Effects of Photo-oxidizing Analogs of Fluorescein on the Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase

FUNCTIONAL CONSEQUENCES FOR SUBSTRATE HYDROLYSIS AND EFFECTS ON THE PARTIAL REACTIONS OF THE HYDROLYTIC CYCLE\textsuperscript{+}

(Received for publication, May 22, 1995, and in revised form, March 25, 1996)

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Erythrosin B was used to photo-oxidize the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase. The ATPase activity is rapidly and irreversibly inhibited by photo-oxidation with erythrosin. This inhibition is protected by the presence of ATP during the photo-oxidation period. After photo-oxidation, the steady-state phosphorylation by ATP remains almost unchanged, whereas phosphorylation by inorganic phosphate is impaired. The pseudo-first order rate constants for phosphorylation by 15 \textmu M ATP at 25 °C are strongly inhibited when starting from either a Ca\textsuperscript{2+}-bound or a Ca\textsuperscript{2+}-free enzyme form, decreasing from 145 to 23 s\textsuperscript{-1} for the Ca\textsuperscript{2+}-bound form and from 50 to 18 s\textsuperscript{-1} for the Ca\textsuperscript{2+}-free form. Concurrently, the rate constants for dephosphorylation are also severely inhibited, changing from a fast double exponential to a very slow single exponential decay in the reverse direction and from a moderately slow single to a very slow single exponential decay in the forward direction. Ca\textsuperscript{2+}-binding data show that the phosphorylated intermediate formed by the photo-oxidized enzyme contains two occluded Ca\textsuperscript{2+}, and TNP-ATP fluorescence measurements indicate that it accumulates in a E\textsubscript{2}P- Ca\textsuperscript{2+}-like conformation. Protection by ADP against glutaraldehyde-induced cross-linking indicates that ADP binding to Ca\textsuperscript{2+}-ATPase is not impaired by photo-oxidation nor by free erythrosin. These data support the view that an ADP-insensitive, Ca\textsuperscript{2+}-bound, slowly interconverting phosphoenzyme is formed. Thus, photo-oxidation with erythrosin B leads to impairment of phosphoryl transfer reactions and related conformational changes.

The sarcoplasmic reticulum (SR) \textsuperscript{1}Ca\textsuperscript{2+}-ATPase is a 115-kDa transmembrane protein that couples ATP hydrolysis to removal of Ca\textsuperscript{2+} from the cytosol in skeletal muscle fibers, triggering the end of muscular contraction (Hassellbach and Makino, 1961; Ebashi and Lipmann, 1962). The primary structure of this enzyme has been determined and has led to predictions of secondary structure and enzyme topology (Brandl et al., 1986; Clarke et al., 1989). Molecular imaging of ATPase crystals seems to agree with general structural predictions (Stokes et al., 1994), but questions concerning the role of specific amino acids in binding and transporting Ca\textsuperscript{2+} as well as the structure of the nucleotide binding site(s) remain unanswered. Efforts to map the enzyme functionally have used chemical modification by specific reagents and site-specific mutagenesis. This latter technique has been used recently to identify amino acids that affect kinetic parameters related to the catalytic site and to the Ca\textsuperscript{2+} binding sites (Maruyama and Maclennan, 1988; Maruyama et al., 1989; Clarke et al., 1990; Vilisen et al., 1991).

In addition, many amino acids have been labeled, identified, and located within the primary structure using a variety of probes for the nucleotide binding site. These experiments have almost always involved probes that bind covalently to the enzyme, such as FITC (Mitchinson et al., 1982), 8-azido-ATP (Lacapère et al., 1993), pyridoxal 5\textsuperscript{-}-phosphate (Yamagata et al., 1993), oxidized ATP (Mignaco et al., 1990), or chemical reagents that cause radical structural changes, such as glutaraldehyde (Mntosh, 1992) or dithiodreitol (Dahm and Kanazawa, 1994). Thus, kinetic parameters such as those involved in enzyme phosphorylation and dephosphorylation could not always be assessed with the labeled enzyme, and the precise functional role of the labeled amino acids remains uncertain.

It has been reported that Ca\textsuperscript{2+}-ATPase modified with FITC loses all the activities related to ATP binding (Pick, 1981; Pick and Bassilian, 1981). Because Lys-515, which is the point of attachment of FITC to the enzyme, is neither unusually reactive toward FITC (Murphy, 1988) nor essential for ATP hydrolysis (Maruyama et al., 1989), it has been suggested that it is the fluorescein moiety of FITC that directs the reaction toward the ATP site. Thus, affinity binding of fluorescein would bring the isothiocyanate group close to Lys-515, promoting a specific attachment, and fluorescein would then remain anchored to the putative ATP binding site, thus leading to inhibition of the Ca\textsuperscript{2+}-ATPase activity.

In this study we use erythrosin B, a halogenated derivative of fluorescein that is considered to bind to enzyme nucleotide binding sites with high affinity and specificity (Lundblad and Noyes, 1984; Neslund et al., 1984), to photo-oxidize residues on SR Ca\textsuperscript{2+}-ATPase, and we examine its influence on the partial reactions of the hydrolytic cycle. Erythrosin is capable of promoting selective photo-oxidation of amino acids, which can be useful in the identification of groups involved in catalysis.

\textsuperscript{+}This work was supported by grants from Financiadora de Estudos e Projetos, Conselho Nacional de Desenvolvimento Científico e Tecnológico, and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\textsuperscript{1}The abbreviations used are: SR, sarcoplasmic reticulum; FITC, fluorescein isothiocyanate; MES, 3-(N-morpholino)propane-sulfonic acid; MOPS, 3-(N-morpholino)propane-sulfonic acid; TNP-ATP, 2',3'-O-(2,4,6-trinitrocyclohexadienyldiene)adenosine 5'-triphosphate.
Photo-oxidation of Ca\(^{2+}\)-ATPase by Fluorescein Analogs

**Lundblad and Noyes, 1984; Neslund et al., 1984; Halliwell and Gutteridge, 1989,** and has two advantages: even after photo-oxidation the sites would still be able to bind ATP, and no bulky group would remain covalently bound to the enzyme.

**MATERIALS AND METHODS**

Enzyme Preparations—Sarcoplasmic reticulum vesicles were obtained from rabbit hind leg skeletal muscle as described by Eleter and Inesi (1972). Ca\(^{2+}\)-ATPase was purified by method number 2 of Messner et al. (1973) and stored in liquid nitrogen. Protein determinations were performed according to Lowry et al. (1951), using bovine serum albumin as standard.

Photo-oxidation of Ca\(^{2+}\)-ATPase—Unless otherwise stated, Ca\(^{2+}\)-ATPase was incubated with the dyes for 10 min at 25 \(^{\circ}\)C under room light in medium containing 20 mM Tris-HCl (pH 7.4), 0.05 mM CaCl\(_2\), 5 mM MgCl\(_2\), and the dye concentrations specified in the legends before starting the reactions. The protein concentration on the assays could vary from 10 to 50 \(\mu\)g/mL. In this range there was no significant variation in the sensitivity of the ATPase toward photo-inhibition by erythrosin. However, when the protein concentration was raised to 0.2 mg/mL for the experiments of phosphorylation by Pi, approximately three times as much erythrosin was needed to reach the same extent of inhibition.

Enzyme Hydrolytic Activities—ATPase activities were assayed at 37 \(^{\circ}\)C in reaction mixture containing 20 mM Tris-HCl (pH 7.4), 80 mM KCl, 5 mM MgCl\(_2\), and 0.05 mM [\(^{32}\)P]ATP, and the reaction was quenched in 25% perchloric acid and 5 mM Pi. Samples were then filtered through Millipore filtration, and the retained radioactivity was counted. Blanks obtained either by processing the reaction at pH 5.0 or by omission of the enzyme were discounted to correct for nonspecific binding of \([\text{Ca}\text{]}^{2+}\) to the enzyme and for the dead volume of the filters, respectively. Both blanks were identical, and the radioactivity corresponding to specific binding was always equivalent to approximately twice the radioactivity of the blanks.

Cross-linking with Glutaraldehyde—After photo-oxidation with 1 \(\mu\)M erythrosin for 60 min, the Ca\(^{2+}\)-ATPase was further incubated with glutaraldehyde for 90 min in the presence of 0–100 \(\mu\)M ADP as described by McIntosh (1992). The medium contained 0.2 mg/mL Ca\(^{2+}\)-ATPase, 20 mM MOPS-Tris (pH 7.0), 80 mM KCl, 5 mM MgCl\(_2\), 0.05 mM CaCl\(_2\), and 0.142 mM glutaraldehyde. The cross-linking reaction was quenched with one volume of a denaturing buffer containing 2% SDS, 5 mM urea, 200 mM Tris-HCl (pH 6.8), and 500 mM 2-mercaptoethanol, and submitted to SDS polyacrylamide gel electrophoresis (Laemmli, 1970). In another set of experiments, photo-oxidation was omitted, and ADP was replaced by equivalent concentrations of erythrosin.

Fluorescence of TNP-ATP—Fluorescence was measured at room temperature in a continuously stirred 1-cm light path quartz cuvette using a Hitachi F-3010 spectrofluorimeter. Excitation was set at 400 nm, and emission was set at 540 nm. The emission values were corrected for erythrosin fluorescence and filter effects.

**RESULTS**

Photo-inhibition of Ca\(^{2+}\)-ATPase with Erythrosin—After a 10 min incubation with the enzyme, erythrosin B proved to be the best photo-inhibitor when compared with fluorescein and eosin Y (Fig. 1A). Erythrosin B showed the lowest K\(_{0.5}\) (0.5–1.0 \(\mu\)M), followed by eosin (K\(_{0.5}\) of 4–5 \(\mu\)M) and fluorescein (K\(_{0.5}\) > 100 \(\mu\)M). The extent of inhibition by erythrosin increased with the time of exposure to light and also increased with dye concentration. The addition of ATP (Fig. 1B) or ADP (not shown) during exposure to light partially protected the enzyme against photo-oxidation with erythrosin. The photo-inhibition elicited by erythrosin was irreversible, because several centrifugation-washing cycles or dilution of the phospholipids with large excess of detergents such as polyoxyethylene 10-cmethylbenzene did not restore the ATPase activity (not shown). Erythrosin and eosin (not shown) also induced the inhibition of ATP hydrolysis in the absence of light. The K\(_{0.5}\) for this effect was 5–10-fold higher than in the light (Fig. 1C). Unlike the photo-inhibition, the inhibition in the dark was not time-dependent and was almost fully reversible after the washing or detergent treatments indicated above (not shown). To determine whether the protein had indeed been covalently modified by photo-oxidation with erythrosin, we measured the differential absorbance of the protein samples prior to and after reaction in the light. The differential absorbance spectrum resulting from the photo-oxi-
Fig. 1. Concentration dependence of photo-inhibition with erythrosin, eosin, or fluorescein. A, Ca\(^{2+}\)-ATPase was photo-oxidized for 10 min under fluorescent room light in presence of either fluorescein (●), eosin (○), or erythrosin (●). In this and the following experiments, activity was measured in medium containing 20 mM Tris-HCl (pH 7.4), 80 mM KCl, 5 mM MgCl\(_2\), 0.05 mM total or free CaCl\(_2\), 10 μM Ca\(^{2+}\)-ATPase, 2 mM ATP, and the indicated concentration of dye at 37°C. B, erythrosin was fixed at 1 μM, and oxidation was done for the designated times in presence of 0.5 mM EGTA (●) or 0.5 mM EGTA + 2 mM ATP (○). Hydrolysis was started by the addition of either 0.55 mM Ca\(^{2+}\) + 2 mM ATP (●) or 0.55 mM Ca\(^{2+}\) (○). C, inhibition of ATPase activity induced by erythrosin with (●) or without (○) photo-oxidation.

![Graph](image)

Table I

| Dye, μM | Time (min) | Erythrosin, μM |
|---------|------------|---------------|
| 0.01    | 0          | 0             |
| 0.1     | 0          | 0.05          |
| 0.2     | 0          | 0.1           |
| 0.5     | 0          | 1             |

The values are calculated from the best fit to the experimental points, considering a biphasic response to the ATP concentration using the nonlinear regression program Enzfitter. The curve was generated assuming a model of two interconverting ATP binding sites, one with high affinity that behaves as the catalytic site and one with low affinity that acts as the regulatory site. The rates and affinities for ATP hydrolysis are calculated according to the equation \( v = \frac{V_{m1}}{K_{m1} + S} \times \frac{S}{K_{m2}} + \frac{V_{m2}}{K_{m2}} \times \frac{1}{1 + \frac{S}{K_{m2}}}, \) where \( V_{m1} \) is the maximal velocity at the catalytic site and \( V_{m2} \) is the velocity calculated for the sum of the effects of both sites.

The affinity of the enzyme for Ca\(^{2+}\) was not modified by photo-inhibition, considering both the high and low affinity Ca\(^{2+}\) binding sites, although the apparent cooperativity may have been slightly decreased (Fig. 3). Equilibrium binding of Ca\(^{2+}\) to the photo-oxidized enzyme reached the same values as in the control, attaining approximately 8 nmol of \([\text{CaCl}_{2}]\text{Ca}^{2+}\) bound per milligram of Ca\(^{2+}\)-ATPase with 10 μM Ca\(^{2+}\) in the medium. This result rules out the possibility that the observed decrease in ATPase activity could be due to destruction of the Ca\(^{2+}\) binding sites transforming the inhibited enzyme into an incompetent silent form. It also establishes that inhibition is not a result of nonsaturation of these sites due to a decrease in affinity.

![Graph](image)

Fig. 2. Differential absorbance spectrum of Ca\(^{2+}\)-ATPase after photo-oxidation. The absorbance spectrum of a sample containing a protein concentration of 0.2 mg/ml, 1 μM erythrosin, 20 mM Tris-HCl (pH 7.4), 80 mM KCl, 5 mM MgCl\(_2\), and 50 μM CaCl\(_2\) was recorded. After irradiation for 60 min, the spectrum of the sample was re-run, and the differential spectrum was calculated.

Table II

| Erythrosin | 0.01 | 0.1 | 0.2 | 0.5 |
|-----------|------|-----|-----|-----|
| \( K_{m1} \) | μM   | μM  | μM  | μM  |
| \( V_{m1} \) | μmol/mg/min | μmol/mg/min | μmol/mg/min | μmol/mg/min |
| \( K_{m2} \) | μM   | μM  | μM  | μM  |
| \( V_{m2} \) | μmol/mg/min | μmol/mg/min | μmol/mg/min | μmol/mg/min |
| 0         | 1.17 | 1.33 | 500 | 10.5 |
| 0.2       | 1.00 | 1.11 | 833 | 9.0 |
| 0.5       | 1.11 | 0.91 | 1250| 7.7 |

The ATP concentration dependence of Ca\(^{2+}\)-ATPase with 10 min under fluorescent room light in presence of either fluorescein (●), eosin (○), or erythrosin (●). In this and the following experiments, activity was measured in medium containing 20 mM Tris-HCl (pH 7.4), 80 mM KCl, 5 mM MgCl\(_2\), 0.05 mM total or free CaCl\(_2\), 10 μM Ca\(^{2+}\)-ATPase, 2 mM ATP, and the indicated concentration of dye at 37°C. B, erythrosin was fixed at 1 μM, and oxidation was done for the designated times in presence of 0.5 mM EGTA (●) or 0.5 mM EGTA + 2 mM ATP (○). Hydrolysis was started by the addition of either 0.55 mM Ca\(^{2+}\) + 2 mM ATP (●) or 0.55 mM Ca\(^{2+}\) (○). C, inhibition of ATPase activity induced by erythrosin with (●) or without (○) photo-oxidation.

The ATP concentration dependence of Ca\(^{2+}\)-ATPase was studied using low erythrosin concentrations (0.2 and 0.5 μM) for photo-inhibition in order to achieve measurable ATP hydrolysis together with observable kinetic effects. The photo-inhibition of the ATPase activity induced by erythrosin was not reversed by increasing the ATP concentration. Both \( V_{max1} \) and \( V_{max2} \) were significantly and proportionally reduced, with no change in the affinity for ATP at the catalytic site, however, a significant decrease was observed in the apparent affinity for the secondary activation induced by ATP. These data are summarized in Table I. A similar inhibitory behavior with erythrosin was observed for the erythrocyte Ca\(^{2+}\)-ATPase by Mugica et al. (1984). Those authors concluded that inhibition with erythrosin was noncompetitive with ATP for the catalytic site but that the dye modified the affinity and competed with ATP for the regulatory site of the enzyme.

**Ca\(^{2+}\)-Binding to Ca\(^{2+}\)-ATPase**—The affinity of the enzyme for Ca\(^{2+}\) was not modified by photo-inhibition, considering both the high and low affinity Ca\(^{2+}\) binding sites, although the apparent cooperativity may have been slightly decreased (Fig. 3). Equilibrium binding of Ca\(^{2+}\) to the photo-oxidized enzyme reached the same values as in the control, attaining approximately 8 nmol of \([\text{CaCl}_{2}]\text{Ca}^{2+}\) bound per milligram of Ca\(^{2+}\)-ATPase with 10 μM Ca\(^{2+}\) in the medium. This result rules out the possibility that the observed decrease in ATPase activity could be due to destruction of the Ca\(^{2+}\) binding sites transforming the inhibited enzyme into an incompetent silent form. It also establishes that inhibition is not a result of nonsaturation of these sites due to a decrease in affinity.

**Effects of Photo-inhibition on the Steady-state Levels of Phosphoenzyme**—The same steady-state values for phosphorylation with ATP were obtained with control enzyme and photo-inhibited Ca\(^{2+}\)-ATPase after illumination with 5 μM erythrosin during 10 min. Because ATPase activity was inhibited up to 80% in these conditions, this observation indicates that photo-oxidation led to a slower turnover rate. A slow and probably nonspecific reaction finally impaired phosphorylation by ATP (not shown).

Table II shows the steady-state values obtained for phosphorylation with \( [\text{CaCl}_{2}]\text{ATP} \) and \([\text{CaCl}_{2}]\text{IP}_{\text{p}} \). For comparison, the effects elicited by fluorescein are also shown. Although the
control and photo-oxidized enzyme were preincubated in the presence of Ca\(^{2+}\) and Mg\(^{2+}\), and the phosphorylation reaction was started by the addition of ATP, whereas in Fig. 4, C and D, enzyme phosphorylation was initiated by the addition of ATP and Ca\(^{2+}\) to the enzyme previously exposed to light in the presence of EGTA and Mg\(^{2+}\) with or without the dye. In both cases, a marked reduction of the phosphorylation rate was observed in the photo-inhibited enzyme. However, inhibition with the dye induced a proportionally greater decrease (k\(^i\) = 145 s\(^{-1}\); k\(^f\) = 23 s\(^{-1}\)) in the initial apparent phosphorylation rate when the phosphorylation reactions were started with the Ca\(^{2+}\)-bound enzyme (Fig. 4, A and B) than when enzyme was preincubated in EGTA (k\(^i\) = 50 s\(^{-1}\); k\(^f\) = 18 s\(^{-1}\)) beforehand (Fig. 4, C and D). Conversely, maximal phosphorylation levels similar to control tended to be reached both by the Ca\(^{2+}\)-incubated and EGTA-incubated enzyme. It must be stressed that the reaction rates observed for phosphorylation of the photo-oxidized enzyme are very similar regardless of the initial (i.e. whether Ca\(^{2+}\) was bound or not) enzyme conformation. At the concentrations used in this work, Ca\(^{2+}\) and ATP binding to the E\(_1\) form of the enzyme are expected to be very fast when compared with the E\(_2\)-E\(_1\) conformational transition rate (Scaiano et al., 1979). Thus it is assumed that isomerization from E\(_2\) to E\(_1\) would be the rate-limiting step for phosphorylation of the native Ca\(^{2+}\)-ATPase when beginning with the E\(_2\) form of the enzyme. However, after photo-oxidation this rate-limiting step seems to be superseded due to slowing of a subsequent step, which makes the overall phosphorylation reaction rates very slow, and similar irrespective of the initial conformer. Changing the rate of ATP binding would be expected to change the k\(_{50}\) for ATP and shift the k\(_{mn}\) to higher values, a change that was not observed for the catalytic site of the photo-oxidized enzyme (see Table I). Thus, it is likely that the phosphoryl transfer reaction was affected by reaction with erythrosin.

Photo-oxidation had a pronounced effect on the dephosphorylation rates of ATP-formed phosphoenzyme. The dephosphorylation reaction was much slower than control both for the forward (EGTA-induced) and backward (ADP-sensitive) directions. The EGTA-induced phosphoenzyme decay showed a pronounced decrease in the observed rate constants for the photo-oxidized enzyme, decreasing from k\(_{D}\) = 3.36 s\(^{-1}\) to k\(_{D}\) = 1.1 s\(^{-1}\) (Fig. 5, A and B). The extent of inhibition was higher the higher the dye concentrations used (not shown). Although photo-inhibition severely impaired phosphoenzyme decay in both the forward and reverse directions, the inhibition was much more accentuated on the ADP-sensitive pathway, and the typical two-exponential, fast ADP-sensitive decay (k\(_{D}\) = 183 s\(^{-1}\); k\(_{D}\) = 20 s\(^{-1}\)) became so inhibited that it could be simply fitted with a slow (k\(_{D}\) = 0.75 s\(^{-1}\)), single exponential decay (Fig. 5, C and D). Binding of ADP to the phosphoenzyme was probably not impaired by photo-oxidation, because in these experiments the enzyme was already phosphorylated by ATP (evidencing nucleotide binding) and also because ADP was still able to protect against the well described glutaraldehyde-induced intramolecular cross-linking (Mdnios, 1992) in the same conditions and to the same extent for both the control and the photo-oxidized enzyme (Fig. 6A). Interestingly, erythrosin, in the absence of illumination, efficiently protected against this glutaraldehyde-induced cross-linking (Fig. 6B), suggesting that both ADP and erythrosin (and probably ATP also), may bind to a common site or induce similar enzyme conformational changes. Therefore, our results point toward the accumulation of a trapped, ADP-insensitive phosphoenzyme. This could be the E\(_2\)-P-Ca\(_2\) or the E\(_1\)-P form or a modified form of E\(_1\)-P-Ca\(_2\), which binds ADP but can not efficiently resynthesize ATP due to the lack of one or more amino acids essential to the synthesis reaction.
Determination of the Conformational Phosphoenzyme Intermediate—As cited earlier in this text, \[^{45}\text{Ca}]\text{Ca}^{2+}\) binding at equilibrium to a static, nonhydrolyzing enzyme showed the same levels for control and erythrosin photo-inhibited enzyme. If the accumulated phosphoenzyme generated upon ATP phosphorylation were a \(\text{Ca}^{2+}\)-free \(E_2\)-P form, one would expect a decrease in the total bound \[^{45}\text{Ca}]\text{Ca}^{2+}\) and also in the ratio between bound \(\text{Ca}^{2+}\) and phosphoenzyme formed. This indeed was not the case, because in all the conditions tested, with varying degrees of inhibition, the ratio of \(\text{Ca}^{2+}\) bound to the phosphoenzyme formed was always around 2. This ratio was maintained even when ATPase activity was reduced to less than 10% of the control and even when the levels of phosphorylation with ATP were already partially impaired by photo-oxidation (Table III). Therefore, a possible accumulation of a \(\text{Ca}^{2+}\)-free phosphoenzyme can be discarded, and the observed phosphoenzyme must thus be a \(\text{Ca}^{2+}\)-bound intermediate.

TNP-ATP, a fluorescent ATP derivative that binds strongly but noncovalently to \(\text{Ca}^{2+}\)-ATPase, was used to distinguish among the possible \(\text{Ca}^{2+}\)-bound phosphoprotein forms. TNP-ATP fluorescence is enhanced upon binding to the enzyme, and fluorescence is further enhanced when phosphorylation by ATP or \(P_i\) occurs. This phenomenon, classically known...
as superfluorescence, is attributed to the accumulation of E2-P-Ca2- and E2-P forms (Berman, 1986; Bishop et al., 1987). Binding of TNP-ATP to the Ca2+-ATPase was not disabled by the photo-inhibition nor by the remaining free erythrosin, because the addition of 2 μM TNP-ATP to both control and modified enzyme caused a fluorescence enhancement much greater than that observed following addition of the probe to enzyme-free solutions and because the fluorescence of the bound TNP-ATP was entirely suppressed when an 80% phosphorylatable but less than 10% catalytically competent photo-oxidized Ca2+-ATPase was phosphorylated with ATP (Fig. 7), showing that perhaps a hydrophilic/hydrophobic transition in the TNP-ATP binding site, linked to the transition from E2-P-Ca2- to E2-P-Ca2 and responsible for the superfluorescence, was impaired. Indeed, the addition of ATP to the photo-oxidized enzyme (Fig. 7, right panel) induced a small decrease in fluorescence that could be due to binding of the nucleotide and accumulation of E2-P-Ca2, because (as cited above) the superfluorescence is observed when the accumulation of E2-P-Ca2 or E2-P occurs.

**Discussion**

The purpose of this study was to correlate the modification of amino acids of the SR Ca2+-ATPase by a dye that behaves as a nonhydrolyzable substrate analog with the effects observed on the different steps of substrate hydrolysis. This was achieved using the halogenated fluorescein derivative erythrosin B, which can induce photo-inhibition of the Ca2+-ATPase in a reaction that has been described as oxidizing residues located in particular environments, possibly within putative nucleotide binding site(s) (Lundblad and Noyes, 1984; Ray and Koshland, 1962).

Photo-oxidation with methylene blue (Yu et al., 1967) or rose bengal (Yu et al., 1974; Coffey et al., 1975) has been used to investigate ATP hydrolysis and Ca2+ transport by the Ca2+-ATPase, and early findings led to the conclusion that histidyl residues, which in these cases were shown to be the main target, are critical for Ca2+ binding and participate in ATP hydrolysis and phosphoenzyme formation and turnover. Some xanthene dyes are structurally similar to adenine nucleotides and have been shown to bind to several nonmembranous enzymes that have nucleotide binding sites, such as lactate dehydrogenase (Wassarman and Lentz, 1971), aspartate transcarbamylase (Jacobsberg et al., 1975), hexokinase (Yip and Rudolph, 1976), and creatine kinase (Sommerville and Quichocho, 1977). They also bind to membraneous ion-transporting enzymes, including shark rectal gland Na+-K+-ATPase (Skou and Esmann, 1981; Skou and Esmann, 1983), pig stomach H+-K+-ATPase (Helmiich-de Jong et al., 1986), mitochondrial F1 ATPase (Neslund et al., 1984), erythrocyte Ca2+-ATPase (Mugica et al., 1984; Gatto and Milanick, 1993), plant plasma membrane Ca2+-ATPase (De Michels et al., 1993), yeast plasma membrane H+-ATPase (Wach and Gräber, 1991), and SR Ca2+-ATPase (Coffey et al., 1975; Morris et al., 1982; Murphy, 1988). For most of these ATPases, xanthene dyes were shown to reversibly inhibit ATP hydrolysis in the micromolar range, provided that photo-oxidation was avoided. The structural similarity of erythrosin with adenine nucleotides is well discussed in Neslund et al. (1984). Therefore, it is quite possible that fluorescein and its derivatives are binding to nucleotide sites of these enzymes. The nucleotide site or sites in different P-type ion-transporting ATPases are likely to be very similar in structure, as evidenced by amino acid homologies within P-ATPases, which reveal a highly conserved glycine-rich fragment in the segments that are predicted to form the ATP catalytic site (Taylor and Green, 1989).
A reagent frequently used for the study of ion-transporting pumps is the fluorescein derivative FITC, which in most cases binds covalently and stoichiometrically to a lysine residue in the putative nucleotide binding site, inducing a concomitant loss of the ability to bind ATP (Pick, 1981). Murphy (1988) demonstrated that Lys-515, which is specifically labeled in SR Ca\(^{2+}\)-ATPase (Maruyama et al., 1989), is not unusually reactive toward FITC, indicating that attachment of the affinity label must be directed by the fluorescein moiety of the compound. However, Murphy’s data and our own results show that the Ca\(^{2+}\)-ATPase affinity for free fluorescein is not particularly high, being strongly increased (at least two orders of magnitude) by the addition of halogenated substitutes to the molecule. Many authors have used eosin isothiocyanate (Papp et al., 1987; Munkonge et al., 1988) and erythrosin isoithiocyanate (Papp et al., 1987; Birmachu and Thomas, 1990; Voss et al., 1991) rather than FITC, due to their higher affinity and particular fluorescence characteristics, to study rotational movements of the SR Ca\(^{2+}\)-ATPase. These authors concluded that both compounds bind to the same site and react with the same lysozyme residue as FITC (Papp et al., 1987; Birmachu and Thomas, 1990). Consequently, it is expected that erythrosin itself must bind noncovalently and with high affinity to a nucleotide binding cleft. Furthermore, as the oxygen species generated upon excitation of the dye with light is rapidly deactivated in aqueous solutions (overall rate constant 5 \times 10^9 \text{M}^{-1} \text{s}^{-1} at pH 7.0, Halliwell and Gutteridge, 1989), the free radical must be produced very near the target modified amino acid. Thus, our reasoning is that erythrosin exerts photo-inhibition by modification of some residue(s) within the nucleotide binding site(s) of the SR Ca\(^{2+}\)-ATPase, through a reaction that depends on the generation of singlet oxygen reactive species inside the site(s) by the light-excited, noncovalently bound dye (Halliwell and Gutteridge, 1989).

Yu et al. (1974) demonstrated that photo-inhibition of SR Ca\(^{2+}\)-ATPase by rose bengal was due to the modification of histidyl residues, which were preferentially lost upon photo-oxidation, and not to peroxidation of phospholipids, consistent with Martonosi et al. (1972). However, Yu et al. (1974) reported that Ca\(^{2+}\) transport was more sensitive than ATPase activity to photo-inhibition with rose bengal. Morris et al. (1982) found a similar behavior using erythrosin but assumed that no photo-oxidation occurred in their experiments. We find that net Ca\(^{2+}\) transport is indeed inhibited more effectively than ATPase activity, but this is due to a Ca\(^{2+}\) leakage induced after some minutes of incubation with erythrosin, even in the absence of light. Therefore, it is not clear whether the apparently higher sensitivity of Ca\(^{2+}\) transport to halogenated dyes is due to uncoupling of the enzyme by photo-oxidation of amino acids directly related to Ca\(^{2+}\) transport or to membrane permeabilization.

Our results show that the events related to phosphoryl transfer to and from the Ca\(^{2+}\)-ATPase are drastically affected by photo-oxidation. Coffey et al. (1974) had already observed that both phosphorylation and dephosphorylation rates were impaired by photo-oxidation of SR vesicles. It does not seem that the residue(s) modified in our work, are directly involved in either Ca\(^{2+}\) binding (Coan and DiCarlo, 1990) or substrate binding (Lacapere et al., 1990), because neither the Ca\(^{2+}\) dependence of ATP hydrolysis nor the K_m for nucleotide hydrolysis is impaired by photo-oxidation. This is further supported by the fact that photo-oxidation also impairs phosphorylation by P_i, which does not depend on Ca\(^{2+}\) binding and does not need a tight fit to a nucleotide cleft.

The time course for phosphorylation of the photo-oxidized Ca\(^{2+}\)-ATPase by ATP is adequately simulated by a model in which only the phosphoryl transfer and the rate constants related to the E_2\rightarrow E_1 transition are changed. These simulations were based on the hydrolytic cycle of the Ca\(^{2+}\)-ATPase presented in Scheme I, as originally proposed by Carvalho et al. (1976) and do not distinguish transitional subconformations, such as the sequential binding of Ca\(^{2+}\) ions (Inesi, 1987), and

![Scheme I](image)

**Table IV**

| Step | Reaction | k_{in} | k_{n} | k_{r} | k'_{in} | k'_{n} |
|------|----------|-------|------|------|--------|-------|
| 1    | E_1 + 2Ca^{2+} ↔ E_1 + Ca^{2+} | 1 \times 10^{13} \text{M}^{-2} \text{s}^{-1} | - | - | 10 \text{s}^{-1} | - |
| 2    | E_1 + Ca^{2+} + ATP ↔ E_1 + Ca^{2+} + ATP | 2 \times 10^{7} \text{M}^{-1} \text{s}^{-1} | - | - | 40 \text{s}^{-1} | - |
| 3    | E_1 - Ca^{2+} + ATP ↔ E_1 - Ca^{2+} + ATP | 40 \text{s}^{-1} | - | - | 400 \text{s}^{-1} | - |
| 4    | E_1 - P + Ca^{2+} + ADP ↔ E_1 - P + Ca^{2+} + ADP | 3 \times 10^{4} \text{s}^{-1} | - | - | 1 \times 10^{5} \text{M}^{-1} \text{s}^{-1} | - |
| 5    | E_1 - P + Ca^{2+} ↔ E_1 - P + Ca^{2+} | 20 \text{s}^{-1} | - | - | 100 \text{s}^{-1} | - |
| 6    | E_1 - P + Ca^{2+} ↔ E_1 - P + 2Ca^{2+} | 25 \text{s}^{-1} | - | - | 6.2 \times 10^{4} \text{M}^{-1} \text{s}^{-1} | - |
| 7    | E_1 - P + Ca^{2+} ↔ E_1 - P + Ca^{2+} | 60 \text{s}^{-1} | - | - | 200 \text{s}^{-1} | - |
| 8    | E_1 - P + Ca^{2+} ↔ E_2 | 100 \text{s}^{-1} | - | - | 1 \times 10^{5} \text{M}^{-1} \text{s}^{-1} | - |
| 9    | E_2 ↔ E_1 | 40 \text{s}^{-1} | - | - | 53 \text{s}^{-1} | - |

[a] Pickart and Jencks, 1984.
[b] Alonso and Hedt, 1990 (with modifications from Teruel et al., 1987).
[c] Pethiyoh and Jencks, 1988.
[d] Fujimori and Jencks, 1988.
[e] Pickart and Jencks, 1982.
[f] Nakamura et al., 1986.
[g] Inesi et al., 1982.
[h] This work.
[i] Stahl and Jencks, 1987.
[j] Inesi and de Meis, 1989.
intermediate steps after nucleotide binding and before the phosphoryl transfer reactions (Stahl and Jencks, 1987; Petithory and Jencks, 1988). Most of the rate constants used in Table IV for the unmodiﬁed Ca²⁺-ATPase are based on other publications (see Table IV), and after small adjustments are adequate for the simulation of the curves shown in this work (see Figs. 4 and 5).

All of the results obtained with the photo-oxidized Ca²⁺-ATPase presented in Figs. 4 and 5 can be reasonably simulated by assuming that the rate constants related to the phosphoryl transfer reactions (steps 3 and 7 in Scheme I) and to the interconversions between the E₁-P and E₂-P and E₂ and E₁ (steps 5 and 9 on Scheme I) are lower than in the control (Table IV). These values also predict the steady-state ATPase activity for the photo-oxidized enzyme at 15% of the control values, which is in good agreement with the observed experimental values (15–20%, see Fig. 1). The values for the constants after photo-oxidation were obtained by considering: (a) the low phosphorylation rate of E₁-Ca²⁺ by ATP (k₅); (b) the very slow dephosphorylation induced by ADP and EGTA (k₃); (c) the accumulation of a Ca²⁺-bound phosphoenzyme together with the observed loss of the superﬂuorescence of TNP-ATP (k₉ and k₇); and (d) the inhibition of phosphorylation by P₁ (k₉ and k₈). The values of k₉ and k₈ were adjusted so as to account for the differences in phosphorylation rates between the Ca²⁺-bound enzyme and the Ca²⁺-depleted enzyme (estimated as 25% of the enzyme initially in the E₂ conformation).

There are several ways to explain the results obtained with the photo-oxidized Ca²⁺-ATPase. One or more of the oxidized residues could be involved in: (a) the coordination of the phosphoryl and/or aspartyl group(s), which is the basis for phosphoryl transfer or hydrolysis. Taken together with results obtained elsewhere using halogenated photo-oxidants, our data are consistent with the notion that one or more amino acids susceptible to photo-oxidation by xanthene dyes is involved in substrate hydrolysis in the catalytic site of some, if not all, P-type ATPases.