Identification of Arginyl Residues Located at the ATP Binding Site of Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase

MODIFICATION WITH 1,2-CYCLOHEXANEDIONE*

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Sarcoplasmic reticulum vesicles were treated with 1,2-cyclohexanediene in sodium borate (pH 8.0). The Ca\(^{2+}\)-ATPase activity was completely inhibited. Inhibition of Mg-ATP and Mg-ADP binding to the high affinity ATP binding site as well as inhibition of phosphorylation with ATP occurred simultaneously with the inhibition of the Ca\(^{2+}\)-ATPase activity. Phosphorylation with acetyl phosphate was not inhibited. The Ca\(^{2+}\)-ATPase was strongly protected by Mg-ATP, Mg-ADP, and MgAMP against this inhibition. Binding of acetyl phosphate or Pi to the enzyme gave no protection. Phosphorylation with acetyl phosphate also had no protective effect. Peptide mapping of the tryptic digests, detection of peptides containing CHD-modified arginyl residues with Girard’s reagent T, and sequencing revealed that Arg-489, Arg-505, and Arg-678 were modified with CHD. Mg-ATP against this modification, but partially protected by prelabeling with fluorescein 5-isothiocyanate, which occupies the adenosine binding region in the ATP binding site. In contrast, Arg-505 was slightly protected by Mg-ATP and almost completely protected by prelabeling with fluorescein 5-isothiocyanate. Taken together, these findings suggest that Arg-489 and Arg-678 are located in or near the region occupied by the triphosphate moiety of ATP, either or both of these residues being in close to the region occupied by the \(\alpha\)-phosphoryl group in the high affinity ATP binding site and involved in the CHD-induced inhibition of this enzyme and that Arg-505 is very close to (but slightly out of) this adenine binding region in the ATP binding site. The acetyl phosphatase activity and phosphorylation with Pi were also inhibited by the CHD treatment, but the inhibitions were considerably slower than those described above. This suggests that the arginyl residues involved in these inhibitions are distinct from that involved in the inhibition of the Ca\(^{2+}\)-ATPase activity.

The SR\(^1\) Ca\(^{2+}\)-ATPase catalyzes Ca\(^{2+}\) transport coupled to ATP hydrolysis (1, 2). This enzyme consists of a single 110-kDa polypeptide chain, and its primary structure has been revealed (3). The enzyme has one high affinity ATP binding site (catalytic site) in the cytoplasmic domain and two high affinity Ca\(^{2+}\)-binding sites in the transmembrane domain (4). In the catalytic cycle, the enzyme is activated by Ca\(^{2+}\) binding to the Ca\(^{2+}\)-binding sites, and then the \(\gamma\)-phosphoryl group of Mg-ATP bound to the ATP binding site is transferred to Asp-351 (3, 5–7) to form an EP intermediate (8, 9). Acetyl phosphate can also be hydrolyzed as a substrate through EP formation with the Ca\(^{2+}\)-activated enzyme (10, 11). In addition, EP is formed from Pi in the absence of Ca\(^{2+}\) and presence of Mg\(^{2+}\) by reversal of the late step of the catalytic cycle (12, 13).

Some residues located at or near the ATP binding site of this enzyme were previously identified by chemical modifications. The identified residues include Lys-492 (14–18), Lys-515 (19, 20), Thr-532 (21), Thr-533 (21), and Lys-684 (14, 22). It is well known (23–27) that a number of enzymes contain arginyl residues that are located at the catalytic site and closely involved in substrate binding and catalysis. Murphy (28) carried out a modification of the SR Ca\(^{2+}\)-ATPase with 2,3-butanedione (a specific reagent for modification of arginyl residues) and showed that one or more arginyl residues play a role in the substrate-active site interaction. Bishop (29) further suggested on the basis of a modification study with phenylglyoxal (another specific reagent for modification of arginyl residues) that the ATP binding site of this enzyme contains a single arginyl residue at the \(\alpha\)-phosphate position. On the other hand, modifications of the plasma membrane Ca\(^{2+}\)-ATPase from erythrocytes (30) and smooth muscle (31) with phenylglyoxal suggested that an essential arginyl residue is present at the low affinity (but not high affinity) ATP binding site (30) or that an arginyl residue(s) is involved in a functionally important interaction of the acidic lipids with the enzyme protein (31). However, the arginyl residues predicted in the above studies have not yet been identified. Site-directed mutagenesis studies made hitherto showed that Arg-604 (32), Arg-615 (33), and Arg-620 (33) are not essential.

For a better understanding of structure-function relationships in the catalytic site of the Ca\(^{2+}\)-ATPase, it is now critically important to identify arginyl residues located at the catalytic site of this enzyme. Previously, McIntosh and co-workers (34, 35) showed that cross-linking between Lys-492 and Arg-678 by glutaraldehyde decreases the affinity of this enzyme for nucleotides and that nucleotide binding inhibits formation of this cross-link. Those findings suggested that Arg-678 is a likely candidate for arginyl residues located at the ATP binding site, although the possibility cannot be excluded that only Lys-492 in the cross-linked Lys-492 and Arg-678 is located at the ATP binding site.

In this study, the SR Ca\(^{2+}\)-ATPase has been modified with
CHD under the conditions in which CHD reacts specifically with arginyl residues to form a single stable product, DHCH-Arg (36). The Ca²⁺-ATPase activity, binding of MgATP or MgADP to the high affinity ATP binding site, and phosphorylation of the enzyme with ATP have been almost completely inhibited. Peptide mapping of the tryptic digests and sequencing of peptides containing DHCH-Arg have been performed. The results suggest that Arg-489 and Arg-678 are located in or near the region occupied by the triphosphate moiety of ATP, either or both of these residues being in or close to the region occupied by the α-phosphoryl group in the high affinity ATP binding site and involved in the CHD-induced inhibition of this enzyme, and that Arg-505 is very close to (but slightly out of) the adenosine binding region in the ATP binding site.

**EXPERIMENTAL PROCEDURES**

**Preparation of SR Vesicles**—SR vesicles were prepared from rabbit skeletal muscle and stored at −80°C as described previously (37). The content of phosphorylation site determined with [γ-³²P]ATP according to Barrabin et al. (38) was 4.08 ± 0.07 nmol/mg (n = 3).

**Chemical Modification with CHD**—Modification was started at 37°C by adding CHD to a suspension of the SR vesicles. The reaction was quenched at 0°C by one of the following methods. Method I, the mixture was diluted 95 times with a solution (pH 7.0) containing 7.4 mM MgCl₂, 0.53 mM CaCl₂, 0.43 mM EGTA, 85 mM KCl, 1.8 µM A23187, 21 mM MOPS, and 53 mM sodium borate. Method II, the mixture was applied twice to centrifuge columns (5-ml disposable syringe filled with coarse Sephadex G-50) pre-equilibrated with a solution (pH 7.0) containing 80 mM KCl, 4 mM MOPS, and 10 mM sodium borate. Method III, the mixture was centrifuged, and the pellet was washed three times with 50 mM sodium borate (pH 8.0) and suspended in this buffer. Method I was used for the assay of ATPase activity, Method II used for the assays of acetyl phosphate activity, enzyme phosphorylation, and binding of ATP and ADP, and Method III used for proteolysis.

**Modification of FITC-Prelabelled SR Vesicles with CHD**—Prelabeling with FITC was performed as described previously (17). This labeling caused 95% inhibition of the Ca²⁺-ATPase. The content of bound FITC determined at 496 nm with an extinction coefficient of 80,000 (39) in 10% SDS at pH 8.8 was 11.3 nmol/mg. The FITC-prelabelled SR vesicles were then modified with CHD as described above.

**Proteolysis, Peptide Mapping, Detection of Peptides Containing DHCH-Arg, and Sequencing**—The CHD-modified SR vesicles as well as the FITC-prelabelled 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 CaCl₂ and 50 mM sodium borate (pH 8.0). After centrifugation, the supernatant was subjected to reversed phase HPLC, which was performed at a flow rate of 1 ml/min as described previously (17). The absorbance of peptides was monitored at 214 nm and the fluorescence of FITC monitored with excitation at 485 nm and emission at 525 nm. Fractions of 1 ml each were collected, unless otherwise stated. Peptides containing DHCH-Arg in the fractions were detected at 325 nm by the method of Patthy et al. (40) using Girard’s reagent T. Sequencing of isolated peptides was performed with an Applied Biosystems 477A/120A sequencer. It was difficult to determine the content of DHCH-Arg in the other experiments for ligand-induced protection, the vesicles were treated with CHD in the presence of 10 mM MgCl₂ and 10 mM EGTA, otherwise as described under “Experimental Procedures.” The Ca²⁺-ATPase activity was determined. Dashed and dotted lines drawn under solid circles and crosses, respectively, show least squares fit to a single exponential, in which the first order rate constants were 0.070 and 0.066 min⁻¹, respectively. C, the SR vesicles were treated with CHD for 25 min in the presence of various concentrations of MgATP (○), MgADP (▲), acetyl phosphate (▲), or Pi (×), respectively, and the Ca²⁺-ATPase activity was determined. The Ca²⁺-ATPase activity in the control (3.06 ± 0.04 µmol/min/mg (n = 6)), in which the vesicles were treated without CHD, was set at 100%.

**FIG. 1. CHD-induced inhibition of Ca²⁺-ATPase activity and of phosphorylation with ATP and protection of Ca²⁺-ATPase by various ligands against the inhibition.** A, the SR vesicles were treated with (●, □) or without (○, △) CHD for various times. In the experiments for ligand-induced protection, the vesicles were treated with CHD in the presence of 10 mM MgATP (○), 9.3 mM MgADP (▲), 23 mM MgAMP (△), or 0.1 mM CaCl₂ without EGTA (×) under the otherwise same conditions as above. The reaction was quenched at the indicated times after the addition of CHD, and the Ca²⁺-ATPase activity (○, △, ▲, □) and phosphorylation with ATP (●, □) were determined. Dashed, dotted, and solid lines drawn under solid circles, crosses, and solid squares, respectively, show least squares fit to a single exponential, in which the first order rate constants were 0.082, 0.076, and 0.052 min⁻¹, respectively. B, the SR vesicles were treated with (●, △) or without (○, □) CHD in the presence (×) or absence (○, △) of 1.0 mM acetyl phosphate in a medium containing 5 mM CaCl₂ and 10 µM A23187 without MgCl₂ and EGTA, otherwise as described under “Experimental Procedures.” The Ca²⁺-ATPase activity was determined. Dashed and dotted lines drawn under solid circles and crosses, respectively, show least squares fit to a single exponential, in which the first order rate constants were 0.070 and 0.066 min⁻¹, respectively.
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CHD-modified vesicles by the above method of Patthy et al. (40), because the background level of the absorbance was too high even after solubilization of the vesicles with SDS.

\textbf{Ca\(^{2+}\)-ATPase Activity—}The total ATPase activity was determined at 25 °C in a mixture (pH 7.0) containing 0.1 mg of SR vesicles/ml, 1 mM acetyl phosphate, 5 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), 0.4 mM EGTA, 80 mM KCl, 1.7 mM A23187, 20 mM MOPS, and 50 mM sodium borate. The Ca\(^{2+}\)-ATPase activity was obtained by subtracting the Ca\(^{2+}\)-independent ATPase activity (determined in the presence of 5 mM EGTA without added CaCl\(_2\)) from the total ATPase activity.

\textbf{Acetyl Phosphatase Activity—}Hydrolysis of acetyl phosphate was performed at 25 °C in a mixture (pH 7.0) containing 0.1 mg of SR vesicles/ml, 1 mM acetyl phosphate, 5 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), 0.4 mM EGTA, 80 mM KCl, 2 μM A23187, 20 mM MOPS, and 50 mM sodium borate. The reaction was quenched by addition of a neutralized hydrazine solution, and the amount of acetyl phosphate remaining was determined by the method of Lipmann and Tuttle (41). The acetyl phosphatase activity was obtained by subtracting the rate of acetyl phosphate hydrolysis determined in the presence of 5 mM EGTA without added CaCl\(_2\) from that determined as described above.

\textbf{Phosphorylation of Ca\(^{2+}\)-ATPase—}Phosphorylation of the SR vesicles (1 mg/ml) with 0.1 mM [γ-\(^{32}\)P]ATP was performed at 25 °C (pH 8.0) for 5 s in 5 mM MgCl\(_2\), 10 mM CaCl\(_2\), 80 mM KCl, and 50 mM sodium borate. Phosphorylation of the SR vesicles (0.2 mg/ml) with 1 mM acetyl[\(^{32}\)P]phosphate was performed at 25 °C (pH 7.0) for 1 min in 5 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), 0.4 mM EGTA, 80 mM KCl, 2 μM A23187, 20 mM MOPS, and 50 mM sodium borate. The phosphorylation of the SR vesicles (0.25 mg/ml) with 1 mM [\(^{32}\)P]ATP was performed at 25 °C (pH 7.0) for 10 min in 20 mM MgCl\(_2\), 2 mM EGTA, 40% (v/v) Me\(_2\)SO, 30 mM MOPS, and 7 mM sodium borate. The amount of \(\mathrm{PF_2}\) formed was determined as described previously (42).

\textbf{Binding of ATP and ADP—}The SR vesicles (0.1 mg/ml) were incubated at 25 °C (pH 7.0) for 5 s in various concentrations of [\(^{32}\)P]ATP or [\(^{32}\)P]ADP, 5 mM MgCl\(_2\), 5 mM EGTA, 0.1 mM KCl, 30 mM MOPS, and 7 mM sodium borate. The amount of [\(^{32}\)P]ATP or [\(^{32}\)P]ADP bound to the vesicles was determined as described previously (42).

\textbf{Miscellaneous Methods—}CHD was purchased from Aldrich. Girard's reagent T was from Nacalai Tesque (Kyoto, Japan). ATP, ADP, and AMP were obtained from Yamasa Biochemicals (Choshi, Japan). Acetyl phosphate was from Kohjin (Tokyo, Japan). TPCK-treated trypsin and FITC were from Sigma. [γ-\(^{32}\)P]ATP and [α-\(^{32}\)P]ADP were obtained from DuPont NEN. [α-\(^{32}\)P]ADP was prepared by hydrolysis of [\(^{32}\)P]ATP with SR vesicles and purified by the method of Cohn (43). Acetyl[\(^{32}\)P]phosphate was prepared by Procedure B in the method of Stadman (44). [\(^{32}\)P]ATP was purified according to Kanazawa and Boyer (12). The steady-state intensity of the trypophan fluorescence was measured as described previously (37). Protein concentrations were determined by the method of Lowry et al. (45) with bovine serum albumin as a standard. Calculation of the concentrations of free ligands and complexes was performed as described previously (42). Data were analyzed by the nonlinear least squares method with the program RS/1 (BBN Software Corp., Cambridge, MA).

\textbf{RESULTS—}CHD-induced Inhibition of Ca\(^{2+}\)-ATPase Activity and of Phosphorylation with ATP—When the SR vesicles were treated with CHD for various times, the Ca\(^{2+}\)-ATPase activity decreased with pseudo-first order kinetics and completely disappeared in 60 min (Fig. 1A). Phosphorylation of the enzyme with ATP was also completely inhibited, although the inhibition was somewhat slower than that of the Ca\(^{2+}\)-ATPase activity.

\textbf{Protection of Ca\(^{2+}\)-ATPase by Various Ligands against CHD-}
induced Inhibition—The Ca\textsuperscript{2+}-ATPase was strongly protected by Mg-ATP against the CHD-induced inhibition (Fig. 1A). The enzyme was also protected by Mg-ADP and Mg-AMP, but these nucleotides appeared to be somewhat less effective than Mg-ATP. The presence of Ca\textsuperscript{2+} gave no protection (Fig. 1, A and B). Phosphorylation of the enzyme with acetyl phosphate also had no protective effect under the conditions in which the maximum steady-state EP level is attained (11) (Fig. 1B and cf. Fig. 3B).\textsuperscript{2}

The SR vesicles were treated with CHD in the presence of various concentrations of ligands. The protection of the enzyme was increased with increasing concentrations of Mg-ATP, Mg-ADP, and Mg-AMP (Fig. 1C). The protection followed saturation kinetics. The degrees of protection by Mg-ATP, Mg-ADP, and Mg-AMP decreased in that order. The concentrations of Mg-ATP and Mg-ADP giving a half-maximum protection were much higher than the previously reported dissociation constants for these nucleotides in the ATP binding site (46). This is most probably due to the fact that the CHD-induced inhibition is irreversible. The concentration of Mg-AMP giving a half-maximum protection was seemingly close to the previously reported dissociation constant for AMP in the ATP binding site (47). However, the maximum degree of protection and the concentration of Mg-AMP giving a half-maximum protection estimated from the least squares fitting in Fig. 1C are likely unreliable because of the very low affinity of the ATP binding site for AMP. Acetyl phosphate or P\textsubscript{i} in the absence of Ca\textsuperscript{2+} gave no protection.

CHD-induced Inhibition of ATP and ADP Binding—The SR vesicles were treated with CHD for 60 min, and binding of ATP or ADP to the vesicles was determined at various concentrations of Mg-ATP or Mg-ADP (Fig. 2, A and B). In the control, in which the vesicles were treated without CHD, the dissociation constants for Mg-ATP and Mg-ADP were in agreement with those previously reported for Mg-ATP and Mg-ADP in the high affinity ATP binding site (46). The maximum amounts of bound ATP and ADP were somewhat larger than the content of phosphorylation site (cf. “Experimental Procedures”). Binding of ATP or ADP to the vesicles was greatly reduced by the CHD treatment. The residual binding to the CHD-treated vesicles is probably due to nonspecific adsorption. These results show that binding of Mg-ATP or Mg-ADP to the high affinity ATP binding site was almost completely inhibited by the CHD treatment. When the vesicles were treated with CHD for various times (Fig. 2, C and D), inhibition of ATP and ADP binding followed pseudo-first order kinetics that agreed fairly well with the kinetics of inhibition of the Ca\textsuperscript{2+}-ATPase activity under the same conditions (cf. Fig. 1A).

CHD-induced Inhibition of Acetyl Phosphatase Activity and Lack of Inhibition of Phosphorylation with Acetyl Phosphate—When the SR vesicles were treated with CHD for various times, the acetyl phosphatase activity decreased with pseudo-first order kinetics (Fig. 3A). The inhibition was considerably slower than that of the Ca\textsuperscript{2+}-ATPase activity (cf. Fig. 1A). The acetyl phosphatase activity was protected by Mg-ATP against the CHD-induced inhibition, but the protection was much less effective than that against the inhibition of the Ca\textsuperscript{2+}-ATPase activity (cf. Fig. 1A).

Phosphorylation with acetyl phosphate was not inhibited by the CHD treatment (Fig. 3B). This finding shows that the phosphoryl transfer from the bound substrate to Asp-351 in the forward reaction is not blocked by modification of arginyl residues with CHD. Actually, the steady-state level of EP formed with acetyl phosphate was markedly increased by the CHD treatment. This increase was partially repressed by Mg-ATP.

The Ca\textsuperscript{2+}-induced tryptophan fluorescence change, which reflects a conformational change involved in the Ca\textsuperscript{2+}-induced activation of the Ca\textsuperscript{2+}-ATPase (48), was unaffected by the CHD
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Mg\(^{2+}\) fluorescence of FITC (containing DHCH-Arg at 325 nm) with CHD. Vesicles and effects of Mg\(^{2+}\) on ATP (Fig. 1A), phosphorylation with ATP (cf. Fig. 1A), and acetyl phosphatase activity (cf. Fig. 3A). The inhibition was partially repressed by Mg-ATP.

Peptide Mapping of Tryptic Digests of CHD-modified SR Vesicles—CHD modification was performed in the absence (Fig. 5A) and presence (Fig. 5B) of Mg-ATP. In another experiment, the modification was performed with the FITC-prelabeled vesicles (Fig. 5, C and D). The tryptic digests of the modified vesicles were subjected to reversed phase HPLC. The peptide maps at 214 nm of these digests (Fig. 5, A–C, lower traces) agreed closely with each other. On the other hand, the absorbance at 325 nm of peptides containing DHCH-Arg in fraction I, fraction II, and fraction III shown in Fig. 5A (upper trace) was substantially reduced by Mg-ATP (Fig. 5B, upper trace) and FITC (Fig. 5C, upper trace). This reduction was more clearly revealed by the profiles that represent the Mg-ATP-sensitive part (Fig. 5E) and FITC-sensitive part (Fig. 5F) of the absorbance at 325 nm.

FITC-labeled peptides in two major peaks of the FITC fluorescence (Fig. 5D, peak 1 and peak 2) were purified and sequenced. These peaks contained a single FITC-labeled peptide with a sequence of MVFXGAEPEGVIDR (peak 1) or VX-GAPEGVDR (peak 2), in which X represents FITC-labeled Lys-515 of the Ca\(^{2+}\)-ATPase. These results show that Lys-515 was exclusively labeled with FITC, being in agreement with the findings reported by Mitchinson et al. (20). The content of bound FITC was about twice (strictly speaking, somewhat more than twice) the content of phosphorylation site (cf. “Experimental Procedures”). This stoichiometry of FITC binding is in agreement with our previous results (17) that were obtained under the same conditions as in the present experiments. This is also in accord with our observations suggesting that the stoichiometry of the FITC binding site (Lys-515) to the phosphorylation site in the Ca\(^{2+}\)-ATPase of SR vesicles is 2:1. The observed FITC labeling somewhat in excess of this stoichiometry is likely due to nonspecific adsorption to the vesicles. The above findings are in line with the our previously proposed view (49, 50) that only half of the Ca\(^{2+}\)-ATPase molecules present in SR vesicles are phosphorylatable.

Purification of Peptides Containing DHCH-Arg—Fraction I, fraction II, and fraction III in Fig. 5 (A–C) were collected separately and subjected to the second reversed phase HPLC (Fig. 6). Fraction I in Fig. 5A gave a single major peak at 325 nm (upper trace of Fig. 6A, fraction I). Fraction I in Fig. 5B again gave a single major peak at 325 nm (upper trace of Fig.

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Phosphorylation by ATP (or P) or labeling by FITC (15 µM FITC (pH 8.0), 25°C, 240 min) was performed with SR vesicles under the same conditions as in the present experiments. The resulting vesicles were solubilized in lithium dodecyl sulfate and then the Ca\(^{2+}\)-ATPase was isolated by size exclusion HPLC. Peptide mapping of the tryptic digest and sequencing showed that Lys-515 of the Ca\(^{2+}\)-ATPase was exclusively labeled. The content of EP formed from ATP (4.57 nmol/mg of Ca\(^{2+}\)-ATPase) or from P (4.94 nmol/mg of Ca\(^{2+}\)-ATPase) in the Ca\(^{2+}\)-ATPase is approximately half the content of bound FITC (8.17–8.25 nmol/mg of Ca\(^{2+}\)-ATPase) in the isolated Ca\(^{2+}\)-ATPase and also half the content of the Ca\(^{2+}\)-ATPase molecule (9.06 nmol/mg of Ca\(^{2+}\)-ATPase) calculated from the molecular mass of this enzyme (110, 331 Da) (S. Nakamura, H. Suzuki, and T. Kanazawa, unpublished observations).
FIG. 6. Purification of peptides containing DHCH-Arg. Fractions containing DHCH-Arg shown in Fig. 5 were subjected to reversed phase HPLC. Fractions subjected to HPLC: A, fraction I in Fig. 5A; B, fraction I in Fig. 5B; C, fraction I in Fig. 5C; D, fraction II in Fig. 5A; E, fraction II in Fig. 5B; F, fraction II in Fig. 5C; G, fraction III in Fig. 5A; H, fraction III in Fig. 5B; I, fraction III in Fig. 5C. Elution was performed with the following linear gradient of acetonitrile in 1 mM acetic acid: A–C, 0% from 0 to 10 min, 9% at 70 min, and 100% at 85 min; D–F, 0% at 0 min,
6B, fraction I), but this peak was slightly smaller than the corresponding peak in Fig. 6A. Fraction I in Fig. 5C gave only a trivial peak at 325 nm at the corresponding retention time (Fig. 6C, upper trace). Fraction II in Fig. 5A gave a single major peak at 325 nm (upper trace of Fig. 6D, fraction II), whereas fraction II in Fig. 5B gave no peak at the corresponding retention time (Fig. 6E, upper trace). Fraction II in Fig. 5C gave a small but appreciable corresponding peak (Fig. 6F, upper trace). Fraction III in Fig. 5A gave four major peaks at 325 nm (Fig. 6G, upper trace). One of these peaks (fraction III) was almost completely missing (Fig. 6H, upper trace) when HPLC was performed with fraction III shown in Fig. 5B. In contrast, this peak was only partially reduced (Fig. 6I, upper trace) when HPLC was performed with fraction III shown in Fig. 5C.

Peptides containing DHCH-Arg in fraction I, fraction II, and fraction III in Fig. 6 were further purified by the third reversed phase HPLC (Fig. 7, A–D). Fraction I in Fig. 6A gave a major broad peak at 325 nm (Fig. 7A, upper trace). When monitored at 214 nm, this peak split into two close peaks (lower trace of Fig. 7A, peak Ia and peak Ib). When HPLC was performed with fraction I shown in Fig. 6B, the split peaks (lower trace of Fig. 7B, peak Ia’ and peak Ib’) were only slightly smaller than the corresponding peaks in Fig. 7A. Fraction II in Fig. 6D gave three peaks at 325 and 214 nm (upper and lower traces of Fig. 7C, peak IIa, peak Ib, and peak IIc). Fraction III in Fig. 6G gave a broad peak at 325 and 214 nm indicated by an arrow (Fig. 7D, upper and lower traces). Peptides in this peak were further purified by the fourth reversed phase HPLC (Fig. 7E). This HPLC gave a broad peak at 325 nm, which consisted of two very close peaks at 214 nm (peak IIIa and peak IIIb).

**Sequencing of Peptides Containing DHCH-Arg**—The peptides containing DHCH-Arg isolated as above were sequenced (Table I). Peak Ia, peak Ib, peak Ia’, and peak Ib’ gave the same sequence (Ser-503 to Lys-511) in the Ca$^{2+}$-ATPase, in which a missing arginyl residue (X) corresponded to Arg-505. Peak IIa, peak IIb, and peak IIc gave a sequence (Ala-673 to Lys-684), in which X corresponded to Arg-678. Peak IIIa and peak IIIb gave a sequence (Glu-482 to Arg-491), in which X corresponded to Arg-489. These results show that Arg-489, Arg-505, and Arg-678 in the Ca$^{2+}$-ATPase were modified with CHD. The observed resistance of carboxyl-terminal peptide bonds of CHD-modified arginyl residues to trypsin cleavage is consistent with the previously reported findings (36). The reason why peptides with the same sequence gave different retention times in HPLC remains obscure.

**DISCUSSION**

**Effects of CHD Modification on Enzyme Activities**—The saturable protection by Mg-ATP, MgADP, and MgAMP against the CHD-induced inhibition of the Ca$^{2+}$-ATPase activity (Fig. 1, A and C), together with the CHD-induced inhibition of high affinity binding of MgATP and MgADP (Fig. 2, A and B), demonstrates that a CHD-modified arginyl residue(s) responsible for these inhibitions is located at the high affinity ATP binding site, being in or close to the region occupied by the AMP moiety of bound ATP. The fair agreement on the kinetics of inhibition among the Ca$^{2+}$-ATPase activity (Fig. 1A), phosphorylation with ATP (Fig. 1A), and binding of MgATP and MgADP (Fig. 2, C and D) under the same conditions most probably indicates that the inhibition of MgATP binding to the high affinity binding site is a primary event in these inhibitions. It is likely that the seemingly slower inhibition of phosphorylation (Fig. 1A) is due to enhanced accumulation of EP during the ATPase reaction. This enhanced accumulation should occur because the reaction step(s) following EP formation is inhibited somewhat more slowly than the ATPase activity (compare Fig. 1A with Fig. 3A and cf. the discussion in the next paragraph). The observed first order kinetics of inhibition (Fig. 1, A and B, and Fig. 2, C and D) is consistent with the
The inhibition of phosphorylation with Pi is also distinct from that involved in the inhibition of the Ca\(^{2+}\) binding to the enzyme (Fig. 1A) as well as by binding of acetyl phosphate or P\(_i\) to the enzyme (Fig. 1C) indicates that CHD-modified arginyl residues are not located at the phosphorylation site and the P\(_i\) binding site. This conclusion is consistent with the lack of CHD-induced inhibition of phosphorylation with acetyl phosphate (Fig. 3B).

The CHD-modified arginyl residue responsible for the inhibition of the acetyl phosphatase activity is likely distinct from that responsible for the inhibition of the Ca\(^{2+}\) -ATPase activity, because the inhibition of the acetyl phosphatase activity is considerably slower than that of the Ca\(^{2+}\) -ATPase activity (Figs. 1A and 3A). This view is further supported by the observations (Figs. 1A and 3A) showing a large difference in the extent of the Mg-ATP-induced protection between the acetyl phosphatase activity and the Ca\(^{2+}\) -ATPase activity. The progressive increase in the steady-state EP level by the CHD treatment (Fig. 3B) is most probably due to modification of an arginyl residue(s) involved in some reaction step(s) following phosphorylation. This probability is supported by the finding (Fig. 4) that phosphorylation with P\(_i\) (reversal of EP hydrolysis) is inhibited by the CHD treatment. This residue again seems distinct from that involved in the inhibition of the Ca\(^{2+}\) -ATPase activity, because the insufficient protection of this residue by Mg-ATP (Fig. 3B) is in contrast to the much more effective protection of the Ca\(^{2+}\) -ATPase activity by Mg-ATP (Fig. 1A). The CHD-modified arginyl residue(s) responsible for the inhibition of phosphorylation with P\(_i\) is also distinct from that responsible for the inhibition of the Ca\(^{2+}\) -ATPase activity, because the kinetics of the former inhibition (Fig. 4) is very different from that of the latter (Fig. 1A). Further characterization of these residues is in progress.

The lack of effect of the CHD modification on the Ca\(^{2+}\) -induced activation of the enzyme (see “Results”) is consistent with the previously reported findings that high affinity Ca\(^{2+}\) binding to the plasma membrane Ca\(^{2+}\) -ATPase from erythrocytes (30) and smooth muscle (31) is unaffected by phenylglyoxal modification.

**Identification of Arginyl Residues Located at the ATP Binding Site**—The data from peptide mapping (Figs. 5–7) and sequencing (Table I) suggest that Arg-489, Arg-505, and Arg-678 are located at or near the ATP binding site, because these residues are almost completely protected against the CHD modification by Mg-ATP or by prelabeling with FITC, which occupies the adenosine binding region in the ATP binding site (51, 52). It is very likely that at least one of these residues is located at the high affinity ATP binding site, because modification of these residues causes an almost complete loss of the capacity for high affinity Mg-ATP binding (Fig. 2).

The almost complete protection of Arg-489 and Arg-678 against the CHD modification by Mg-ATP (Fig. 6, E and H), together with the only partial protection by prelabeling with FITC (Fig. 6, F and I), suggests that Arg-489 and Arg-678 are located in or near the triphosphate binding region and apart from the adenosine binding region in the ATP binding site. In contrast, the only slight protection of Arg-505 by Mg-ATP (Fig. 6B) and the almost complete protection by prelabeling with FITC (Fig. 6C) suggest that Arg-505 is located very close to (but slightly out of) the adenosine binding region.

It is very probable that Arg-489 or Arg-678 or both are involved in the CHD-induced inhibition of the Ca\(^{2+}\) -ATPase activity, because the almost complete protection of Arg-489 and Arg-678 (but not Arg-505) by Mg-ATP against the CHD modification (Fig. 6, E and H) corresponds well to the observed very effective protection of the enzyme by Mg-ATP against the CHD-induced inhibition of the Ca\(^{2+}\) -ATPase activity (Fig. 1A). This leads to the conclusion that either or both of these residues are located in or close to the region occupied by the α-phosphoryl group of ATP in the high affinity ATP binding site, because the residue(s) should be situated in or close to the region occupied by the AMP moiety of bound ATP and apart from the adenosine binding region as discussed above.

The present results show that the ATP binding site is made up from residues (Arg-489 to Arg-678) that are widely separated in the linear sequence. This is consistent with the previously proposed model of the ATP binding site (4, 53), which is constituted of several discrete segments in the Ca\(^{2+}\) -ATPase. The presence of the two remote arginyl residues (Arg-489 and Arg-678) at the ATP binding site is in harmony with the previously reported findings that Lys-492 and Arg-678 are cross-linked by glutaraldehyde at the high affinity ATP binding site (35) and that both Lys-492 and Lys-684 are labeled with adenosine triphosphopyridoxal (14).

The presence of Arg-489 at the ATP binding site is consistent with the results of other chemical modifications (14–18) showing the location of Lys-492 in the close vicinity of the ATP binding site, particularly with our previous conclusion (17, 18) that Lys-492 is located in or near the region occupied by the α-phosphoryl group of bound ATP. It should be noted that Arg-678 is situated at the amino-terminal beginning of the hinge domain (3, 54), which was previously suggested to inter-

| Cycle no. | Residues determined at the given cycle and their location in the sequence of Ca\(^{2+}\) -ATPase |
|-----------|---------------------------------------------------------------------------------------------------|
| 1         | Ser (60) Ser (137) Ser (34) Ser (98)                                                             |
| 2         | Ser (51) Ser (95) Ser (31) Ser (73)                                                              |
| 3         | X         X         X         X                                                                 |
| 4         | Ala (390) Ala (786) Ala (206) Ala (546)                                                         |
| 5         | Ala (399) Ala (843) Ala (227) Ala (590)                                                         |
| 6         | Val (508) Val (954) Val (281) Val (703)                                                         |
| 7         | Gly (430) Gly (817) Gly (230) Gly (596)                                                         |
| 8         | Asn (163) Asn (349) Asn (78) Asn (252)                                                          |
| 9         | Lys (131) Lys (227) Lys (53) Lys (195)                                                          |
| 10        | Ser (12) Ser (21) Ser (19)                                                                       |
| 11        | His (6)  His (7)  His (8)                                                                       |
| 12        | Lys (45)  Lys (76)  Lys (88)                                                                     |

**Table I**

**Sequences of peptides containing DHCH-Arg**

The purified peptides containing DHCH-Arg shown in Fig. 7 were sequenced. The numbers in parentheses indicate picomoles of amino acid at the given cycle. X and — represent, respectively, the expected DHCH-arginyl residues and cysteinyl residues which cannot be detected by the method of sequencing used.

| Cycle no. | Peptide sequence |
|-----------|-----------------|
| 1         | Ser-(DHCH-Arg)   |
| 2         | Ser-(DHCH-Arg)   |
| 3         | Ser-(DHCH-Arg)   |
| 4         | Ser-(DHCH-Arg)   |
| 5         | Ser-(DHCH-Arg)   |
| 6         | Ser-(DHCH-Arg)   |
| 7         | Ser-(DHCH-Arg)   |
| 8         | Ser-(DHCH-Arg)   |
| 9         | Ser-(DHCH-Arg)   |
| 10        | Ser-(DHCH-Arg)   |
| 11        | Ser-(DHCH-Arg)   |
| 12        | Ser-(DHCH-Arg)   |
act with the phosphorylation domain (55). The location of Arg-505 slightly out of the adenine binding region is in harmony with the previously reported findings (56, 57) that the carboxyl-terminal peptide bond of Arg-505 is highly susceptible to tryptic even when ATP or its nonhydrolyzable analog is present. Arg-489 is most likely nonessential because this residue is not conserved in other P-type ATPases, including the Na⁺,K⁺-ATPase (58, 59), H⁺-K⁺-ATPase (60, 61), and plasma membrane Ca²⁺-ATPase (62). However, the possibility of a steric hindrance by modified Arg-489 cannot be excluded. On the other hand, Arg-678 is conserved in all P-type ATPases (3, 58–62), but not conserved in bacterial (63) and fungal (64, 65) P-type ATPases. Animal P-type ATPases have high affinities for ATP (Kₘ is 0.2–5 μM) (46, 66–68), whereas bacterial and fungal P-type ATPases have much lower affinities for ATP (Kₘ is 0.1–4.9 mM) (69–71). It appears possible that Arg-678 plays a role in conferring high affinities for ATP on animal P-type ATPases.

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