Modification of Arginine-198 in Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase by 1,2-Cyclohexanedione Causes Inhibition of Formation of the Phosphoenzyme Intermediate from Inorganic Phosphate*

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Sarcoplasmic reticulum vesicles were modified with 1,2-cyclohexanedione (CHD), a specific arginine-modifying reagent, in sodium borate (pH 8.0 or 8.8). Phosphoenzyme formation from P\(_i\) in the Ca\(^{2+}\)-ATPase (reversal of hydrolysis of the phosphoenzyme intermediate) was almost completely inhibited by the modification with CHD. Tight binding of F\(^-\) and Mg\(^{2+}\) and high affinity binding of vanadate in the presence of Mg\(^{2+}\), either of which produces a transition state analog for phosphoenzyme formation from the magnesium-enzyme-phosphate complex, were also markedly inhibited. In contrast, phosphoenzyme formation from acetyl phosphate in the forward reaction was unaffected. The enzyme was appreciably protected by tight binding of F\(^-\) and Mg\(^{2+}\) or by high affinity binding of vanadate in the presence of Mg\(^{2+}\), but not by the presence of 20 mM MgCl\(_2\) alone or 150 mM P\(_i\), alone, against the CHD-induced inhibition of phosphoenzyme formation from P\(_i\). Peptide mapping of the tryptic digests, detection of peptides containing CHD-modified arginyl residues with Girard's reagent T, sequencing, and mass spectrometry showed that Arg-198 was a single major residue protected by tight binding of F\(^-\) and Mg\(^{2+}\) against the modification with CHD. These results indicate that modification of Arg-198 with CHD is responsible for at least a part (the portion reduced by the transition state analogs) of the CHD-induced inhibition of phosphoenzyme formation from P\(_i\), and suggest that Arg-198 is located in or close to the catalytic site in the transition state for phosphoenzyme formation from the magnesium-enzyme-phosphate complex.

The SR\(^{2+}\)-ATPase is a 110-kDa membrane-bound protein, the primary structure of which has been revealed (1). This enzyme catalyzes Ca\(^{2+}\) transport coupled to ATP hydrolysis (2, 3). The enzyme is activated by Ca\(^{2+}\) binding to the high affinity Ca\(^{2+}\) binding sites, and then the \(\gamma\)-phosphoryl group of Mg-ATP bound to the ATP binding site is transferred to Asp-351 (1, 4–6) to form an EP intermediate (7, 8). A subsequent conformational change of the EP results in Ca\(^{2+}\) release to the lumen (9). Finally, the EP is hydrolyzed to form P\(_i\) and the dephosphoenzyme. Acetyl phosphate also serves as a substrate through EP formation (10, 11). The EP can be formed from P\(_i\) in the presence of Mg\(^{2+}\) and absence of Ca\(^{2+}\) by reversal of the late step of the catalytic cycle (12, 13). This EP formation occurs through a magnesium-enzyme-phosphate complex that is formed by random binding of Mg\(^{2+}\) and P\(_i\) to the enzyme (14, 15).

CHD is a chemical modification reagent that reacts specifically with the guanidino group of arginyl residues to produce a stable product, DHCH-Arg (16). Recently, we have shown that binding of MgATP or MgADP to the ATP binding site of the Ca\(^{2+}\)-ATPase is completely inhibited by the modification with CHD and that Arg-489 and Arg-678 are involved in this inhibition (17). The findings have led to the conclusion that these arginyl residues are located at the ATP binding site. We have further shown that EP formation from P\(_i\) has also been inhibited by the modification with CHD and suggested that the arginyl residue(s) involved in this inhibition is distinct from the above residues.

It was previously shown (18–20) that F\(^-\) and Mg\(^{2+}\) bind simultaneously and tightly to the catalytic site of this enzyme to form a stable transition state analog for EP formation from the magnesium-enzyme-phosphate complex. Vanadate also binds with high affinity to the enzyme in the presence of Mg\(^{2+}\) to form a transition state analog for this EP formation (21, 22). In the present study, to identify the arginyl residue(s) involved in the CHD-induced inhibition of EP formation from P\(_i\), we have modified the SR Ca\(^{2+}\)-ATPase with CHD and examined effects of the above transition state analogs on this modification. We have found that the enzyme has been appreciably protected by the transition state analogs against the CHD-induced inhibition of EP formation from P\(_i\). Peptide mapping of tryptic digests of the CHD-modified enzyme, sequencing, and mass spectrometry have shown that Arg-198 is a single major residue protected by tight binding of F\(^-\) and Mg\(^{2+}\) against the modification with CHD. The results indicate that modification of Arg-198 with CHD is responsible for at least a part (the portion reduced by the transition state analogs) of the inhibition of EP formation from P\(_i\), and suggest that Arg-198 is located in or close to the catalytic site in the transition state for EP formation from the magnesium-enzyme-phosphate complex.

EXPERIMENTAL PROCEDURES

Preparation of SR Vesicles—SR vesicles were prepared from rabbit skeletal muscle and stored at \(-80^\circ\)C as described previously (23). The content of phosphorylation site determined with \([\gamma\ss\text{32P}]\text{ATP}\) according to Barraboin et al. (24) was 4.00 ± 0.06 nmol/mg (\(n = 6\)).

 Pretreatment of SR Vesicles with F\(^-\) and Mg\(^{2+}\)—Pretreatment of the SR vesicles with F\(^-\) and Mg\(^{2+}\) was performed as described previously (20) with slight modifications. The vesicles (2 mg/ml) were incubated at 25 °C for 3 h in 1 mM KF, 10 mM MgCl\(_2\), 1 mM EGTA, 20% (v/v) Me\(_2\)SO, 4% (w/v) 2-mercaptoethanol, 0.25 M sucrose, 10 mM Hepes, pH 7.0.
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A 20% (v/v) glycerol, 0.1 M KCl, and 40 mM imidazole/HCl (pH 7.5), unless otherwise stated. The reaction was quenched by diluting the mixture twice with an ice-cold solution containing 0.1 mM CaCl\textsubscript{2}, 0.1 M KCl, 0.3 M sucrose, and 5 mM MOPS/Tris (pH 7.0). The resulting vesicles were washed by centrifugation once with this solution.

Modification with CHD—Modification was started at 37 °C by adding CHD (dissolved in 50 mM sodium borate (pH 8.0 or 8.8)) to a suspension of the SR vesicles to give a final composition as described in the figure legends. The modification was quenched at 0 °C by one of the following methods. In Method I, the mixture was applied to a centrifuge column (10-ml disposable syringe filled with coarse Sephadex G-50) preequilibrated with a 0.1 M solution (Solution A) containing 0.1 M KCl, 20 mM MOPS, and 5 mM sodium borate (pH 7.0). In Method II, the mixture was centrifuged, and the pellet was washed with Solution A and suspended in this solution. In Method III, the mixture was centrifuged, and the pellet was washed with 50 mM sodium borate (pH 8.0) and suspended in this buffer.

Determination of Tightly Bound F\textsuperscript{−} and Mg\textsuperscript{2+}—The SR vesicles were treated with F\textsuperscript{−} and Mg\textsuperscript{2+} as described above. In the control samples, CaCl\textsubscript{2} was added to the incubation medium to give 0.1 mM free Ca\textsuperscript{2+} under the otherwise same conditions as above (tight binding of F\textsuperscript{−} and Mg\textsuperscript{2+} is prevented by 0.1 mM Ca\textsuperscript{2+} (20)). The treated vesicles were washed by centrifugation four times with a solution containing 1 mM A23187, 2 mM EDTA, 10% (v/v) Me\textsubscript{2}SO, 0.1 M KCl, and 5 mM MOPS/NaOH (pH 8.0) and suspended in deionized water. Magnesium bound to the vesicles was extracted with 0.8 N H\textsubscript{2}SO\textsubscript{4}, and the concentration of magnesium in the extract was determined by atomic absorption spectrophotometry as described previously (19). The content of tightly bound Mg\textsuperscript{2+} was obtained by subtracting the content of magnesium in the extract from the control sample. Fluoride bound to the vesicles was extracted by incubating the vesicles at 95 °C for 5 min in 5 mM HEPES/KOH (pH 7.8). The sample was then centrifuged to remove insoluble materials, and KNO\textsubscript{3} was added to the supernatant to give 0.1 M. The sample was adjusted to pH 3.0 with citric acid, and the concentration of F\textsuperscript{−} was measured by use of a fluoride-selective electrode as described.

FIG. 1. CHD-induced inhibition of EP formation from P\textsubscript{i}, and protection by pretreatment with F\textsuperscript{−} and Mg\textsuperscript{2+}, by the presence of vanadate and Mg\textsuperscript{2+}, or by EP formation from P\textsubscript{i}, against the inhibition. A, the SR vesicles were pretreated with KF and MgCl\textsubscript{2} (○), without KF and with MgCl\textsubscript{2} (■), with KF and without MgCl\textsubscript{2} in the presence of 5 mM EDTA and absence of EGTA (△, □), or with neither KF nor MgCl\textsubscript{2} in the presence of 5 mM EDTA and absence of EGTA (◇, ▽), otherwise as described under "Experimental Procedures." The vesicles (2 mg/ml) were then incubated with (□, △, ▽) or without (○, ■, △, ▽) 4 mM CHD for various times in 2 mM EDTA, 10% (v/v) Me\textsubscript{2}SO, and 30 mM sodium borate (pH 8.8). The reaction was quenched by Method I. The vesicles were further incubated at 25 °C for 60 min in 20 mM CaCl\textsubscript{2}, 0.1 M KCl, 20 mM MOPS, and 5 mM sodium borate (pH 7.0) (tightly bound F\textsuperscript{−} and Mg\textsuperscript{2+} are entirely released by incubation with 20 mM Ca\textsuperscript{2+} (19)) and then passed through a centrifuge column preequilibrated with a solution (Solution B) containing 40 mM MgCl\textsubscript{2}, 10 mM EGTA, 20 mM MOPS, and 5 mM sodium borate (pH 7.0). EP formation from 32P\textsubscript{i} was determined. Solid and dashed lines show least squares fits to a single exponential in which the first order rate constants were 0.024 and 0.012 min\textsuperscript{-1}, respectively. B, the SR vesicles (2.1 mg/ml) were preincubated at 37 °C for 30 min in 1.04 mM EGTA, 20.8% (v/v) Me\textsubscript{2}SO, and 31.3 mM sodium borate (pH 8.8) in the presence of 0.52 mM vanadate and 2.1 mM EDTA (◇, ▽), in the absence of vanadate and presence of 0.024 and 0.012 min\textsuperscript{-1}, respectively. The final composition was 2 mg of the vesicles/ml, 0 or 4 mM CHD, 0 or 2 mM EDTA, 0 or 2 mM EGTA, 30% (v/v) Me\textsubscript{2}SO, and 30 mM sodium borate (pH 7.0) (bound vanadate and Mg\textsuperscript{2+} were prevented by 0.1 mM Ca\textsuperscript{2+} (20)). The treated vesicles were further incubated for various times. The final composition was 2 mg of the vesicles/ml, 0 or 4 mM CHD, 0 or 0.5 mM vanadate, 0 or 20 mM MgCl\textsubscript{2}, 0 or 2 mM EDTA, 0 or 1 mM EGTA, 20% (v/v) Me\textsubscript{2}SO, and 30 mM sodium borate (pH 8.8). The reaction was quenched by Method I. The vesicles were then incubated with 3 mM CaCl\textsubscript{2} and 2 mM ATP at 25 °C for 30 min in 0.1 M KCl, 20 mM MOPS, and 5 mM sodium borate (pH 7.0) (bound vanadate and Mg\textsuperscript{2+} are entirely released by this incubation (45 and 46)). The vesicles were passed through two successive centrifuge columns, the first column being preequilibrated with Solution A and the second column with Solution B. EP formation from 32P\textsubscript{i} was determined. Solid and dashed lines show least squares fits to a single exponential in which the first order rate constants were 0.023 and 0.011 min\textsuperscript{-1}, respectively. C, the SR vesicles (2.1 mg/ml) were preincubated in the presence of 1.04 mM P\textsubscript{i}, and 8.33 mM MgCl\textsubscript{2} at 37 °C for 15 min in 2.08 mM EGTA, 31.3% (v/v) Me\textsubscript{2}SO, and 31.3 mM sodium borate (pH 8.0) (○). The amount of EP formed from P\textsubscript{i} by this preincubation was 4.1 nmol/mg. In other experiments, the vesicles were preincubated in the absence of P\textsubscript{i} and presence of 8.33 mM MgCl\textsubscript{2} (■), in the presence of 156 mM P\textsubscript{i}, and 2.1 mM EDTA and absence of MgCl\textsubscript{2} and EGTA (△, □), or in the absence of P\textsubscript{i}, MgCl\textsubscript{2}, and EGTA and presence of 2.1 mM EDTA (◇, ▽). The amount of EP formed under these conditions). After the addition of sodium borate (pH 8.0) with (○, □, △, ▽) or without (■, ■, △, ▽) CHD, the vesicles were further incubated for various times. The final composition was 2 mg of the vesicles/ml, 0 or 4 mM CHD, 0 or 0.5 mM vanadate, 0 or 20 mM MgCl\textsubscript{2}, 0 or 2 mM EDTA, 0 or 1 mM EGTA, 20% (v/v) Me\textsubscript{2}SO, and 30 mM sodium borate (pH 8.8). The reaction was quenched by Method I. The vesicles were then incubated with 3 mM CaCl\textsubscript{2} and 2 mM ATP at 25 °C for 30 min in 0.1 M KCl, 20 mM MOPS, and 5 mM sodium borate (pH 7.0) (bound vanadate and Mg\textsuperscript{2+} are entirely released by this incubation (45 and 46)). The vesicles were passed through two successive centrifuge columns, the first column being preequilibrated with Solution A and the second column with Solution B. EP formation from 32P\textsubscript{i} was determined. Solid and dashed lines show least squares fits to a single exponential in which the first order rate constants were 0.023 and 0.011 min\textsuperscript{-1}, respectively.
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previously (19). The content of tightly bound F\(^-\) was obtained by subtracing the content of F\(^-\) in the extract from the control sample.

**Determination of Bound Vanadate**—The SR vesicles (0.2 mg/ml) were incubated at 25 °C for 30 min in various concentrations of vanadate, 40% (v/v) Me\(_2\)SO, 0.1 M KCl, 30 mM MOPS, 7 mM sodium borate (pH 7.0), and other conditions described in the legend to Fig. 3. The mixture was centrifuged, and the pellet was dissolved in 2% (w/v) SDS and 0.1 N NaOH. The solution was neutralized with HCl, and the concentration of vanadate was measured by the method of Goodno (25).

**Proteolysis, Peptide Mapping, Detection of Peptides Containing DHCH-Arg, Sequencing, and Mass Spectrometry**—The CHD-modified SR vesicles (1 mg/ml) were digested with TPCK-treated trypsin (0.2 mg/ml) at 40 °C for 4 h in 10 mM CaCl\(_2\) and 50 mM sodium borate (pH 8.0). After centrifugation, the supernatant was subjected to reversed phase HPLC that was performed at a flow rate of 1 ml/min as described previously (26). The absorbance of peptides was monitored at 214 nm, and fractions of 0.3–1.4 ml each were collected. Peptides containing DHCH-Arg in the fractions were detected at 325 nm by the method of Patthy (et al.) (27) using Girard’s reagent T. It was difficult to determine the content of DHCH-Arg in the CHD-modified vesicles by this method because the background level of the absorbance was too high even after solubilization of the vesicles with SDS. Sequencing of isolated peptides was performed with an Applied Biosystems 477A/120A sequencer. For sequencing, the isolated peptides were dissolved in 0.1% trifluoroacetic acid, 50% methanol, and 0.3% 1-thioglycerol in water (v/v). The accelerating voltage was 10 kV, and ions were analyzed in the positive mode as a function of their m/z ratio.

**Phosphorylation of Ca\(^{2+}\)-ATPase**—Phosphorylation of the SR vesicles (0.4 mg/ml) with 1 mM \[^{32}\text{P}\]ATP was performed at 25 °C for 10 min in 20 mM MgCl\(_2\), 5 mM EGTA, 40% (v/v) Me\(_2\)SO, 30 mM MOPS, and 7 mM sodium borate (pH 7.0). The reaction was quenched with trichloroacetic acid containing P\(_i\). Phosphorylation of the vesicles (1 mg/ml) with 3 mM acetyl phosphate was performed at 25 °C in 5 mM MgCl\(_2\), 2 mM CaCl\(_2\), 50 mM KCl, 50 mM Tris, and 20 mM sodium borate (pH 8.0). The reaction was quenched with trichloroacetic acid containing nonradioactive acetyl phosphate. The amount of EP formed was determined as described previously (19).

**Miscellaneous Methods**—CHD was purchased from Aldrich. Girard’s reagent T and KF were from Nacalai Tesque (Kyoto, Japan). ATP was from Sigma. \[^{32}\text{P}\]ATP was obtained from NEN Life Science Products. Vanadate solutions were prepared from Na\(_3\)VO\(_4\) according to Goodno (25) just before use. P\(_i\) was purified according to Kanazawa and Boyer (12). Acetyl \[^{32}\text{P}\]phosphate was prepared by Procedure B in the method of Stadtman (28). Protein concentrations were determined by the method of Lowry et al. (12) with bovine serum albumin as a standard. Data were analyzed by the nonlinear least squares method according to the algorithm of Marquardt (30) as described previously (19).

**RESULTS**

**CHD-induced Inhibition of EP Formation from P\(_i\) and Protection by Pretreatment with F\(^-\) and Mg\(^{2+}\) against the Inhibition**—As shown in Fig. 1A, when the SR vesicles were pre-treated with neither F\(^-\) nor Mg\(^{2+}\) and then treated with CHD, EP formation from P\(_i\) was inhibited with pseudo-first order kinetics and fell to 13% of the original level in 90 min. When the vesicles were pretreated with F\(^-\) and Mg\(^{2+}\) and then treated with CHD, the enzyme was protected appreciably against the CHD-induced inhibition of EP formation (Fig. 1B). The pretreatment without F\(^-\) or Mg\(^{2+}\) provided no protection. The observed first order kinetic of the CHD-induced inhibition of EP formation (Fig. 1A–C, solid lines) is consistent with the possibility that this inhibition was caused by modification of a single residue. However, it is also possible that more than one vital arginyl residue was modified at an approximately equal rate.

**Protection by Presence of Vanadate and Mg\(^{2+}\) against CHD-induced Inhibition of EP Formation from P\(_i\)**—As shown in Fig. 2.
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Fig. 3. CHD-induced inhibition of vanadate binding and protection by pretreatment with F\(^{-}\) and Mg\(^{2+}\) against the inhibition. The SR vesicles were pretreated with KF and MgCl\(_2\) (○, ●) or without KF and with MgCl\(_2\) (□, ▲, △, ▲), otherwise as described under “Experimental Procedures.” A, the pretreated vesicles were incubated with (○, ●, ▲, △) or without (▲) 4 mM CHD for 90 min in 2 mM EDTA, 20% (v/v) Me\(_2\)SO, and 30 mM sodium borate (pH 8.8). The reaction was quenched by Method II. The vesicles were further incubated with 20 mM CaCl\(_2\) and others as in Fig. 1A (to remove tightly bound F\(^{-}\) and Mg\(^{2+}\)) and then washed with centrifugation with Solution A. Vanadate binding to the vesicles was determined at various concentrations of vanadate in the presence of 5 mM MgCl\(_2\) and 2 mM EGTA (○, □, △) or in the presence of 5 mM EDTA (○, ▲, △), otherwise as described under “Experimental Procedures.” The vesicles were further incubated with 20 mM CaCl\(_2\) and others as in A. The vesicles were phosphorylated with acetyl \([\text{32P}]\) phosphate for 5 min, and the amount of EP formed was determined. B, the pretreated vesicles were incubated with (○, □ or without (●, ▲) 4 mM CHD for 90 min, as in A. The reaction was quenched by Method II. The vesicles were further incubated with 20 mM CaCl\(_2\) and others as in A and then washed with Solution A. The resulting vesicles and native SR vesicles (×) were phosphorylated with acetyl \([\text{32P}]\) phosphate for various times, and the amount of EP formed was determined.

Fig. 4. Lack of CHD-induced inhibition of EP formation from acetyl phosphate. The SR vesicles were pretreated with KF and MgCl\(_2\) (○, ●) or without KF and with MgCl\(_2\) (□, ▲), otherwise as described under “Experimental Procedures.” A, the pretreated vesicles were incubated with (○, □ or without (●, ▲) 4 mM CHD for various times in 2 mM EDTA, 20% (v/v) Me\(_2\)SO, and 30 mM sodium borate (pH 8.8). The reaction was quenched by Method II. The vesicles were further incubated with 20 mM CaCl\(_2\) and others as in Fig. 1A (to remove tightly bound F\(^{-}\) and Mg\(^{2+}\)) and then washed by centrifugation with Solution A. The vesicles were phosphorylated with acetyl \([\text{32P}]\) phosphate for 5 min, and the amount of EP formed was determined. B, the pretreated vesicles were incubated with (○, □ or without (●, ▲) 4 mM CHD for 5 min, as in A. The reaction was quenched by Method II. The vesicles were further incubated with 20 mM CaCl\(_2\) and others as in A and then washed with Solution A. The resulting vesicles and native SR vesicles (×) were phosphorylated with acetyl \([\text{32P}]\) phosphate for various times, and the amount of EP formed was determined.

Lack of CHD-induced inhibition of EP formation from acetyl phosphate—The SR vesicles were pretreated with or without F\(^{-}\) in the presence of Mg\(^{2+}\). In the experiments shown in Fig.
FIG. 5. Peptide mapping of tryptic digests of CHD-modified SR vesicles and effects of pretreatment with F\(^-\) and Mg\(^{2+}\), of the presence of vanadate and Mg\(^{2+}\), or of EP formation from P\(_i\), on the modification with CHD. In the first series of experiments (A–D), the
4A, the pretreated vesicles were treated with or without CHD for various times. After tightly bound F$^{-}$ and Mg$^{2+}$ were removed, EP formation from acetyl phosphate was determined. CHD caused no substantial inhibition of the EP formation when the vesicles were pretreated without F$^{-}$ (compare ○ with □). The pretreatment with F$^{-}$ and Mg$^{2+}$ exerted a slight protective effect during the control incubation without CHD (compare ● with △). In the experiments shown in Fig. 4B, the pretreated vesicles were treated with or without CHD for 90 min and then phosphorylated with acetyl phosphate for various times. When the vesicles were pretreated without F$^{-}$ and treated with CHD, the rate of EP formation was essentially the same as that of EP formation in native SR vesicles (compare □ with ○). The pretreatment with F$^{-}$ and Mg$^{2+}$ had virtually no effect on the kinetics of EP formation in the CHD-treated vesicles (compare ○ with □. When the vesicles were pretreated without F$^{-}$ and treated without CHD, EP formation was appreciably faster than that in the native vesicles for unknown reasons (compare ● with △).

**Peptide Mapping of Tryptic Digests of CHD-modified SR Vesicles**—In the first series of experiments (Fig. 5, A–D), the SR vesicles were pretreated with either F$^{-}$ or Mg$^{2+}$ (A), with F$^{-}$ and without Mg$^{2+}$ (B), without F$^{-}$ and with Mg$^{2+}$ (C), or with F$^{-}$ and Mg$^{2+}$ (D) and then treated with CHD. After tightly bound F$^{-}$ and Mg$^{2+}$ were removed, the vesicles were digested with TPCK-treated trypsin and subjected to reversed phase HPLC. The peptide maps at 214 nm of the digests (Fig. 5, A–D, lower traces) agreed closely with each other. The absorbance at 325 nm of peptides containing DHCH-Arg in the peaks indicated by arrows were strongly reduced by the pretreatment with F$^{-}$ and Mg$^{2+}$ (compare upper trace of Fig. 5D with upper trace of Fig. 5A) but not by the pretreatment without F$^{-}$ or Mg$^{2+}$ (compare upper traces of Fig. 5, A-D, lower traces) agreed closely with each other. The absorbance at 325 nm of peptides containing DHCH-Arg in the peaks indicated by arrows were strongly reduced by the pretreatment with F$^{-}$ and Mg$^{2+}$.

In the second series of experiments (Fig. 5, E–G), the SR vesicles were treated with CHD in the absence of vanadate and presence of Mg$^{2+}$ (E), in the presence of vanadate and absence of Mg$^{2+}$ (F), or in the presence of vanadate and Mg$^{2+}$ (G). After bound vanadate and Mg$^{2+}$ were removed, the vesicles were digested with TPCK-treated trypsin and subjected to reversed phase HPLC. The peaks indicated by arrows were substantially reduced only when both vanadate and Mg$^{2+}$ were present (compare upper trace of Fig. 5G with upper traces of Fig. 5, E and F).

In the third series of experiments (Fig. 5, H–J), the SR vesicles were preincubated in the absence of P$_i$ and presence of 8.33 mM MgCl$_2$ (H), in the presence of 156 mM P$_i$ and absence of Mg$^{2+}$ (I), or in the presence of 1.04 mM P$_i$ and 8.33 mM MgCl$_2$ (J) and then treated with CHD. The vesicles were digested with TPCK-treated trypsin and subjected to reversed phase HPLC. The preincubination in the presence of P$_i$ and Mg$^{2+}$ (4.1 nmol of EP per mg of SR vesicles was formed by this preincubation) induced a small but definite reduction in the peaks indicated by arrows (compare upper trace of Fig. 5J with upper trace of Fig. 5H). Binding of P$_i$ to the catalytic site in the presence of 150 mM P$_i$ and absence of Mg$^{2+}$ had no effect on these peaks (compare upper trace of Fig. 5I with upper trace of Fig. 5H).

It is clear from the chromatographic profiles that the peaks reduced by the presence of vanadate and Mg$^{2+}$ (Fig. 5, E–G, arrows) and by the presence of P$_i$ and Mg$^{2+}$ (Fig. 5, H–J, arrows) corresponded to those reduced by the pretreatment with F$^{-}$ and Mg$^{2+}$ (Fig. 5, A–D, arrows).

**Purification of Peptides Containing DHCH-Arg**—The SR vesicles were pretreated without F$^{-}$ and with Mg$^{2+}$ (Fig. 6A) or with F$^{-}$ and Mg$^{2+}$ (Fig. 6B) and then treated with CHD. After tightly bound F$^{-}$ and Mg$^{2+}$ were removed, the vesicles were digested with TPCK-treated trypsin and subjected to the first reversed phase HPLC (Fig. 6, A and B).

Fraction I and fraction II in the figures were pooled separately and subjected to the second reversed phase HPLC (Fig. 6, C–F). Fraction I in Fig. 6A gave a single major peak at 325 nm (upper trace of Fig. 6C, fraction I). Fraction I in Fig. 6B gave a major peak at 325 nm at the same retention time as in Fig. 6C (upper trace of Fig. 6D, fraction I), but this peak was much smaller than the corresponding peak in Fig. 6C. Fraction II in Fig. 6A gave two close peaks at 325 nm (upper trace of Fig. 6E, fraction II). These two peaks were greatly reduced (upper trace of Fig. 6F, fraction II) when HPLC was performed with fraction II in Fig. 6B.

Peptides containing DHCH-Arg in fraction I in Fig. 6 (C and D) and fraction II in Fig. 6 (E and F) were further purified by the third reversed phase HPLC (Fig. 6, G–J). Fraction I in Fig. 6C gave a major peak and a minor peak at 325 nm (upper trace of Fig. 6G, peak Ia and peak Ib). Fraction I in Fig. 6D also gave a major peak and a minor peak at 325 nm (upper trace of Fig. 6H, peak Ia and peak Ib), but these peaks were much smaller than the corresponding peaks in Fig. 6G. Fraction II in Fig. 6E gave two major peaks at 325 nm (upper trace of Fig. 6I, peak IIa and peak Iib). Fraction II in Fig. 6F also gave two major peaks at 325 nm (upper trace of Fig. 6J, peak IIa and peak Iib), but these two peaks were much smaller than the corresponding peaks in Fig. 6I.

**Sequencing and Mass Analysis of Peptides Containing DHCH-Arg**—The peptides containing DHCH-Arg isolated as above (peak Ia and peak Ib in Fig. 6, G and H, and peak IIa and peak IIb in Fig. 6, I and J) were sequenced (Table I). All the isolated peptides gave the same sequence (His-190 to Lys-204) in the Ca$^{2+}$-ATPase, in which a missing residue (X) corresponded to Arg-198. These results suggest that Arg-198 in the Ca$^{2+}$-ATPase was modified with CHD. This is consistent with the previously reported findings (16) that carboxyl-terminal peptide bonds of CHD-modified arginyl residues are resistant...
to tryptic cleavage. The molecular masses of these peptides determined by mass spectrometry were in good agreement with the monoisotopic mass calculated from the above sequence on the assumption that X is DHCH-Arg (Table II). These findings show that Arg-198 in the Ca\textsuperscript{2+}-ATPase was modified with CHD and that this modification of Arg-198 was strongly inhibited by the pretreatment with F\textsuperscript{−} and Mg\textsuperscript{2+}. Furthermore, it is very likely that the modification of Arg-198 with CHD was specifically inhibited also by the presence of vanadate and Mg\textsuperscript{2+} (Fig. 5, E–G, arrows) or by the presence of P\textsubscript{i} and Mg\textsuperscript{2+} (Fig. 5, H–J, arrows). The reason why peptides with the same sequence and same modification gave different retention times in HPLC remains obscure.

**DISCUSSION**

The observed protection by tight binding of F\textsuperscript{−} and Mg\textsuperscript{2+} (Fig. 1A) or by high affinity binding of vanadate (Fig. 1B) against the CHD-induced inhibition of EP formation from P\textsubscript{i}...
indicates that no phenylthiohydantoin derivatives were detected. Protection by tight binding of F
modification with CHD (Figs. 2 and 3) and that the enzyme is binding of the transition state analogs is inhibited by the mod-
transition state. This view is consistent with the findings that 
X is also in harmony with the observed lack of protection by the
induced inhibition of high affinity vanadate binding (Fig. 3). It
different from the structure of the transition state for
formation from the magnesium-enzyme-phosphate complex.
and Mg
residue(s) is located close to the essential components (bound
arginyl residue(s) protected by these transition state analogs contributes toward formation of the transi-
suggests that an arginyl residue(s) protected by these transition state analogs plays a key role in the formation of the transition state for EP.

Masses of the peptides containing DHCH-Arg in Table I were deter-
Masses of peptides containing DHCH-Arg shown in Fig. 6 (G–J) were sequenced. The numbers in parentheses indicate pmol of amino acid at the given cycle. X represents the expected DHCH-arginyl residues, which cannot be detected by the method of sequencing used. The dash (—) indicates that no phenylthiohydantoin derivatives were detected.

| Sequence of peptides containing DHCH-Arg | Mass of peptides containing DHCH-Arg |
|-----------------------------------------|------------------------------------|
| 1 | His (22) | His (27) | His (13) | His (50) | His (19) | His (7) | His (128) | His (24) |
| 2 | Thr (591) | Thr (249) | Thr (173) | Thr (239) | Thr (215) | Thr (54) | Thr (288) | Thr (261) |
| 3 | Glu (373) | Glu (219) | Glu (245) | Glu (348) | Glu (201) | Glu (52) | Glu (712) | Glu (245) |
| 4 | Pro (1811) | Pro (678) | Pro (644) | Pro (880) | Pro (700) | Pro (112) | Pro (1234) | Pro (932) |
| 5 | Val (2485) | Val (1144) | Val (1173) | Val (1422) | Val (1409) | Val (271) | Val (2022) | Val (1283) |
| 6 | Pro (1488) | Pro (590) | Pro (494) | Pro (686) | Pro (614) | Pro (84) | Pro (953) | Pro (653) |
| 7 | Asp (155) | Asp (59) | Asp (94) | Asp (82) | Asp (48) | Asp (8) | Asp (129) | Asp (113) |
| 8 | Pro (1152) | Pro (372) | Pro (329) | Pro (341) | Pro (382) | Pro (61) | Pro (701) | Pro (487) |
| 9 | X | X | X | X | X | X | X | X |
| 10 | Ala (823) | Ala (362) | Ala (272) | Ala (257) | Ala (359) | Ala (59) | Ala (444) | Ala (437) |
| 11 | Val (1012) | Val (409) | Val (407) | Val (406) | Val (467) | Val (91) | Val (623) | Val (525) |
| 12 | Asn (244) | Asn (73) | Asn (68) | Asn (56) | Asn (108) | Asn (13) | Asn (62) | Asn (126) |
| 13 | Gln (340) | Gln (125) | Gln (85) | Gln (72) | Gln (171) | Gln (16) | Gln (130) | Gln (165) |
| 14 | Asp (129) | Asp (37) | Asp (48) | Asp (54) | Asp (15) | Asp (4) | Asp (76) | Asp (85) |
| 15 | Lys (320) | Lys (93) | Lys (99) | Lys (119) | Lys (90) | Lys (6) | Lys (184) | Lys (185) |
| 16 | — | — | — | — | — | — | — |
| 17 | — | — | — | — | — | — | — |
| 18 | — | — | — | — | — | — |

| TABLE I | Sequences of peptides containing DHCH-Arg |
|-----------------------------|--------------------------------------------------|
| Cycle No. | Peptides in Fig. 6 (G and I) | Peptides in Fig. 6 (H and J) |
|-----------------------------------------|-----------------------------------------|
| 1 | His (22) | His (27) | His (13) | His (50) | His (19) | His (7) | His (128) | His (24) |
| 2 | Thr (591) | Thr (249) | Thr (173) | Thr (239) | Thr (215) | Thr (54) | Thr (288) | Thr (261) |
| 3 | Glu (373) | Glu (219) | Glu (245) | Glu (348) | Glu (201) | Glu (52) | Glu (712) | Glu (245) |
| 4 | Pro (1811) | Pro (678) | Pro (644) | Pro (880) | Pro (700) | Pro (112) | Pro (1234) | Pro (932) |
| 5 | Val (2485) | Val (1144) | Val (1173) | Val (1422) | Val (1409) | Val (271) | Val (2022) | Val (1283) |
| 6 | Pro (1488) | Pro (590) | Pro (494) | Pro (686) | Pro (614) | Pro (84) | Pro (953) | Pro (653) |
| 7 | Asp (155) | Asp (59) | Asp (94) | Asp (82) | Asp (48) | Asp (8) | Asp (129) | Asp (113) |
| 8 | Pro (1152) | Pro (372) | Pro (329) | Pro (341) | Pro (382) | Pro (61) | Pro (701) | Pro (487) |
| 9 | X | X | X | X | X | X | X | X |
| 10 | Ala (823) | Ala (362) | Ala (272) | Ala (257) | Ala (359) | Ala (59) | Ala (444) | Ala (437) |
| 11 | Val (1012) | Val (409) | Val (407) | Val (406) | Val (467) | Val (91) | Val (623) | Val (525) |
| 12 | Asn (244) | Asn (73) | Asn (68) | Asn (56) | Asn (108) | Asn (13) | Asn (62) | Asn (126) |
| 13 | Gln (340) | Gln (125) | Gln (85) | Gln (72) | Gln (171) | Gln (16) | Gln (130) | Gln (165) |
| 14 | Asp (129) | Asp (37) | Asp (48) | Asp (54) | Asp (15) | Asp (4) | Asp (76) | Asp (85) |
| 15 | Lys (320) | Lys (93) | Lys (99) | Lys (119) | Lys (90) | Lys (6) | Lys (184) | Lys (185) |
| 16 | — | — | — | — | — | — | — |
| 17 | — | — | — | — | — | — | — |
| 18 | — | — | — | — | — | — |

Arg-198 is conserved in sarco(endo)plasmic reticulum Ca

Modification of Arg-198 in SR Ca\(^{2+}\)-ATPase by Cyclohexanedione

ATPases (1, 34–36) but not in other P-type ATPases including the Na\(^+\),K\(^+\)-ATPase (37, 38), H\(^+\),K\(^+\)-ATPase (39, 40), and plasma membrane Ca\(^{2+}\)-ATPase (41). Therefore, although it is possible that Arg-198 is directly involved in the catalytic process of EP formation from P\(_i\), it appears more likely that the observed inhibition (at least the portion reduced by the transition state analogs) is due to steric hindrance induced by CHD-modified Arg-198.

The secondary structural model for the Ca\(^{2+}\)-ATPase suggests that the enzyme is composed of 10 transmembrane \(\alpha\)-helices (M\(_1\)–M\(_{10}\)) and a cytoplasmic globular fragment that is divided into two main domains, a small cytoplasmic loop (Ala-132–Asp-237 between M\(_2\) and M\(_3\)) and a large cytoplasmic loop (Asn-330–Phe-740 between M\(_4\) and M\(_5\)) (1). The large cytoplasmic loop contains the phosphorylation site and the ATP binding site. The functional role of the small cytoplasmic loop is less clear. However, in the previous studies on the function of this region, it was shown that the conformational change of EP is blocked by site-directed mutations of Thr-181, Gly-182, Glu-183 (42), and Gly-233 (43) and by a tryptic cleavage at Lys-234 or Arg-236 (44). It was also shown (43) that the affinity for P\(_i\) in EP formation from P\(_i\) is reduced in the Gly-233 mutants. Accordingly, it is likely that this small cytoplasmic loop including Arg-198 also contributes in part to the catalytic site.

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