The Importance of Carboxyl Groups on the Luminal Side of the Membrane for the Function of the Ca\(^{2+}\)-ATPase of Sarcoplasmic Reticulum*

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The conventional model for transport of Ca\(^{2+}\) by the Ca\(^{2+}\)-ATPase of skeletal muscle sarcoplasmic reticulum (SR) involves a pair of binding sites for Ca\(^{2+}\) that change upon phosphorylation of the ATPase from being high affinity and exposed to the cytoplasm to being low affinity and exposed to the lumen. However, a number of recent experiments suggest that in fact transport involves two separate pairs of binding sites for Ca\(^{2+}\), one pair exposed to the cytoplasmic side and the other pair exposed to the luminal side. Here we show that the carbodiimide 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide (EDC) is membrane-impermeable, and we use EDC to distinguish between cytoplasmic and luminal sites of reaction. Modification of the Ca\(^{2+}\)-ATPase in sealed SR vesicles with EDC leads to loss of ATPase activity without modification of the pair of high affinity Ca\(^{2+}\)-binding sites. Modification of the purified ATPase in unsealed membrane fragments was faster than modification in SR vesicles, suggesting the presence of more quickly reacting luminal sites. This was confirmed in experiments measuring EDC modification of the ATPase reconstituted randomly into sealed lipid vesicles. Modification of sites on the luminal face of the ATPase led to loss of the Ca\(^{2+}\)-induced increase in phosphorylation by P\(_{i}\). It is concluded that carboxyl groups on the luminal side of the ATPase are involved in Ca\(^{2+}\) binding to the luminal side of the ATPase and that modification of these sites leads to loss of ATPase activity. The presence of MgATP or MgADP leads to faster inhibition of the ATPase by EDC in unsealed membrane fragments than in sealed vesicles, suggesting that binding of MgATP or MgADP to the ATPase leads to a conformational change on the luminal side of the membrane.

The conventional view of Ca\(^{2+}\) pumping by the sarcoplasmic/endoplasmic reticulum class of Ca\(^{2+}\)-ATPases, as expressed in the alternating Ca\(^{2+}\)-binding site model, is that a pair of Ca\(^{2+}\)-binding sites change upon phosphorylation of the ATPase from a state in which they face the cytoplasm to a state in which they face the lumen of the endoplasmic or sarcoplasmic reticulum (1). The change in orientation of the sites is linked to a change in the affinity of the sites, from high affinity when facing the cytoplasm to low affinity when facing the lumen. Thus the unphosphorylated ATPase binds two Ca\(^{2+}\) ions from the cytoplasmic side of the membrane, where the Ca\(^{2+}\) ion concentration is \(\mu\)M, and, following phosphorylation by ATP, transports the two Ca\(^{2+}\) ions across the membrane, releasing them on the luminal side where the Ca\(^{2+}\) concentration is mM. However, recent experiments have identified problems with this model. In particular, the alternating site model predicts that binding of Ca\(^{2+}\) from the cytoplasmic side of the membrane should be competitive with binding of Ca\(^{2+}\) from the luminal side, but a variety of equilibrium and kinetic experiments have shown that binding from the two sides of the membrane is, in fact, noncompetitive (2–4). These data are consistent with a four-site model for the ATPase, with separate pairs of binding sites for Ca\(^{2+}\) on the two sides of the membrane; transport of Ca\(^{2+}\) by the Ca\(^{2+}\)-ATPase then corresponds to transfer of Ca\(^{2+}\) from the cytoplasmic pair of sites to the luminal pair of sites, driven by phosphorylation of the ATPase by ATP (Fig. 1) (2, 3).

The 10 putative transmembrane \(\alpha\)-helices in the Ca\(^{2+}\)-ATPase contain four negatively charged residues, in helices M4, M5, M6, and M8, and mutation of these residues blocks high affinity Ca\(^{2+}\) binding to the ATPase. These residues are therefore presumed to constitute the pair of binding sites to which Ca\(^{2+}\) ions bind from the cytoplasmic side of the membrane (5). It was first assumed that two of these acidic residues would be present at each of the two Ca\(^{2+}\) ion-binding sites and that the two binding sites would be located one above the other because binding of Ca\(^{2+}\) to the ATPase is sequential (5, 6). Subsequent studies have, however, suggested a peripheral role for helix M8 in Ca\(^{2+}\) binding (7, 8) consistent with a side-by-side arrangement for the two Ca\(^{2+}\) ion-binding sites (8); a side-by-side arrangement is also consistent with recent cryo-electron microscopic studies of the Ca\(^{2+}\)-ATPase (9). As well as the residues in the transmembrane region, it has been suggested that Asp residues in the loop joining helices M6 and M7 on the cytoplasmic side of the membrane could make up part of the cytoplasmic pair of Ca\(^{2+}\)-binding sites (10).

Because the only four acidic residues in the transmembrane region of the ATPase are involved in the cytoplasmic pair of binding sites, acidic residues at the pair of low affinity sites must correspond to residues on the luminal side of the membrane. Only about 10% of the mass of the Ca\(^{2+}\)-ATPase is located on the luminal side (9), and this is predicted to consist mainly of small loops between the transmembrane \(\alpha\)-helices but with a relatively large loop between helices M7 and M8 (Fig. 1). Mutagenesis of polar residues on the luminal side of the ATPase failed to identify any whose mutation blocked accumulation of Ca\(^{2+}\) driven by hydrolysis of ATP (7).

Chemical labeling of residues has often been found to have larger effects on activity than mutagenesis, presumably because of steric effects associated with the relatively bulky labeling reagents; good examples are provided by studies of the labeling of Cys residues in lac permease (11). Here we have used labeling by a carbodiimide to show the presence of amino

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Acid residues on the luminal side of the ATPase, critical for function. Labeling of the Ca\(^{2+}\)-ATPase with the hydrophobic carbodiimides dicyclohexylcarbodiimide and N-cyclohexyl-N'-(4-dimethylamino-1-naphthyl)carbodiimide is competitive with binding of Ca\(^{2+}\) at the high affinity sites, and so these carbodiimides are likely to modify carboxyl groups at the high affinity Ca\(^{2+}\)-binding sites (12–14). The Ca\(^{2+}\)-ATPase is also inactivated by reaction with the water-soluble 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide (EDC)\(^1\) (15). Here we make use of the membrane impermeability of EDC to distinguish between the effects of modifying carboxyl groups on the cytoplasmic and luminal sides of the Ca\(^{2+}\)-ATPase and show that modification of carboxyl groups on the luminal side of the membrane inhibits ATPase activity and Ca\(^{2+}\)-dependent phosphorylation by P\(_i\). These results are consistent with the four-site model for the Ca\(^{2+}\)-ATPase.

**MATERIALS AND METHODS**

Octaethylene glycol-n-dodecyl ether (C\(_n\)_E\(_k\)) and n-octyl-\(\beta\)-D-glucoside were obtained from Calbiochem and Sigma, respectively. Dioleoylphosphatidylcholine (DOPC) was from Avanti Polar Lipids, and 4-bromomethyl-6,7-dimethoxycoumarin (Br-DMC) was from Molecular Probes. Sarcoplasmic reticulum (SR) vesicles were prepared from rabbit skeletal muscle as described in Ref. 16, and Ca\(^{2+}\)-ATPase was reconstituted in skeletal muscle as described in Ref. 16, and Ca\(^{2+}\) was added by incubation in 50 mM Tris, 200 mM sucrose, and the required concentration of Ca\(^{2+}\) at pH 7.0 for 2 h at a protein concentration of 40 mg protein/ml.

ATPase activities were determined at 25 °C using a coupled enzyme assay in a medium containing 40 mM Hepes/KOH, pH 7.2, 100 mM KCl, 5 mM MgSO\(_4\), 2.1 mM ATP, 1.1 mM EGTA, 0.53 mM phosphoenolpyruvate, 0.15 mM NADH, pyruvate kinase (7.5 IU), and lactate dehydrogenase (18 IU) in a total volume of 2.5 ml. The reaction was initiated by the addition of an aliquot of a 25 mM CaCl\(_2\) solution to a cuvette containing the ATPase and the other reagents to give a maximally stimulating concentration of Ca\(^{2+}\) (free Ca\(^{2+}\) concentration was ~10 \(\mu\)M).

Large unilamellar vesicles were prepared by suspending 18 mg of DOPC in 1.1 ml of buffer (10 mM Pipes, pH 4.0, 100 mM K\(_2\)SO\(_4\)) and extruding 15 times through a 100-nm pore polycarbonate filter in an Avivin mini-extruder. Fluorescein or EDC was trapped in the vesicles by including them in the original suspension buffer; untrapped reagent was removed by passing the vesicles through two 5-ml columns of coarse Sephadex G50. Samples were diluted 12-fold into buffer, and the fluorescence was recorded, exciting fluorescence at 481 nm, recording the fluorescence intensity at 507 nm. When required, vesicles were made leaky by addition of 1.2 mM C\(_{12}\)E\(_8\). ATPase Labeling—SR or ATPase (6 mg protein/ml) were incubated with EDC (2.4 mM) at 37 °C in buffer (100 mM Mops, pH 6.8, 2 mM EGTA, 80 mM KCl, 1.8 mM CaCl\(_2\)) for the stated times. The reaction was stopped either by dilution into the ATPase assay mix or by centrifugation of the reaction mixture through two columns containing coarse Sephadex G-50, pre-equilibrated in buffer. The Ca\(^{2+}\)-ATPase was labeled with Br-DMC by addition of a stock solution of Br-DMC in dimethylformamide to SR (8 mg protein/ml) in buffer (50 mM Tris, pH 7.0, 200 mM sucrose) to an initial molar ratio of DMC/SR of 10:1. The mixture was incubated at room temperature, and the unreacted Br-DMC was removed by centrifugation through two Sephadex G-50 columns. The labeling ratio was estimated using extinction coefficients of 1.2 liter g\(^{-1}\) cm\(^{-1}\) and 12,900 m\(^{-1}\) cm\(^{-1}\) for protein and DMC, respectively, in 1% (w/v) SDS (19).

Fluorescence intensities were recorded by using an SLM 8000C spectrophotometer. DMC fluorescence was excited at 350 nm and observed at 425 nm; tryptophan fluorescence was excited at 290 nm and observed at 340 nm; and fluorescein fluorescence was excited at 495 nm and observed at 525 nm.

**RESULTS**

**EDC Is Membrane-impermeable**—The positive charge on the water-soluble EDC would be expected to make it membrane-impermeable. This was confirmed by studying the reaction between EDC and the carboxyl-containing fluorescence probe fluorescein, with either EDC or fluorescein trapped in unilamellar lipid vesicles. The presence of the -OH and -COOH groups on fluorescein (for structure see Fig. 2) make its fluorescence intensity pH-sensitive; addition of NaOH to increase the pH of a solution of fluorescein from four to ten results in a ~24-fold increase in fluorescence intensity (Fig. 2, curve a). Addition of 2 mM EDC immediately before the addition of NaOH leads to a 32% decrease in the response to NaOH (Fig. 2, curve c). Increasing the time of reaction with EDC or increasing the concentration of EDC had no significant effect on the change in response to NaOH; we therefore conclude that formation of the acylisourea derivative of fluorescein results in a 32% lower fluorescence intensity at high pH. It should also be noted that the rate of reaction of EDC with carboxyl groups is fastest in the pH range 3.5–4.5 (20).

Fig. 2A also shows the results of an experiment in which 200 mM EDC was trapped inside large unilamellar vesicles, which were then diluted 12-fold into pH 4.0 buffer containing 1 \(\mu\)M fluorescein. The sample was incubated for 1 h, and then NaOH was added to increase the pH to 10; as shown (curve b), the observed increase in fluorescence intensity was the same as for fluorescein alone in the absence of EDC (curve a), showing that over the 1-h time period of the incubation EDC did not leak out...
of the vesicles. If the detergent C12E8 was added immediately before the addition of NaOH to make the vesicles leaky to EDC, the response to NaOH was reduced (curve d) as seen previously (curve c). The experiment was repeated with fluorescein (0.1 mM) trapped in the vesicles (Fig. 2B), using carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) to equilibrate protons across the membrane. Dilution of the vesicles into buffer at pH 4.0 followed by addition of NaOH resulted in only a small increase in fluorescence intensity, showing that the majority of the fluorescein was trapped in the vesicles; subsequent addition of FCCP (0.25 μM) resulted in a large increase in fluorescence intensity, showing that the majority of the fluorescein was trapped in the vesicles; subsequent addition of FCCP and NaOH at the time marked by the arrow. Curve d shows an experiment in which vesicles containing EDC were diluted 12-fold into buffer containing fluorescein at pH 4.0 and incubated for 1 h before addition of NaOH at the time marked by the arrow. Curve c shows an experiment in which NaOH was added to a solution of fluorescein (1 μM) in buffer at pH 4.0 to increase the pH to 10, in the absence (a) or presence (c) of 2 mM EDC. Curve b shows an experiment in which vesicles containing EDC were diluted 12-fold into buffer containing fluorescein at pH 4.0 and incubated for 1 h before addition of NaOH, B, fluorescein (0.1 mM) was trapped inside vesicles at pH 4.0 and then diluted 12-fold into buffer at pH 4.0. Curve c shows an experiment in which NaOH was added followed by FCCP to make the vesicles leaky to protons. Curve a shows an experiment in which EDC was added first followed by NaOH. Curve b shows an experiment in which the vesicles were diluted into buffer at pH 4.0 containing 2 mM EDC and incubated for 1 h before addition of FCCP and NaOH at the time given by the arrow.

**EDC Does Not React with the High Affinity Ca2+-binding Sites on the Ca2+-ATPase—Ca2+ binding to the high affinity sites on the Ca2+-ATPase can be conveniently monitored from the change in tryptophan fluorescence intensity observed on Ca2+ binding to the ATPase (21–24). Labeling the ATPase with excess EDC had no significant effect on the measured affinity for Ca2+ (Fig. 3). Maximal levels of Ca2+ binding to the ATPase were determined by incubation with 50 μM 45Ca2+ at pH 7.2, as described in Starling et al. (25). The levels of binding observed for unlabeled and EDC-labeled ATPase were 7.5 and 6.0 nmol/mg protein, respectively.

**Effect of EDC on ATPase Activity—Coan et al. (15) have shown that incubation of SR vesicles with EDC leads to loss of ATPase activity. This is confirmed by the data shown in Fig. 4. SR vesicles were incubated with 2.4 mM EDC at pH 6.8 and 37 °C for the given periods of time, and then 2 μl of the sample were diluted into 3 ml of the ATPase assay mixture to stop the reaction and to determine the remaining ATPase activity. Reaction with EDC leads to almost complete loss of ATPase activity, at a rate of 0.07 ± 0.01 min−1 (Fig. 4). When the same experiment was repeated but with the purified Ca2+-ATPase, present as unsealed membrane fragments, inhibition was found to be somewhat faster, with a rate of 0.10 ± 0.01 min−1. These experiments suggest the presence of more quickly reacting sites on the Ca2+-ATPase, accessible to EDC in unsealed membrane fragments but inaccessible in sealed SR vesicles. This possibility was explored further by studying the reaction with the Ca2+-ATPase reconstituted into sealed phospholipid vesicles.

Reconstituted vesicles were prepared by dissolving DOPC in buffer containing a relatively high (40 mM) concentration of octyl-β-D-glucoside and then mixing with SR solubilized in C12E8. Removal of detergent by the addition of SM2 Bio-Beads led to the formation of tightly sealed vesicles containing the Ca2+-ATPase (26). The reconstituted vesicles are impermeable to ATP so that only outwardly facing ATPase molecules hydrolyze ATP added to the external medium. The reconstitution procedure gives a random incorporation of ATPase molecules between the two halves of the bilayer, as shown by a doubling of ATPase activity upon addition of low concentrations of C12E8 (0.8 mg/ml) to make the vesicles leaky to ATP (26); C12E8 at this concentration had no significant effect on the activity of the purified Ca2+-ATPase (27). Reconstituted vesicles were incubated with EDC and then diluted into the ATPase assay mixture to determine the ATPase activity of the outward facing
ATPase molecules. C_{12}E_8 (0.8 mg/ml) was then added to give the total ATPase activity; subtraction of the activity measured in the absence of C_{12}E_8 from that measured in the presence of C_{12}E_8 gave the activity of the inward facing ATPase molecules. The time course of inhibition of the ATPase activity of the outward facing ATPase molecules was found to be identical to that of the ATPase in SR vesicles (0.06 ± 0.01 min^{-1}), but for the inward facing ATPase molecules the maximum inhibition was only 80%, and the time course of modification was faster, with a rate (0.11 ± 0.02 min^{-1}) the same as that for the purified ATPase (Fig. 4). The observation that the inward facing ATPase molecules are inhibited by EDC shows that sites on the luminal side of the ATPase react with EDC, leading to inhibition of the ATPase.

Reaction of a carbodiimide with an acidic amino acid residue gives an initial unstable O-acylisourea, which can then undergo further reaction, including reaction with an endogenous nucleophile to give an intramolecular cross-linked product (28). To reduce the possibility of intramolecular cross-linking, reactions with EDC were also performed in the presence of 5 mM glycine ethyl ester; as shown in Fig. 4, this had no effect on the rate or level of inhibition of ATPase activity.

Effect of EDC on Ca^{2+} Dependence of Phosphorylation of the ATPase by P_i—The Ca^{2+}-ATPase can be phosphorylated by P_i in a Mg^{2+}-dependent reaction in the absence of Ca^{2+} ions on the cytoplasmic side of the membrane; binding of Ca^{2+} to the high affinity binding sites on the Ca^{2+-ATPase inhibits this phosphorylation (1). In contrast, binding of Ca^{2+} to the low affinity, luminal pair of Ca^{2+}-binding sites on the ATPase leads to increased levels of phosphorylation by P_i, an effect particularly marked at neutral pH values where the levels of phosphorylation are otherwise low (2, 4, 29). Levels of phosphorylation can either be measured directly using ^{32}P_{i} or through the decrease in the fluorescence intensity of DMC-labeled ATPase, which occurs upon phosphorylation (4). DMC-labeled SR vesicles were loaded with Ca^{2+} and diluted to 0.12 mg protein/ml in buffer containing 40 mM Tris, pH 7.0, 6 mM EGTA, and 20 mM Mg^{2+}, and the change in EDC fluorescence upon addition of 10 mM P_i was recorded. As shown in Fig. 5 the magnitude of the fluorescence response increased with increasing Ca^{2+} load, a maximal change of about 4.8% being observed in the presence of 4 mM Ca^{2+}.

Coan et al. (15) have reported that modification of SR vesicles with EDC (at cytoplasmic sites) blocks phosphorylation with ^{32}P_{i}, and in agreement, addition of P_i to DMC-labeled EDC-modified SR resulted in no significant change in fluorescence intensity (data not shown). To study the effect of EDC modification of the luminal sites on the response of phosphorylation to Ca^{2+}, DMC-labeled ATPase was reconstituted into sealed vesicles of DOPC in the presence of 10 mM P_i and 10 mM Mg^{2+}, trapping P_i and Mg^{2+} within the reconstituted vesicles. Reconstituted vesicles were then diluted into buffer (10 mM Pipes, pH 7.1, 100 mM K_2SO_4), and the response to the addition of Ca^{2+} was recorded. Under these conditions, binding of Ca^{2+} to luminal sites on the inward facing ATPase molecules would lead to increased phosphorylation, observed as a decrease in DMC fluorescence intensity, and binding of Ca^{2+} to the cytoplasmic sites on the outward facing ATPase molecules would lead to dephosphorylation of the small proportion of these ATPase molecules phosphorylated under these conditions. As shown in Fig. 5, the net result is that addition of Ca^{2+} leads to a decrease in fluorescence intensity of up to about 5%, the concentration of Ca^{2+} giving a half-maximal effect being 1.2 mM, comparable with the luminal concentration of Ca^{2+} giving half-maximal effect for SR vesicles.

The Ca^{2+}-ATPase can bind and transport divalent metal ions such as Ca^{2+} and Mg^{2+} instead of Ca^{2+} (30); Mn^{2+} will also replace Mg^{2+} in phosphorylation of the ATPase by P_i (31). Light scattering problems prevented the use of Co^{2+}, but as shown in Fig. 5, we found that addition of Mn^{2+} produced a larger phosphorylation-induced change in DMC fluorescence intensity than did Ca^{2+}, and so Mn^{2+} was used in subsequent experiments. Effects of Mn^{2+} were only observed in the presence of luminal P_i, and the concentration of Mn^{2+} giving a half-maximal effect was about 0.5 mM (Fig. 5).

To determine the effect of modification with EDC on the fluorescence change caused by Mn^{2+}, vesicles were reconstituted as described above and then incubated with 3 mM EDC at 37°C. At various times samples were taken and diluted into buffer, and the response to the addition of 1 mM Mn^{2+} was recorded. As shown in the inset to Fig. 6, whereas a ~10% decrease in fluorescence intensity was observed upon addition...
of 1 mM Mn2+ to unreacted vesicles, after 60-min reaction with EDC, the fluorescence response was reduced to ~2%, after correcting for dilution effects. The time course of the loss of fluorescence response fitted to a single exponential process with a rate of 0.05 ± 0.01 min⁻¹ comparable with the time course of the loss of ATPase activity. Modification with EDC was also found to block the fluorescence response to addition of Ca²⁺ over the same time period (data not shown).

Effect of ATP on Reaction with EDC—As reported by Coan et al. (15), the presence of Mg²⁺ in the external medium results in some protection for SR against inactivation by EDC; in the presence of 5 mM Mg²⁺, reaction with 2.4 mM EDC for 60 min leads to 81% inhibition of ATPase activity (Fig. 7) compared with 90% in the absence of Mg²⁺ (Fig. 4). A similar effect of Mg²⁺ is observed for the purified ATPase (Figs. 4 and 7). Addition of ATP in the presence of 5 mM Mg²⁺ had no significant effect on the level of inhibition of SR by EDC, but for the purified ATPase, inhibition increased to 95% at high concentrations of ATP (Fig. 7). The half-maximal effect of ATP was observed at 5 μM ATP; this is similar to the Kᵦ value for MgATP, estimated as between 2 and 6 μM at pH 7.2 in the presence of 2 mM Mg²⁺ (32). ADP in the presence of Mg²⁺ was observed to have the same effect as ATP but, in the absence of Mg²⁺, neither ADP nor ATP at concentrations up to 5 mM had any effect on inactivation of the ATPase by EDC. We conclude that binding of MgATP or MgADP to the catalytic site on the cytoplasmic domain of the ATPase leads to a conformational change on the luminal side of the ATPase, resulting in increased inhibition on reaction with EDC.

**DISCUSSION**

The mechanism of the Ca²⁺-ATPase has usually been described in terms of the alternating sites, E1-E2 model (1). This proposes that in the E1 conformation, the ATPase has two outward facing Ca²⁺-binding sites of high affinity. Following the binding of two Ca²⁺ ions and MgATP, the ATPase becomes phosphorylated to give E1PCa₂. This then undergoes a conformational change to the E2PCa₂ state in which the two Ca²⁺-binding sites are inward facing and low affinity. Following loss of Ca²⁺ from these sites, the ATPase can be dephosphorylated to give E2, which can then undergo a conformational change back to E1. However, this model predicts that binding of Ca²⁺ to the ATPase from the luminal side of the membrane (to E2) will be competitive with binding of Ca²⁺ to the ATPase from the cytoplasmic side of the membrane (to E1), whereas experimentally binding from the two sides of the membrane has been found to be noncompetitive (2–4). A four-site model for the ATPase has therefore been proposed in which the Ca²⁺-ATPase contains two pairs of sites for Ca²⁺, a high affinity pair of sites facing the cytoplasm and a low affinity pair of sites facing the lumen (Fig. 1). Phosphorylation of the ATPase results in closing of the cytoplasmic pair of sites with transfer of the two bound Ca²⁺ ions to the pair of luminal sites (2, 3).

The luminal side of the ATPase consists mostly of small loops between transmembrane α-helices with a larger loop between helices M7 and M8 (Fig. 1). The small loop between helices M1 and M2 and the larger loop between helices M7 and M8 are predicted to be rich in acidic residues. The two low affinity Cu²⁺-binding sites could be organized side-by-side as suggested in Fig. 1 because release of Ca²⁺ from the phosphorylated ATPase is sequential (33, 34). Release of Ca²⁺ from the luminal sites must involve a conformational change on the ATPase because release of Ca²⁺ is slow (~20 s⁻¹; see Ref. 34) despite the low affinity of the sites for Ca²⁺; this conformational change could correspond to movement of the M7-M8 loop, as suggested in Fig. 1.

If a pair of binding sites for Ca²⁺ do exist on the luminal side of the membrane, then chemical modification of these residues would be expected to block binding of Ca²⁺ on the luminal side of the membrane and, possibly, block transfer of Ca²⁺ from the cytoplasmic to the luminal sites and thus block ATPase activity. Here we have shown that the charged, water-soluble carbodiimide EDC is membrane-impermeable, and we have used this membrane impermeability to distinguish between acidic groups on the cytoplasmic and luminal sides of the ATPase. Homogenization of muscle gives a preparation of sealed SR vesicles in which the ATPase molecules are oriented the right way round; purification of the Ca²⁺-ATPase from the SR membrane gives a preparation of ATPase in unsealed membrane-fragments (1). Thus in SR vesicles only carboxyl groups in the ATPase exposed on the cytoplasmic side are available for reaction with EDC, whereas for the purified Ca²⁺-ATPase both cytoplasmic and luminal sides are available for reaction. As shown in Fig. 4, inhibition of ATPase activity by EDC is faster for the purified ATPase than for SR, suggesting that inhibitory
sites on the luminal side react more quickly than those on the cytoplasmic side. This was confirmed by experiments with reconstituted membranes in which the Ca$^{2+}$-ATPase is randomly distributed between the two faces of the membrane; outward facing ATPase molecules are inhibited more slowly than inward facing ATPase molecules (Fig. 4).

The observation that inward facing ATPase molecules are inhibited by EDC (Fig. 4) shows that modification of sites accessible from what would normally be the luminal side of the ATPase causes inhibition of the ATPase. The modified sites are not the pair of high affinity Ca$^{2+}$ ion-binding sites because modification of the ATPase with EDC does not result in any change in affinity of the high affinity sites for Ca$^{2+}$ (Fig. 3); modification of the high affinity sites with hydrophobic carbodiimides blocks Ca$^{2+}$ binding to these site (12–14). That the luminal sites modified by EDC could be the luminal Ca$^{2+}$-binding sites is suggested by experiments measuring the effect of divalent metal ions on the level of phosphorylation of the ATPase by Pi. The level of phosphorylation of the ATPase can be measured from changes in the fluorescence intensity of DMC-labeled ATPase (4). Binding of Ca$^{2+}$ or Mn$^{2+}$ to luminal sites on the ATPase leads to increased levels of phosphorylation by P$_{i}$ (Fig. 5), but modification of luminal sites by EDC leads to a loss of this stimulatory effect of divalent metal ions (Fig. 6). Sites labeled by EDC on the luminal side of the membrane therefore include sites involved in Ca$^{2+}$ binding on the luminal side of the membrane.

Extensive mutagenesis of luminal residues by Andersen (7) failed to identify any residues whose mutagenesis blocked uptake of Ca$^{2+}$ driven by hydrolysis of ATP. It is possible that more sensitive assays of protein function would have detected defects resulting from mutagenesis of key residues on the luminal side of the ATPase. It is also possible that the inhibitory effects of EDC observed here follow from steric effects rather than from simple removal of the negative charge resulting from modification of a carboxyl group.

The Ca$^{2+}$-binding sites on the ATPase must be linked functionally to the cytoplasmic domain of the ATPase because phosphorylation of the ATPase by ATP leads to transfer of Ca$^{2+}$ from the cytoplasmic to the luminal pair of binding sites. Evidence for such a link is provided by the experiment shown in Fig. 7, showing that binding of MgATP to its binding site on the cytoplasmic domain leads to increased inhibition of the purified ATPase but not Sr; this implies that binding of MgATP results in increased reactivity of luminal sites to EDC. In the absence of ligands for the Ca$^{2+}$-ATPase, the Ca$^{2+}$-ATPase is present as an equilibrium mixture of the two conformations E1 and E2, with the E1-E2 equilibrium being pH-dependent, high pH favoring the E1 conformation (reviewed in Ref. 24). Lacapere et al. (32) have shown that the affinity of the ATPase for ATP in the absence of Ca$^{2+}$ increases with increasing pH, consistent with stronger binding of ATP to the E1 than to the E2 conformation of the ATPase. Stronger binding of ATP to the E1 than to the E2 conformation of the ATPase means that addition of ATP will shift the E1-E2 equilibrium toward E1. The observed increase in inhibition of the purified ATPase by EDC in the presence of ATP would then be consistent with a greater reactivity for the luminal sites in the E1 conformation of the ATPase than in the E2 conformation.

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