol/acetic acid/water/pyridine (15:3:12:10 by vol.).

Protein concentration was calculated from the A_{280} in 1% SDS using $A^{1\%}_{cm}$ = 10.0 for SR protein and $A^{1\%}_{cm}$ = 12.0 for 'purified ATPase' [19]. ATPase activity was determined by following oxidation of NADH using a coupled assay system [14]. The hydrolysis of *p*-nitrophenyl phosphate was measured spectrophotometrically (at 400 nm) in 10 mM PNPP, 0.1 mM $CaCl_2$, 5 mM $MgCl_2$, 75 mM KCl, 50 mM Tris–HCl (pH 7.5), 20% glycerol at 33°C after addition of ~50 μg protein [20]. Gel electrophoresis, amino acid analysis and sequencing of the purified peptides by the dansyl Edman method were performed as in [13].

3. RESULTS

3.1. *Stoicheiometry of inhibition by FITC*

We confirmed the observation of a rapid and complete inhibition of the Ca^{2+}-dependent ATPase activity of SR and of purified ATPase which was prevented by bound nucleotides [10,27]. The Ca^{2+}-independent activity was not inhibited.

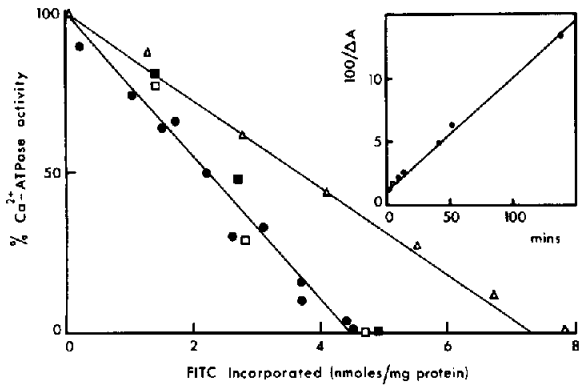


Fig.1. Inhibition of SR and of purified ATPase by FITC. SR (1.0 mg/ml) in 50 mM bicine (pH 8.8), 0.5 mM $CaCl_2$ was reacted with FITC in the absence (•) and in the presence of $C_{12}E_9$ at 1 mg/ml (▪) or 5 mg/ml (□). In the case of detergent-purified ATPase (△) the reaction was performed in the elution buffer (pH 8.25). The inset shows a second-order plot of the reaction at pH 7.0 (1.0 mg SR/ml, 4.8 μM active FITC, 50 mM Tris–HCl, 0.5 mM $CaCl_2$). ΔA was the difference between the measured and final activity. The rate constant was 300 $M^{-1} \cdot s^{-1}$.

ATPase inhibited by FITC retained 60% of its original nitrophenylphosphatase activity and this was not inhibited by ATP (0.1 mM), whereas that of the parent enzyme was inhibited. The inhibition of sarcoplasmic reticulum and of purified detergent solubilised ATPase is shown in fig.1. The linearity of the inhibition curve suggests a stoicheiometric reaction between FITC and the ATPase and shows that incorporation of 4.5 nmol reagent/mg protein was required for complete inactivation. The stoicheiometric reaction was confirmed by the finding that all of the active material in the FITC was incorporated into the ATPase. Only the 15–30% of inactive impurity was removed by the treatment with Dowex II. When equimolar amounts of reagent and protein were used the kinetics of the inhibition were second order (fig.1, inset).

The second curve in fig.1 shows that the detergent-purified ATPase reacted similarly and that incorporation of 7.4 nmol FITC/mg protein was required for complete inhibition.

The molar concentration of ATPase protein was estimated by scanning SDS gels of the SR. These showed that 70% of the protein ran in the position of the ATPase. Since almost all of this band was cleaved rapidly by trypsin to give subfragments A and B [23] we concluded that there was < 5% of any other protein in the band. From M_r 115 000 ± 5000 [1,2] we calculated that the SR contained 6 ± 0.5 nmol ATPase/mg protein. Similar experiments with the detergent-purified ATPase showed that 95% of the protein ran as a single band, implying a maximum of 8.3 ± 0.6 nmol ATPase/mg protein. These results are consistent with the presence of 1 FITC reaction site/peptide chain of the ATPase, making allowance for 25% and 9% of inactive protein in the 115 000 M_r band of the 2 preparations. The levels of inactive protein would be decreased to 20% and 5% if the extinction coefficient of bound fluorescein were taken as 76 000.

Another result of interest illustrated in fig.1 is the lack of effect of non-ionic detergent on the FITC-inhibition of ATPase. The presence of sufficient $C_{12}E_9$ to dissociate the ATPase to monomer [24] (□,▪) did not increase the amount of FITC required for inactivation, nor was any increase in activity observed when detergent was added to FITC-inhibited SR (not shown). This is contrary to expectation if the ATPase were dimeric and required only 1 mol FITC to inactivate the dimer.

3.2. *Identification of the site of labelling*

ATPase vesicles purified by the method of Meissner were used in these experiments. In a typical experiment the ATPase, incubated with

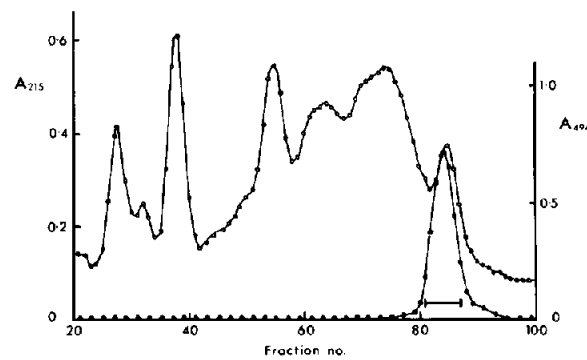


Fig.2. Gel filtration of tryptic peptides. The peptides from 34 mg FITC-labelled ATPase were freeze-dried, dissolved in ammonium bicarbonate (50 mM) and separated (2 ml fractions) on a column (1.6 × 90 cm) of Sephadex G-50.

Table 1

Amino acid composition of fluorescein-labelled peptides

Amino acid	Short peptide		
	Lobster	Rabbit (1)	Rabbit (2)
Asp	1.2 (1)	1.3 (1)	1.2 (1)
Thr	0.3	0.3	0.2
Ser	0.5[a]	1.0[a]	0.9[a]
Glu	1.5[a] (1)	1.6[a] (1)	1.5[a] (1)
Pro	1.2 (1)	0.8 (1)	0.9 (1)
Gly	2.0 (2)	3.2[a] (2)	2.9[a] (2)
Ala	0.9 (1)	1.3 (1)	0.8 (1)
Val	1.1 (1)	1.5 (2)	1.5 (2)
Met	0.6 (1)	0.6 (1)	0.4 (1)
Ileu	1.0 (1)	0.7 (1)	0.7 (1)
Leu	1.2 (1)	0.4	0.4
Tyr	0.1	0.2	0.2
Phe	0.9 (1)	0.9 (1)	1.0 (1)
His	0.1	0.2	0.1
Lys	0.9 (1)	1.1 (1)	1.1 (1)
Arg	1.0 (1)	1.0 (1)	1.0 (1)

The numbers of residues are expressed relative to arginine (1.0). The numbers in parentheses were determined from the sequence. The high analytical values (a) were caused by impurities picked up during the TLC separation. A blank sample of the silica gave significant amounts of Gly, Ser and Glu. The two peptides from the rabbit were derived from the double TLC spot

10 nmol active FITC/mg, gave a product containing 7.6 nmol fluorescein/mg protein. Digestion with trypsin released 74% of the fluorescein as soluble peptides which were fractionated on Sephadex G-50 (fig.2). Most of the label (90%) emerged in a single peak near to the total column volume. The labelled peptides were recovered quantitatively from the immunoadsorbent column and further purified by TLC. Only a single fluorescent peptide was detected. Although this gave a double fluorescent spot (R_F = 0.52, 0.58), both components had the same amino acid composition (table 1). The composition was close to that of a 13-residue tryptic peptide beginning at Met_{187} of sequence 3, 7 residues from the N-terminus of the tryptic fragment B [25]. This is the same fragment that was labelled in [9]. The sequence of amino acids in the peptide confirmed this identification (fig.3) and cleavage of a fluorescent residue from

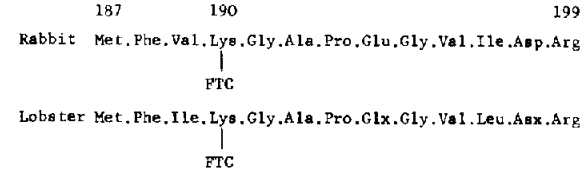


Fig.3. Sequences of fluorescein-labelled peptides from the Ca^{2+}-ATPases of rabbit and lobster.

the peptide on the fourth cycle showed that Lys_{190} of sequence 3 was the reactive residue. The relatively late emergence of the 13-residue peptide on the G-50 column was due to adsorption of the fluorescein residue; free label emerged after 1.8 column vol. Several experiments in which the ATPase was labelled with up to 8 nmol FITC/mg protein showed only this single labelled peptide.

When higher concentrations of FITC were used for labelling, introducing up to 15 nmol fluorescein/mg protein small amounts of several other labelled peptides appeared on the G-50 column, but these were not identified.

The ATPase from the lobster sarcoplasmic reticulum gave a similar peptide (table 1, fig.3), showing only 2 conservative replacements in 13 residues. This degree of homology is significantly higher than the average (70%) observed between glyceraldehyde phosphate dehydrogenase of pig and lobster but is similar to that between the active site regions of these enzymes [26].

Some ATPase preparations which were carboxymethylated and succinylated before triptic digestion [25] gave a longer 19-residue peptide because the tryptic cleavage site was shifted to Arg_{180} by blocking of Lys_{186}. This method was avoided since analyses of the peptide were deficient in lysine and had an extra peak in the tyrosine position, also the yields from the Edman degradation were very low. These anomalies disappeared when the succinylation was omitted, and were probably a consequence of unexpected by-products from transformations of an *S*-succinyl thioureido derivative.

4. DISCUSSION

Our observation that 4.5 mol FITC/mg SR protein were sufficient for complete inhibition of the ATPase is close to the value obtained in [12] (and

more recently in [27]). By assuming a lower M_T for the ATPase and a higher ATPase content for the sarcoplasmic reticulum, it was concluded [12] that only 0.5 mol FITC was sufficient to inhibit the ATPase. This required convoluted arguments to explain the absence of any activating effect when the hypothetical ATPase dimer, inhibited by 1 mol FITC, was dissociated into monomers [12].

We conclude that 0.78 and 0.89 mol FITC/mol were required for complete inactivation of SR and detergent-purified ATPase respectively and consider that the most likely explanation of the apparent deviation form exact 1:1 stoicheiometry resides in the difficulty of determining the active ATPase content of the preparations. Although our results are not compatible with the suggestion that the ATPase is an oligomer which exhibits half-of-sites reactivity with respect to ATP or FITC, they do not exclude a dimeric functional unit for Ca^{2+} transport. Although the labelled lysine is 164 residues from the site of phosphorylation [28], it is likely to be close to it in the tertiary structure in view of the protection against inhibition by ATP and the abolition of the high affinity nucleotide site in the fluorescein-labelled ATPase [27,29]. Although the binding site was blocked, phosphorylation by phosphate and by acetyl phosphate was not prevented [10]. Thus, it appears that one can distinguish 2 sites located on neighbouring domains of the protein. This is supported by our observation that occupancy of the nucleotide site by ATP completely inhibits nitrophenylphosphatase activity, whereas occupancy by a fluorescein residue does not.

From these results and from a detailed kinetic study of the reaction of FITC with the ATPase, to be published elsewhere, we conclude that there is a single highly reactive site for FITC on each active polypeptide chain. The labelling of a specific lysine residue is a consequence of its location near to a binding site for fluorescein and probably for other xanthene dyes [30], which are known to bind to the nucleotide domains of several enzymes [31,32]. The predicted secondary structure of the ATPase in the region immediately following the reactive lysine contains several hydrophobic β-strands, alternating with α-helices, a characteristic feature of such a nucleotide-binding fold. This hypothesis, together with further supporting evidence will be developed in more detail elsewhere.

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