MODIFICATION OF THE ATP BINDING SITE OF THE Ca^{2+}-ATPase FROM SARCOPLASMIC RETICULUM BY FLUORESCEIN ISOTHIOCYANATE

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Received 21 November 1980

1. Introduction

Fluorescein isothiocyanate, generally used as a non-specific fluorescent probe for labeling of proteins, was demonstrated to be an efficient inhibitor of the Na^+, K^+-ATPase [1] and of the Ca^{2+}-ATPase from sarcoplasmic reticulum [2]. Incubation of the Ca^{2+}-ATPase from sarcoplasmic reticulum with μM levels of FITC at alkaline pH led to complete inhibition of the activity within a few minutes. Binding of 1 fluorescein molecule/2 Ca^{2+}-ATPase polypeptides (M_r 105 000) resulted in complete inhibition of the activity which may suggest that the active form of the enzyme is at least a dimer. ATP effectively protected against FITC inhibition both the Na^+, K^+-ATPase and the Ca^{2+}-ATPase [1,2].

Taken together these results may suggest that FITC is a selective affinity probe for the ATP binding site probably labeling a lysine group at or close to the ATP binding site in both ATPases.

Here, we demonstrate that FITC specifically blocks the ATP binding site of the Ca^{2+}-ATPase from sarcoplasmic reticulum and does not prevent the phosphorylation of the enzyme by P_i or Ca^{2+}-uptake with acetyl phosphate as a substrate. The implications of these findings for the role of the regulatory ATP binding site are discussed. The usefulness of this probe to follow interconversions between phosphorylated and non-phosphorylated conformational states of the Ca^{2+}-ATPase is described in [3].

2. Materials and methods

Fragmented SRV were prepared from rabbits as in [4]. Incubation of SRV with FITC was done at 20°C by suspending 150–225 μg SRV protein in 100 μl containing: 0.25 M sucrose, 25 mM Tris–HCl, 25 mM Na-glycine at the indicated pH and 100 μM EGTA. FITC was added from a freshly made dimethyl formamide solution. The incubation was terminated after 10 min either by addition of 400 μl ice-cold solution containing: 0.2 M sucrose, 50 mM Tris–HCl (pH 7.0) and 5 mM Na-ATP (stopping solution) or by transfering 100 μl samples through a Sephadex G-50 column (in 1 ml tuberculin syringe) pre-equilibrated with ice-cold stopping solution, not containing ATP, as in [5]. The activities were measured within 30 min. Ca^{2+}-uptake was measured by the Dowex procedure [6]. Phosphorylation by [γ-^{32}P]ATP [7] and by P_i [8] were measured as described. FITC (isomer I), nucleotides and other chemicals were obtained from Sigma.

Abbreviations: SRV, sarcoplasmic reticulum vesicles; FITC, fluorescein isothiocyanate; AMP-P(NH)P, adenylyl imidodiphosphate

3. Results

In [2] we have reported that FITC is an extremely potent inhibitor of the Ca^{2+}-ATPase from sarcoplasmic reticulum. We have demonstrated that following incubation at alkaline pH with FITC, one fluorescein equivalent bound per two equivalents of 105 000 M_r polypeptides resulted in a complete inhibition of the Ca^{2+}-ATPase and Ca^{2+}-uptake activities.

The presence of ATP in the incubation mixture protected against the inhibition by FITC and prevented the binding to the enzyme which may suggest competition for a common binding site. An apparent competition between FITC and ATP is indeed demonstrated in fig.1. At higher FITC concentrations a higher ATP concentration was required for effective protection. Even though the kinetic interpretation of these data is not straight-forward because FITC is an

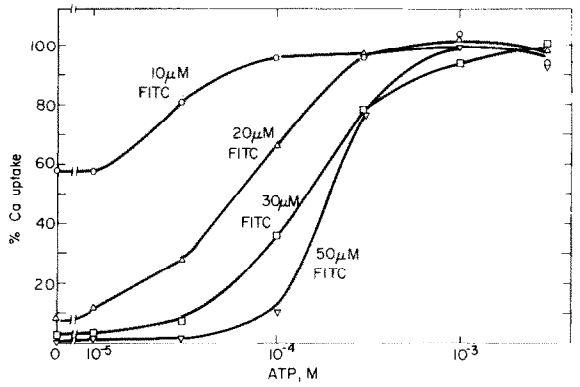

Fig.1. Competition between ATP and FITC. SRV were incubated 10 min at pH 8.0 with ATP and FITC at the indicated concentrations and assayed for Ca^{2+}-uptake as in section 2. The control rate was 1240 nmol Ca^{2+} . mg protein^{-1} . min^{-1}.

irreversible inhibitor it is quite evident that a 5-fold increase of the inhibitor concentration requires ~5-fold higher ATP concentrations for effective protection. It should be noted that the incubations with FITC were done at pH 8.0 and not at pH 10 which is optimal for FITC inhibition in order to test the interaction of the enzyme with ATP under more physiological conditions.

Fig.2 shows that protection against FITC is very specific for adenine nucleotides and does not involve phosphorylation of the enzyme since both ADP (in the presence of glucose and hexokinase) and the non-hydrolyzable ATP analog AMP P(NH)P effectively

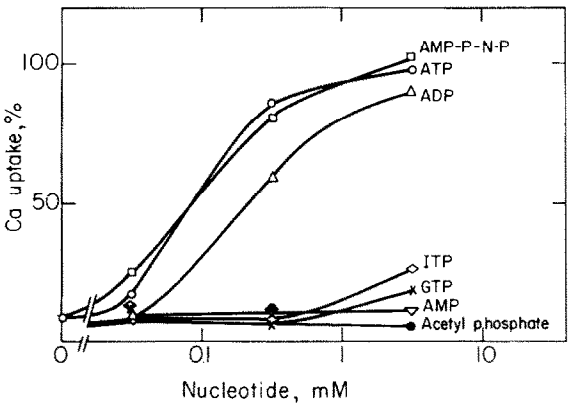

Fig.2. Protection against FITC is specific to adenine nucleotides. SRV were incubated 20 min at pH 8.0 with 30 μM FITC and the additions indicated in the figure and assayed for Ca^{2+}-uptake. The control rate was 1275 nmol Ca^{2+} . mg protein^{-1} . min^{-1}.

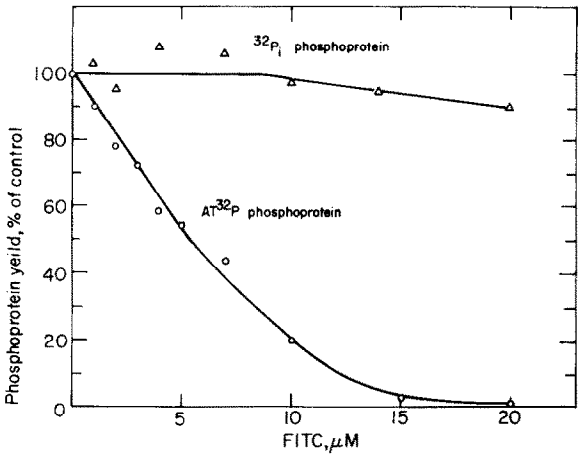

Fig.3. The effect of FITC on phosphoprotein formation from [^{32}P]ATP and $^{32}P_i$ SRV (2.2 mg/ml) was incubated 10 min at pH 10 with FITC at the indicated concentrations. The incubation was terminated by transferring samples through Sephadex G-50 columns to remove the unreacted reagent. Samples containing 330 μg protein were taken for the phosphorylation assays. The control extent of $^{32}P_i$ incorporation were 3.4 nmol/mg protein and 2.9 nmol/mg protein with [γ-^{32}P]ATP and $^{32}P_i$, respectively. Other conditions were as in section 2.

protected the enzyme. Adenine monophosphate and 3′,5′-cyclic AMP (not shown) were completely ineffective. ITP and GTP which can phosphorylate the enzyme and catalyze Ca^{2+}-uptake gave a slight protection at mM levels and acetyl phosphate was completely ineffective. The results suggest that 5′-ADP is the minimal structure recognized by the adenine nucleotide binding site which competes with FITC.

Fig.3 demonstrates that the FITC modified Ca^{2+}-ATPase cannot be phosphorylated by ATP but can be readily phosphorylated by P_i. Since it is assumed that a common aspartyl residue is phosphorylated by both ATP and phosphate it can be concluded that the phosphorylation site is unaffected by FITC. The conclusion is in line with our finding that FITC binds to the 45 000 M_r tryptic fragment of the Ca^{2+}-ATPase whereas the phosphorylation site was identified on the 55 000 M_r tryptic fragment [2].

Fig.4 demonstrates that Ca^{2+}-uptake in FITC modified sarcoplasmic reticulum vesicles with acetyl phosphate (B) as substrate is only partly inhibited even though the Ca^{2+}-uptake catalysed by ATP is completely inhibited (A). The FITC modification increased the initial lag in Ca^{2+}-uptake from 30 s to 2 min and inhibited the steady state rate of Ca^{2+}-uptake by

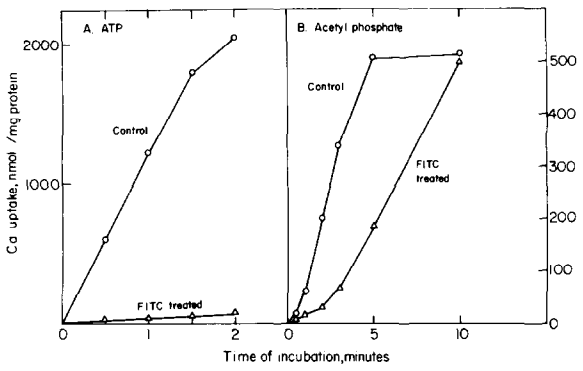

Fig.4. The effect of FITC on Ca^{2+}-uptake with ATP and acetyl phosphate. SRV (2 mg/ml) was incubated for 10 min with or without 20 μM FITC at pH 9.0 and the reaction was terminated by removing the unreacted FITC on Sephadex G-50 columns. Ca^{2+}-uptake was assayed in the presence of 5 mM ATP or 10 mM acetyl phosphate and the protein concentrations in the assay mixtures were 32 μg/ml with ATP and 160 μg/ml with acetyl phosphate. Other conditions were as in section 2.

~50%. This initial lag probably reflects the very slow rate of phosphorylation by acetylphosphate that becomes the rate limiting step in Ca^{2+}-uptake [9]. Similar partial inhibitions of Ca^{2+}-uptake by FITC were obtained with other substrates such as carbamyl phosphate (65%) and with *p*-nitrophenylphosphate but with ITP an almost complete inhibition was obtained (not shown). These results strongly indicate that FITC modifies specifically the ATP binding site of the Ca^{2+}-ATPase.

4. Discussion

These results strongly suggest that FITC competes with ATP for a common binding site and it points to a structural similarity between fluorescein 5-isothiocyanate and ATP. The possibility that the apparent competition between ATP and FITC is a result of cooperative interactions between two different sites seems very unlikely because FITC inhibits also the binding of ATP to the enzyme (U. P., unpublished). It was also demonstrated that protection against FITC is very specific for 5′-adenine nucleotides (fig.2).

The observations that FITC inhibits also the mammalian Na^+, K^+-ATPase [1] and the chloroplast proton ATPase (unpublished) and that ATP protects these 3 enzymes against the inhibition points to a conservative structure of ATP binding sites in widely different ion-transporting ATPase and to the presence of an essential lysine group involved in ATP binding.

The location of the phosphorylation site on the 55 000 M_r tryptic fragment of the Ca^{2+}-ATPase [10] is different from the FITC binding site which was identified in the 45 000 M_r tryptic fragment [2]. This observation may suggest that the catalytic site is made up of peptide segments from both the 55 000 and 45 000 M_r tryptic fragments of the enzyme or alternatively, that there are two physically different ATP binding sites as will be discussed below. The large changes in the fluorescence yield of the bound chromophore in response to Ca^{2+}-binding and to phosphorylation [2,3] indicate that the microenvironment of the chromophore is very sensitive to changes in the conformation of the enzyme. This makes FITC a very useful tool to follow conformational changes in the Ca^{2+}-ATPase from sarcoplasmic reticulum.

Evidence for the existence of more than one type of ATP binding site in the Ca^{2+}-ATPase from sarcoplasmic reticulum was presented before based on kinetic measurements [11–14].

A low affinity ATP binding site (K_m 30–500 μM) was identified which presumably regulates the Ca^{2+}-dependent conformational transition in the dephosphorylated enzyme and differs from the high affinity phosphorylation site. Our results indicate that the properties of the ATP binding sites involved in protection against FITC resemble in many respects the low affinity regulatory sites:

(1) In specificity: it was demonstrated that substrates like ITP, GTP and acetyl phosphate which phosphorylate the enzyme at mM levels were not effective in protection against FITC. Similarly, AMP P(NH)P which has a low affinity for the phosphorylation site (K_i 90 μM) compared to ATP (K_m 3 μM) but has a similar affinity as ATP for the regulatory site [13] is also as effective as ATP in protection against FITC inhibition (fig.2).

(2) The apparent low affinity for ATP for protection is at least an order of magnitude higher than the K_m for phosphorylation. Since the apparent affinity for ATP protection may give an overestimation because of the competition with an irreversible inhibitor we have also measured the initial rates of inactivation by different FITC concentrations in the presence of several concentrations of ATP and from the plot of $1/v$ (v = the initial rate of inactivation) vs FITC concentration obtained a binding constant of 25 μM for ATP which is

much higher than the affinity of the phosphorylation site for ATP (not shown). Since FITC inhibits all the interactions of the enzyme with ATP at the phosphorylation sites (fig.3) the question arises what are the interrelations between the two ATP binding sites. The two different affinities for ATP may represent a single site at two different conformations of the enzyme – in absence of Ca^{2+} the enzyme may exist in a conformation which binds ATP with a low affinity but in the presence of Ca^{2+} which induces a conformational change the site may be converted to the high affinity binding site. In other words, ATP which binds to the enzyme in the absence of Ca^{2+} would become the phosphorylating substrate.

This interpretation is consistent with random binding of ATP and Ca^{2+} to the enzyme which was suggested in [15]. The other alternative is that the two sites are physically different sites and affect each other by cooperative interactions. Our recent observation that the affinity for AMP-P(NH)P in protection against FITC is higher in the presence of Ca^{2+} (unpublished) is in line with the former interpretation.

5. Conclusions

(i) FITC inhibits the Ca^{2+}-ATPase from sarcoplasmic reticulum by specifically modifying the ATP binding site.

(ii) That the fluorescein-labeled enzyme can take up Ca^{2+} from the medium with acetyl phosphate as a substrate demonstrates that the enzyme can perform the complete turnover in the absence of ATP.

(iii) The low affinity and high specificity for adenine nucleotides in protection against FITC imply that the regulatory ATP binding sites are involved in the protection and it suggests a close interrelation or identity of the phosphorylation and the regulatory ATP binding sites.

Acknowledgements

We thank Professors M. C. Berman and S. J. D. Karlish for helpful discussions.

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