Maleimidylsalicylic acid reacts with the Ca\(^{2+}\)-ATPase of skeletal muscle sarcoplasmic reticulum with high affinity and inhibits the ATPase activity following a pseudo-first-order kinetic with a rate constant of 8.3 m\(^{-1}\) s\(^{-1}\). Calcium binding remains unaffected in the maleimide-inhibited ATPase. However, the presence of ATP, ADP, and, to a lesser extent, AMP protects the enzyme against inhibition. Furthermore, ATPase inhibition is accompanied by a concomitant decrease in ATP binding. The stoichiometry of the nucleotide-dependent maleimidylsalicylic acid binding is 6–10 nmol/mg ATPase, which corresponds to the binding of up to one molecule of maleimide/molecule of ATPase. The stoichiometry of maleimide binding is decreased in the presence of nucleotides and in the ATPase previously labeled with fluorescein-5'-isothiocyanate or N-ethylmaleimide A fluorescent peptide was isolated by high performance liquid chromatography after trypsin digestion of the maleimide-bound peptide. Analysis of the sequence and mass spectrometry of the peptide leads us to propose Cys\(^{344}\) as the target for maleimidylsalicylic acid in the inhibition reaction. The effect of Cys\(^{344}\) modification on the nucleotide site is discussed.

The Ca\(^{2+}\)-ATPase of skeletal muscle SR\(^1\) catalyzes calcium transport coupled to ATP hydrolysis (1, 2), a process that is triggered by calcium binding. Afterward, phosphate transfer from bound ATP to Asp\(^{351}\) leads to the formation of the phosphoenzyme intermediate (3–6) and the occlusion of calcium from the cytoplasmic side to the lumen of the SR vesicles. The binding sites for Ca\(^{2+}\) on the Ca\(^{2+}\)-ATPase have been located in the transmembrane region of the protein, involving several residues from different segments, such as M4, M5, M6, and M8 (7–9). It has been proposed that these membrane segments are clustered, forming a channel where two calcium ions can be accommodated in a single row (10, 11).

The ATP-binding site has been probed with different reagents, one of the best characterized being FITC, which reacts with Lys\(^{515}\) to inhibit ATP binding with high specificity (12, 13). However, FITC binding does not alter the catalysis of smaller substrates such as acetylphosphate (14). Other chemical modifications in residues close to Lys\(^{515}\) also modify ATP binding, e.g. Lys\(^{492}\) with glutaraldehyde, pyridoxal derivatives, and azo derivatives of ATP, which also bind to Thr\(^{532}\) and Thr\(^{533}\) (15–17). Single mutations of amino acids of that peptide region do not completely inhibit ATP binding probably because the ATP-binding site is defined by several residues from different segments of the primary structure of the protein (18).

There are 24 cysteine residues in the Ca\(^{2+}\)-ATPase that are susceptible to react with sulhydryl reagents. Some of them are protected by ATP, suggesting that they might be involved in the ATP-binding site (19, 20). The most common reagents used for cysteine residues are iodoacetamides, maleimides, and NBD-Cl. The first reacts with Cys\(^{670}\) and Cys\(^{674}\) and does not alter ATPase activity (21). Among the maleimides used on the Ca\(^{2+}\)-ATPase, NEM has the smallest chemical structure. Under certain experimental conditions it can react with Cys\(^{344}\) and Cys\(^{864}\) or with Cys\(^{777}\) and Cys\(^{814}\) (22–24). Fluorescent maleimides have also been used to probe the maleimide reaction site (25, 26). Fluoresceine maleimide has been reported to bind to Cys\(^{777}\) and Cys\(^{864}\) and 4-dimethylyarnino-phenylazophenyl-4'-maleimide to Cys\(^{864}\) (20). Phenylmaleimide has been shown to inhibit Ca\(^{2+}\)-ATPase activity with high specificity by inhibiting the phosphorylation reaction (27).

In this work a fluorescent maleimide of a similar size to phenylmaleimide, maleimidylsalicylic acid, has been used on the Ca\(^{2+}\)-ATPase. It is shown that the inhibition behavior of this maleimide is different from other known maleimides because it binds near the phosphorylation site, Asp\(^{351}\), inhibiting ATP binding but not the incorporation of phosphate, thus altering the relationship between the nucleotide-binding site and the phosphorylation site.

**EXPERIMENTAL PROCEDURES**

**Materials**—Maleimidylsalicylic acid was from Molecular Probes (Leiden, The Netherlands). Pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, ATP, AMP-PNP and NADH were from Roche Molecular Biochemicals. The radiochemicals \[^{32}P\]ATP, \[^{32}P\]Pi, and \[^{3}H\]glucose were from Amersham Pharmacia Biotech. Angiotensin I and cytochrome c were from Sigma. Calcium ionophore, A23187, was obtained from Calbiochem (Madrid, Spain). All other chemicals were purchased from Sigma. The sarcoplasmic reticulum vesicles were prepared from rabbit hind leg muscle, as described previously by Eletr and Inesi (28) and stored in frozen aliquots until use.

**Fluorescence Spectra of MSA**—The fluorescence measurements were performed in a Shimadzu RF-540 fluorescence spectrometer. The excitation and emission spectra were obtained in 100 mM MOPS/TEA, pH 8.0, and 50 mM L-cysteine. The excitation and emission wavelengths were 307 and 435 nm, respectively.

**MSA and SR Concentration**—The MSA was dissolved in dimethyl sulfoxide, and its concentration was measured at 307 nm in a methanolic solution containing 1% β-mercaptoethanol, 1% sodium acetate. In
these conditions the molar absorption coefficient is $3.4 \times 10^{3} \text{ M}^{-1} \text{cm}^{-1}$.

The protein concentration was determined according to the procedure of Lowry et al. (29) using bovine serum albumin as standard.

**Chemical Modification with MSA**—1 mg/ml SR was incubated at room temperature in darkness with 100 mM MOPS/TEA, pH 9.0, 0.1 mM EGTA, and 5 mM MgCl₂, at different MSA concentrations for different periods of time. The reaction was stopped by adding 10 mM L-cysteine.

**ATPase Activity**—ATPase activity was measured by a coupled enzyme system (30). The reaction medium contained 20 mM MOPS, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM CaCl₂, 3 mM iodonophore A23187, 0.1 mM ATP, 1 mM phosphoenol pyruvate, 0.24 mM NADH, 5 units/ml pyruvate kinase, and 5 units/ml lactate dehydrogenase. The SR concentration used was 0.02 mg/ml. The absorbance was recorded at 340 nm to measure the decrease in NADH concentration. ATP hydrolysis activity was calculated using an $e_{340}$ (NADH) = 6220 $\text{M}^{-1} \text{cm}^{-1}$.

**SR Phosphorylation by Inorganic Phosphate**—The inorganic phosphate was purified before use (31). Phosphorylation of sarcoplasmic reticulum vesicles was achieved as described previously by Inesi et al. (1). Briefly, the protein was incubated for 5 min in a medium containing 20 mM MES, pH 6.0, 5 mM MgCl₂, 1 mM EGTA, and different [32P]Pi concentrations ranging from 0 to 10 mM. The protein concentration used was 0.5 mg/ml. The phosphorylation reaction was quenched by addition of 2 ml of 0.25 M perchloric acid and 4 mM P<sub>I</sub>. The quenched samples were cooled on ice, washed, and centrifuged repeatedly. The final pellets were dissolved in 0.25 ml of 0.1 M NaOH, 2% Na<sub>2</sub>CO<sub>3</sub>, 2% SDS, and 5 mM Na<sub>2</sub>PO<sub>4</sub>, prior to determination of radioactivity and protein concentration.

**SR Phosphorylation by ATP**—The reaction medium contained 40 mM MOPS, pH 6.8, 160 mM KCl, 10 mM MgCl₂, 0.2 mM CaCl₂, 0.2 mM EGTA, and 5 mM A23187. The protein concentration used was 0.06 mg/ml. Protein phosphorylation was carried out at room temperature and started by addition of 0.4 mM $[^{32}P]$ATP for 10 s. The reaction was stopped by adding 4 volumes of an ice-cold solution of 0.8 M perchloric acid and 40 mM P<sub>I</sub>. The quenched samples were filtered through 0.65-μm pore size Millipore filters. The filters were then washed five times with 5 ml of 0.125 M perchloric acid and 20 mM P<sub>I</sub>, and the radioactivity was counted.

**ATP Binding**—The ATP binding assay was carried out in the absence of calcium to prevent ATP splitting. ATP binding to unmodified and modified SR with 75 μM MSA was measured by a double labeling radioactive technique using a filtration method (32, 33). Briefly, a Millipore DAWP 0.65-μm filter was loaded with 0.4 mg of protein and centrifuged repeatedly. The final pellets were dissolved in 0.25 ml of 0.1 M NaOH, 2% Na<sub>2</sub>CO<sub>3</sub>, 2% SDS, and 5 mM Na<sub>2</sub>PO<sub>4</sub>, prior to determination of radioactivity and protein concentration.

**Labeling of ATPase with FITC**—SR vesicles were labeled with FITC (12) as follows: the SR vesicles (2 mg of protein/ml) were incubated in a medium containing 0.2 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 25 mM Tris, 0.25 mM glycerine, 0.1 mM EGTA, and 30 μM FITC for 10 min at 25 °C in darkness. The labeling reaction was stopped by centrifuging the sample through a Sephadex G-50 column pre-equilibrated with ice-cold stopping solution containing 20 mM MOPS, pH 6.8, 80 mM KCl, and 5 mM MgCl₂. The filtered vesicles were then centrifuged at 105,000 × g for 45 min at 5 °C. The pellet was resuspended with 10 mM MOPS, pH 7.0, and 30% sucrose, and the protein concentration was measured.

**Labeling of ATPase with N-Ethylmaleimide**—SR was labeled with NEM after the procedure of Kawakita and Yamashita (34). 3 mg/ml of SR was incubated for 50 min in a medium containing 40 mM MOPS, pH 6.8, 0.1 M KCl, 5 mM MgCl₂, 50 mM CaCl₂, and 0.4 mM NEM, at 30 °C. The labeling reaction was stopped by 3-fold dilution with ice-cold solution containing 1 mM Tris, 1 mM EGTA, and 1 mM NEM. The sample was then quenched by addition of 1 mM Tris, 1 mM EGTA, and 1 mM cysteine residues in the ATPase. The corresponding kinetic rate constant was calculated from the slope of the curve of calcium to prevent ATP splitting. ATP binding to unmodified and modified SR with 75 μM MSA was measured by a double labeling radioactive technique using a filtration method (32, 33). Briefly, a Millipore DAWP 0.65-μm filter was loaded with 0.4 mg of protein and centrifuged repeatedly. The final pellets were dissolved in 0.25 ml of 0.1 M NaOH, 2% Na<sub>2</sub>CO<sub>3</sub>, 2% SDS, and 5 mM Na<sub>2</sub>PO<sub>4</sub>, prior to determination of radioactivity and protein concentration.

Maleimidylsalicylic acid is soluble in water at neutral pH and has low fluorescence until reacted with cysteine residues in proteins, a characteristic that makes it potentially useful for thiol quantification and characterization.

**Kinetics of MSA Inhibition**—SR was preincubated with MSA in 10 mM MOPS/TEA at pH 8.0. Aliquots were withdrawn at different times to assay the residual ATP hydrolysis activity. When the MSA concentration in the preincubation medium was 75 μM, the ATPase inhibition occurred in a time scale of minutes, 75% inhibition being reached after 15 min of reaction (Fig. 1). Longer incubation times or higher MSA concentrations led to a nonspecific effect of MSA on the enzyme. Therefore, these conditions (75 μM MSA and 15 min of incubation time) were selected for most of the following inhibition experiments.

To study the kinetics of MSA inhibition, the same experiment was performed at different MSA concentrations, and the results were plotted on a logarithmic scale, as shown in Fig. 2A. All curves could be fitted to single exponential equations, from which an apparent first-order rate constant was obtained ($k_{app}$). This constant was subsequently plotted versus MSA concentration (Fig. 2B). A linear relationship was observed between the $k_{app}$ and MSA concentration, suggesting a pseudo-first-order kinetic in the cofavalent reaction between the MSA and the cysteine residues of the ATPase. The corresponding kinetic rate constant, as calculated from the slope of the curve...
from Fig. 2B, was 8.3 $\text{m}^{-1} \text{s}^{-1}$.

**Calcium Binding**—The residual activity of partially inhibited ATPase was assayed at different calcium concentrations. In the experiment of Fig. 3, the ATPase was inhibited with 75 $\mu\text{M}$ MSA for 15 min. No difference was seen between the native and inhibited ATPase, and the calcium dependent activity was not modified by MSA. The presence of calcium in the inhibition medium did not alter the inhibition pattern (not shown), indicating that calcium binding does not affect the reactive cysteine residues.

**Nucleotide Binding**—The presence of different nucleotides in the inhibition medium appeared to strongly block the inhibition reaction (Fig. 4). The nucleotides used were ATP, AMP-PNP, ADP, and AMP at a fixed concentration of 1 $\text{mM}$. All of them were capable of slowing down the inhibition reaction, with AMP being the least effective. However, ADP, AMP-PNP, and ATP completely abolished the inhibition in the experimental conditions used. The different effect of these nucleotides is not surprising because their affinities for ATPase are also different, the apparent dissociation constant of ATP, AMP-PNP, and ADP being in the micromolar range and in the millimolar range in the case of AMP (35). Therefore, AMP is not at a saturating concentration unlike the rest of the nucleotides used, which explains the lower protection it affords. These results indicate that binding of the adenine moiety of the nucleotide is the main feature in blocking MSA inhibition, and the ionic moieties of the $\beta$ and $\gamma$ phosphates are not involved in the obstruction of MSA interaction.

Binding of ATP was determined by incubating SR with $[^{\gamma-32}\text{P}]$ATP in the absence of calcium and including $[^{3}\text{H}]$glucose in the medium. Aliquots of 0.4 mg of protein were filtered, and the radioactivity retained in the filters was counted. Bound ATP was calculated by subtracting the free $[^{\gamma-32}\text{P}]$ATP concentration contained on the filters, calculated from the radioactivity corresponding to $[^{3}\text{H}]$glucose, from total $[^{\gamma-32}\text{P}]$ATP. In Fig. 5 it can be seen that ATP binding was strongly and progressively reduced when the ATPase was previously modified by MSA at increasing time periods.

**Enzyme Phosphorylation**—Once it was determined that nucleotide binding affects the inhibition reaction and vice versa and that the MSA reaction interferes with ATP binding, other intermediates of the catalytic cycle, which might be involved in the inhibition of ATPase activity by MSA, were studied. In the experiment shown in Fig. 5 for EP, an aliquot was withdrawn from the inhibition reaction at different times and added to a medium containing 0.4 $\text{mM}$ $[^{\gamma-32}\text{P}]$ATP and calcium to measure the phosphoenzyme level reached in steady-state conditions. It is clear from Fig. 5 that ATP phosphorylation was inhibited during the MSA reaction. This was not unexpected because the ATP phosphorylation reaction depends on previous partial reactions, such as ATP binding. Therefore the inhibition of EP formation appears as a result of the ATP binding inhibition. However, a comparison of ATP binding and EP levels in Fig. 5 shows that ATP inhibition takes place faster than enzyme phosphorylation.

Enzyme phosphorylation with inorganic phosphate was performed in equilibrium conditions using $[^{32}\text{P}]$P$_i$ as phosphate donor at pH 6.0. No appreciable difference was observed in P$_i$ phosphorylation up to 10 $\text{mM}$ P$_i$ concentration in SR modified with 75 $\mu\text{M}$ MSA (Fig. 6A), and only a slight decrease was seen at very high MSA concentrations (Fig. 6B). This might reflect the reaction of MSA with other less specific residues when higher MSA concentrations are used.

**MSA Binding**—Free MSA has a very low fluorescence, which
different MSA concentrations. The EP was formed with 5 mM Pi.

SR (presence of different inorganic phosphate concentrations using native m l labeled with 75 m to irreversibly prevent nucleotide binding and the latter to differentiate between specific and nonspecific MSA binding. These experiments are depicted in Fig. 7, where the MSA fluorescence units are converted to bound MSA. In the presence of AMP-PNP, MSA binding was not completely inhibited in our experimental conditions. Despite this, in these conditions the inhibition of the ATPase activity was fully protected (Fig. 4). This may be the result of nonspecific binding to the ATPase, which would not affect the ATP hydrolysis activity of the enzyme.

The stoichiometry of MSA binding was 6–10 nmol MSA/mg of protein, which corresponds to the reaction of up to one cysteine residue per ATPase molecule. FITC-modified SR cannot bind ATP, and, as can be seen in Fig. 7, the MSA binding capacity is reduced by more than 50%, confirming the involvement of the nucleotide-binding site.

NEM is a much smaller maleimide than MSA because it lacks the aromatic ring provided by the salicylic group, thus minimizing any steric effects it may have. Furthermore, NEM labeling was performed in conditions in which NEM reacts with Cys344 and Cys364 (22). In this case MSA binding was fully inhibited (Fig. 7A). MSA binding performed on ATPase prelabeled with different NEM concentrations is shown in Fig. 7B, the result clearly indicating the involvement of some of these cysteine residues in the reaction with MSA.

Protein Digestion and Fluorescent Fraction Isolation—To isolate a small peptide fragment containing the MSA label responsible for the inhibition of the ATPase activity, the SR was labeled in the presence and in the absence of AMP-PNP to differentiate between specific and nonspecific MSA binding. Labeling was performed with 75 mM MSA for 15 min. The labeled SR was subjected to extensive tryptic digestion with a trypsin/ATPase ratio of 0.25 (w/w) for 5 min at 37 °C. The digestion was stopped by addition of 1% trifluoroacetic acid, which precipitates the membrane fraction, maintaining the soluble peptides in the supernatant. Both fractions then separated by ultracentrifugation at 105,000 × g for 45 min. The pellet was resuspended in 20 mM MOPS, pH 6.8, and 80 mM KCl and the MSA fluorescence was measured in the pellet and in the supernatant. Only the soluble fraction was fluorescent, and this was subsequently subjected to reverse phase HPLC using a C18 column. Fig. 8A shows the chromatogram of SR labeled in the absence of AMP-PNP, in which different fluorescent peaks can be observed. However, when MSA-SR was obtained in the presence of AMP-PNP to inhibit specific MSA binding, the same peaks were obtained except the one appearing at 23 min (Fig. 8B). It seems then, that this peptide might be specific for ATPase inhibition because it did not appear when MSA labeling was performed in the presence of AMP-PNP. Consequently, the fraction retained at 23 min was eluted and lyophilized for peptide sequencing and mass spectrometry.

Peptide Sequencing and Mass Spectrometry—The lyophilized peptide was submitted to six cycles of peptide sequencing from the N-terminal residue, resulting in one major sequence starting by GFNPPD and another in the background starting by SLPSE. Both peptides can be matched to the ATPase sequence, the first one corresponding to Gly508 and the second to Ser335. To ascertain the length of the peptide, the molecular mass was determined by MALDI-TOF mass spectrometry. The sample (lyophilized eluted fraction) was dissolved in acetonitrile.
tides separated by ultracentrifugation were subjected to C\textsubscript{18} HPLC in the presence of AMP-PNP (\textit{B}), and digested with trypsin. The soluble peptides separated by ultracentrifugation were subjected to C\textsubscript{18} HPLC using the acetonitrile/water/trifluoroacetic acid system. The fluorescence of the eluates was recorded at 330-nm excitation wavelength and 435-nm emission wavelength.

FIG. 8. HPLC of trypsin digest of labeled ATPase. SR was derivatized with 75 \textmu M MSA for 15 min in the absence (\textit{A}) and in the presence of AMP-PNP (\textit{B}), and digested with trypsin. The soluble peptides separated by ultracentrifugation were subjected to C\textsubscript{18} HPLC using the acetonitrile/water/trifluoroacetic acid system. The fluorescence of the eluates was recorded at 330-nm excitation wavelength and 435-nm emission wavelength.

FIG. 9. MALDI mass spectrum of the eluted fraction. MALDI-TOF mass spectrum of the fraction eluted at 23 min shown in Fig. 8A.  

trile/water (30\%) containing 0.1\% trifluoroacetic acid and mixed with a saturated solution of \textalpha-\textgamma-cyano-\textdelta-hydroxycinnamic acid in the same solvent as the sample. Fig. 9 shows a representative spectrogram. Two peaks are observed with mass to charge ratios of 1738 and 2069 Da. When the possibility that each peak might correspond to one of each sequenced peptide was checked, it resulted that the sequence \texttt{G} was checked. It was concluded that the fluorescent peak eluted from Gly\textsubscript{808}–Arg\textsubscript{822} had a molecular mass of 1740 Da, and the sequence \texttt{DRPPR} had a molecular mass of 1740 Da, and the sequence \texttt{GFNPPDLDIM}–Arg\textsubscript{822} had a molecular mass of 1839 Da. If this last peptide had one MSA molecule attached to one of its cysteine residues, its mass would increase to 2072 Da. Thus, both sequences correlate quite well with the mass observed in the spectrogram (Fig. 9), although other possibilities were also taken into account. For example, it was considered that both peaks might correspond to only one peptide with different degree of degradation and/or with different bound MSA molecules. However, these possibilities were not discounted. The possibility of a different charge ratio was also excluded because masses of [M + 2H\textsuperscript{+}] were not detected. None of the possibilities analyzed fitted as well as the one proposed above. Therefore, it was concluded that the fluorescent peak eluted from the HPLC column is composed of two major peptides, with only one being fluorescent, because the peptide Gly\textsubscript{808}–Arg\textsubscript{822} has no cysteine residues in the sequence.

**DISCUSSION**

The present work shows that labeling with MSA is accompanied by strong inhibition of the Ca\textsuperscript{2+}-ATPase, and that an exclusive prevention of MSA binding protects against the Ca\textsuperscript{2+}-ATPase inhibition. This protection may be achieved by occupancy of the nucleotide-binding site. Conversely, MSA labeling inhibits ATP binding and consequently EP formation by ATP, resulting in the inhibition of ATP hydrolysis activity.

We chose maleimidysalicylic acid to inhibit the Ca\textsuperscript{2+}-ATPase because it is similar in size to phenylmaleimide, which we studied previously (27). Nevertheless, MSA has polar substituents in the phenyl ring provided by a carboxylic anion and a hydroxyl group, which presumably cause differences in the interaction with the enzyme. In fact, both maleimides inhibit ATPase activity in the micromolar concentration range, indicating their high affinity interaction with the enzyme. However, phenylmaleimide mainly inhibits the phosphorylation reaction and does not affect ATP or calcium binding. In contrast, MSA does not significantly affect EP formation by inorganic phosphate but strongly inhibits ATP binding. Furthermore, the proposed reactive residue is also different. It has been proposed that phenylmaleimide reacts with Cys\textsuperscript{377} (27), but we do not find evidence that this cysteine reacts with MSA. Nevertheless, it is possible that the binding pockets of both maleimides are close because of the proximity in the primary structure of the cysteine residues involved, as will be discussed below. Therefore, it seems that the differences in the polar characteristics of both maleimides result in important differences in their interaction with the enzyme to obstruct the catalytic cycle.

The localization of MSA proposed in this work is based on the amino acid sequencing of fluorescent tryptic fragments in combination with their molecular mass obtained by MALDI-TOF mass spectrometry. It has to be stressed that the main sequence obtained starts at Gly\textsubscript{808} but does not contain cysteine residues. It is somewhat surprising to have found the sequence Gly\textsuperscript{808}–Arg\textsuperscript{822} as a result of a tryptic digestion, because the cleavage at Leu\textsuperscript{807}–Gly\textsuperscript{808} does not correspond to an expected tryptic digestion, which occurs preferentially at basic residues (e.g., Arg and Lys).

In our experimental conditions, some unspecific cleavage seems to be produced by trypsin. Nevertheless, it is of particular interest that this peptide was supposed to be a membrane peptide located in M6, as predicted by Brand et al. (3). Our results indicate, however, that this peptide is accessible to trypsin, and therefore both cleavage sites are located outside of the membrane bilayer.

A result similar to ours was previously reported using proteinase K instead of trypsin (36), suggesting that the cytoplasmic loop linking the membrane segments M6 and M7 extends from Gly\textsuperscript{808} to Leu\textsuperscript{833} and is accessible to the action of proteinases. Thus, it may also be speculated that a trypsin to membrane interaction takes place, bringing Gly\textsuperscript{808} closer to the catalytic site of trypsin because of its proximity to the membrane portion of this residue.

Although the peptide Gly\textsuperscript{808}–Arg\textsuperscript{822} has the capacity to bind calcium and although the mutation of aspartyl residues to alanine in this peptide results in a substantial loss of ATP hydrolysis activity (36), it cannot be proposed as a target of MSA because it lacks cysteine residues, and our experimental conditions used for labeling are sufficiently mild to prevent its reaction to other residues (e.g., Ser and Thr). Therefore, it seems more feasible that Gly\textsuperscript{808}–Arg\textsuperscript{822} peptide appears in the fluorescent fraction sensitive to AMP-PNP because of its similar hydrophobicity to the fluorescent peptide, which would explain why it is equally retained in the chromatographic column.

The sequence of the tryptic fragment containing the reactive cysteines can be read in the background of the sequence analysis as a secondary sequence. This fragment starts at Ser\textsuperscript{335} and contains two cysteine residues, Cys\textsuperscript{344} and Cys\textsuperscript{349}, both
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near Asp$^{351}$, the phosphorylation site. However, despite the closeness of these sites, no evidence was found to support EP inhibition by inorganic phosphate (Fig. 6). The present study leads us to propose that Ser$^{325}$–Lys$^{352}$ peptide is labeled by MSA. This suggestion is based, on the one hand, on the amino acid analysis of the sequences obtained of the fluorescent tryptic fraction eluted from the HPLC, which determines the starting amino acid sequence of the peptide, and, on the other hand, on its molecular mass, which establishes the length and therefore the C-terminal residue. This peptide contains two cysteine residues as possible targets for MSA, Cys$^{344}$ and Cys$^{349}$. Apart from phenylmaleimide discussed above, other maleimides have been reported to react with proximal cysteines, such as Cys$^{344}$ and Cys$^{364}$, both of which are labeled with NEM, fluorescein-maleimide, 2-(4-maleimidoanilino)naphtalene-6-sulfonic acid, and 4-dimethylamino-phenylazophenyl-4'-maleimide (20, 22, 25, 34). It was reported that enzyme phosphorylation by ATP is not affected by labeling of these maleimides despite the proximity of these labels to the phosphorylation site, Asp$^{351}$ (25). We found that NEM fully inhibits MSA binding, indicating that MSA would react with Cys$^{344}$ and/or Cys$^{364}$ (Fig. 7B). Taking into account that the label peptide comprises amino acid residues from Ser$^{325}$ to Lys$^{352}$, we can exclude Cys$^{364}$, and so the reactive cysteine must be Cys$^{344}$. It has been shown that modification of Cys$^{344}$ and Cys$^{349}$ by NEM results in the inhibition of EP formation by inorganic phosphate and the inhibition of phosphoenzyme decomposition formed with ATP (37–39). When ATP binding is compared with the level of EP formed (Fig. 5), the EP level is constantly greater than the extent of ATP binding, indicating that EP decomposition is partially inhibited. It is of interest that NEM also inhibits EP decomposition. Thus, the EP level is a result of the inhibition of ATP binding and EP decomposition, although some inhibition of phosphate transfer from ATP to Asp$^{351}$ cannot be completely ruled out. Our result is in agreement with this observation because MSA could react with one of these Cys residues with no effect on the phosphorylation reaction by inorganic phosphate (Fig. 6).

Similar results have been published for NBD-Cl (42), which specifically labels Cys$^{344}$, whereas a concomitant ATP hydrolysis inhibition takes place. Nucleotides, such as Mg-ATP, Ca-ATP, and even Mg-ADP, protected the Ca$^{2+}$-ATPase. However, we found that MSA binding inhibits ATP binding and, conversely, ATP binding and even FITC labeling inhibit MSA binding. Besides, MSA inhibits the binding of any adenosine nucleotide regardless of the phosphate groups in the molecule, which would exclude that MSA could inhibit ATP binding by disruptions of electrostatic interactions with the γ-phosphate of ATP. It is then difficult to explain these results because long distance interactions because the signal should go in both directions, that is, occupancy of the adenosine moiety should alter the phosphorylation domain, and MSA binding should be transmitted to the nucleotide site. This could be possible if the network of hydrogen bonds or electrostatic charges necessary for domains to interact is disrupted. Another possible explanation is a competitive interaction between both sites, although this would require the phenyl ring of MSA to be closer to the nucleotide site than the 25 Å distance predicted. In any case, these results indicate a mutual binding interaction and that alterations of one domain would be transmitted to the other.

In conclusion, this study shows a maleimide derivative that specifically interferes with nucleotide binding but that is attached close to the phosphorylation site, thus establishing a relationship between the nucleotide-binding site and the phosphorylation pocket. This observation is in agreement with a certain approach and interrelationship of both sites, which is a prerequisite to allow phosphate transfer from ATP to Asp$^{351}$ and accordingly the operation of the catalytic cycle of the Ca$^{2+}$-ATPase.

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