Functional Consequences of Proline Mutations in the Cytoplasmic and Transmembrane Sectors of the Ca\(^{2+}\)-ATPase of Sarcoplasmic Reticulum*

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Site-specific mutagenesis was used to investigate whether Pro\(^{190}\), Pro\(^{195}\), Pro\(^{304}\), Pro\(^{312}\), Pro\(^{303}\), and Pro\(^{612}\) play essential roles in the function of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase. All six prolines were substituted with alanine, and in addition, Pro\(^{308}\) was replaced by glycine and Pro\(^{314}\) by glycine as well as by leucine. Mutant CDNAs were expressed in COS-1 cells, and mutant Ca\(^{2+}\)-ATPases located in the isolated microsomal fraction were examined with respect to Ca\(^{2+}\) uptake activity, Ca\(^{2+}\) dependence of phosphorylation from ATP, and the kinetic properties of the phosphoenzyme intermediates formed from both ATP and P\(_{i}\). The enzymatic cycle was little affected by substitution of Pro\(^{190}\), Pro\(^{195}\), and Pro\(^{312}\), which are located in the hydrophobic domain; but replacement of Pro\(^{308}\), Pro\(^{314}\), and Pro\(^{610}\), in the putative transmembrane helices, had a profound impact on the function of the enzyme. All mutations of Pro\(^{308}\) and Pro\(^{303}\) led to ATPases which were characterized by a reduced affinity for Ca\(^{2+}\). These prolines may therefore be involved in the structure of the high affinity Ca\(^{2+}\)-binding sites in the enzyme. Substitution of Pro\(^{314}\) with alanine or glycine gave rise to mutants unable to transport Ca\(^{2+}\) even though their apparent affinities for Ca\(^{2+}\) in the phosphorylation reaction with ATP were increased. In these enzymes, the ADP-sensitive phosphoenzyme intermediate was stable for at least 5 min at 0°C, whereas the ADP-insensitive phosphoenzyme intermediate decayed at a rate similar to that of the wild type. Thus, the inability to transport Ca\(^{2+}\) could be accounted for by a block of ADP-sensitive to ADP-insensitive phosphoenzyme intermediate conformational transition. In contrast, substitution of Pro\(^{312}\) with leucine gave rise to a mutant enzyme that retained about 7% of the normal Ca\(^{2+}\) transport rate. Phosphoenzyme turnover in this mutant also occurred at a low but significant rate, suggesting that the leucine side chain can substitute to some extent for proline.

The Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum catalyzes the uphill transport of Ca\(^{2+}\) from the sarcoplasm to the membrane lumen at the expense of free energy derived from the hydrolysis of ATP (Hasselbach, 1964; Martonosi and Beeler, 1983). A structural model for the Ca\(^{2+}\)-ATPase has been proposed on the basis of the amino acid sequence deduced from the cDNA sequence (MacLennan et al., 1985; Brandl et al., 1986). In this model, two globular cytoplasmic domains are separated from a transmembrane helix region consisting of 10 \(\alpha\)-helices by a stalk sector made up of five \(\alpha\)-helices. The formation of an ADP-sensitive phosphoenzyme intermediate (E\(_{2}\)P) by reaction with ATP is central to the mechanism of energy transduction in the enzyme. This form is then converted to an ADP-insensitive phosphoenzyme intermediate (E\(_{2}\)P) during the course of Ca\(^{2+}\) transport (de Meis and Vianna, 1979). These reactions are linked with conformational changes in the enzyme which apparently bring about a reorientation of the two Ca\(^{2+}\)-binding sites, leading to a loss in their affinity for Ca\(^{2+}\). This eventually leads to discharge of two Ca\(^{2+}\) ions into the luminal space. E\(_{2}\)P, the bound Ca\(^{2+}\) ions are in an occluded state characterized by a low rate of isotopic exchange with free Ca\(^{2+}\) on either side of the membrane (Dupont, 1980; Vilsen and Andersen, 1987). It is unclear whether the occluded Ca\(^{2+}\) ions are bound at the primary Ca\(^{2+}\)-binding sites in the enzyme or whether one or both of the ions have been transferred to low affinity sites in a release channel leading to the luminal surface (Green et al., 1986; Pettithory and Jencks, 1988).

One way to improve our understanding of the molecular details of the Ca\(^{2+}\) transport mechanism is to identify the residues involved in the specific partial reactions of the pump cycle through site-directed mutagenesis. This approach has recently proven valuable in the assignment of residues making up the ATP-binding site (Maruyama and MacLennan, 1988; Maruyama et al., 1989) and the high affinity Ca\(^{2+}\)-binding sites (Clarke et al., 1989a, 1989b). Thus, substitution of more than 39 negatively charged and polar residues in the protein pinpointed 6 residues in the putative transmembrane helices as possible candidates for Ca\(^{2+}\) ligands.

Of great interest for our understanding of the mechanism

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† The abbreviations used are: E\(_{2}\)P, ADP-sensitive phosphoenzyme intermediate; E\(_{2}\), ADP-insensitive phosphoenzyme intermediate; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; EGTA, [ethylenebis(β-oxoethylenedinitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate.
of Ca\textsuperscript{2+} transport are proline residues because of their unique structural and functional properties. The structural destabilization induced by prolines located in the middle of \(\alpha\)-helices and the possibility of cis-trans isomerization of peptide bonds between prolines and their preceding residues make prolines important candidates for specific functions related to conformational changes in the protein (Schulz and Schirmer, 1979; Barlow and Thornton, 1988; Brandl and Deber, 1986). Furthermore, increased basicity of the carbonyl oxygen atom in peptide bonds involving proline may be of importance in the creation of cation-binding sites. Indeed, it has been found that the putative transmembrane segments of several membrane transport proteins contain a significantly higher number of proline residues than do the transmembrane portions of nontransport membrane proteins, suggesting some functional role of membrane-buried prolines in the transport reaction (Brandl and Deber, 1986).

In this study, we have mutated 6 proline residues in the Ca\textsuperscript{2+}-ATPase. Three are located in putative transmembrane helices, 2 are in the predicted \(\beta\)-strand sector in the globular cytoplasmic projection, and 1 is in a relatively short loop connecting putative transmembrane helices M6 and M7 (Fig. 1). All of the prolines are substituted initially with alanine. In addition, Pro\textsuperscript{308} was changed to glycine and Pro\textsuperscript{312} to glycine as well as to leucine to test for the importance of \(\alpha\)-helix destabilization. Information about Ca\textsuperscript{2+} binding in the mutants was obtained by studying the Ca\textsuperscript{2+} dependence of Ca\textsuperscript{2+}-ATPase activity and of phosphorylation of the Ca\textsuperscript{2+}-ATPase from ATP. The \(E_\text{P} \to E_\text{P} \) interconversion in the enzyme was analyzed by measurement of ADP sensitivity of the phosphorylated intermediate and of the rate of dephosphorylation, starting from either \(E_\text{P} \) or \(E_\text{P} \).

We have found that all 3 prolines located in the transmembrane sector are important for function. Pro\textsuperscript{308} and Pro\textsuperscript{303} seem to be involved in Ca\textsuperscript{2+} binding, whereas Pro\textsuperscript{312} is essential to the transition between \(E_\text{P} \) and \(E_\text{P} \). On the other hand, the enzyme cycle seems to be little affected by the mutations of prolines in nontransmembrane portions of the protein.

### MATERIALS AND METHODS

**Site-specific Mutagenesis—Oligonucleotide-directed site-specific mutagenesis was carried out according to Kunkel (1985) as described previously (Maruyama and MacLennan, 1988). Mutations were introduced into short restriction fragments that had been excised from the full-length rabbit fast-twitch muscle Ca\textsuperscript{2+}-ATPase cDNA clone and inserted into the Bluescript vector (Stratagene, La Jolla, CA).**

The fragments used were the following: Smal (position 442)-KpnI (position 663) for mutation of Pro\textsuperscript{308} and Pro\textsuperscript{305}, BamHI (position 865)-Smal (position 1600) for Pro\textsuperscript{308} and Pro\textsuperscript{312}, and AucI (position 2353)-BstII (position 2716) for Pro\textsuperscript{308} and Pro\textsuperscript{305}. Mutant clones were selected by hybridization screening with radiolabeled mutant oligonucleotides according to standard procedures (Maniatis et al. 1982). In order to verify that mutations were appropriate and to ensure that no unwanted mutations or deletions had occurred, sequencing was performed using the dideoxynucleotide chain termination method (Sanger et al., 1977) with either Sequenase (IBI Inc.) or T7-DNA polymerase I (Pharmacia LKB Biotechnology Inc.).

To solve compressions sometimes occurring in (G + C)-rich regions, the dITP or 7-deaza-dGTP analogues of dGTP were used (Barnes et al. 1983; Mizusawa et al., 1986). For two mutations, it was possible to design the mutant oligonucleotides so that a new restriction site (PvuI site) was generated, allowing additional confirmation of these mutations (Fig. 2).

The mutated fragments were excised from the Bluescript vector and religated back into their original positions in the full-length clone contained in the pHSF4 vector (Maruyama and MacLennan, 1988). It is important that the vector fragment used in the religation be uncontaminated by a single digested vector which would readily circularize to reform the wild type. Therefore, the vector fragment was purified by agarose gel electrophoresis followed by extraction with GeneClean (Bio/Can Scientific Inc.) before use in the religation reaction. For expression in COS-1 cells (Ghuzman, 1981), the entire Ca\textsuperscript{2+}-ATPase cDNA containing the mutation was cloned into the EcoRI site of vector pR1023(B) (Wong et al., 1985).

**Expression of Mutant DNA—Transfection of COS-1 cells by the DEAE-dextran chloroquine shock method (Sompayrac and Danna, 1981; Gorman, 1985) and isolation of the microsomal fraction containing the expressed Ca\textsuperscript{2+}-ATPase were carried out as described previously (Maruyama and MacLennan 1988; Clarke et al., 1989b).**

Expression levels were examined and quantitated by immunoblotting following SDS gel electrophoresis and by sandwich enzyme-linked immunosorbent assay (Clarke et al., 1989b) using the highly specific monoclonal antibody A52 (Zubryzka-Gaarn et al., 1984). In these assays, the standard enzyme used was the deoxycholate-purified fast-switch skeletal muscle enzyme (MacLennan, 1970).

**Ca\textsuperscript{2+} Transport Activity—For measurement of ATP-driven uptake of Ca\textsuperscript{2+} in the isolated microsomes, the reaction mixture contained 0.05–0.2 \(\mu\)g of Ca\textsuperscript{2+}-ATPase protein/ml, 20 mM MOPS, pH 6.8, 80 mM KC\textsubscript{1}, 5 mM MgCl\textsubscript{2}, 5 mM ATP, 0.5 mM EGTA, 5 mM potassium oxalate, 2 \(\mu\)Ci/ml \(\text{3}^{21}\text{Ca}\), and various concentrations of Ca\textsuperscript{2+} to obtain the desired concentrations of free Ca\textsuperscript{2+}, calculated according to published stability constants for CaEGTA, MgEGTA, CaATP, and MgATP (Vianna, 1975; Dupont, 1982). Incubation was performed at 27°C for 5, 10, and 25 min and terminated by transferring 150-\(\mu\)l aliquots to 3 ml of quench solution containing 0.15 M KCl and 1 mM LaCl\textsubscript{3}. The quenced samples were filtered through 0.3-\(\mu\)m PHWP or 0.45-\(\mu\)m HAWP Millipore filters. The filters retaining the calcium-loaded microsomes were washed with 15 ml of quench solution, and the radioactivity on the filters was measured by liquid scintillation counting.**

The Ca\textsuperscript{2+} uptake referable to the expressed fast-twitch Ca\textsuperscript{2+}-ATPase or mutants was calculated after subtraction of the background (usually less than 5%) corresponding to microsomes derived from nontransfected COS-1 cells. The linear part of the Ca\textsuperscript{2+} uptake curve (the first 5 min) was used in this calculation. The specific rate of Ca\textsuperscript{2+} uptake was expressed as the molecular turnover number calculated assuming a stoichiometry of two Ca\textsuperscript{2+} ions transmembrane transport proteins of the protein.

**FIG. 1.** Simplified representation of location of mutated proline residues based on structural model for Ca\textsuperscript{2+}-ATPase proposed by Brandl et al. (1986). M1–M10 correspond to the predicted transmembrane \(\alpha\)-helices.

**FIG. 2.** PvuI digestion pattern of Pro\textsuperscript{308} \(\to\) Ala and Pro\textsuperscript{312} \(\to\) Gly mutant cDNAs. Full-length Ca\textsuperscript{2+}-ATPase cDNA containing the Pro\textsuperscript{308} \(\to\) Ala or Pro\textsuperscript{312} \(\to\) Gly mutation cloned into the EcoRI site of expression vector pR1023(B) was digested with PvuI and EcoRI. Lane a, Pro\textsuperscript{308} \(\to\) Ala mutant; lane b, Pro\textsuperscript{308} \(\to\) Gly mutant; lane c, wild type. The positions of markers (in kilobases (kb)) are indicated. The mutant DNA contains a PvuI site, permitting cleavage of the full-length clone of approximately 3 kilobases to 1- and 2-kilobase fragments.
ported per enzymatic cycle (Inesi, 1985) and a site concentration as determined by the steady-state level of phosphorylation from ATP.

*Analysis of Phosphoenzyme Intermediates—Phosphorylation from either ATP or inorganic phosphate to obtain BIP or was performed as described (Clarke et al., 1989b). Studies of the intermediate reaction steps were carried out as described in the accompanying paper (Andersen et al., 1989).

**RESULTS**

**Expression of Mutants**—Fig. 3 shows examples of immunoblots of the wild-type fast-twitch Ca\(^{2+}\)-ATPase and of the proline mutants expressed in COS-1 cell microsomes. As shown, the mutants are expressed to approximately the same level as that of the wild type. As described previously, the antibody used reacts specifically with epitopes on the fast-twitch Ca\(^{2+}\)-ATPase (Clarke et al., 1989b). There is no cross-reactivity with the endogenous slow-twitch endoplasmic reticulum Ca\(^{2+}\)-ATPase of the COS-1 (monkey kidney) cells. For each of the mutants, the average expression level in four to six microsomal preparations was estimated to be between 80 and 100% of the average wild-type level, suggesting that no major structural perturbation was introduced by substitution of the proline residues.

**Ca\(^{2+}\) Uptake**—Table I presents the molecular turnover rates calculated from measurements of the rates of Ca\(^{2+}\) transport for the wild-type and mutant Ca-ATPases at pCa 5.0. To extend the time period in which the Ca\(^{2+}\) uptake showed a linear time dependence beyond seconds, oxalate was included in the reaction mixture to precipitate transported calcium ions inside the microsomes (Maruyama and MacLennan, 1988). For the wild-type enzyme in the COS-1 cell microsomes, the observed turnover rate of 12.5 s\(^{-1}\) agrees well with the value measured under the same conditions for the Ca\(^{2+}\)-ATPase in sarcoplasmic reticulum vesicles. The Pro195 → Ala, Pro195 → Ala, Pro106 → Ala, Pro106 → Ala, Pro852 → Ala, and Pro852 → Ala mutants all transported Ca\(^{2+}\) at maximum rates close to or identical to that of the wild type. In contrast to the Pro852 → Ala mutant, a decrease of the transport activity to 12% of the wild type at pCa 5.0 was observed with the Pro106 → Gly mutant. The Pro312 → Ala and Pro312 → Gly mutants were completely unable to transport Ca\(^{2+}\), and very little transport activity was detected with the Pro312 → Leu mutant (7% of the wild type).

For those mutants which were able to transport Ca\(^{2+}\) at measurable rates, it was possible to examine the Ca\(^{2+}\) affinity by titration of the Ca\(^{2+}\) dependence of Ca\(^{2+}\)-transport (Fig. 4). As seen in Fig. 4, the Pro852 → Ala, Pro852 → Gly, and Pro852 → Ala mutants all displayed a lower Ca\(^{2+}\) affinity than that of the wild type, with the pCa at which half-saturation occurs being shifted about 0.5 unit. A less significant deviation from the wild-type Ca\(^{2+}\) affinity was seen for the Pro851 → Ala mutant. For the Pro590 → Ala mutant, Ca\(^{2+}\) dependence was identical to that of the wild type. This was also the case for the Pro106 → Ala mutant (data not shown).

**Phosphorylation of Mutants from ATP**—The finding of a reduced Ca\(^{2+}\) affinity for the Pro106 and Pro852 mutants and a complete loss of Ca\(^{2+}\) transport activity for the Pro591 → Ala and Pro592 → Gly mutants led us to examine the partial reactions of Ca\(^{2+}\)-transport. The initial transfer of the terminal phosphate group of ATP to the enzyme, forming the phosphorylated intermediate, is a Ca\(^{2+}\)-dependent reaction which requires the binding of two Ca\(^{2+}\) ions at high affinity sites in the wild-type enzyme (de Meis and Vianna, 1979; Pettithory and Jencks, 1988). The proline mutants examined in this study all showed the same level of phosphorylation from ATP as the wild-type enzyme when the reaction was carried out at a free Ca\(^{2+}\) concentration of approximately 100 μM.

**TABLE I**

| Mutants   | Calcium affinity(Km/μM) | Turnover rate(s⁻¹) |
|-----------|------------------------|-------------------|
| Wild type | 0.45                   | 12.5              |
| Pro195 → Ala | 0.63            | 9.5               |
| Pro195 → Ala | 0.52            | 12.4              |
| Pro106 → Ala | 1.30             | 10.6              |
| Pro106 → Ala | 0.16             | 0                 |
| Pro591 → Ala | 1.11            | 11.3              |
| Pro592 → Ala | 0.79             | 12.5              |
| Pro592 → Gly | 1.40            | 1.5               |
| Pro592 → Gly | 0.19             | 0                 |
| Pro592 → Leu | 0.23            | 0.9               |

* Determined from the calcium dependence of phosphorylation from ATP.

* Calculated on the basis of the initial Ca\(^{2+}\) uptake rate (nanomoles of Ca\(^{2+}\) transported/s/mg total microsomal protein) measured at pCa 5.0 and the active site concentration obtained by measurement of phosphorylation (nanomoles of phosphoprotein/mg of total microsomal protein). Since two Ca\(^{2+}\) ions are transported per molecule of ATP hydrolyzed, the Ca\(^{2+}\) uptake rate was divided by 2 to obtain the turnover rate.

**Fig. 4. Ca\(^{2+}\) dependence of Ca\(^{2+}\)-transport catalyzed by mutant Ca\(^{2+}\)-ATPases.** Ca\(^{2+}\)-ATPase corresponding to the wild type (C) and the Pro195 → Ala (D), Pro106 → Gly (E), Pro106 → Ala (E), Pro852 → Ala (A), and Pro592 → Ala (Δ) mutants was incubated at 27 °C for 5 min in a reaction mixture containing 20 mM MOPS, pH 6.8, 80 mM KCl, 5 mM MgCl₂, 5 mM ATP, 0.5 mM EGTA, 5 mM potassium oxalate, and various concentrations of "CaCl₂ (0.01–0.51 mM) to produce the pCa values indicated. The amount of Ca\(^{2+}\) taken up by the microsomes after 5 min was measured after Millipore filtration as described under "Materials and Methods." The specific Ca\(^{2+}\) uptake activity (calculated per milligram of protein) is shown relative to that measured for the wild type at saturating Ca\(^{2+}\) concentration.
was saturated with Ca\(^{2+}\) at pCa 6, the Pro\(^{310}\)
PM.

Examples of this are seen in Fig. 5, which presents autoradiographs of phosphorylation data obtained with the Pro\(^{308}\) \(\rightarrow\) Ala and Pro\(^{312}\) \(\rightarrow\) Ala mutants. Whereas the wild-type enzyme was saturated with Ca\(^{2+}\) at pCa 6, the Pro\(^{308}\) \(\rightarrow\) Ala mutant required a pCa close to 5.5 for saturation, confirming the reduced affinity observed by measurement of Ca\(^{2+}\) uptake. On the other hand, the Pro\(^{312}\) \(\rightarrow\) Ala mutant showed a higher apparent affinity for Ca\(^{2+}\) relative to that of the wild type, with saturation occurring at pCa 6.2–6.5.

Fig. 6 shows quantitation of similar data obtained when Pro\(^{308}\) and Pro\(^{312}\) were substituted with glycine instead of alanine. Again, an increase of apparent affinity was observed after substitution of Pro\(^{312}\). We also replaced Pro\(^{312}\) with leucine, the residue which is present at the homologous position in the Na\(^{+}, K^{+}\)-ATPase (Shull et al., 1985). This substitution induced an increase in affinity similar to that observed with the other two substitutions of Pro\(^{312}\).

The Ca\(^{2+}\) titration data for all of our proline mutants are presented in Table I as the Ca\(^{2+}\) concentration at which half-maximum phosphorylation occurred (K\(_{o,3}\)). The relative affinities of mutants and the wild type measured in the phosphorylation experiments are consistent with those observed in the Ca\(^{2+}\) uptake measurements (Fig. 4). A comparison of the results presented in Table I with Fig. 4, however, shows that for both the wild type and mutants, the activation occurred at higher Ca\(^{2+}\) concentrations in the phosphorylation experiments than in the Ca\(^{2+}\) uptake experiments. This can be accounted for by the higher concentration of free Mg\(^{2+}\) present in the phosphorylation experiments (ATP concentration of only 2 \(\mu\)M since Mg\(^{2+}\) competes with Ca\(^{2+}\) at the high affinity Ca\(^{2+}\)-binding sites (Vilsen and Andersen, 1987).

Dephosphorylation Experiments—The inability of the fully phosphorylated Pro\(^{312}\) mutants to pump Ca\(^{2+}\) suggests that a partial reaction following phosphorylation is critically dependent on this proline residue. Therefore, we set up assay conditions with the purpose of testing the later steps in the reaction cycle. As seen in the first lanes of Fig. 7 and illustrated by the leftward reaction pathway in Scheme 1, all of the phosphoenzyme disappeared within 5 s when 1 mM ADP was added, indicating that no E-P was accumulated under these conditions. When EGTA was added to E-P in the absence of ADP, the Ca\(^{2+}\)-dependent phosphorylation from ATP ceased, and the dephosphorylation occurring through conversion to E:P and its subsequent hydrolysis could be observed (see Scheme 1, rightward reaction pathway). As seen in Fig. 7, EGTA-induced dephosphorylation was almost completed within 20 s for the wild-type enzyme. By contrast, dephosphorylation in the absence of ADP was very slow in the Pro\(^{312}\) mutants. Quantitation of the radioactivity associated with the protein in the gel showed that for the Pro\(^{312}\) \(\rightarrow\) Ala mutant, more than 80% of the phosphoenzyme remained 5 min after the addition of EGTA. For the Pro\(^{312}\) \(\rightarrow\) Leu mutant, less than 30% of the phosphoprotein was left after 30 s. Analogous experiments conducted with the Pro\(^{312}\) \(\rightarrow\) Gly mutant demonstrated a very low dephosphorylation rate, similar to that observed with the Pro\(^{312}\) \(\rightarrow\) Ala mutant (data not shown). The differences between mutants and the wild type cannot be ascribed to Ca\(^{2+}\) accumulated in the wild-type microsomes since all experiments were conducted in the presence of ionophore A23187.

In order to examine separately the dephosphorylation of the “low energy” phosphoenzyme intermediate (E:P), phosphorylation was performed with inorganic phosphate as substrate (Fig. 7, right panels). The Pro\(^{312}\) mutants (as well as the other proline mutants examined in this study) reacted with P\(_{i}\) to the same extent as the wild-type enzyme. When the phosphorylation by P\(_{i}\) was terminated at 0 °C by dilution, the subsequent decay of the phosphoenzyme in the mutants occurred at a rate similar to that of the wild-type enzyme. This rate was much higher than the rate of dephosphorylation observed after the EGTA quench of E:P. This shows that the E:P to E:P interconversion, rather than E:P hydrolysis, is
rate-limiting for phosphoenzyme turnover and that the inhibition of dephosphorylation from E1P in the Pro\textsuperscript{312} mutants is caused mainly by an effect on the E1P to E2P transition.

To substantiate this conclusion, phosphorylation experiments with ATP were performed at pH 8.35 in the presence of 10 mM Mg\textsuperscript{2+} and in the absence of alkali metal ions. This medium composition promotes the E1P to E2P interconversion and slows dephosphorylation of E2P (Shigekawa et al., 1983; Andersen et al., 1985), leading to steady-state accumulation of E1P in the wild-type enzyme (Fig. 8, left panel). No E1P accumulated with the Pro\textsuperscript{312} → Gly mutant, however, as demonstrated by the full dephosphorylation obtained in the presence of ADP, even 5 min after addition of EGTA (Fig. 8, right panel). Similar results were obtained with the Pro\textsuperscript{312} → Ala mutant, but the Pro\textsuperscript{312} → Leu mutant accumulated E1P to a level intermediate between the wild type and the Pro\textsuperscript{312} → Gly mutant (data not shown).

**DISCUSSION**

Of the proline mutants examined in this study, none of those located in the predicted cytoplasmic region of the Ca\textsuperscript{2+}-ATPase behaved strikingly differently from the wild-type enzyme. Surprisingly, Pro\textsuperscript{196}, which is highly conserved among the cation-transporting ATPases (Serrano, 1988; Green et al.), could be replaced by alanine without any major consequences for the function of the enzyme. Pro\textsuperscript{196} has been suggested to be part of a Ca\textsuperscript{2+}-binding torus (Gangola and Shamoo, 1986), but such a role seems to be excluded by the normal behavior of the Pro\textsuperscript{306} → Ala mutant as well as by the earlier finding that the acidic residues in the proposed torus could be mutated without loss of Ca\textsuperscript{2+} transport function (Clarke et al., 1989b).

By contrast, mutations of those prolines located in the transmembrane region of the molecule had profound effects on the function of the Ca\textsuperscript{2+}-ATPase. The transmembrane mutants fell into two functional groups. The Pro\textsuperscript{308} and Pro\textsuperscript{309} mutants were characterized by a reduced affinity for Ca\textsuperscript{2+} as judged from the Ca\textsuperscript{2+} dependence of Ca\textsuperscript{2+} transport and of phosphorylation, whereas the Pro\textsuperscript{311} mutants were unable to transport Ca\textsuperscript{2+}, but displayed a higher Ca\textsuperscript{2+} affinity than that of the wild-type enzyme in the phosphorylation assay. Prolines 308 and 803 are located in the middle of predicted transmembrane helices M4 and M6, respectively, close to carboxylic groups which, in our previous mutagenesis study (Clarke et al., 1989a), were assigned as Ca\textsuperscript{2+} ligands. On the basis of the reduced Ca\textsuperscript{2+} affinity that we observed after replacement of Pro\textsuperscript{308} and Pro\textsuperscript{803}, it seems likely that these prolines are also involved in formation of one or both high affinity Ca\textsuperscript{2+}-binding sites, either by donating the electrons associated with the carbonyl group in the preceding peptide sequence for the function of the enzyme. Pro\textsuperscript{19} has been suggested to be part of a Ca\textsuperscript{2+}-binding torus (Gangola and Shamoo, 1986), but such a role seems to be excluded by the normal behavior of the Pro\textsuperscript{306} → Ala mutant as well as by the earlier finding that the acidic residues in the proposed torus could be mutated without loss of Ca\textsuperscript{2+} transport function (Clarke et al., 1989b).

2 N. M. Green et al., unpublished data.
from the measured $K_{m}$ values (Table I), are $-8.0$, $-7.4$, and $-7.5$ kcal/mol for the wild type and the Pro$^{308}$ $\rightarrow$ Ala and Pro$^{308}$ $\rightarrow$ Ala mutants, respectively. Thus, the contribution of each of these proline residues to the total binding energy of the Ca$^{2+}$ sites would lie in the range of 6-7%.

It is noteworthy that Pro$^{308}$ is highly conserved among cation-transporting ATPases (Serrano, 1988; Green et al.$^{3}$). Pro$^{308}$, on the other hand, is conserved only in the Na$^{+}$/K$^{+}$-ATPase. In the plasma membrane Ca$^{2+}$-ATPase, the residue located in the homologous position is an alanine (Shull and Greeb, 1988; Verma et al., 1988), the residue used to replace proline in this study. Since both Pro$^{308}$ and Pro$^{309}$ can be substituted with alanine without a major reduction of the expression level or of the maximum turnover rate, it seems unlikely that these prolines are involved in formation of intramembranous $\beta$-turns or other loop structures in the transmembrane sector (Lodish, 1988). It also seems unlikely that cis-trans isomerization of the peptide bonds at Pro$^{308}$ and Pro$^{312}$ contributes to conformational changes associated with Ca$^{2+}$ transport.

In contrast to the mutants discussed above, the Pro$^{312}$ $\rightarrow$ Ala and Pro$^{312}$ $\rightarrow$ Gly mutants were unable to transport Ca$^{2+}$, even at Ca$^{2+}$ concentrations permitting full phosphorylation from ATP. We have shown that these mutants are defective in the $E_1P$ to $E_2P$ transition, which accounts for their inability to translocate Ca$^{2+}$. Our evidence was based on kinetic experiments carried out with the ADP-sensitive and -insensitive phosphoenzyme intermediates formed by phosphorylation from ATP and P, respectively. As a consequence of the block of the $E_1P$ to $E_2P$ step in the reaction cycle, the ADP-sensitive phosphoenzyme intermediate accumulated in the Pro$^{312}$ $\rightarrow$ Ala and Pro$^{312}$ $\rightarrow$ Gly mutants. This kinetic effect can explain the fact that the mutants display a higher apparent affinity for Ca$^{2+}$ in the phosphorylation reaction than does the wild type.

It is remarkable that the Pro$^{312}$ residue is, by itself, conserved only in other Ca$^{2+}$-ATPases and in the yeast H$^{+}$-ATPase (Serrano, 1988), it is located in a highly conserved region which forms a physical link between the cation-binding domain and the phosphorylated aspartic acid residue (Asp$^{373}$). This may be of importance from the point of view of a general mechanism for energy transduction in transport ATPases. We have proposed a model in which the $E,F$ to $E,P$ conformational change comprises a rotation or tilting of one or more of the transmembrane helices, disrupting the Ca$^{2+}$-binding domain and thereby making the previously occluded Ca$^{2+}$ ions accessible to a release channel (Brandl et al., 1986; Clarke et al., 1989).

On the basis of our observation that there is a crucial dependence of the $E,P$ to $E,P$ transition on the Pro$^{312}$ residue, we now suggest that movement of helix $\alpha$ in which this proline is located, forms the central element in the conformational change. By introducing a defect in helix packing in the transmembrane sequences, the Pro$^{312}$ residue may be decisive in signal transduction leading to channel opening.

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