Mutants of individual residues of the plasma membrane Ca\(^{2+}\)-pump were made in the highly conserved region that (in related P-type ATPases) has been associated with nucleotide binding. Alteration of the strictly conserved Asp\(^{672}\) to Glu nearly eliminated the ability of the pump to transport Ca\(^{2+}\), while alteration at Val\(^{674}\), Arg\(^{675}\), and Lys\(^{686}\) reduced the activity. High levels of ATP (25 \(\mu\)M) did not overcome the reduced activity, indicating that it could not be due to a reduction in the affinity for ATP. Effects not directly related to ATP binding seemed to result from mutations in this area. For instance, the amount of phosphorylated intermediate in the most severely inhibited mutant, Asp\(^{672}\)→Glu, was nearly as high as that in the wild type, a much larger amount of phosphorylated intermediate than was expected from its low activity. However, the rate of decomposition of this intermediate was much slower than that of the wild type, indicating that the inhibition of this mutant resulted from an inhibition of the E→P step in the enzyme cycle.

The P-type ATPases of animals are divided into three main classes: 1) the Ca\(^{2+}\)-pumps of sarco-/endoplasmic reticulum; 2) the Na\(^{+}\),K\(^{+}\)-pumps (and their close relatives, the H\(^{-}\),K\(^{-}\)-pumps); and 3) the plasma membrane Ca\(^{2+}\)-pumps. Certain regions of the pump show high identity between the different classes. These conserved regions appear to be essential for pump function. One region that displays some conservation is the "nucleotide-binding" region. It received this name because of the reaction of a lysine in this region with the nucleotide analog, fluorescein isothiocyanate and because of a supposed similarity between its secondary structure and that of the nucleotide-binding region of adenylate kinase and phosphofructokinase (Taylor and Green, 1989). We have studied mutants of five residues in crucial parts of the nucleotide-binding region. The locations of the mutants are shown in Fig. 1, which also shows the alignment of hPMCA4b with several other P-type ATPases. Since most of the amino acids altered were highly conserved among the different P-type ATPases, their alteration caused a substantial reduction in the enzyme's activity. We tested whether the reduction in activity was due to a change in the affinity of the enzyme for ATP and found no change in the apparent ATP affinity. Instead we found that the most severely inhibited mutant had a lower rate of dephosphorylation, which accounted for its low activity.

**MATERIALS AND METHODS**

The construction of the hPMCA4b full-length cDNA was described in detail previously (Adamo et al., 1992b). Mutations were performed using the Altered Site mutagenesis kit (Promega Corp.). Specific oligonucleotides containing the desired changes were hybridized with the single-strand hPMCA4b cDNA contained in the pAlter-1 vector. After transformation and ampicillin selection the clones containing the mutations were identified by double-strand sequencing. The 426-base pair Aval-Aval fragment containing the mutations was sequenced and then cloned into the pMM2 vector containing hPMCA4b for expression in COS-1 cells (Gluzman, 1981). The pMM2 vector was previously called pMT2-m (Adamo et al., 1992b). The construction of the C-terminally truncated mutant hPMCA4b( ct120) was described previously (Enyedi et al., 1993). To obtain the truncated Asp\(^{672}\)→Glu( ct120) the Aval-Aval fragment was excised from the full-length Asp\(^{672}\)→Glu DNA and cloned into hPMCA4b( ct120). The cells were transfected by the DEAE-dextran-chloroquine method (Adamo et al., 1992b) and harvested after 48 h. For the isolation of the microsomal fraction (Enyedi et al., 1993) the cells were swollen at 4°C for 10 min in 10 mM Tris-HCl (pH 7.5 at 37°C), 1 mM MgCl\(_2\), 0.1 mM phenylmethylsulfonyl fluoride, 4 \(\mu\)g/ml aprotinin, and 1 \(\mu\)g/ml leupeptin and then homogenized with 30 strokes in a glass Dounce homogenizer. The homogenate was diluted with an equal volume of a solution of 0.5 M sucrose, 4 mM dithiothreitol, 0.3 mM KCl, 10 mM Tris-HCl (pH 7.5 at 37°C) and the suspension was rehomogenized with 20 strokes in the same homogenizer. The suspension was centrifuged at 5,100 \(\times\) g for 40 min, and the pellet was homogenized in 0.25 M sucrose, 0.15 M KCl, 10 mM Tris-HCl (pH 7.5 at 37°C) and 20 \(\mu\)M CaCl\(_2\) with 20 strokes in a Teflon pestle glass homogenizer. The final pellet was aliquoted and kept in liquid nitrogen.

Ca\(^{2+}\) transport activity was measured as described (Enyedi et al., 1993). The reaction mixture contained 28 mM KCl, 25 mM Tris-HCl (pH 7.5 at 37°C), 7 mM MgCl\(_2\), 20 mM phosphate, 5 mM NaN\(_3\), 0.5 mM ouabain, 4 \(\mu\)g/ml oligomycin, 400 mM thapsigargin, 250 mM calmodulin, 0.1 mM CaCl\(_2\), and enough EGTA to give the free Ca\(^{2+}\) the wanted. Five micrograms of membranes were preincubated in the reaction mixture for 5 min at 37°C, and the reaction was initiated by the addition of 6 \(\mu\)M ATP. Unless stated otherwise, Ca\(^{2+}\) uptake proceeded in the presence of ATP for 5 min, and the reaction was terminated by filtering the samples through a 0.45- \(\mu\)M Millipore filter. The \(4^t\)Ca taken up by the vesicles was then determined by counting in a scintillation counter. The calcium uptake was linear for more than 10 min.

The ATP dependence of the Ca\(^{2+}\) transport was measured in the same medium but with the addition of 1 \(\mu\)M phosphocreatine, 5 units/ml creatine phosphokinase, and 0.1 mg/ml of bovine serum albumin. Because the ATP concentration was being varied, it was also necessary to vary the total Mg\(^{2+}\) in order to assure a constant level of free Mg\(^{2+}\). Therefore, the concentration of Mg\(^{2+}\) in the absence of ATP was 4 \(\mu\)M, and ATP and Mg\(^{2+}\) were added in equimolar amounts.

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† To whom correspondence should be addressed: Guggenheim 16, Mayo Clinic, 200 First Street South West, Rochester, MN 55905. Tel.: 507-284-2295; Fax: 507-284-9759.

1. The abbreviations used are: hPMCA4b, human plasma membrane Ca\(^{2+}\)-pump, isoform 4b; SERCA1, sarco/endoplasmic reticulum Ca\(^{2+}\)-pump, isoform 1.
The phosphorylation reaction was carried out at 4 °C in a medium containing 28 mM KCl, 25 mM Tris-HCl (pH 7.35 at 4 °C), 20 mM phosphate, 5 mM NaN₃, 0.5 mM glutamin, 4 μM diglycerin, 400 mM thapsigargin, 250 mM calmodulin in the presence of 0.05 mM EGTA or of 0.02 mM CaCl₂ plus 0.02 mM LaCl₃. The reaction was initiated by the addition of 15 μM (γ-32P)ATP and stopped after 60 s with 10% trichloroacetic acid, 10 mM MgCl₂, 0.02 mM CaCl₂, and 1 μM cold ATP. The denatured proteins were collected by centrifugation at 20,000 × g for 10 min, washed once, and separated by using SDS-electrophoresis according to Sarkadi et al. (1986).

In the dephosphorylation experiments the enzyme was phosphorylated at 4 °C for 30 s in the presence of 100 mM KCl, 25 mM Tris-HCl (pH 7.35 at 4 °C), 400 mM thapsigargin, 5 mM MgCl₂, 0.02 mM CaCl₂, and 1 μM (γ-32P)ATP. Dephosphorylation was initiated by adding cold ATP to give a final concentration of 0.6 mM, and the reaction was stopped at different times with 10% trichloroacetic acid and 10 mM P₁ + 1 mM cold ATP.

To quantify the expressed Ca²⁺-ATPase a sandwich enzyme-linked immunosorbent assay was used (Enyedi et al., 1993). In this assay, the enzyme was adsorbed to the plates by using the monoclonal antibody 5F10 (Borke et al., 1989), and the amount of Ca²⁺-ATPase was quantitated using a polyclonal antibody (Verma et al., 1984).

Protein concentration was estimated by the method of Bradford using bovine serum albumin as a standard. SDS-electrophoresis and immunoblotting were carried out as described previously (Magocsi and Penniston, 1991). For the Western blot shown in Fig. 2, 5 μg of protein from membranes were loaded in each well and separated by SDS-electrophoresis on a 12.5% gel. Proteins were transferred to Millipore Immobilon membranes, and nonspecific binding was blocked by phospho-buffered saline containing 1% bovine serum albumin for 1 h at 37 °C. For staining, biotinylated anti-mouse immunoglobulin G and avidin-horseradish peroxidase conjugate were used.

**RESULTS**

The size of the expressed Ca²⁺-pump was assessed by Western blot, and the level of expression was assessed by means of an enzyme-linked immunosorbent assay. The Western blot, shown in Fig. 2, was done utilizing monoclonal antibody J9, which reacts with the extreme amino terminus of hPMCA4b and does not react with the endogenous plasma membrane Ca²⁺-pump (Adamo et al., 1992a) and does not react with the endogenous plasma membrane Ca²⁺-pump of COS cells. This portion of the pump was not altered in any of the mutations reported in this paper. These data show that all of the mutants caused the expression of full-size Ca²⁺-pump. The amount of expression was tested more quantitatively by sandwich enzyme-linked immunosorbent assay, which showed (Table I) that all of the mutants were expressed in a quantity nearly equal to that of hPMCA4b.

**Ca²⁺ Transport Activity**—The presence of a functional Ca²⁺-pump in the microsomal fractions from transfected cells was investigated by measuring the component of Ca²⁺ uptake that was dependent on the presence of ATP. Phosphate was included in the uptake medium to enhance the linearity and amount of Ca²⁺ uptake, and thapsigargin was added to specifically inhibit the endoplasmic reticulum Ca²⁺-pump as described previously (Enyedi et al., 1993). The response to calmodulin of the Ca²⁺-uptake of the mutants was the same as that of the wild type (not shown), indicating that none of the mutations interfered with the mechanism of calmodulin stimulation. Since the activity of the pump is very low in the absence of calmodulin the Ca²⁺ uptake experiments were done in the presence of optimal concentrations of calmodulin. The effect of each mutation on the Ca²⁺ transport activity of the pump is shown in Table II. Since most of the residues altered are highly conserved, it is not surprising that substantial inhibition was caused by all of the alterations except Arg⁶⁷⁵ → Ile. The other mutants showed a wide range of degrees of inactivation, ranging from nearly complete inactivation, when the strictly conserved Asp⁶⁷² was converted to Glu, to very substantial activity remaining (Lys⁶⁸⁶ → Ile).

**ATP Dependence of Ca²⁺ Uptake**—In order to detect any changes in their response to ATP, three of the partially inactivated mutants were compared with the wild type hPMCA4b. For two of the mutants (Val⁶⁷⁴ → Pro and Arg⁶⁷⁵ → Lys), it was possible to measure the ATP dependence at low ATP, but the third (Asp⁶⁷² → Glu) had too low an activity to make such measurements. Fig. 3 shows the ATP dependence for concentrations of ATP up to 1.5 mM. All three of the samples tested approach saturation at about 1 mM ATP. Even with enough ATP to saturate the enzyme, the activity of the mutants remained lower than that of hPMCA4b. In a separate experiment (Table III) the effect of higher levels of ATP was tested on the activity of the same three enzymes as were shown in Fig. 3, and on Asp⁶⁷² → Glu. Even 24 mM ATP did not overcome the inhibition caused by these three mutations.

Phosphorylation by ATP—The ability of the mutants to be phosphorylated by ATP, forming an acylphosphate, was inves-
tigated. The phosphorylation reaction was carried out in the presence of Ca\textsuperscript{2+} and La\textsuperscript{3+} with Mg\textsuperscript{2+} omitted. These conditions inhibit the reactions subsequent to phosphorylation, strongly facilitating observation of the acylphosphate (Schatzmann and Burgin, 1978). A significant amount of phosphorylated intermediate was shown by all of the mutants treated (Fig. 4); in the absence of La\textsuperscript{3+}, the nearly inactive mutant Asp\textsuperscript{672} → Glu showed about the same amount of acylphosphate as the wild type (Fig. 5), much more than would have been expected from its very low activity.

Dephosphorylation of the Asp\textsuperscript{672} → Glu Mutant—To investigate further the cause of the inhibition of this mutant, we studied the decomposition of its phosphoenzyme. To facilitate the observation of the phosphoenzyme of the expressed pump, a new mutant Asp\textsuperscript{672} → Glu(ct120) was constructed. In this mutant, the carboxyl-terminal 120 residues were deleted from the mutant Asp\textsuperscript{672} → Glu. Enyedi et al. (1993) had previously shown that a similar deletion of the carboxyl terminus from the wild type had produced the fully activated enzyme hPMCA4b(ct120). The activity of the Asp\textsuperscript{672} → Glu(ct120) mutant was about 10% of the activity of the hPMCA4b(ct120) enzyme (not shown). Comparison with the degree of inhibition for the mutant Asp\textsuperscript{672} → Glu shown in Table II indicates that the carboxyl-terminal deletion did not change the degree of inhibition caused by the alteration of Asp\textsuperscript{672}. As was expected from their size, the truncated proteins migrated faster during electrophoresis, and hence the bands that represent them were clearly separated from the bands formed by the endogenous Ca\textsuperscript{2+}-pump. This allowed a clear demonstration of the dephosphorylation without interference from that of the endogenous pump. After phosphorylation with 1 \muM ATP for 30 s, the dephosphorylation was initiated by adding 0.8 mM cold ATP. The decay of the wild type phosphoenzyme was rapid, with 10% of the initial phosphoenzyme remaining after 5 s, the shortest time that was possible to measure (Fig. 5). In an experiment done at the same time, the decay of the Asp\textsuperscript{672} → Glu(ct120) phosphorylated intermediate was considerably slower, taking about 15 s to achieve the low intensity observed after 5 s (or less) for the wild type; two other experiments like this gave similar results.

**DISCUSSION**

As is shown in Fig. 1, most of the residues that were altered in this study are highly conserved among the different P-type ATPases, even those that are phylogenetically quite distant from one another. Our results confirmed that the activity of the pump is quite sensitive to changes in these conserved residues. Particularly sensitive to change was Asp\textsuperscript{672}. Even the relatively conservative change to Glu nearly inactivated the enzyme. The corresponding Asp has also been altered in the Ca\textsuperscript{2+}-pump of SERCA1 (Clarke et al., 1990) and in the H\textsuperscript{+}-pump of yeast (Portillo and Serrano, 1988). Changing Asp\textsuperscript{672} to Met also produced an enzyme with substantial remaining activity. This residue is of substantial importance to the activity of the enzyme, but a misfitting substitute does not destroy the activity.

The alteration of Val\textsuperscript{674} to Pro is interesting because the corresponding residue in other P-type ion pumps is Pro. Because we had restored the consensus residue, we expected this change to produce a fully active enzyme, but the mutant had only about 24% of the activity of the wild type. This result shows that the occurrence of Val in this position in the plasma membrane Ca\textsuperscript{2+}-pump is probably due to a difference in function. This may indicate the existence of differences in the structural organization of this region in the plasma membrane Ca\textsuperscript{2+}-pump or compensating differences in other regions that

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**TABLE II**

Activities of hPMCA4b mutants

| Mutant       | Percentage of hPMCA4b activity |
|--------------|--------------------------------|
| hPMCA4b      | 100                            |
| Asp\textsuperscript{672} → Glu | 15 ± 3 (4)                     |
| Val\textsuperscript{674} → Pro  | 24 ± 3 (6)                     |
| Arg\textsuperscript{675} → Lys  | 47 ± 2 (6)                     |
| Arg\textsuperscript{675} → Asp  | 20 ± 7 (4)                     |
| Arg\textsuperscript{675} → Leu   | 24 ± 7 (7)                     |
| Lys\textsuperscript{686} → Ile   | 63 ± 20 (3)                    |
| Arg\textsuperscript{693} → Ile   | 95 ± 12 (5)                    |

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**Fig. 3.** Activity of hPMCA4b and mutants versus ATP. The activity of a control (membranes from COS cells transfected with the empty plasmid pMM2) was subtracted from each point.
TABLE III
Activities of mutants at high ATP levels

| Mutant      | Ca\(^{2+}\) uptake, nmol \cdot mg\(^{-1}\) \cdot min\(^{-1}\) |
|-------------|--------------------------------------------------|
|             | 6 mM ATP | 24 mM ATP |
| hPMCA4b     | 1.54     | 1.50      |
| Asp\(^{672}\) → Glu | 0.12 | 0.10 |
| Arg\(^{675}\) → Lys | 1.10 | 1.05 |
| Val\(^{674}\) → Pro | 0.33 | 0.34 |

We found a substantial amount of acylphosphate formation in all of the mutants tested, even in the mutant Asp\(^{672}\) → Glu, which showed very low Ca\(^{2+}\) uptake. This differed from the results of Clarke et al. (1990) on SERCA1, which reported that certain of the mutations in this region showed no phosphorylation. In particular, their mutation of the corresponding Asp\(^{601}\) to Glu showed no phosphorylation from ATP.

When chased with unlabeled ATP, the acylphosphate formed by the mutant Asp\(^{672}\) → Glu decomposed at a slower rate than did the wild type. In the normal cycle of partial reactions of the plasma membrane Ca\(^{2+}\)-pump (Rega and Garrahan, 1986), this would occur if the alterations slowed the conformational change from E\(_1\)-P to E\(_2\)-P or if it slowed the hydrolysis of E\(_2\)-P to E\(_2\) and P. The latter step is probably the one that is slowed, although the experiments described here do not allow a definitive choice between them.

Previous studies on mutations to this region of P-type ion pumps have found complex effects on various aspects of pump function. The most extensive study was by Clarke et al. (1990) on SERCA1. Their data suggested that alteration of some residues in this region affected the rate of the E\(_2\)-P to E\(_2\)-P transition, while alteration of others totally prevented formation of the phosphorylated intermediate. Another report from the same laboratory (MacLennan et al., 1992) showed that mutations in this region prevented cross-linking by glutaraldehyde. Since ATP also prevented such cross-linking, they infer some connection between this region and ATP binding. The residues whose mutation prevented cross-linking were Asp\(^{601}\) and Pro\(^{603}\) of SERCA1a, which correspond to Asp\(^{672}\) and Val\(^{674}\) of hPMCA4b.

The results presented here do not support a central role in ATP binding of residues Asp\(^{672}\), Val\(^{674}\) and Arg\(^{675}\) of the plasma membrane Ca\(^{2+}\)-pump. Rather, they indicate that the alteration of these residues changes the rate of the catalytic events that occur after phosphorylation.

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Mutants in the Putative Nucleotide-binding Region of the Plasma Membrane Ca\textsuperscript{2+} -Pump: A REDUCTION IN ACTIVITY DUE TO SLOW DEPHOSPHORYLATION
Hugo P. Adamo, Adelaida G. Filoteo, Agnes Enyedi and John T. Penniston

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