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ATPase and phosphatase activities are differentially inhibited by photo-oxidation of the sarcoplasmic reticulum Ca^{2+}-ATPase

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Abstract

We have already described that photo-oxidation of the sarcoplasmic reticulum Ca^{2+}-ATPase with the halogenated dye erythrosin B produces inhibition of the ATPase activity (J.A. Mignaco et al., Biochemistry 35 (1996) 3886–3891). We now show that the Ca^{2+}-dependent and Ca^{2+}-independent *p*-nitrophenylphosphatase activities are also inhibited by this treatment. Modification of rapidly (< 10 min) oxidized residue(s) is responsible for the major loss of ATPase activity, whereas photo-inhibition of the phosphatase activities occurs more slowly ($t_{1/2}$ 20–30 min). Here we have focused on photo-inhibition of the Ca^{2+}-independent *p*NPPase activity, and the counteracting effects of ATP and FITC. Following photo-oxidation, the Ca^{2+}-independent *p*NPPase activity decreases monotonically. ATP partially protects against the inactivation of the *p*NPPase, whereas labeling the enzyme with FITC does not. However, the protective effect of ATP is completely abolished by the attached FITC. These data are interpreted in terms of two different sites that are susceptible to photo-oxidation and are involved in different events related to substrate hydrolysis. © 1997 Elsevier Science B.V.

Keywords: Nucleotide site; ATPase, Ca^{2+}-; Phosphatase activity; Erythrosin; FITC

1. Introduction

The sarcoplasmic reticulum Ca^{2+}-ATPase has been extensively characterized kinetically [1–3], but the underlying structural correlates of catalysis and its coupling to Ca^{2+} transport are relatively unknown. Despite elucidation of its primary structure [4,5], a detailed three-dimensional image of the enzyme is still lacking. Since a direct X-ray imaging study of the binding of substrate to this enzyme cannot be predicted for the near future [6], many researchers have used genetic engineering and/or modification with chemical reagents in attempts to pinpoint

Abbreviations: SR, sarcoplasmic reticulum; FITC, fluorescein isothiocyanate; EGTA, ethylene glycol bis(β-amino-ethyl ether) *N,N,N′,N′*-tetraacetic acid; MES, (3-[*N*-morpholino]ethane-sulfonic acid); Tris, Tris(hydroxymethyl)aminomethane; *p*NPP, *p*-nitrophenylphosphate

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residues that are important for the kinetics of the enzyme. Of particular importance was the identification of Asp-351 as the residue that is phosphorylated during the hydrolytic cycle of the enzyme [4], and which serves also to identify a region that is part of the catalytic site. Covalent modification with group-selective chemical reagents has led to the detection of reactive lysyl [7–11], cysteinyl [12,13], histidyl [14,15], threonyl [16], and carboxylic [17] residues, which upon labeling affect substrate binding or hydrolysis and/or Ca^{2+} transport. Mapping of the Ca^{2+}-binding site and of additional residues involved in catalysis has been developed with the use of point mutations [18–22]. Much work has been done with compounds designed to specifically probe the ATP binding site [8,11,16,23]. We have been studying the involvement of different amino-acids in the structure and the catalytic function of this site using the photo-sensitizing xanthene dye erythrosin B. This compound binds non-covalently to the nucleotide binding sites of various enzymes [24–32], and it has the advantage that it can modify neighboring amino-acids with relatively high specificity, via active-center-generated singlet oxygen [33,34]. Therefore, erythrosin B is a substrate analog that can be used to modify the ATP-binding site of an enzyme, yet without leaving a bulky group anchored within this site.

In a previous study [35] we showed that photo-oxidation by singlet oxygen species generated in the catalytic site decreased the rate of several partial reactions of the catalytic cycle of the Ca^{2+}-ATPase. Here we used the pseudosubstrate *p*-nitrophenylphosphate (*p*NPP) to further characterize the effects of photo-oxidation towards the enzyme hydrolytic capacity. *p*NPP hydrolysis has many steps in common with the mechanism for ATP hydrolysis [36,37]. Similarly to other pseudosubstrates, *p*NPP presents only one measurable K_M for hydrolysis in the millimolar range [36,38]. This compound can be hydrolyzed in a Ca^{2+}-dependent activity coupled to Ca^{2+} transport involving E_1–E_2 transitions, and in a Ca^{2+}-independent pathway attributed to the E_2 form of the enzyme [39,40].

In this study we show that at least two residues of the SR Ca^{2+}-ATPase are susceptible to photo-oxidation with erythrosin B, and that their modification differentially affects the hydrolysis of the substrates ATP and *p*-nitrophenylphosphate.

2. Materials and methods

2.1. Enzyme preparation

Sarcoplasmic reticulum vesicles (SRV) were prepared from rabbit hind leg skeletal muscle according to Eletr and Inesi [41]. SR Ca^{2+}-ATPase was purified using Method 2 described by Meissner et al. [42] and stored in liquid nitrogen. The specific activity of the purified preparations was typically between 4 to 6 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$ under standard ATPase reaction conditions. The protein concentration was determined following the method of Lowry et al. [43], using bovine serum albumin as standard.

2.2. pNPP hydrolysis

p-Nitrophenylphosphatase (*p*NPPase) activity was measured at 37°C in media containing 20 mM Tris-HCl (pH 7.4), 0.5 mM EGTA or 0.05 mM $CaCl_2$, 120 mM KCl, 10 mM $MgCl_2$, 10–20 μg/ml Ca^{2+}-ATPase, 3 mM *p*NPP, and the different dye concentrations specified for each experiment, in a final volume of 0.5 ml. After 20 to 40 min, the reactions were quenched with 0.1 ml of 0.3 N NaOH, followed by addition of 0.4 ml deionized water. The *p*-nitrophenol produced was estimated by measuring the absorbance at 425 nm, assuming an extinction coefficient of 1.1×10^4 M^{-1} cm^{-1}. Note that in the protection experiments and others described throughout the text, ATP and erythrosin were not washed from the enzyme prior to the addition of *p*NPP to the assay mixtures. This resulted in a moderate inhibition of the phosphatase activity, even in the control without erythrosin, probably as a result of competition between ATP and *p*NPP for binding to the enzyme.

2.3. Labeling with FITC

The Ca^{2+}-ATPase was labeled with FITC by a modification of previously described procedures [44]. The enzyme was labeled in the dark at room temperature, in a medium containing 20 mM Tris-HCl (pH 8.5), 80 mM KCl, 0.05 mM $CaCl_2$, 5 mM $MgCl_2$, 0.01 mM FITC and 1.0 mg protein/ml. After 20 min, labeling was stopped by neutralizing the medium with addition of an equal volume of Tris-HCl 50 mM (pH 7.0). This mixture was further diluted to a pro-

tein concentration of 20 μg/ml for assays of *p*NPPase activity. Ca^{2+}-dependent ATPase activity was always reduced to less than 5% of the control value, while phosphatase activity was unaffected by the labeling.

2.4. Photo-oxidation of the Ca^{2+}-ATPase

The enzyme was photo-oxidized as described elsewhere [35]. Briefly, unless otherwise specified, ATPase (20 μg/ml) was incubated for 20 min at 25°C under roomlight in a medium containing 20 mM Tris-HCl (pH 7.4), 120 mM KCl, 10 mM $MgCl_2$, different concentrations of erythrosin and either 0.5 mM EGTA or 0.5 mM EGTA + 0.55 mM $CaCl_2$.

2.5. Reagents

p-nitrophenylphosphate (dicyclohexylammonium salt), ATP (disodium salt), Tris, MES, EGTA, and 5′-fluorescein isothiocyanate (FITC) were from Sigma; erythrosin B was from Riedel-de-Häen. All other reagents used were of analytical grade.

3. Results

We have previously described that photo-oxidation of the SR Ca^{2+}-ATPase with erythrosin results in inhibition of the Ca^{2+}-activated ATP hydrolysis [35]. Fig. 1 shows that the *p*NPPase activity of the SR Ca^{2+}-ATPase is also inhibited by photo-oxidation. However, a marked difference is noted for the inhibition of *p*NPPase activity when compared with inhibition of the ATPase activity: a much longer period of photo-oxidation is needed in order to inhibit *p*NPP hydrolysis (20–30 min vs. ≅ 10 min for the Ca^{2+}-ATPase activity — Fig. 1A). In these experiments, photo-oxidation was performed in an EGTA-containing medium. As a control, Fig. 1A shows that preincubation with EGTA for as long as 40 min does not inhibit either the *p*NPPase or the Ca^{2+}-ATPase activity.

The *p*NPPase activity of the SR Ca^{2+}-ATPase is expressed in two different ways: a Ca^{2+}-dependent activity that is coupled to Ca^{2+} uptake [37–39], and a Ca^{2+}-independent, futile hydrolysis, attributed solely to the E_2 form of the enzyme [39,40]. Both activities are inhibited by photo-oxidation with erythrosin (Fig. 1B). The effect of photo-oxidation with erythrosin on the Ca^{2+}-dependent phosphatase is, however, biphasic: low concentrations of erythrosin enhance this activity, while higher concentrations of the dye result in inhibition. The reason for this enhancement of the Ca^{2+}-phosphatase activity is not so far understood, but is most probably due to a stimulatory effect on the turnover rate of the enzyme that low concentra-

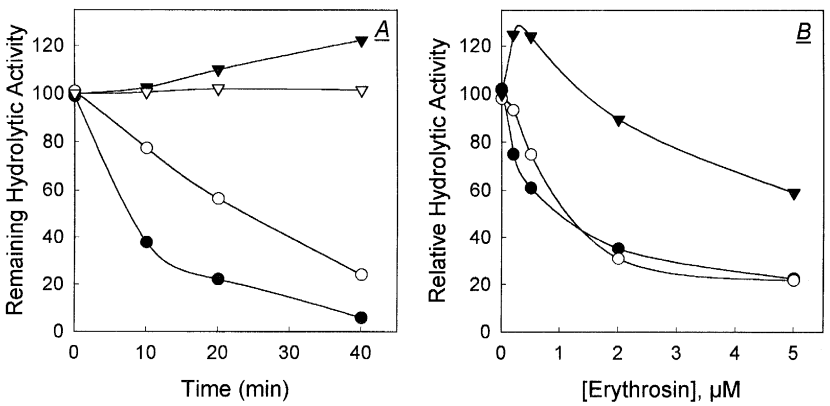

Fig. 1. Photo-inhibition of the *p*NPPase and ATPase activities with erythrosin. (A) Ca^{2+}-ATPase was photo-oxidized with 1 μM erythrosin in a medium containing 120 mM KCl, 20 mM Tris-Cl (pH 7.4), 10 mM $MgCl_2$, 0.5 mM EGTA, and 20 μg/ml protein. At the indicated times, aliquots were withdrawn and either 2 mM ATP + 0.55 mM $CaCl_2$ (●) or 3 mM *p*NPP (○) were added. The activities were assayed as indicated in Section 2. The Ca^{2+}-ATPase (▼) and Ca^{2+}-independent *p*NPPase (▽) activities of samples treated similarly but in absence of erythrosin are also shown. (B) After photo-oxidation of the Ca^{2+}-ATPase for 20 min with different concentrations of erythrosin in the medium described in (A) but with either 0.5 mM EGTA (○) or 0.5 mM EGTA + 0.55 mM $CaCl_2$ (▼), 3 mM *p*NPP was added and the *p*NPPase activity measured. The Ca^{2+}-ATPase activity measured with 2 mM ATP after a 10 min photo-oxidation period (●) is shown for comparison.

Table 1
Photo-inhibition of the *p*NPPase activity and protection by ATP. The enzyme (40 μg/ml) was exposed to light for 30 min in a medium containing 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM EGTA, and 20 mM Tris-Cl (pH 7.4), in the presence or absence of 5 mM ATP and with or without (control) 2 μM erythrosin. Hydrolysis was initiated by addition of 3 mM *p*NPP. Values are mean ± S.D. of three experiments

Pre-incubation		Activity (nmol *p*NP/mg/min)	Relative activity (%)
Without ATP	Control	12.1 ± 0.4	100
	Photo-oxidized	2.2 ± 0.3	18.2 ± 2.5
With 5 mM ATP	Control	7.1 ± 0.2	100
	Photo-oxidized	3.1 ± 0.1	43.7 ± 1.4

tions of erythrosin and eosin have on the phosphatase activity under non-photo-oxidizing conditions [32]. Further experiments were focused on the inhibition of the Ca^{2+}-independent phosphatase activity, which occurs progressively and monotonically with increasing incubation times (Fig. 1A) and increasing concentrations of erythrosin (Fig. 1B). For comparison, we show the inhibition of the Ca^{2+}-ATPase activity after a period of 10 min of photo-oxidation with the same dye concentrations.

It should be noted that in Fig. 1B the enzyme was photo-oxidized either in the absence or in the presence of Ca^{2+}. The same results were obtained regardless of whether photo-oxidation was performed in the presence of Ca^{2+} and the reactions started by addition of substrate, or whether photo-oxidation was performed in the absence of Ca^{2+} and the reactions initiated by addition of substrate and $CaCl_2$ (data not shown), indicating that the concentration of Ca^{2+} in the photo-oxidation media did not modify the pattern of photo-inhibition.

The addition of ATP to the medium prior to photo-oxidation had a limited, yet reproducible, protective action against the loss of the Ca^{2+}-independent phosphatase activity (Table 1), as observed previously for the ATPase activity [35]. Erythrosin is considered to be a nucleotide analog due to its morphological similarity to AMP and its affinity for proteins with ATP-sites [45]. Protection by ATP suggests that steric hindrance of the erythrosin photo-oxidatable residues would be induced by the binding of ATP to the enzyme. In order to rule out a possible non-specific photo-oxidation of the enzyme, we repeated the ATP-protection experiments in the presence of 1–10 mM histidine, which is known to quench singlet oxygen species in aqueous solutions [34]. The presence of free histidine did not affect either the extent or the time-course of photo-inhibition, and did not alter the protection by ATP (data not shown). Therefore, it is apparent that photo-oxidation of the Ca^{2+}-ATPase with erythrosin is a selective and site-specific phenomenon.

In order to localize the residue responsible for photo-inhibition of the *p*NPPase, we studied the effects of photo-oxidation on the FITC-labeled Ca^{2+}-ATPase. Pick [46,47] reported that the SR Ca^{2+}-ATPase labeled with FITC is unable to hydrolyze ATP, although it can hydrolyze *p*NPP and other small pseudo-substrates at rates similar to the control. The FITC-labeled enzyme is also normally phospho-

Table 2
Photo-inhibition of the *p*NPPase activity after labeling with FITC. After labeling with FITC (see Section 2), the Ca^{2+}-ATPase was incubated under light as in Table 1, in the absence (control) or presence of 2 μM erythrosin. *p*NPPase activity was measured as in Table 1. Similar results were obtained with two different preparations

		Activity (nmol *p*NP/mg/min)	Relative activity (%)
− FITC	Control	18.4	100.0
	10 min photo-oxidation	11.0	59.8
	20 min photo-oxidation	6.7	36.4
+ FITC	Control	18.4	100.0
	10 min photo-oxidation	11.0	59.8
	20 min photo-oxidation	7.4	40.2

rylated by [^{32}P]P_i. These observations mean that the binding of FITC, and the binding of a pseudo-substrate or P_i are not mutually exclusive. There appears to be a consensus that FITC, which covalently binds at Lys-515 [4,5], intrudes into the nucleotide binding domain of the catalytic site, yet leaving a portion or domain with catalytic activity mostly unaffected.

Remarkably, prior labeling of the Ca^{2+}-ATPase with FITC did not protect the enzyme against photo-oxidation with erythrosin and the resulting inhibition of the phosphatase activity (Table 2). This is an indication that the FITC-labeled ATPase can bind erythrosin with similar affinity and at the same site as the control. The data are thus indicative that FITC and erythrosin can bind to the enzyme concurrently.

A dramatic effect of FITC was observed, however, on the protection by ATP against photo-oxidation with erythrosin: protection was completely abolished in the FITC-labeled enzyme (Table 3). It is noteworthy that labeling with FITC also abolished the inhibition of the phosphatase activity by ATP, which was consistently observed whenever ATP was present (compare the initial activities in Table 3). Evidence has been presented to show that ATP and FITC bind at the same enzymatic site of the sarcoplasmic reticulum Ca^{2+}-ATPase [7,46,47], and of some other P-type ATPases [48–53]. As shown, the enzyme can accommodate both FITC and erythrosin at the same time, but the binding of either one of these molecules blocks the entry of ATP. Distinctly from the labeling with FITC, however, photo-oxidation with erythrosin blocks the hydrolysis of both ATP and *p*NPP with different inactivation rates for each hydrolytic activity.

4. Discussion

Our data show that at least two residues of the SR Ca^{2+}-ATPase can be oxidized by singlet oxygen species generated by illumination in the presence of erythrosin, and we present evidence for the involvement of these residues in the hydrolysis of different substrates. One type of these residues, which is oxidized rapidly, is responsible for the initial rapid loss of the ATPase activity, and does not seem to be stringently required for the phosphatase activity, since *p*NPP hydrolysis is inhibited much more slowly. Further photo-oxidation of the enzyme also produces the inhibition of the phosphatase activity of the enzyme, in a reaction that is attenuated by ATP. Miara et al. [54] reported that the Na^+,K^+-ATPase isolated from eel electroplax also possesses at least two different residues which can be modified by photo-oxidation with the analog reagent rose Bengal or by ethoxyformylation with diethylpyrocarbonate (DEPC). In their experiments, as in ours, a rapidly photo-oxidized residue was responsible for the loss of the ATPase activity, while a second residue was oxidized more slowly and induced the loss of the phosphatase activity. These observations emphasize the similarity of the ATP sites in these two transporting enzymes. In the case of Na^+,K^+-ATPase, the two modified amino-acids were identified as histidyl residues.

In a previous paper [35], we characterized the kinetic effects of the photo-oxidation of the SR Ca^{2+}-ATPase with erythrosin within the first 10 min, which is responsible for the inhibition of ATP hydrolysis. In that study, photo-oxidation was demon-

Table 3
Effects of labeling with FITC on the protection by ATP against photo-oxidation. Ca^{2+}-ATPase was labeled with FITC as in Table 2 and later illuminated for 30 min in the presence or absence of 2 μM erythrosin, with or without 5 mM ATP. *p*NPPase assay conditions are as in Table 1. Results shown are means ± S.D. from three different preparations

		Activity (nmol pNP/mg/min)		Relative activity (%)	
		− ATP	+ ATP	− ATP	+ ATP
− FITC	Control	14.4 ± 1.1	10.4 ± 1.8	100	100
	Photo-oxidized	1.8 ± 0.2	4.2 ± 1.2	12.5 ± 1.4	40.4 ± 11.5
+ FITC	Control	12.8 ± 1.7	12.6 ± 1.8	100	100
	Photo-oxidized	2.6 ± 0.8	3.0 ± 1.0	20.3 ± 6.3	23.8 ± 7.9

strated to drastically decrease the rate of all the phosphoryl transfer reactions to and from the enzyme. We now show that the *p*NPPase activity is scarcely affected by short periods of photo-oxidation when low concentrations of erythrosin are used. Thus, a rapidly-oxidized residue seems not to be essential or rate-determining for hydrolysis of *p*NPP (even though this residue determines the rate of decay of the phosphoenzyme formed with ATP, see [35]), and the oxidation of a second target is needed in order to impair the hydrolysis of *p*NPP. These data strongly suggest that the rate-limiting step of *p*NPP hydrolysis in the absence of Ca^{2+} is not dephosphorylation of a putative phosphoenzyme. An alternative is that the substrate binding step would be impaired. *p*NPP binding was also suggested by Nakamura and Tonomura [36] to be the rate-limiting step for hydrolysis in the presence of Ca^{2+}. Therefore, it is reasonable to propose that the slowly-reacting residue would be directly involved in the binding of the substrate *p*NPP and that its modification (oxidation) would affect the overall rate of pseudo-substrate hydrolysis.

The binding of FITC does not modify photo-oxidation of the slowly-reacting amino-acid. Since the singlet-oxygen generated by erythrosin is short lived in aqueous solution [34], the slowly reacting residue should necessarily be located in a site directly involved in substrate catalysis which is not blocked by FITC. This is a clear evidence that both fluorescein derived molecules can bind simultaneously to the Ca^{2+}-ATPase. Furthermore, we have also measured (not shown) that the FITC labeled Ca^{2+}-ATPase hydrolyzes 3-*O*-methylfluoresceinphosphate, indicating that other substrate analogs, besides erythrosin, can also bind simultaneously to the enzyme molecule.

Our kinetic measurements do not show whether the oxidation of the rapidly-reacting residue that affects ATP hydrolysis is protected or not by prior labeling of the ATPase with FITC. The difference in rate of oxidation of the two amino-acids could be due to different micro-environments surrounding similar residues, or to structural differences in the residues themselves. One possibility is that both targets for photo-oxidation are histidyl residues, which are broadly described as the main target of oxidation by halogenated xanthene dyes [33,34]. In fact, when we measured the differential absorbance spectrum of the SR Ca^{2+}-ATPase after photo-oxidation a single peak at 248 nm was obtained, which is typical for the oxidation of histidyl residues in proteins [35]. We consider that the photo-oxidation reactions and the results described here will be useful for identifying different residues of the Ca^{2+}-ATPase that are directly involved on the catalytic mechanism of the enzyme.

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