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Oxidation of thiols in the Ca^{2+}-ATPase of sarcoplasmic reticulum microsomes

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We recently showed that oxidative stress impairs the function of the sarcoplasmic reticulum to transport and retain calcium. Inhibition results primarily from oxidation of one or more thiol groups in the Ca^{2+}-ATPase. We now report that thiol oxidation does not result in disulfide formation. Oxidative inhibition of Ca^{2+}-ATPase activity was not reversed by dithiothreitol. Also, arsenite, which crosslinks dithiols, only mildly inhibited Ca^{2+}-ATPase activity and protected against inhibition by peroxydisulfate. These data suggest the thiols susceptible to oxidation are not spatially close enough to form a disulfide. Furthermore, these thiols appear to be involved in some aspect of phosphoenzyme formation. ATP, in the presence of calcium and magnesium, protected against inhibition of Ca^{2+}-ATPase activity by both oxidants and thiol-binding agents. Both inhibitors also decreased binding of the nucleotide analogue TNP-AMP after phosphorylation by P_i. Dithiothreitol and arsenite were protective. In conclusion, reversible redox regulation of the Ca^{2+}-ATPase of sarcoplasmic reticulum by thiol-disulfide exchange does not occur. However, some other mechanism of redox regulation may operate because the enzyme is sensitive to oxidants, thiol-binding agents and activity can be enhanced by prolonged exposure to dithiothreitol.

Introduction

Oxidant toxicity may result from changes in the cellular redox potential [1–4]. A major consequence could be inhibition of calcium-transporting enzymes thereby allowing intracellular calcium to rise. We tested this mechanism of oxidant toxicity in vitro by assessing the effect of oxidation on the Ca^{2+}-ATPase of sarcoplasmic reticulum isolated from lobster striated muscle.

Several studies indicated that one or two thiols of the Ca^{2+}-ATPase must be maintained in a reduced state for catalysis to occur [5–10], although there is some disagreement on this point [11–14]. Evidence for oxidative inhibition of the Ca^{2+}-ATPase due to modification of thiols includes the ability of reducing agents to prevent oxidative inhibition [15–17], a decline in thiol content which is correlated with the decline in enzyme activity [15,18], a loss of cysteine (but not other amino acids) after oxidation [7,11], and, indirectly, the ability of thiol-binding agents to inhibit enzyme activity [5–10,12,15,18–20].

In a previous report [15], we showed that moderate levels of oxidative stress reduced the function of the sarcoplasmic reticulum primarily by oxidation of thiols in the Ca^{2+}-ATPase rather than by causing lipid peroxidation or crosslinking proteins. In this study, we were particularly inter-

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TNP-AMP, 2′,3′-*O*-(2,4,6-trinitrocyclohexyldienylidine)adenosine monophosphate.

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ested in determining whether the thiols were oxidized to a disulfide. This would open the possibility of regulation of enzyme activity by reversible thiol-disulfide exchange, as has been proposed for the sarcoplasmic reticulum Ca^{2+}-ATPase by Robillard and Konings [21]. We found that this mechanism does not appear to regulate Ca^{2+}-ATPase activity. However, some other form of redox regulation involving changes in the oxidation state of thiols is probable.

We also examined the functional role of the thiols susceptible to oxidation. This was done by assessing substrate protection of Ca^{2+}-ATPase activity and binding of the fluorescent nucleotide analogue, TNP-AMP after exposure of sarcoplasmic reticulum to oxidants or thiol-binding agents. The data indicate that these thiols are involved in phosphoenzyme formation.

Materials and Methods

Chemicals of analytical grade were obtained from Sigma Chemical Co., St. Louis, MO, unless noted otherwise. Ammonium peroxydisulfate, Coomassie dye reagent and Bio-Gel P-100 were purchased from Bio-Rad, Richmond, CA, and 5,5′-dithiobis(2-nitrobenzoic acid) from Boehringer Mannheim, Indianapolis, Indiana. TNP-AMP was a gift of Drs. Nakamoto and Inesi, University of Maryland School of Medicine, Baltimore, MD.

Isolation of sarcoplasmic reticulum and determination of enzyme activity. Sarcoplasmic reticulum microsomes were isolated from lobster abdominal muscle (fast-flexor type) according to the method of Hanna et al. [22]. This preparation is a mixture of heavy and light sarcoplasmic reticulum with a substantial amount of T-tubules [23]. Ca^{2+}-ATPase activity was measured by a linked-enzyme assay which measures the decrease in absorbance by NADH or by the change in pH due to calcium-stimulated ATP hydrolysis, as described previously [15]. Protein concentration was estimated by the Coomassie blue assay [24] using bovine serum albumin as a standard.

Method of oxidation. Microsomes were suspended in a buffer consisting of 50 mM Tes, 4 mM $MgCl_2$, 100 mM KCl and 0.1 mM EGTA, pH 7.0 (buffer A) at a concentration of 1 mg/ml. The sarcoplasmic reticulum was oxidized by exposure for 1 or 15 min to 5 to 40 mM ammonium peroxydisulfate; 2 mM hydrogen peroxide; 1 mM $FeCl_3$ and 1 mM ascorbic acid; or by storage for several days at 4°C in either 100% nitrogen, 100% oxygen, or at 0°C exposed to air [15].

Assay for reversal of oxidative inhibition. To determine whether oxidative inhibition of the Ca^{2+}-ATPase was reversible, 0.5–1 mM dithiothreitol, 1 mM glutathione, or 0.5–1 mM β-mercaptoethanol was added to sarcoplasmic reticulum that had been oxidized by hydrogen peroxide, iron/ascorbate, or peroxydisulfate. The level of reducing agents used in these experiments was lower than the 5 to 10 mM concentrations used in other experiments in order to maintain a constant ratio of sarcoplasmic reticulum to reducing agent. In these experiments, reducing agents were added after the sarcoplasmic reticulum was diluted from the standard concentration of 1 mg/ml to 50 to 100 μg/ml.

In the second protocol, 5 mM dithiothreitol was added to sarcoplasmic reticulum followed by oxidation of the sarcoplasmic reticulum by 5 to 10 mM peroxydisulfate. 1 ml aliquots of oxidized sarcoplasmic reticulum were sampled over a period of 10 s to 15 min after the addition of peroxydisulfate. Aliquots were filtered through Bio-Gel P-100, using the centrifugation method of Fry et al. [25], to separate the peroxydisulfate from the sarcoplasmic reticulum. The eluant containing the sarcoplasmic reticulum was divided into two fractions. Dithiothreitol (5 mM) was added to one fraction and then both fractions were assayed for Ca^{2+}-ATPase activity.

Measurement of TNP-AMP binding. Binding of the fluorescent nucleotide analogue, 2′,3′-*O*-(2,4,6-trinitrocyclohexyldienylidine)adenosine monophosphate (TNP-AMP) to the Ca^{2+}-ATPase, was used to indicate phosphoenzyme formations following the method of Nakamoto and Inesi [26]. Although it is an AMP derivative, TNP-AMP acts similarly to the ATP analogue but has the advantage of giving a stronger fluorescent signal [26]. Upon phosphorylation of the Ca^{2+}-ATPase by either ATP or P_i, TNP-nucleotides appear to bind strongly and specifically to a nucleotide binding site [26–28]. Both ATP and P_i compete with TNP-nucleotide binding. The exact nature of the

nucleotide binding site occupied by TNP-nucleotide is unclear. The site may be the catalytic site, or to both a catalytic and regulatory nucleotide binding site, which are physically distinct, or one site which undergoes transformation upon phosphorylation [27]. The increase in the fluorescence of the TNP-AMP has been shown to be a good index of phosphoenzyme formation by both [^{32}P]ATP and P_i for the Ca^{2+}-ATPase of skeletal sarcoplasmic reticulum [26–30]. We measured TNP-AMP binding after phosphorylation of the Ca^{2+}-ATPase by P_i, rather than ATP because the fluorescence enhancement of the TNP-AMP-enzyme complex is greatest under these conditions [26].

The effect of oxidants and thiol-binding agents on phosphoenzyme formation by P_i was tested either by premixing the reagents with the sarcoplasmic reticulum before the addition of the TNP-AMP or adding them after the TNP-AMP was mixed with the sarcoplasmic reticulum and the sarcoplasmic reticulum phosphorylated. In the first protocol, sarcoplasmic reticulum at a concentration of 1 mg/ml in buffer A (pH 7.0), with or without antioxidants or reducing agents, was exposed to peroxydisulfate for 1 min. A 0.2-ml aliquot of sarcoplasmic reticulum was added to 1.8 ml of buffer A (pH 6.0) containing 1 μM TNP-AMP. The fluorescence of the probe in the presence of the non-phosphorylated enzyme was scanned from 500 to 650 nm at an excitation wavelength of 410 nm. The fluorescence of the TNP-AMP under these conditions was negligible. Phosphorylation of the Ca^{2+}-ATPase was initiated by the addition of 50 mM inorganic phosphate, diluted from phosphoric acid and adjusted to pH 6.0 with KOH. In the second protocol, the sarcoplasmic reticulum was prepared as described above except the oxidant, antioxidant or reducing agent was added after sarcoplasmic reticulum was mixed with TNP-AMP and inorganic phosphate.

Results

Irreversibility of inhibition caused by oxidants

Exposure of sarcoplasmic reticulum to peroxydisulfate results in a decease of Ca^{2+}-ATPase activity and calcium transport [15]. If the decline were due to oxidation of thiols to a disulfide, then

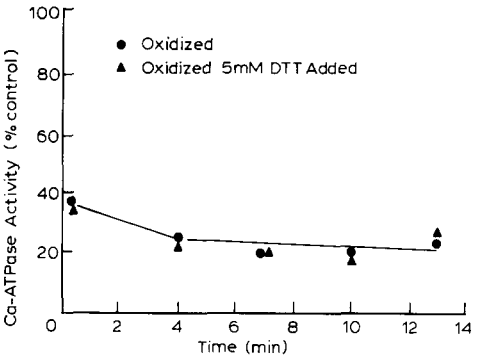

Fig. 1. Inhibition by peroxydisulfate was not reversible. Peroxydisulfate (9 mM) was added to sarcoplasmic reticulum (1 mg/ml) at the zero-time point. At the indicated times, a 0.5-ml aliquot was filtered through Bio-Gel P-100 to remove the oxidant, 5 mM dithiothreitol added to half the eluant and Ca^{2+}-ATPase activity measured. The data are representative of three separate experiments.

reducing agents should reverse oxidative inhibition. However, the reducing agents, dithiothreitol (0.5 or 1 mM), glutathione (1 mM), or β-mercaptoethanol (0.5 or 1 mM), did not reverse the inhibition of the Ca^{2+}-ATPase caused by hydrogen peroxide, iron/ascorbate or peroxydisulfate when the reducing agents were added to the reaction mixture containing the oxidant ($n = 23$).

The inability of reducing agents to reverse inhibition could have been due to the continued presence of the oxidant in the reaction mixture. In a second set of experiments, the sarcoplasmic reticulum was oxidized by peroxydisulfate followed by removal of the oxidant by gel filtration. The first aliquot was sampled as quickly as possible after the addition of peroxydisulfate because oxidation may be reversible during the early stages when disulfides exist, but not later due to the formation of higher oxidation states [31,32]. Eluant containing sarcoplasmic reticulum appeared after two 15 s centrifugations. Dithiothreitol was added to half of the eluant and both fractions assayed for Ca^{2+}-ATPase activity. Inhibition of Ca^{2+}-ATPase activity was not reversed by dithiothreitol, even during the early stages of oxidation (Fig. 1). This suggests that the inhibition did not involve disulfide formation.

Protection of aged sarcoplasmic reticulum by dithiothreitol

It is commonly observed that the Ca^{2+}-ATPase

TABLE I

EFFECT OF STORAGE-RELATED OXIDANT STRESS ON ATPase ACTIVITY

Six separate preparations of sarcoplasmic reticulum (SR) (about 10 mg protein/ml) were held on ice for 1, 2 or 6 days before dilution to 1 mg protein/ml in buffer A and measurement of Ca^{2+}-ATPase activity. SR was then stored for 3 days at 4°C under 100% nitrogen or oxygen in the presence or absence of 5 mM dithiothreitol (DTT). Data are given as a percentage of the Ca^{2+}-ATPase activity at the beginning of the storage period (the 'initial' activity), and presented as the mean + S.E., $n = 3$.

Days since isolation	Initial activity (μmol/mg per min)	Storage atmosphere	Ca^{2+}-ATPase activity (% initial activity)	
			Control	5 mM DTT
6	0.61	oxygen	84 ± 3	186 ± 8
		nitrogen	111 ± 2	198 ± 3
2	0.90	oxygen	66 ± 1	100 ± 2
1	1.02	oxygen	62 ± 2	105 ± 0
		nitrogen	63 ± 2	110 ± 3
1	1.16	oxygen	78 ± 3	101 ± 2
		nitrogen	98 ± 3	123 ± 3
1	1.48	oxygen	47 ± 3	62 ± 0
		nitrogen	65 ± 1	72 ± 1
1	1.74	oxygen	73 ± 2	87 ± 0
		nitrogen	76 ± 3	80 ± 1

activity of sarcoplasmic reticulum declines over time. The activity of sarcoplasmic reticulum stored for 3 days under oxygen was less than that of the corresponding samples stored under nitrogen, as would be expected if the decline in activity resulted from an oxidative process (Table I). Sarcoplasmic reticulum which was stored with dithiothreitol under either nitrogen or oxygen had a higher Ca^{2+}-ATPase activity than control sarcoplasmic reticulum stored without dithiothreitol. The protective effect of dithiothreitol was titratable (data not shown).

The degree of activation of different sarcoplasmic reticulum preparations stored with dithiothreitol varied inversely with the activity of the sarcoplasmic reticulum measured at the beginning of the storage period (the initial Ca^{2+}-ATPase activity). In some cases, the Ca^{2+}-ATPase activity of aged sarcoplasmic reticulum stored for 3 days with dithiothreitol was higher than the initial activity.

Protection of oxidized sarcoplasmic reticulum by arsenite

We measured Ca^{2+}-ATPase activity after exposure of sarcoplasmic reticulum to arsenite as an additional test of whether the thiols susceptible to oxidation formed a disulfide. Arsenite has an especially high affinity for dithiols. Enzymes having essential thiols spatially close enough to form a disulfide are strongly inhibited by low (10^{-5} M) concentrations of arsenite [33]. In addition, arsenite is reported to reduce monothiols which have been oxidized to sulfenic acid (SOH) to thiols [34,35]. Arsenite does not reduce disulfides [33].

Ca^{2+}-ATPase activity was only mildly inhibited by arsenite, and the degree of inhibition was time dependent. Sarcoplasmic reticulum exposed to 2 mM sodium arsenite for 50 min had a Ca^{2+}-ATPase activity 90% of control, declining to 58% of control after 124 min. In contrast, much lower levels of arsenite would be expected to cause an immediate decline in activity if the essential thiols of the Ca^{2+}-ATPase existed as dithiols.

Although arsenite had relatively little effect on Ca^{2+}-ATPase activity in non-oxidized sarcoplasmic reticulum, it protected the Ca^{2+}-ATPase activity of oxidized sarcoplasmic reticulum. Addition of 2 mM arsenite to sarcoplasmic reticulum immediately before exposure for 1 min to various concentrations of peroxydisulfate increased Ca^{2+}-ATPase activity by an average of 27 ± 3% ($n = 6$) over the activity of sarcoplasmic reticulum samples oxidized in the absence of arsenite. The observation that arsenite protected the Ca^{2+}-ATPase activity against oxidation by peroxydisulfate suggests that the thiols might be oxidized to sulfenic acid. However, the possibility that arsenite protected the enzyme by reduction of peroxydisulfate, the sulfate radical, or peroxymonosulfate, cannot be excluded. We consider this unlikely because peroxydisulfate was present in stoichiometrically much higher amounts than arsenite, typically a 10-fold excess. In addition, the inability of antioxidants to protect against oxidative inhibition [15] argues against the protective effect of arsenite being caused by inactivation of the oxidant.

Effect of oxidation on phosphoenzyme formation

We used two approaches to determine if phos-

TABLE II

ADENINE NUCLEOTIDE PROTECTS Ca^{2+}-ATPase

The Ca^{2+}-ATPase activity was measured in sarcoplasmic reticulum (SR, 1 mg/ml) in buffer A plus 2 mM ATP, 5 mM $MgCl_2$; 2 mM ATP, 5 mM $MgCl_2$, and/or 1 mM hydrogen peroxide, 9 mM peroxydisulfate, 0.1 mM DTNB, or stored for 4 days in air. The data are representative of three trails using hydrogen peroxide, four trials using peroxydisulfate, two trials using DTNB, and three trials in which the SR was aged. Data are presented as the mean ± S.E., $n = 3$.

Treatment	Ca^{2+}-ATPase activity (% non-oxidized control)			
Inhibitor:	H_2O_2	$S_2O_8^{4-}$	aged	DTNB
No additions	53±2	52±1	10±0	49±18
Ca^{2+}	52±1	–	6±1	44± 5
Mg^{2+}-ATP	50±5	67±1	62±1	68±10
(Ca^{2+}-Mg^{2+})-ATP	69±3	73±3	63±3	88±18

phoenzyme formation was affected by oxidation: (1) assess whether substrates of the Ca^{2+}-ATPase would protect against oxidative inhibition, and (2) measure the effect of oxidation on TNP-AMP fluorescence. We considered that oxidative modification of the Ca^{2+} binding sites was unlikely because the K_a for Ca^{2+} binding ($\bar{x} = 4.0 \pm 0.5$ μmol; range 1.3–8.2 μmol) was not correlated with the degree of oxidative inhibition of Ca^{2+}-ATPase activity (correlation coefficient = −0.25, $n = 18$).

In order to test substrate protection, the sarcoplasmic reticulum was preincubated with (Ca^{2+}-Mg^{2+})-ATP, Mg^{2+}-ATP or Ca^{2+} before oxidation by peroxydisulfate, hydrogen peroxide or before storage. The Ca^{2+}-ATPase activity of oxidized or aged sarcoplasmic reticulum was protected by Ca^{2+}-Mg^{2+}-ATP and, except in the case of oxidation by hydrogen peroxide, by Mg^{2+}-ATP (Table II). However, Ca^{2+} alone was not protective. If ATP protected the catalytic site by masking thiols, then it should also protect the Ca^{2+}-ATPase from inactivation by the thiol-binding agent, DTNB. This was the case, and again Ca^{2+} alone was ineffective. The ability of (Ca^{2+}-Mg^{2+})-ATP to reduce the inhibition of the Ca^{2+}-ATPase by oxidants, storage or DTNB suggests that oxidants and thiol-binding agents both modified the nucleotide binding site.

This possibility was tested further by measuring the effect of oxidation on TNP-AMP fluorescence

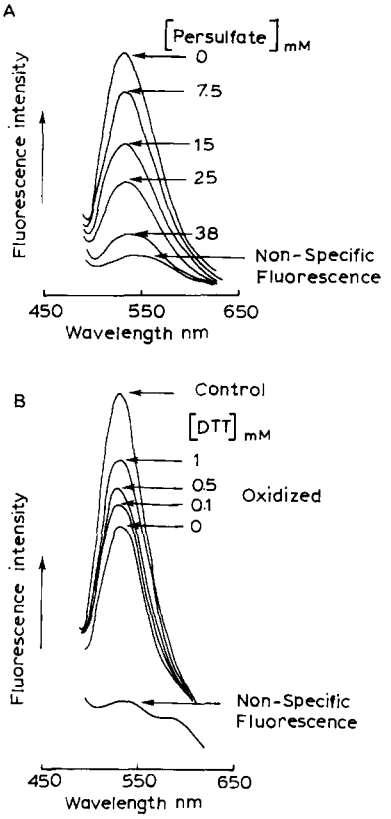

Fig. 2. Oxidation decreased TNP-AMP binding. The fluorescence spectrum of 1 μM TNP-AMP in buffer A is shown. Non-specific fluorescence was measured before phosphorylation of the Ca^{2+}-ATPase was initiated by the addition of 50 mM P_i. In (A), the sarcoplasmic reticulum was exposed to various concentrations of peroxydisulfate. This trace is representative of three trials in which peroxydisulfate was added before the sarcoplasmic reticulum was mixed with TNP-AMP and four trials in which the oxidant was added after phosphorylation. Identical results were obtained regardless of the time of addition. In (B), the sarcoplasmic reticulum was preincubated with various concentrations of dithiothreitol before exposure to 25 mM peroxydisulfate. This trace is representative of nine trials.

after phosphorylation by P_i. Preincubation of the sarcoplasmic reticulum with peroxydisulfate before the addition of TNP-AMP, or addition of the oxidant to the phosphoenzyme in the presence of TNP-AMP, decreased the fluorescence relative to the fluorescence of non-oxidized sarcoplasmic reticulum in a titratable manner (Fig. 2A). Peroxydisulfate had no effect on TNP-AMP fluorescence in the absence of sarcoplasmic reticulum.

The decrease in fluorescence caused by per-

oxydisulfate was prevented by preincubation of the sarcoplasmic reticulum with dithiothreitol and this protective effect was titratable (Fig. 2B). The sarcoplasmic reticulum was routinely incubated with dithiothreitol for 30 min before the addition of peroxydisulfate in order to obtain maximal protection. Addition of dithiothreitol after peroxydisulfate did not reverse the inhibition. Also the antioxidants thiourea (10 mM), butylated hydroxytoluene (20 μM), and α-tocopherol (10 mM) failed to prevent the decline in fluorescence caused by the oxidant (data not shown). The ability of dithiothreitol but not antioxidants, to increase the TNP-AMP fluorescence of oxidized sarcoplasmic reticulum suggests that thiols are necessary for phosphoenzyme formation by P_i.

The TNP-AMP fluorescence and Ca^{2+}-ATPase activity were measured in samples of sarcoplasmic reticulum stored with dithiothreitol, butylated hydroxytoluene, α-tocopherol, α-tocopherol, or Mn^{2+}. None of the antioxidants prevented the decline in Ca^{2+}-ATPase activity or fluorescence which occurred during storage; however, dithiothreitol was protective (Table III).

Preincubation of sarcoplasmic reticulum with arsenite before exposure to peroxydisulfate enhanced TNP-AMP fluorescence of enzyme phosphorylated by P_i. The fluorescence of sarcoplasmic reticulum oxidized by 40 mM peroxydisulfate in the presence of 4 mM or 8 mM arsenite was 70% and 96%, respectively, of the fluorescence of non-oxidized sarcoplasmic reticulum. This compares to a value of 44% in sarcoplasmic reticulum oxidized in the absence of arsenite. Higher levels of arsenite were not as effective in preventing the decrease in fluorescence of oxidized sarcoplasmic reticulum.

TABLE III

TNP-AMP FLUORESCENCE OF AGED SARCOPLASMIC RETICULUM

Sarcoplasmic reticulum (1 mg/ml) was stored in buffer A or buffer A plus either dithiothreitol, butylated hydroxytoluene, $MnCl_2$, or α-tocopherol (the concentration is approximate because this concentration exceeds the solubility) for 4 days at 0°C. At the end of the period of Ca^{2+}-ATPase activity and TNP-AMP fluorescence were assayed in triplicate on each sample. Data are presented as the mean ± S.E., $n = 3$.

Incubation conditions	Fluorescence intensity (relative units)	Ca^{2+}-ATPase activity (μmol/mg per min)
Control	100	1.95 ± 7
5 mM dithiothreitol	215 ± 19	2.63 ± 7
20 μM butylated hydroxytoluene	124 ± 13	2.05 ± 9
10 mM α-tocopherol	128 ± 12	1.99 ± 19
250 μM $MnCl_2$	58 ± 13	1.42 ± 3

The TNP-AMP fluorescence of sarcoplasmic reticulum exposed to thiol-binding agents was measured to determine the effect of direct modification of thiols. Exposure of sarcoplasmic reticulum phosphorylated by P_i to 5 μM *p*-chloromercuriphenylsulfonic acid resulted in a 54 ± 2% ($n = 4$) decline in TNP-AMP fluorescence. Subsequent addition of dithiothreitol increased the fluorescence to 93 ± 1% ($n = 4$) of control. Similar results were obtained when the sarcoplasmic reticulum was exposed to 5 μM Ag^+ (data not shown). Therefore, direct modification of thiols can prevent the fluorescence increase following phosphorylation by P_i.

Discussion

Both peroxydisulfate and thiol-blocking agents inhibited Ca^{2+}-ATPase activity and TNP-AMP binding. Prior incubation of sarcoplasmic reticulum with dithiothreitol, (Ca^{2+}-Mg^{2+})-ATP or arsenite reduced the level of both types of inhibition. These data are consistent with inhibition by peroxydisulfate occurring primarily by thiol oxidation. We do not suggest that peroxydisulfate only oxidized thiols, however, we previously showed that lipid peroxidation and crosslinking of proteins are not correlated with inhibition of sarcoplasmic reticulum function under these conditions [15].

The thiols are probably not oxidized to a disulfide because: (1) dithiothreitol did not reverse inhibition caused by oxidants, and (2) Ca^{2+}-ATPase activity was only mildly inhibited by millimolar levels of arsenite. Moreover, arsenite protected against oxidative inhibition. This is consistent with oxidation of the thiols to sulfenic acid, however, we did not measure the oxidation state of the sulfur. Arsenite has previously been shown to reverse oxidative inhibition of enzymes [34,35], but to our knowledge has not been reported to

protect enzymes from oxidative inhibition.

The conclusion that inhibition of the Ca^{2+}-ATPase does not involve disulfide formation is supported by previous reports which determined the sensitivity of the Ca^{2+}-ATPase to mercurials with different chain lengths [11] and changes in cysteine content during isolation of the sarcoplasmic reticulum [7].

We found that storage of sarcoplasmic reticulum isolated form lobster with dithiothreitol preserved Ca^{2+}-ATPase activity. This confirms the findings of Van der Kloot [17]. In contrast, Sreter et al. [16] found no protection by dithiothreitol of rabbit white sarcoplasmic reticulum or cardiac sarcoplasmic reticulum, although there was modest protection of rabbit red muscle sarcoplasmic reticulum.

The degree of activation of Ca^{2+}-ATPase activity by dithiothreitol was generally greater in preparations with low Ca^{2+}-ATPase activities at the start of the experimental storage period. In these samples storage with dithiothreitol could increase Ca^{2+}-ATPase activity above the initial rate. We interpret the variation in the ability of dithiothreitol to enhance Ca^{2+}-ATPase activity to reflect the extent of inhibition which occurred during isolation and storage of the sarcoplasmic reticulum before the experiment began. Stimulation of Ca^{2+}-ATPase level above the initial rate suggests that prolonged exposure to reductants can re-activate the enzyme.

The thiols susceptible to oxidation appear to be involved in some aspect of phosphoenzyme formation. TNP-AMP fluorescence following phosphorylation by P_i was reduced in sarcoplasmic reticulum exposed to peroxydisulfate or thiol-binding agents. Also, (Ca^{2+}-Mg^{2+})-ATP partially protected Ca^{2+}-ATPase activity from inhibition by oxidants, aging and thiol-binding agents. This is the first report of nucleotide protection of Ca^{2+}-ATPase activity against oxidative inhibition, although protection against thiol-binding agents is well known [7,10–12]. Yamada and Ikemoto reported that thiol groups are essential for phosphorylation and dephosphorylation of the Ca^{2+}-ATPase of skeletal muscle, based on *N*-ethylmaleimide binding [5].

The interpretation that the susceptible thiols are involved in phosphoenzyme formation is reasonable based on the structure of the phosphorylation domain of the Ca^{2+}-ATPase. A cysteine is located one amino acid residue way from the aspartate which is phosphorylated [36]. The ($Na^+ + K^+$)-ATPase from dog kidney, the ($H^+ + K^+$)-ATPase from dog gastric mucosa and the corn-root ATPase also have a cysteine at this position [37], and so may be inhibited by oxidation in the same manner.

The effect of oxidation on phosphoenzyme formation in a Ca^{2+}-ATPase has been examined in one other study. Okabe et al. [38] examined changes in the Ca^{2+}-ATPase of cardiac sarcoplasmic reticulum after oxidation by superoxide. They reported that oxidation did not decrease amount of phosphoenzyme formation by [^{32}P]ATP, although there was a decline in enzyme turnover. In contrast, we found that oxidation decreased TNP-AMP binding after phosphorylation by P_i. The difference between our results may be due to ATP and P_i phosphorylating different conformations of the enzyme or at different sites [39]. It is not possible to rule out that oxidation interfers directly with TNP-AMP binding without affecting phosphorylation because there is still some question as to the binding site of the probe [27].

Another key to the location of oxidized thiols is their reactivity to oxidants. Benisek [40] suggested that the oxidant, permanganate, selectively reacts with thiol groups located at the phosphorylation site of an enzyme because permanganate is structurally similar to P_i. This would explain the extreme sensitivity of the Ca^{2+}-ATPase to inhibition by permanganate reported by Ariki and Shamoo [11]. In our study it is possible that peroxydisulfate and its breakdown products (sulfate, the sulfate radical and peroxymonosulfate) act as structural analogues of phosphate and preferentially oxidize thiols at the phosphorylation site. Fig. 3 shows a possible scheme of oxidant species involved in these reactions.

In summary, these results suggest that the Ca^{2+}-ATPase of striated muscle has one or more thiols which must be in the reduced state for maximal enzyme activity. These thiols appear to be involved in some aspect of phosphoenzyme formation. Oxidation does not result in disulfide formation, thereby ruling out thiol-disulfide ex-

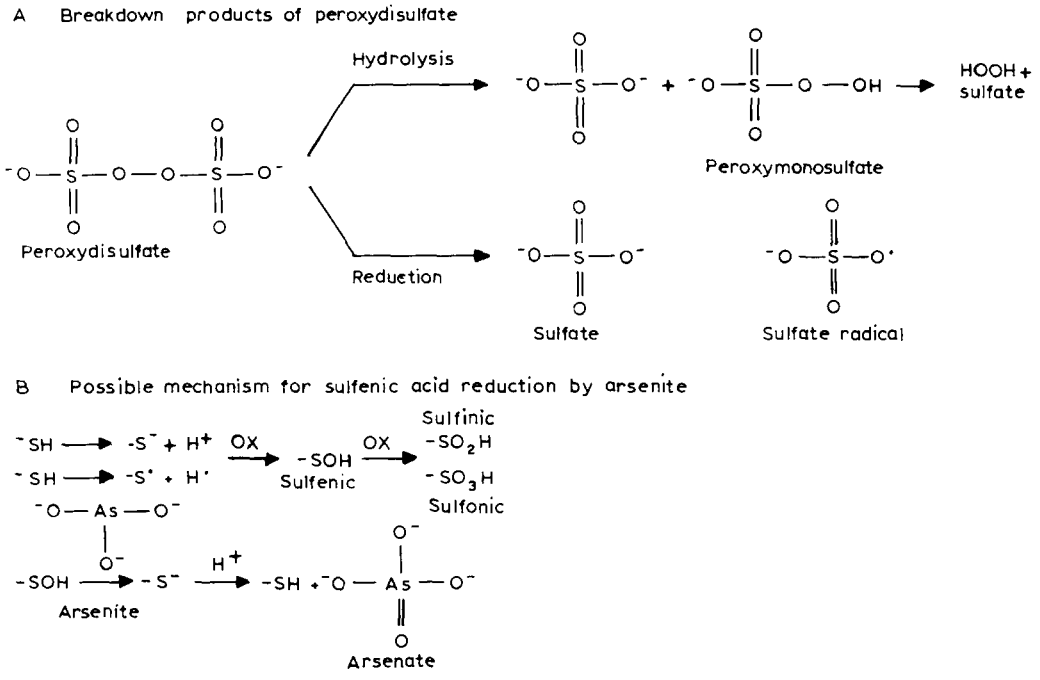

Fig. 3. Scheme for radical formation and reduction. (A) Peroxydisulfate can undergo either hydrolysis or reduction to form peroxides or sulfate radicals. (B) Thiols can be oxidized sequentially to sulfenic acid, sulfonic and sulfonic acid. Arsenite may protect against this form of oxidant stress by reduction of sulfenic groups to thiols.

change as a mechanism for reversible regulation of enzyme activity. However, the stimulatory effect of dithiothreitol on aged sarcoplasmic reticulum suggests that some other form of redox regulation of the Ca^{2+}-ATPase may occur.

Acknowledgments

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