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Affinity labeling of the active site of the Ca^{2+}-ATPase of sarcoplasmic reticulum

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The inactivation of sarcoplasmic reticulum ATPase by fluorescein isothiocyanate (FITC) was shown to have a hyperbolic dependence on the concentration of FITC. The results were quantitatively accounted for by a model in which the reagent first binds reversibly ($K_I = 70\ \mu M$) to the ATPase and then reacts irreversibly ($k_{max} = 0.8$ and 2 min^{-1} in the absence and presence of 1 mM Mg^{2+}, respectively) to form inactive enzyme. Comparison with the rate constant for the reaction of the model compound α-acetyllysine with FITC showed that the FITC-reactive lysyl side-chain of the ATPase is not unusually reactive, indicating that the specificity of the reaction is due to affinity labeling behavior of the reagent. This was supported by protection experiments using ATP, ADP, Ado*PP*[NH]*P*, ITP, and TNP-ATP, all of which displayed protection constants similar to their known binding constants to the active site of the ATPase. Both inorganic phosphate and orthovanadate were effective in preventing inactivation by FITC, and calcium only partially reversed the effect of these anions, implying the existence of a ternary complex such as $Ca_2 \cdot E \cdot P_i$. Since all ligands (ATP, ADP and P_i) which bind or react at the catalytic site protect it, only the unliganded form appears to bind and react with FITC. Addition of calcium to the MgATP complex of the ATPase caused an increase in the FITC inactivation rate, implying that during turnover there is a larger fraction of unliganded enzyme present, i.e., substrate binding is weaker (K_s is larger). Protection was also observed with fluorescein and two related dyes, eosin and erythrosin. Like FITC, the isothiocyanates of these dyes were effective inactivators. In separate experiments, these two dyes were shown to promote photoinactivation of the ATPase. ATP exerted a protective effect with a concentration dependence consistent with high-affinity active-site binding.

Introduction

The Ca^{2+}-ATPase of sarcoplasmic reticulum membranes produces accumulation of calcium ions in the SR lumen by coupling this transport to ATP hydrolysis. Prepared from skeletal muscle in the form of vesicles which retain this active transport capability, SR contains in addition to the Ca^{2+}-ATPase less well-characterized proteins (for reviews, see Refs. 1–3). With a molecular weight of $1.1 \cdot 10^5$ and a known amino-acid sequence [4,5], the Ca^{2+}-ATPase appears to constitute 50% of the vesicular protein [6], a result which agrees

Abbreviations: SR, sarcoplasmic reticulum; Mops, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Ado*PP*[NH]*P*, adenyl-5′-yl imidodiphosphate; SDS, sodium dodecyl sulfate; FITC, fluorescein isothiocyanate (isomer I); TNP-ATP, 2′,3′-(2,4,6-trinitrocylohexadienylidene)adenosine 5′-triphosphate; DTT, dithiothreitol; V_i, orthovanadate; EP, phosphorylated enzyme intermediate, NPP *p*-nitrophenyl phosphate; PLP, pyridoxal 5-phosphate.

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with the ATP- and P_i-phosphorylatable active site content of 4–5 nmol per mg of SR protein [7]. The enzyme's transport cycle includes phosphorylation of an aspartate residue (number 351 in the sequence [4]) accompanied by transformation of outwardly oriented, high-affinity calcium sites to inwardly oriented, low-affinity ones.

Essential to understanding this calcium transport protein is the characterization of its active site, including the identity and properties of the amino-acid residues which comprise it. Site-specific labeling is one approach to this problem, and reaction of the ATPase with FITC is an example of its application [8]. Although it seems clear that this reagent labels a nucleotide site, because of what appeared to be a substrate protection requirement of 100 μM ATP [9], it is not clear whether the active site ($K_d = 1$–10 μM) or a distinct, presumably regulatory site ($K_d > 100$ μM) is labeled. It also remains to be determined whether the specificity of this reaction is due to (a) a hyperreactive protein group, (b) high-affinity binding followed by reaction with a group of normal reactivity (affinity labeling), or (c) a combination of these. In this paper we present evidence that FITC and other xanthene isothiocyanates are affinity labels of the active site, and that the site's amino group which is labeled has a normal reactivity, the selectivity of modification being primarily due to the affinity of the reagent for the site.

Materials and Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit hind leg muscle [10]. FITC (isomer I), rabbit muscle lactate dehydrogenase (type II), and rabbit muscle pyruvate kinase (type II) were from Sigma; erythrosin isothiocyanate, eosin isothiocyanate and TNP-ATP were obtained from Molecular Probes; all other chemicals were reagent grade or better.

ATPase assays were carried out at 37°C in solutions containing 80 mM KCl, 50 mM Mops (pH 7.0), 5 mM $MgCl_2$, 1.1 mM $CaCl_2$, 1 mM EGTA, 0.5 μg/ml calcium ionophore (A23187) and 2.5 mM MgATP. Hydrolysis of ATP was followed as previously described either by measuring P_i liberation with acid molybdate [11] or by coupling ATP regeneration to oxidation of NADH [12].

Reaction of SR vesicles with FITC was done at 25°C in 50 mM Mops (pH 7.0), 1 mM EGTA, and 1–2 mg SR protein per ml. At various times, mixtures were quenched by dilution to 10 μg SR protein per ml into the above-described assay buffer at 0°C. Reaction of α-acetyllysine (200 mM) with FITC (20 μM) was followed spectrophotometrically at 510 nm in 50 mM sodium borate (pH 8.7) at 25°C.

Rate constants (k_{obs}) were obtained using a nonlinear regression algorithm by computer fit to

$$A = A_0(\exp(-k_{obs}t))$$

where A and A_0 are the absorbance or ATPase activity at time $= t$ and 0, respectively. In experiments where inactivation was measured as a function of [FITC] (Fig. 3), k_{obs} in this equation was replaced by the expression

$$k_{max}[F]/([F] + K_F)$$

where K_F and k_{max} are respectively the dissociation and inactivation rate constants, and [F] is the FITC concentration. These and other parameters were obtained by analogous computer fit to the appropriate equation. For the reaction of FITC with α-acetyllysine, the unitary rate constant (k_u; mole fraction units) was calculated from

$$k_u = (10^{7.0-8.7})(278)(k_{obs})$$

where k_{obs} is the pseudo-first-order rate constant at pH 8.7; the first factor on the right side of the equation derives from the unprotonated amino group being the presumed reactive species, so that the rate constant is inversely proportional to $[H^+]$ when $pH \ll pKa$; the second factor is required for the conversion to mole fraction (unitary) units, i.e., division by 1/278 (the mole fraction of a 0.2 M aqueous solution).

Photoinactivation experiments were carried out by placing a 100 W incandescent lamp 4 cm from 1 ml reaction mixtures immersed in a 25°C bath.

Polyacrylamide gel electrophoresis was done according to Laemmli [13]. Upon completion of the run, the tubes were scanned at 500 nm with a gel scanner module on a GCA/McPherson spec-

trophotometer. The amount of FITC incorporated was calculated from the FITC added times the fraction of the total area of the gel scan peaks which corresponded to the ATPase.

Results

Stoichiometry of the inactivation of the Ca^{2+} ATPase by FITC

Consistent with the results of Pick and Karlish [8], we found that at pH 10 FITC at low concentration rapidly inactivated the Ca^{2+}-ATPase of SR. The reaction also occurred readily at the more physiologically relevant pH of 7: activity loss was linearly related to the concentration of added FITC. Extrapolating to complete inactivation gave 6.8 nmol FITC added per mg of protein. Not all of the FITC is incorporated into the ATPase protein, however; the FITC linked to the ATPase was obtained from the areas of the peaks on scans of gels. As shown in Fig. 1a, the incorporation was proportional to the concentration of FITC added up to a value of about 5.3 ± 0.5 nmol FITC incorporated per mg of protein. The dependence of the ATPase activity on the FITC incorporated per mg of protein (Fig. 1b) yielded a value of 5.2 ± 0.5 nmol FITC incorporated per mg of protein. There is thus approximate agreement between the high-reactivity and inactivation stoichiometries, consistent with the presence on the enzyme of a uniquely reactive group, which when modified results in loss of ATPase activity. Our stoichiometry thus agrees with previously reported values [14–16]. Analogous experiments using the isothiocyanates of the fluorescein analogs eosin and erythrosin gave similar stoichiometries (data not shown).

Kinetics of the inactivation of the SR ATPase by FITC

We observed that the rates of inactivation of the Ca^{2+}-ATPase of SR were conveniently measurable at pH 7.0 and 25 °C. At FITC concentrations sufficiently high to establish pseudo-first-order conditions, the data were in all cases well-described by a single exponential (Fig. 2), suggesting that reaction at a single site was occurring. The inactivation rate constants (k_{obs}) thus obtained permit a quantitative comparison of the effects of various ligands on the inactivation (see below).

If the inactivation were a consequence of a simple bimolecular reaction, k_{obs} would be directly proportional to [FITC]. We observed, how-

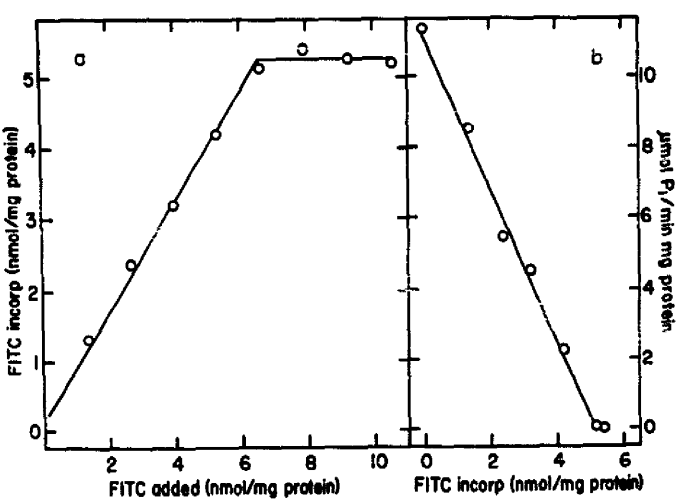


Fig. 1. Stoichiometry of the reaction of SR with FITC. SR vesicles were incubated for 18 h with the indicated amount of reagent (FITC added). Aliquots were then assayed for ATPase activity and subjected to gel electrophoresis as described in Materials and Methods.

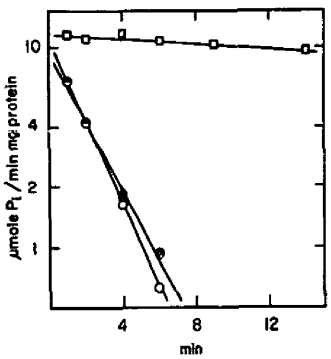
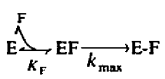


Fig. 2. Time-course of the loss of SR ATPase activity induced by FITC. Conditions were as described in Materials and Methods. [FITC] = 100 μM. Reaction mixtures also contained: ○, 1 mM EGTA; ●, 1 mM $CaCl_2$; □, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM ATP.

ever, that k_{obs} appeared to increase hyperbolically with increasing [FITC] (Fig. 3a). This saturation behavior suggests that reversible binding of the reagent (F) to the enzyme (E) precedes irreversible reaction of the binary complex (EF) to form an inactive product (E-F) (Scheme I):



Scheme I.

Accordingly, if the binding step is rapid compared to the inactivation step, a linear equation which relates k_{obs} to [F] is:

$$k_{obs} = k_{max} - K_F(k_{obs}/[F]) \quad (1)$$

where K_F and k_{max} are respectively the dissociation and inactivation rate constants for the binary complex EF. These parameters, formally equivalent to K_m and V_{max}, respectively, of the Michaelis-Menten equation, are equal to the slope and intercept of the Scatchard plots shown in Fig. 3b. For reaction in the absence and presence of 1 mM Mg^{2+}, K_F was 69 and 68 μM, respectively, while k_{max} was 0.76 and 1.8 min^{-1}. Magnesium thus appears to exert a small but significant increase in reactivity of the ATPase toward FITC. (The Mg^{2+} effect appeared not to be a consequence of its binding to FITC, as judged by the absence of a difference in the visible absorption spectrum of FITC at pH 7 in the presence and absence of 10 mM Mg^{2+}.)

The k_{max} values may be compared with its counterpart for the reaction of α-acetyllysine in aqueous solution. At 25°C and pH 8.7, 20 μM FITC was found to react with 0.2 M of the model compound with a pseudo-first-order rate constant of 0.11 min^{-1}, which corresponds to a rate constant (mole fraction units) of 0.6 min^{-1} at pH 7.0. This is in the same range as k_{max}, which suggests that the FITC-reactive group of the enzyme does not have an anomalous reactivity. The specificity of the reaction is apparently instead a conse-

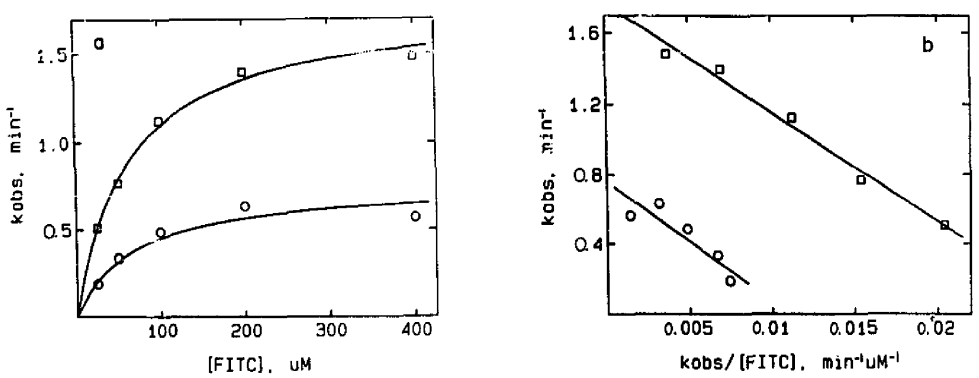
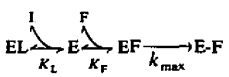


Fig. 3. FITC concentration dependence of the observed rate constant for SR ATPase inactivation. The results are plotted in (a) linear and (b) Scatchard (Eadie-Hofstee) forms. In addition to components described in Materials and Methods, reaction mixtures also contained: ○, 1 mM EDTA; □, 1 mM $MgCl_2$.

quence of the affinity of the reagent for a specific site on the enzyme.

Effect of ligands on the inactivation by FITC

That this site is a nucleotide-binding site is indicated by the protective effect of ATP. In the absence of ATP, the ATPase was inactivated by 100 μM FITC with a k_{obs} of 0.46 min^{-1} (Fig. 2); as shown, essentially the same result was obtained whether or not Ca^{2+} was present. With 1 mM ATP present, k_{obs} was lowered over 30-fold (Fig. 2). ADP was similarly protective, while AMP, which does not bind to the enzyme, was not (Table I). The data were analyzed by modifying Scheme I to include rapid equilibrium competitive binding of a ligand, L:



Scheme II.

According to this scheme, K_L can be calculated by combining Eqn. 1 and its modified form in which K_F is multiplied by $(1+[L]/K_L)$ to obtain:

$$K_L = \frac{[L]}{\left(\frac{[F]}{K_F}+1\right)\left(\frac{k_{obs-L}}{k_{obs+L}}-1\right)} \quad (2)$$

where k_{obs-L} and k_{obs+L} are the observed rate constants in the absence and presence of ligand, respectively. Values of K_L calculated according to this equation for a number of ligands are listed in Table I.

For MgATP, we obtained a K_L value of 5 μM, suggesting that its protective effect is exerted at a high-affinity site, most likely the catalytic site ($K_m = 4.5$ μM [12]; $K_d = 17$ μM at 5 mM $MgCl_2$ [17]. Consistent with this was a K_L of 30 μM for MgADP. It is worth noting that, although MgATP has been implicated as the true substrate of the enzyme, a K_L of 15 μM for ATP itself (EDTA present) implies that the divalent cation-free nucleotide binds almost as strongly to the enzyme as MgATP does.

With both Ca^{2+} and MgATP present, k_{obs} was significantly higher. The effect of adding Ca^{2+} to

TABLE I

INACTIVATION RATE CONSTANTS (k_{obs}) AND PROTECTION CONSTANTS (K_L) FOR THE REACTION OF FITC WITH THE SR ATPase

Reactions were carried out as described in Materials and Methods at 100 μM FITC.

Additions	k_{obs} (min^{-1})	K_L (μM)
1 mM EGTA	0.49	
0.5 mM Ca	0.44	
1 mM Mg	1.1	
10 mM Mg	3.6	
1 mM Mg, 1 mM AMP	0.76	
1 mM Mg, 1 mM ADP	0.082	31
1 mM Mg, 1 mM ATP	0.014	5
1 mM EDTA, 1 mM ATP	0.015	15
1 mM Ca, 2 mM ATP	0.09	> 50
1 mM Mg, 1 mM Ado*PP*[NH]*P*	0.029	10
1 mM Mg, 0.1 mM Ado*PP*[NH]*P*, 1 mM Ca	0.020	0.7
1 mM Mg, 5 μM TNP-ATP	0.011	0.02
1 mM Mg, 5 μM TNP-ATP, 1 mM Ca	0.0065	0.012
5 mM Mg, 5 mM ITP	0.03	950
1 mM EDTA, 5 mM ITP	0.041	220
0.5 mM fluorescein	0.30	170
50 μM eosin	0.051	1.5
10 μM erythrosin	0.13	1.9

EMgATP which is reacting with FITC is shown in Fig. 4: the inactivation rate was increased almost 10-fold. Since it is the unliganded enzyme (E) which reacts with FITC, this result indicates that the fraction of E increased by initiation of turnover. We hypothesize that under these conditions the ATPase has a much lower affinity for the substrate compared to the enzyme with high-affinity calcium sites unoccupied.

For comparison we measured the effect of the nonhydrolyzable analog Ado*PP*[NH]*P*. The K_L value of 10 μM for EMgAdo*PP*[NH]*P* was similar to the ATP counterpart (Table I). In contrast to the effect of Ca^{2+} on EMgATP, however, binding of Ca^{2+} to EMgAdo*PP*[NH]*P* resulted in a more than 10-fold decrease in K_L (Table I). Similar results were obtained with the β,γ-methylene analog. The difference in the time-course of inactivation of EMgAdo*PP*[NH]*P* with and without calcium present is illustrated in Fig. 4. Since Ca^{2+} binding itself had no significant protective effect

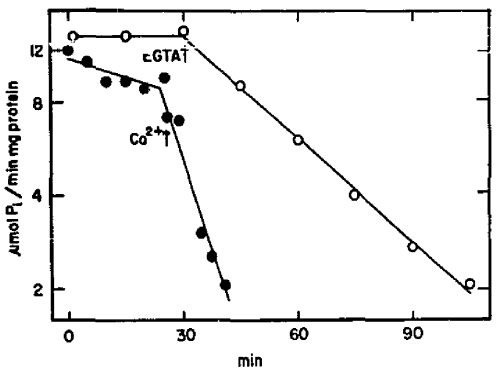


Fig. 4. Effect of nucleotides and calcium on the time-course of the loss of SR ATPase activity induced by FITC. In addition to 100 μM FITC, reaction mixtures contained: ●, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM ATP; at the time indicated by the arrow, 1 mM $CaCl_2$ was added; ○, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM ado*PP*[NH]*P*; at the time indicated by the arrow, 1 mM EGTA was added.

(Fig. 2), the results imply that calcium binding to the transport sites increases the affinity of the nucleotide-binding site. Most of the enhanced protective effect is manifested when the free Ca concentration is 2 μM, making it unlikely that formation and binding of Ca-Ado*PP*[NH]*P* is responsible.

The protective effect of TNP-ATP is quite strong, with k_{obs} being decreased about a 100-fold with as little as 5 μM TNP-ATP present. The K_L values with and without calcium present were about 0.02 and 0.01 μM, respectively (Table I); the smaller calcium effect on K_L than was observed for Ado*PP*[NH]*P* is perhaps a reflection of the overriding contribution of the TNP moiety to TNP nucleotide affinity [18]. The potent protective effect of TNP-ATP suggests it binds specifically with high affinity ($K_d < 0.1$ μM) to the active site, consistent with previously reported binding results [17,19,20].

In contrast to adenine nucleotides, ITP was a relatively ineffective protective agent (Table I). Curiously, ITP (EDTA present) was significantly more effective than MgITP ($K_L = 220$ and 950 μM, respectively). The low affinity of the ATPase for ITP is consistent with the much higher K_m for ITP compared to ATP [21].

Xanthene dyes

We found that the halogenated analogs of FITC, erythrosin isothiocyanate and eosin isothiocyanate, were also effective inactivators of the ATPase. For example, the time-course of ATPase activity loss under conditions where k_{obs} for FITC was 0.49 min^{-1} (no Mg^{2+}; Table I) yielded values for 5 μM erythrosin isothiocyanate and eosin isothiocyanate of 0.21 and 0.14 min^{-1}, respectively. We therefore evaluated the protective effect of the parent compounds towards FITC inactivation. Fluorescein exhibited a weak protective effect ($K_L = 170$ μM), while its tetraiodinated and tetrabrominated derivatives erythrosin and eosin were strongly protective ($K_L = 2$ μM). The former's effect is in agreement with our studies showing it is a competitive inhibitor of the ATPase ($K_i \approx 10$ μM; unpublished results), and with reports of its mimicry of adenosine moiety binding in other enzymes [20,21].

Xanthene dye-mediated photoinactivation

The affinity of the ATPase's nucleotide site for xanthenes is also demonstrated by photoinactivation experiments. As illustrated by Fig. 5, in the presence of 2.5 nmol of eosin or erythrosin per mg of SR protein, the ATPase activity is destroyed with a first-order time dependence. Consistent with its lower affinity for the enzyme, fluorescein was less effective. The rates of inactivation are attenuated in the presence of ATP (Fig. 5). Assuming that the protective effect is a competitive one and that the K_d values for eosin and erythrosin are 2 μM, Eqn. 2 allows the calculation of K_L values for ATP with respect to eosin and erythrosin of 4 and 6 μM, respectively. These values are in agreement with those listed in Table I.

Effect of P_i and V_i

Phosphorylation of the CaATPase by P_i results in a decreased rate of inactivation by FITC (Table II); the K_L calculated from this result is about 1 mM. A similar protective effect is conferred by V_i, a putative P_i analog. Consistent with its previously reported greater affinity for the enzyme [7,24], a much lower concentration of V_i is needed to confer significant protection (Table II); the value of K_L is calculated to be 1 μM.

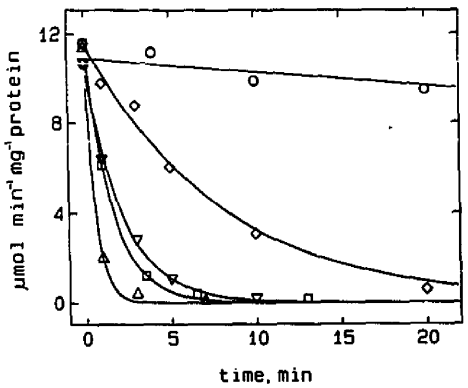


Fig. 5. Inactivation of SR APTase by eosin or erythrosin and protection by ATP. Irradiation was done for the designated times with an incandescent lamp (geometry fixed relative to the sample) at 25°C (pH 7), 1 mM EGTA, 2 mg/ml SR, 5 µM dye. The observed rate constants (in min^{-1}) and symbols are: fluorescein, ○; eosin (0.64), □; eosin + 1 mM MgATP (0.12), ◇; erythrosin (1.7), △; erythrosin + 1 mM MgATP (0.5), ▽.

When the above experiments are carried out with free calcium present, the protective effects of P_i and V_i are only partially overcome (Table II), suggesting that these ligands may still bind to the Ca complex of the enzyme, although not in the form of a covalent phosphorylated (vanadylated) adduct.

TABLE II

EFFECT OF P_i AND VANADATE (V_i) ON THE INACTIVATION RATE CONSTANT OF SR ATPase BY FITC

The reaction was carried out at an FITC concentration of 100 µM. Except as noted, conditions were as described in Materials and Methods. When present (+Ca), the total calcium concentration was 1.1 mM (80–100 µM free).

Ligand [a]	−Ca k_{obs} (min^{-1})	+Ca k_{obs} (min^{-1})
10 mM Mg [b]	2.0	1.8
16 mM Mg, 20 mM NaP_i [b,c]	0.067	0.44
1 mM Mg	1.2	1.3
1 mM Mg, 20 µM V_i	0.13	0.25

[a] Ligand concentrations are total concentrations.
[b] pH 6.75.
[c] Free concentrations are calculated to be Mg, 10 mM; P_i, 14 mM. Calculated from the constants compiled by Smith and Martell [44].

Discussion

The rapid and specific inactivation of the SR ATPase by FITC at first suggested that the enzyme possesses a hyperreactive lysyl side-chain. Among enzymes with a lysine of this type are oxaloacetate decarboxylase [25], aspartate transaminase [26] and glutamate dehydrogenase [27]. Specific labeling attributable to a hyperreactive functional group is best exemplified by the serine proteinases [28]. The SR ATPase as well appears to contain at least one such group, a carboxyl hyperreactive toward dicyclohexylcarbodiimide [11,29].

It is, however, the other common origin of selective inactivation, where the reagent has a high affinity for a site on the protein, which appears to account for our results using FITC. That this reagent acts as an affinity label of the catalytic site of the ATPase is suggested by (a) the saturation kinetic behavior with respect to the FITC concentration; the k_{max} values obtained are similar to the rate constant for the reaction of FITC with acetyllysine, suggesting that specific labeling resulted from the location of a normally reactive lysyl group near the site for which FITC has an affinity (dissociation constant K_F). Affinity labeling is also suggested by (b) the protective effect of ATP and other adenine nucleotides, which displayed protection constants (K_L, Table I) in the concentration range expected for interaction at the active site; (c) the protective effect of xanthene dyes; (d) incorporation of FITC to a level which is approximately stoichiometric. These results support the supposition in fluorescence energy transfer measurements that the FITC reaction site is the active site [16,30,31], there being no need to postulate a second (e.g., regulatory) site.

At least three other enzymes have been reported to bind the xanthene dye erythrosin (tetraiodofluorescein) at their adenine nucleotide site: creatine kinase [22] aspartate transcarbamylase [32] and lactate dehydrogenase [23]. The affinity labeling by FITC and protective effects of erythrosin and eosin (Table I) reported here suggest that the structure of at least a portion of the active site of the SR ATPase is similar to those of these enzymes. Our results using the isothiocyanates of erythrosin and eosin as affinity labels lend further

support to this suggestion, since they also specifically inactivate the ATPase. This enzyme may therefore tentatively be added to the list of proteins possessing a 'nucleotide fold' [33]. Comparison of the nucleotide domain structure of some kinases and dehydrogenases with assignments of secondary and tertiary structure in the putative nucleotide domain of the CaATPase supports this [5].

The small but significant accelerating effect of Mg^{2+} on ATPase reactivity toward FITC supports the existence of Mg^{2+} binding independent of the metal's putative role as part of the MgATP complex.

Although the ATPase activity of SR is destroyed by FITC labeling, the acetylphosphatase activity and P_i binding are retained [34]. It would appear that FITC occupies the adenosine-binding part of the site, and that the catalytic part remains intact and accessible to small substrates. The labeled lysyl residue most likely does not participate directly in catalysis, although it might play a role in ATP binding.

The fact that P_i and V_i protect the ATPase from FITC inactivation (Table II) yet can still bind to the modified enzyme [35,36] suggests that the lysine which is labeled is not involved in phosphorylation. These results also imply that P_i (or V_i) binding causes the lysine to move or in some other way become inaccessible to the reagent. Since the presence of calcium prevents phosphorylation by P_i [37,38], the maintenance of protection from FITC inactivation with calcium present implies that the cation does not prevent noncovalent complex formation ($E \cdot P_i$ or $E \cdot V_i$) and that this complex is also unreactive to FITC. Evidence for the existence of this type of ternary complex has also been obtained by measuring the calcium and vanadate concentration dependence of inhibition of ATPase activity [39].

The results presented here indicate that occupancy of the active site prevents reaction with FITC, so that only the unliganded enzyme binds and reacts with the reagent. Accordingly, the increase in FITC reactivity upon enabling turnover by adding calcium to $E \cdot MgATP$ (Fig. 4) indicates that the fraction of ATPase with an unoccupied catalytic site has increased. This implies that the site's dissociation constant for substrate (K_s) has increased, going from about 5 μM to > 50 μM. The latter value is consistent with the often-reported K_m in the 5–10 μM range and the expectation that phosphorylation and subsequent reactions should result in $K_m < K_s$. These results therefore suggest that during turnover the kinetically significant pathway involves an enzyme form which binds ATP relatively weakly.

It is puzzling that adding calcium to the Ado*PP*[NH]*P* complex of the ATPase has the opposite effect, i.e., it decreases FITC reactivity which reflects an increase in nucleotide affinity. This may be because in this case Ado*PP*[NH]*P* does not mimic ATP very well. A more interesting possibility is that Ado*PP*[NH]*P* binds to a more thermodynamically stable (high nucleotide affinity) enzyme form which is bypassed under turnover conditions. The results reported here may be added to growing evidence for the existence of multiple enzyme forms linked by numerous conformation changes (Ref. 40, and references therein).

Comparison of the present results with those reported earlier using pyridoxal phosphate [41] as an apparent affinity label of the ATPase reveals some interesting differences. In contrast to the FITC reaction, EP formation protected against inactivation by PLP better than did formation of the MgATP complex. We have subsequently found for the PLP reaction that hydrolytic activity toward acetyl phosphate and NPP are lost to about the same extent as is ATPase activity (Murphy, A.J., unpublished results). Compared to the FITC-labeled lysine residue, the PLP-labeled one would thus appear to be more closely associated with the catalytic part of the active site. They also differ in their location in the polypeptide chain; the FITC labels the smaller primary tryptic fragment [8,15], while the PLP label, like the active-site aspartyl residue which is phosphorylated [42], is located in the larger one [41]. The ATP active site might thereby be constituted from portions of different domains represented by the primary tryptic fragments.

The specific labeling of two different lysine residues with pyridoxal phosphate and FITC and of an arginine residue with butanedione [43] suggests there are at least three cationic side-chains with important roles in the function of the SR ATPase.

Acknowledgements

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