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OXIDATION OF REACTIVE SULFHYDRYL GROUPS OF SARCOPLASMIC RETICULUM ATPase

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The role of reactive sulfhydryl groups of sarcoplasmic reticulum ATPase has been investigated. Incubation of ATPase with 17 mol *o*-iodosobenzoic acid per mol ATPase results in a 15% inhibition of Ca^{2+} uptake with only a 5% loss of ATPase activity. When ATPase is treated with 15 mol $KMnO_4$ per mol ATPase, Ca^{2+} uptake is completely inhibited. From the measurement of remaining SH groups using 5,5′-dithiobis-(2-nitrobenzoic acid), it is found that the oxidation of approximately four SH groups per ATPase molecule with $KMnO_4$ leads to a complete loss of Ca^{2+} uptake, while the oxidation of five SH groups per ATPase with *o*-iodosobenzoic acid results in only 15% inhibition of Ca^{2+} uptake. The results of amino acid analysis indicate that $KMnO_4$ oxidizes the reactive SH groups to sulfonic acid groups. Among the five *o*-iodosobenzoic acid-reactive SH groups, at least one shows a distinct Ca^{2+} dependence. Addition of *o*-iodosobenzoic acid to the reaction medium containing $KMnO_4$ does not increase the number of oxidized SH groups, indicating that both *o*-iodosobenzoic acid and $KMnO_4$ oxidize the same SH groups of the enzyme. The different effects of two oxidizing agents on sarcoplasmic reticulum ATPase eliminate the possibility of direct involvement of SH group(s) in the ATPase reaction.

Introduction

Sarcoplasmic reticulum vesicles actively accumulate Ca^{2+} by an ATP-dependent transport system [1,2]. The $(Ca^{2+} + Mg^{2+})$-ATPase accounts for about 70% of total sarcoplasmic reticulum protein and is responsible for active Ca^{2+} transport [3,4]. The ATPase has been shown to contain about 26 cysteine residues from the results of amino acid analysis [5]. It has been reported that both sarcoplasmic reticulum and purified ATPase contain about 20 thiol groups per ATPase [6–8], indicating the presence of three disulfide residues.

The blocking of thiol groups of sarcoplasmic reticulum ATPase with SH-directed agents leads to the loss of ATPase activity and Ca^{2+} transport [9–17]. From the modification studies using *N*-ethylmaleimide, it has been shown that ATPase has two to four reactive SH groups [12,15,17]. Yamada and Ikemoto [15] have suggested that there are at least two functionally important thiols, the blocking of which results in inhibition of different steps of the ATPase reaction. Kawakita et al. [17] also indicated the presence of two SH groups essential for Ca^{2+} transport of sarcoplasmic reticulum

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Abbreviations: SH, sulfhydryl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate; Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

vesicles, one being involved in formation of the phosphorylated intermediate (E-P) and the other in its decomposition. Although it is likely that SH groups are important for the ATPase reaction, no clear evidence for actual involvement of SH groups in the catalytic cylce has ever been obtained.

The present study was undertaken to test the possibility of direct involvement of SH group(s) in the catalytic process of sarcoplasmic reticulum ATPase. For this purpose, SH groups were selectively oxidized to different oxidation states: to sulfenic acid groups (-SOH) by *o*-iodosobenzoic acid [18–22] and to sulfonic acid groups ($-SO_3H$) by $KMnO_4$ [23–25]. The results indicate that SH groups are not catalytically involved in Ca^{2+} transport.

Materials and Methods

Potassium permanganate, *o*-iodosobenzoic acid, and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Sigma. Arsenazo III was obtained from Aldrich and purified according to the methods of Kendrick [26]. The Ca^{2+} ionophore A 23187 was obtained from Calbiochem.

Sarcoplasmic reticulum vesicles were prepared from the white skeletal muscle of rabbit hindlegs according to Eletr and Inesi [27] with minor modifications.

ATPase activity was measured according to the method of Warren et al. [28]. The reaction mixture in a 1 ml cuvette contained 30 mM Mops, pH 6.9, 5 mM ATP, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 100 mM KCl, 0.45 mM NADH, 20 μM A 23187, 0.75 mM phospho*enol*pyruvate, pyruvate kinase (10 units/ml), lactate dehydrogenase (50 units/ml), and sarcoplasmic reticulum vesicles (2 to 5 μg/ml protein). The absorbance decrease at 340 nm was monitored at 28°C in a Cary 219 spectrophotometer, and the specific activity was calculated from this absorbance decrease using a molar extinction coefficient of 6220 [29]. Thus our measurements are for total ATPase activity and that the Mg^{2+}-dependent moiety was less than 10% in all preparations.

Ca^{2+} uptake was measured using Arsenazo III as an indicator of free calcium [30]. The reaction mixture in a 1 ml cuvette contained 20 mM Mops, pH 6.9, 1.25 mM ATP, 5 mM $MgCl_2$, 40 μM $CaCl_2$, 5 mM potassium oxalate, 100 mM KCl, 60 mM sucrose, 25 μM Arsenazo III, and sarcoplasmic reticulum vesicles (15 to 20 μg/ml protein). Absorbance decrease at 655 nm was monitored at 28°C in a Cary 219 spectrophotometer. The Arsenazo III absorption was calibrated as a function of calcium concentration. In the presence of 25 μM Arsenazo III, absorbance changes were linear for calcium concentrations up to 40 μM.

The protein concentration of sarcoplasmic reticulum vesicles was determined either spectrophotometrically at 280 nm in the presence of 1% SDS [6] or by the method of Lowry et al. [31].

The number of thiol groups was determined with DTNB using a value of ϵ at 412 nm of $1.36 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [32] and is expressed per $1.5 \cdot 10^5$ g protein since the ATPase has a molecular weight of 106000 and accounts for about 70% of the protein of sarcoplasmic reticulum [3,4]. The reaction mixture in a cuvette (1 ml) contained 0.1 M Tris-HCl buffer, pH 8.0, 1 mM EDTA, 1% SDS, 1 mM DTNB, and sarcoplasmic reticulum vesicles (30 to 40 μg/ml protein) and the absorbance increase at 412 nm was monitored at 25°C.

Tryptophan was spectrophotometrically determined in the presence of 1% SDS using a value of ϵ at 288 nm of 4815 $M^{-1} \cdot cm^{-1}$ [33].

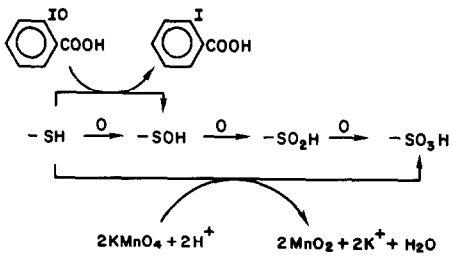
Acid hydrolysis of sarcoplasmic reticulum protein was performed as recommended by Moore and Stein [34]. A Glenco model MM-100 amino acid analyzer was used for analyses of the hydrolysates.

Results

Oxidation of sarcoplasmic reticulum ATPase by o-iodosobenzoic acid and $KMnO_4$

Potassium permanganate ($KMnO_4$), a powerful oxidizing agent, has been used as an active site-directed reagent for phosphate binding sites [23,25]. The basis of its action on phosphate binding sites is its structural similarity to phosphate [35,36]. The reagent has been known to oxidize thiol groups to sulfonic acid groups [23,25] as illustrated in Scheme I.

As shown in Fig. 1, Ca^{2+} uptake activity of sarcoplasmic reticulum vesicles was almost completely inhibited when vesicles were treated with



Scheme I.

160 μM $KMnO_4$. Our time resolution was not fast enough to measure the initial rates of inactivation. However, after the inhibition of 80% of Ca^{2+} uptake, a relatively slow phase with a pseudo first-order rate constant, $k_1 = 3.6 \cdot 10^{-2}$ min^{-1} was observed. In contrast, the inactivation rate was considerably slower ($k_1 = 2.6 \cdot 10^{-3}$ min^{-1}) when sarcoplasmic reticulum vesicles were incubated with the same concentration of *o*-iodosobenzoic acid. As shown in Scheme 1. *o*-iodosobenzoic acid oxidizes thiol groups to sulfenic acid groups [19]. Even after 1 h incubation with *o*-iodosobenzoic acid, sarcoplasmic reticulum vesicles still maintained about 80% of their initial Ca^{2+} uptake activity (Fig. 1). During the oxidation of sarcoplasmic reticulum vesicles with *o*-iodosobenzoic acid, ATPase activity was almost completely retained as shown in Fig. 2A. Increasing concentrations of *o*-iodosobenzoic acid did not cause any further decrease in either Ca^{2+} uptake or ATPase activity. This indicates that the number of SH groups accessible to *o*-iodosobenzoic acid is relatively small and that modification of these SH groups with this oxidant does not cause a progressive denaturation of the ATPase molecule.

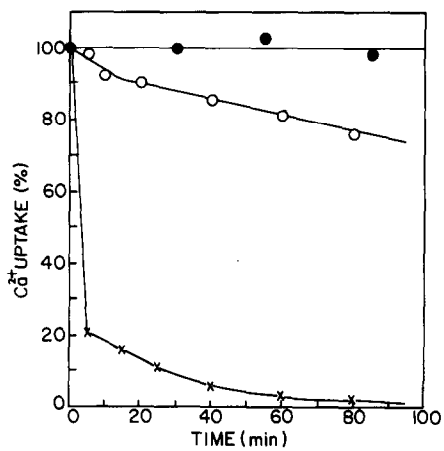


Fig. 1. Effect of SH oxidation by *o*-iodosobenzoic acid and $KMnO_4$ on CA^{2+} uptake of sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles (14.4 μM ATPase) were treated with 160 μM *o*-iodosobenzoic acid (○) or 160 μM $KMnO_4$ (×) at 22°C in 0.5 ml of 30 mM Mops buffer, pH 6.9, containing 100 mM KCl and 115 mM sucrose. At different time intervals after the addition of oxidants, 10-μl aliquots were taken for Ca^{2+} uptake measurement. The specific activity of a control sample (●) was 0.83 μmol Ca^{2+}/min/mg.

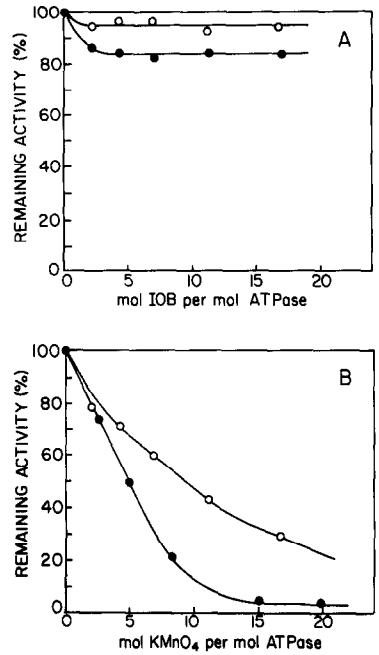


Fig. 2. Effect of oxidant concentration on the inactivation of sarcoplasmic reticulum ATPase. (A) Sarcoplasmic reticulum vesicles (14.2 μM ATPase) were treated with 30 to 240 μM *o*-iodosobenzoic acid (IOB) at 22°C in 0.5 ml of 30 mM Mops buffer, pH 6.9, containing 100 mM KCl and 115 mM sucrose. After 50 min incubation, aliquots were taken for Ca^{2+} uptake measurement and ATPase activity assay. (B) Sarcoplasmic reticulum vesicles (12.1 μM ATPase) were treated with 30 to 240 μM $KMnO_4$ at 22°C in the same medium as indicated in (A). The Ca^{2+} uptake activity and ATPase activity of untreated samples were 0.81 μmol Ca^{2+}/min per mg and 7.3 μmol P_i/min per mg, respectively. Key: ○, Ca^{2+} uptake activity; ●, ATPase activity.

As Fig. 2B shows, the loss of Ca^{2+} transport activity is linear with respect to initial $KMnO_4$ concentration until the latter is eight times the ATPase concentration. As shown in Scheme 1, it requires 2 mol $KMnO_4$ to oxidize 1 mol SH groups to sulfonic acid groups [23]. This suggests that

oxidation of a maximum of 4 SH groups per ATPase molecule is responsible for 80% inhibition of Ca^{2+} transport. Unlike Ca^{2+} uptake, the loss of ATPase activity was somewhat nonlinear with respect to the reagent concentration. Moreover, ATPase activity was less sensitive than Ca^{2+} uptake towards SH oxidation by $KMnO_4$.

Stoichiometry of inactivation. The complete inhibition of Ca^{2+} uptake required $KMnO_4$ oxidation of approx. 4 SH groups per ATPase as illustrated in Fig. 3. Further oxidation of SH groups might be due to denaturation of the protein, resulting in the exposure of buried SH groups. In contrast, the loss of Ca^{2+} uptake activity appeared to be independent of the number of thiol groups oxidized by *o*-iodosobenzoic acid. More than 80% of the original Ca^{2+} transport activity was retained even after oxidation of 5 SH groups by this mild oxidizing agent.

It has been reported that 2 SH groups are essential for ATPase activity [12,15]. Kawakita et al. [17] have shown that only 4 SH groups per ATPase in sarcoplasmic reticulum vesicles are modified by *N*-ethylmaleimide even at a high concentration (1.5 mM) of this alkylating reagent. They have shown that two of those SH groups are essential for Ca^{2+} transport while the other two are apparently nonessential. As shown in Fig. 3, only 5 SH groups are accessible to *o*-iodosobenzoic acid, even with 17 mol of the oxidant per mol ATPase, suggesting that *o*-iodosobenzoic acid-oxidized SH groups might include those which have been modified *N*-ethylmaleimide [17].

In order to see whether *o*-iodosobenzoic acid-oxidized SH groups were idential to those modified with *N*-ethylmaleimide, Ca^{2+} effect on reactivity of SH groups with *o*-iodosobenzoic acid was tested (Table I). DTNB reaction with control samples shows that there are 19.5 thiol groups per $1.5 \cdot 10^5$ g protein (per mol ATPase), in good agreement with about 20 thiol groups per $1.5 \cdot 10^5$ daltons which has been previously reported [6,8]. As shown in Table I, the removal of divalent

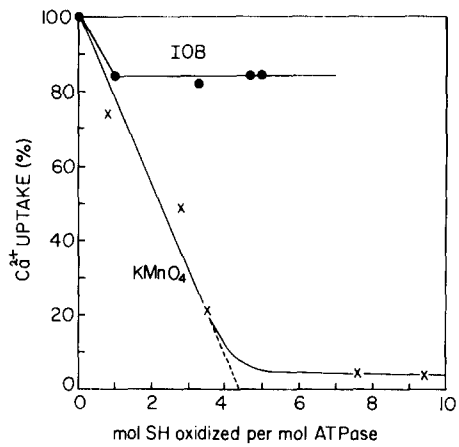


Fig. 3. Stoichiometry of *o*-iodosobenzoic acid and $KMnO_4$ inactivation of sarcoplasmic reticulum ATPase. Sarcoplasmic reticulum vesicles were treated with various concentrations of *o*-iodosobenzoic acid or $KMnO_4$ as described in Fig. 2 and dialyzed twice at room temperature for several hours against 400 ml of 30 mM Mops, pH 6.9, containing 100 mM KCl and 115 mM sucrose with nitrogen bubbling. Protein concentration and thiol content of dialyzed samples were determined as described under Materials and Methods. Remaining Ca^{2+} transport activity of *o*-iodosobensoic acid-treated vesicles (○) and $KMnO_4$-treated vesicles (×) was plotted against the number of modified SH groups. The specific activity of untreated samples was 0.81 μmol Ca^{2+}/min per mg.

TABLE I

EFFECT OF DIVALENT CATIONS ON *o*-IODOSOBENZOIC ACID OXIDATION OF SARCOPLASMIC RETICULUM PROTEIN

SH groups of sarcoplasmic reticulum protein were oxidized by *o*-iodosobenzoic acid as described in Fig. 3. The numbers indicate mean ± S.D. (three separate experiments). Specific activity of control samples was 0.78 μmol Ca^{2+}/min per mg protein.

Additions to reaction medium	Remaining SH groups [a]	Ca^{2+} uptake (%)
Control	19.4 ± 0.8	100
160 μM *o*-iodosobenzoic acid	14.5 ± 0.4	77 ± 5
160 μM *o*-iodosobenzoic acid + 0.4 mM EDTA	15.9 ± 0.3	54 ± 4
160 μM *o*-iodosobenzoic acid + 0.4 mM EDTA + 0.5 mM $CaCl_2$	14.7 ± 0.3	84 ± 3

[a] The values are expressed as number of SH groups per $1.5 \cdot 10^5$ g sarcoplasmic reticulum protein.

cations from the reaction medium by the addition of 0.4 mM EDTA decreased the number of *o*-iodosobenzoic acid-reactive SH groups from 4.9 to 3.5. A significant decrease in Ca^{2+} uptake observed with the samples containing 0.4 mM EDTA might be due to instability of sarcoplasmic reticulum vesicles in the absence of Ca^{2+} since sarcoplasmic reticulum vesicles containing 0.4 mM EDTA showed a gradual loss of Ca^{2+} uptake even without *o*-iodosobenzoic acid during the period of incubation. Duggan and Martonosi [37] reported that incubation of sarcoplasmic reticulum vesicles with 1 mM EDTA at pH 7.0 causes a significant increase in permeability of sarcoplasmic reticulum membrane, accompanied by the higher rate of Ca^{2+} release from sarcoplasmic reticulum. The addition of 0.5 mM $CaCl_2$ to the samples containing 0.4 mM EDTA increased the number of reactive thiol groups by 1.2, regaining approximately the same number of reactive thiol groups obtained for the samples without EDTA. Data not shown where we used 0.4 mM EGTA instead of 0.4 mM EDTA, it was found that 4 mM $MgCl_2$ had no protective effect on Ca^{2+}-uptake and that 0.4 mM $CaCl_2$ with EDTA or EGTA did have the protective effect on Ca^{2+}-uptake. It is of interest to note that both EDTA and EGTA have the same affinity for Ca^{2+}. This clearly demonstrates that the observed change in the number of reactive SH groups is not due to heavy metal binding to SH groups, but to Ca^{2+} binding to ATPase. The results also show that the reactivity of one of the 5 reactive SH groups has a distinct Ca^{2+} concentration dependence. Data not shown indicate that the time course of reaction of SH groups with the reagent is slower in the presence of Ca^{2+} but it reaches the same end-point within 50 min.

Oxidation of amino acid residues in sarcoplasmic reticulum protein by $KMnO_4$. No significant changes in the content of sensitive amino acid residues [23,25] other than cysteine were observed after treating sarcoplasmic reticulum protein with 100 μM $KMnO_4$ (Table II). The results also show the appearance of 3.1 mol of cysteic acid corresponding to the disappearance of 3.5 mol of cysteine, indicating that $KMnO_4$ oxidized the cysteine of sarcoplasmic reticulum protein to cysteic acid residues. Moreover, most of the $KMnO_4$ (8 mol per mol of ATPase) was consumed by cysteine oxidation in this experiment, since 2 mol of $KMnO_4$ is required for oxidation of 1 mol of thiol group to sulfonate group [23]. Thus, it is clear that the observed inactivation by reaction with $KMnO_4$ is a result of the oxidation of three to four reactive thiol groups to sulfonate groups.

TABLE II

EFFECT OF $KMnO_4$ OXIDATION ON SENSITIVE AMINO ACIDS OF SARCOPLASMIC RETICULUM PROTEIN

Sarcoplasmic reticulum vesicles (12.1 μM ATPase) were treated with 100 μM $KMnO_4$ at 22°C in 1 ml of 30 mM Mops buffer, pH 6.9 containing 100 mM KCl and 115 mM sucrose. After 50 min incubation, the reaction was stopped by adding 5 μl of 100 mM dithiothreitol to the reaction medium. At this point, 10 μl aliquots were taken for Ca^{2+} uptake measurement. The specific activity of control samples was 0.83 μmol Ca^{2+}/min per mg. The solutions were dialyzed at room temperature for 2 h against 1 liter of 2 mM Mops buffer, pH 6.9 containing 100 mM KCl and 57 mM sucrose. The samples were, then, extensively dialyzed at 4°C against distilled water. The dialyzed proteins were lyophilyzed and subjected to amino acid analysis as described under Materials and Methods.

Amino acid	Native enzyme	$KMnO_4$-treated enzyme
Methionine [a]	22.8	23.2
Tyrosine [a]	22.8	23.6
Tryptophan [a]	12.0	12.0
Histidine [a]	15.1	15.1
Cysteine [b]	21.0	17.5
Cysteic acid [c]	1.4	4.5
Ca^{2+} uptake (%)	100	11

[a] The values are expressed as number of residues per $1 \cdot 10^5$ g sarcoplasmic reticulum protein.

[b] The values were determined by DTNB method as described under Materials and Methods and are expressed as number of residues per $1.5 \cdot 10^5$ g sarcoplasmic reticulum protein.

[c] The values are expressed as number of residues per $1.5 \cdot 10^5$ g sarcoplasmic reticulum protein.

Effect of reducing agents on oxidized sarcoplasmic reticulum ATPase. In order to see whether the loss of Ca^{2+} uptake observed in $KMnO_4$-oxidized sarcoplasmic reticulum ATPase can be regained by reducing agent, sarcoplasmic reticulum ATPase incubated with 100 μM $KMnO_4$ (8 mol per mol of ATPase) for 5 min was treated with various reducing agents: 0.5 mM dithiothreitol, dithioerythritol, and glutathione. Even after 20 min incubation of $KMnO_4$-oxidized sarcoplasmic reticulum. ATPase with these reducing agents, no

significant reactivation was observed. The results indicate that sulfonic acid is the major species in $KMnO_4$-oxidized thiol groups since sulfonic acid groups are quite stable to reduction [23]. Similarly, neither 0.5 mM dithiothreitol nor 10 mM sodium arsenite [19,38–40] proved to be effective in restoring activity of *o*-iodosobenzoic acid-oxidized sarcoplasmic reticulum ATPase. This suggests that some irreversible changes in local protein conformation near an active site may occur during *o*-iodosobenzoic acid oxidation. Parker and Allison [19] have reported that *o*-iodosobenzoic acid-treated glyceraldhyde-3-phosphate dehydrogenase shows an irreversible inactivation at high temperature, suggesting that the irreversible inactivation is characteristic of *o*-iodosobenzoic acid-inactivated enzymes.

Identification of cysteine residues as a basis for number of reactive SH groups

To see if *o*-iodosobenzoic acid-oxidized SH groups are same as those oxidized with $KMnO_4$, sarcoplasmic reticulum vesicles were treated with *o*-iodosobenzoic acid in the absence and presence of $KMnO_4$. As summarized in Table III, *o*-iodosobenzoic acid alone oxidized 4.6 SH groups while retaining 88% of original Ca^{2+} uptake activity. This number is about the same as that of $KMnO_4$-oxidized SH groups (Table III, line 3). The addition of 240 μM *o*-iodosobenzoic acid to the reaction medium containing 100 μM $KMnO_4$ did not increase the number of oxidized SH groups (Table III, line 4). Moreover, no additional inhibition due to the *o*-iodosobenzoic acid oxidation could be seen. Reversing the order of vesicle treatment whereby *o*-iodosobenzoic acid oxidation was first performed followed by $KMnO_4$ gave similar results in terms of Ca^{2+}-uptake decrease and number of SH groups to those treated with $KMnO_4$ first followed by *o*-iodosobenzoic acid as shown in Table III.

TABLE III

EFFECT OF *o*-IODOSOBENZOIC ACID ON NATIVE AND $KMnO_4$-OXIDIZED SARCOPLASMIC RETICULUM ATPase

Sarcoplasmic reticulum vesicles (14.2 μM ATPase) were incubated with *o*-iodosobenzoic acid and $KMnO_4$ at 22°C. The vesicles were treated with 240 μM *o*-iodosobenzoic acid for 45 min (line 2) or with 100 μM $KMnO_4$ for 50 min (line 3). In line 4, 240 μM *o*-iodosobenzoic acid was added to the reaction medium after 5 min incubation of the vesicles with 100 μM $KMnO_4$ and the reaction was continued for additional 45 min. After incubation for the period indicated above, the samples were dialyzed as described in Fig. 3. The number of SH groups were determined with DTNB titration (see Materials and Methods). The specific activity of control samples was 0.75 μmol Ca^{2+}/min per mg.

Additions to reaction medium	SH groups oxidized	Ca^{2+} uptake (%)
1. Control	0	100
2. 240 μM *o*-iodosobenzoic acid	4.6 ± 0.3	88 ± 1
3. 100 μM $KMnO_4$	4.7 ± 0.1	29 ± 1
4. 100 μM $KMnO_4$ + 240 μM *o*-iodosobenzoic acid	4.7 ± 0.1	29 ± 2

[a] The values are expressed as number of SH groups per $1.5 \cdot 10^5$ g sarcoplasmic reticulum protein.

Discussion

The reaction of SH groups of sarcoplasmic reticulum ATPase with *o*-iodosobenzoic acid and $KMnO_4$ consistently indicates the presence of four to five reactive SH groups. Kawakita et al. [17] have shown that only four SH groups of sarcoplasmic reticulum ATPase are modified with an excess of *N*-ethylmaleimide. They suggested the existence of two essential SH groups for Ca^{2+} transport, one being involved in E-P formation, and the other in its decomposition, which is consistent with the previous results obtained from *N*-ethylmaleimide modification of purified ATPase [15].

While $KMnO_4$ oxidation of SH groups completely inhibited Ca^{2+} uptake, *o*-iodosobenzoic acid did not show a drastic inhibitory effect on either Ca^{2+} uptake of ATPase activity (Fig. 2). Even after oxidation of five SH groups with *o*-iodosobenzoic acid, the enzyme still retained more than 80% of the initial Ca^{2+} uptake activity, as shown in Fig. 3. These results imply that *o*-iodosobenzoic acid oxidation of SH groups has a very little effect on the active site conformation.

A rapid loss of Ca^{2+} uptake upon treatment of sarcoplasmic reticulum ATPase with $KMnO_4$ (Fig. 1) suggests that $KMnO_4$ oxidation has a more drastic effect on active site conformation that *o*-iodosobenzoic acid does. The loss of Ca^{2+} uptake is shown to be correlated with oxidation of reac-

tive SH groups to sulfonic acid groups by $KMnO_4$ (Table II). Thus, inactivation by $KMnO_4$ is likely to be due to electrostatic repulsion between negatively charged $-SO_3^-$ groups. Inactivation by $KMnO_4$ can be also explained by repulsive interactions between newly introduced $-SO_3^-$ groups and other negatively charged groups in the active site, such as carboxyl groups [41,42]. The different effects of $KMnO_4$ oxidation on Ca^{2+} uptake and ATPase activity can not be accounted to phospholipid oxidation since most of $KMnO_4$ in the reaction mixture is consumed for SH oxidation (Table II). It is more likely that conformational changes due to $KMnO_4$ oxidation of SH groups lead to a slight increase in the membrane permiability causing a Ca^{2+} efflux. Probably, some of the SH groups accessible to SH-directed agents may play an important role in the maintenance of the membrane integrity.

In the case of *o*-iodosobenzoic acid oxidation, the retainment of most of the enzyme activity indicates that oxidation of -SH to -SOH does not have a significant effect on the conformation in the proximity of the active site. The extreme stability of the *o*-iodosobenzoic acid-modified enzyme can be interpreted as follows. (i) *o*-Iodosobenzoic acid oxidation does not alter the net charge in the vicinity of the active site since thiol groups are likely to exist in protonated form at pH 6.9 because of their high pK_a values, 7.5–8 [13]. (ii) Since the change in bulkiness due to oxidation of -SH to -SOH is much smaller than those caused by the blocking with bulky agents such as *N*-ethylmaleimide or DTNB, steric interference with substrate binding due to *o*-iodosobenzoic acid oxidation is negligible.

It is not clear, however, whether all of the reactive SH groups exist in the oxidation state of sulfenic acid after *o*-iodosobenzoic acid oxidation. Parker and Allison [19] have suggested, from the results of arsenite reactivation of *o*-iodosobenzoic acid-treated enzyme, that *o*-iodosobenzoic acid oxidizes a reactive SH group of glyceraldehyde-3-phosphate dehydrogenase to a stabilized sulfenic acid. There is no clear evidence for the absence of disulfide bridges in case of *o*-iodosobenzoic acid-oxidized ATPase since no significant reactivation of *o*-iodosobenzoic acid-oxidized enzyme was observed after the treatment with reducing agents. The present results, however, show that the removal of heavy metals from the reaction medium does not have any significant effect on either the number of oxidized SH groups or the remaining Ca^{2+} uptake activity if Ca^{2+} is present during *o*-iodosobenzoic acid oxidation (Table I). Thus, it is unlikely that the partial inactivation during *o*-iodosobenzoic acid oxidation is associated with heavy metal-mediated disulfide bond formation among the reactive SH groups. Recently, Scott [8] found that both $HgCl_2$ and methylmercuric chloride (CH_3HgCl) show the same stoichiometry of Ca^{2+} uptake inhibition with sarcoplasmic reticulum ATPase, indicating that SH groups blocked by $HgCl_2$ exist as $-S-Hg^+$ rather than -S-Hg-S-. These results imply that no two of the reactive SH groups are close enough to each other to form disulfide bonds.

As shown in Table I, at least one out of five *o*-iodosobenzoic acid-reactive SH groups has a distinct Ca^{2+} dependence, suggesting that this SH group might be identical to the one designated '$-SH_2$' by Yamada and Ikemoto [15] or 'SH_D' by Kawakita et al. [17]. Furthermore, the lack of additional effect of *o*-iodosobenzoic acid on $KMnO_4$ oxidation of SH groups clearly demonstrates that both *o*-iodosobenzoic acid and $KMnO_4$ oxidize the same SH groups of the enzyme (Table II). Even though it is difficult to rule out completely the possibility that SH groups or their substitute SOH are involved in the function, the results presented in this study are consistent with the conclusion that the reactive SH groups located near the active site are not directly involved in the catalytic cycle of sarcoplasmic reticulum ATPase, but are important for maintaining the active site conformation.

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