Short and Long Range Functions of Amino Acids in the Transmembrane Region of the Sarcoplasmic Reticulum ATPase

A MUTATIONAL STUDY*

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Mutational analysis of several amino acids in the transmembrane region of the sarcoplasmic reticulum ATPase was performed by expressing wild type ATPase and 32 site-directed mutants in COS-1 cells followed by functional characterization of the microsomal fraction. Four different phenotype characteristics were observed in the mutants: (a) functions similar to those sustained by the wild type ATPase; (b) Ca\(^{2+}\) transport inhibited to a greater extent than ATPase hydrolytic activity; (c) inhibition of transport and hydrolytic activity in the presence of high levels of phosphorylated enzyme intermediate; and (d) total inhibition of ATP utilization by the enzyme while retaining the ability to form phosphoenzyme by utilization of P\(_i\). Analysis of experimental observations and molecular models revealed short and long range functions of several amino acids within the transmembrane region. Short range functions include: (a) direct involvement of five amino acids in Ca\(^{2+}\) binding within a channel formed by clustered transmembrane helices M4, M5, M6, and M8; (b) roles of several amino acids in structural stabilization of the helical cluster for optimal channel function; and (c) a specific role of Lys297 in sealing the distal end of the channel, suggesting that the M4 helix rotates to allow vectorial flux of Ca\(^{2+}\) upon enzyme phosphorylation. Long range functions are related to the influence of several transmembrane amino acids on phosphorylation reactions with ATP or P\(_i\), transmitted to the extramembranous region of the ATPase in the presence or in the absence of Ca\(^{2+}\).

Understanding the topology of functional domains is required to clarify the mechanism of cation translocation by membrane-bound ATPases. With regard to the Ca\(^{2+}\) transport ATPase of sarcoplasmic reticulum (SR),\(^1\) it is known that the catalytic and phosphorylation sites reside within the extramembranous region of the enzyme. On the other hand, the functional relevance of the transmembrane region was revealed by the mutational work of Clarke et al. (1), who suggested that six residues originating from four transmembrane helices (M4, Glu309; M5, Glu771; M6, Asn796; Thr799, and Asp800; M8, Glu908) are involved in Ca\(^{2+}\) binding (Fig. 1). We have now performed a mutational analysis of several amino acids in the transmembrane region to evaluate in detail their roles in Ca\(^{2+}\) binding and, more generally, in the catalytic and transport functions of the Ca\(^{2+}\) ATPase.

EXPERIMENTAL PROCEDURES

Oligonucleotide-directed Mutagenesis and cDNA Expression in COS-1 Cells—The chicken fast muscle SR ATPase cDNA (3) was inserted into the pUC19 plasmid for amplification and then subcloned into the pSELECT-1 vector for site-directed mutagenesis by the Altered Sites in Vitro Mutagenesis System (Promega; Madison, WI) or by overlap extension using the polymerase chain reaction (4). Eleven unique restriction sites were introduced in the cDNA to allow cassette exchange, and a c-myc tag was added to the 3' terminus to monitor ATPase expression as described by Zhang et al. (5). The cDNA constructs were finally transferred into the pCDL-SR296 plasmid (6) for transfection of COS-1 as described by Sumbilla et al. (7).

Microsome Preparation and Immunodetection of Expressed Protein—Four days after transfection the confluent COS-1 cells from 20 plates (150 × 25 mm; 4 × 10\(^6\) cells/plate) were rinsed twice with cold phosphate-buffered saline, scraped with a Teflon spatula, and collected in 80 ml of phosphate-buffered saline. The microsomal fraction was then obtained as described by Zhang et al. (5).

The expressed SERCA-1 ATPase was detected by Western blotting, using monoclonal antibody 9E10 to the c-myc epitope (8) and, in parallel, a monoclonal antibody to the chicken Ca-ATPase, CaF-3-SC3 (9). In addition to Western blots, quantitation of SERCA-1 expression was also obtained by enzyme-linked immunosorbent assays in microtiter plates.

Ca\(^{2+}\) Transport and ATPase Activity—ATP-dependent Ca\(^{2+}\) transport was measured by following the accumulation of radioactive calcium tracer in microsomal vesicles separated from the reaction mixture by filtration (0.45-μm Millipore filters), as described by Zhang et al. (5). ATPase activity was measured by determination of P\(_i\) (Lanzetta et al. (9), also as described by Zhang et al. (5)). The Ca\(^{2+}\)-dependent activity was calculated by subtracting the Ca\(^{2+}\)-independent ATPase from the total ATPase. Both Ca\(^{2+}\) uptake and Ca\(^{2+}\)-dependent ATPase activity were corrected to account for the level of expressed protein in each microsomal preparation as revealed by immunoreactivity and with reference to microsomes obtained from COS-1 cells transfected with wild type cDNA.

Enzyme Phosphorylation with ATP—Steady-state levels of phosphorylated enzyme intermediate were obtained by adding 0.1 ml of 10 μM \(\gamma\)-\[^{32}\]P ATP to 0.4 ml of a reaction mixture containing 20 μM MOPS, pH 7.0, 80 μM KC1, 5 μM MgCl\(_2\), 0.1 mM CaCl\(_2\) and 30–100 μg of microsomal protein (the amount of microsomal protein was varied to approximate the amount of expressed ATPase contained by wild type controls, as revealed by immunoreactivity). The components of the reaction mixture were preincubated in ice, and vortex mixing was carried out in the cold room. The reaction was quenched after 10 s by the addition (vortex mixing) of 0.105 ml of 100 mM perchloric acid. The suspension was transferred immediately onto an Eppendorf tube containing 100 μl of carrier protein and allowed to sit in ice for 5–10 min. After centrifugation at 5,000 rpm for 10 min, the sedimented protein was washed twice with 0.125 mM perchloric acid and once with water. An aliquot of the...
A solubilized sample was then subjected to gel electrophoresis (10) at pH 6.3, and the radioactive phosphoenzyme was detected by autoradiography. The rates of phosphoenzyme decay were determined by first obtaining steady-state levels of phosphoenzyme as explained above. Ten s after the addition of radioactive ATP, 0.5 ml of 1.0 mM nonradioactive ATP was added with rapid mixing, and samples were acid quenched at serial times. A zero time baseline was obtained by acid quenching before the chase. The entire procedure was carried out in ice and in the cold room. Washings, electrophoresis, and autoradiography were performed as described above.

**Molecular Modeling**—Quanta and Hyperchem molecular graphics programs were used for molecular modeling. The valence mapping program was kindly supplied by Prof. E. Di Cera, Washington University, St. Louis.

**RESULTS**

**ATPase Expression in COS-1 Cells**—The studies reported here were performed with wild type and mutated cDNAs encoding the SR ATPase and the mutants listed in Tables I–III. After disruption of the transfected cells and fractionation, the expressed ATPase is recovered with microsomal vesicles and can be detected by Western blots and enzyme-linked immunosorbent assay titrations. The wild type ATPase and the mutants studied in these experiments were expressed at similar, but not identical levels (Fig. 2). Therefore, the relative level of expressed ATPase in each preparation, with reference to a selected wild type ATPase preparation, was used to obtain corrected values for the functional activity of 1 mg of microsomal fraction.
of microsomal protein/min at 30 and 37°C, respectively (in parentheses are the numbers of determinations in two to four microsomal preparations from two batches of plasmid amplified, banded, and sequenced at different times). Northern blot analysis confirmed the presence of an mRNA transcript for this mutant consistent with that of wild type (results not shown). It is of interest that mutation of the same residue to Phe did yield expression, although at low level, and the Lys297 → Met, Lys297 → Arg, and Lys297 → Glu mutants were expressed at normal levels (Fig. 2).

**Ca**\(^{2+}\) Uptake and ATPase Activity—It is shown in Fig. 3 and Table I that several mutations within the M4, M5, M6, and M8 helices sustain levels of Ca\(^{2+}\) transport and ATPase activity which are comparable to those of the wild type ATPase. Therefore, it is possible to introduce point mutations within the transmembrane region without interfering with function. These findings confer a character of specificity to several other mutations that do interfere with function. In fact, not all mutants retaining high ATPase hydrolytic activity retained a correspondingly high Ca\(^{2+}\) uptake. Among the mutants retaining high hydrolytic activity, this differential effect is pronounced in Val314 → Ala, Ile315 → Ala, Trp794 → Ala, Val795 → Ala, and Leu802 → Ala mutants (Table I).

A group of mutations producing inhibition of ATPase hydrolytic activity is shown in Table II. It is of interest that in all of these mutations, Ca\(^{2+}\) transport was inhibited significantly more than hydrolytic activity. Since this uncoupling effect is produced by mutations which do (Table II) or do not (Table I) inhibit hydrolytic activity, it is apparent that a specific structural perturbation is involved in its onset, independent of other perturbations that may affect catalytic activity.

Characterization of the six mutations originally reported by Clarke et al. (1), who proposed that the six native residues participate in Ca\(^{2+}\) binding, is shown in Table III. We confirmed that these mutations produce total inhibition of Ca\(^{2+}\) transport and hydrolytic activity, and then we extended the functional characterization as explained below.

It is noteworthy that the effects of mutations are very specific and dependant both on the residues that are mutated and the side chains that are introduced. An example of the high specificity of mutational effects is found in the total inhibition resulting from the Glu908 → Ala mutation compared with the full activity retained by the Glu908 → Gln mutant (Fig. 3). Of great interest are also the different and specific effects produced by mutating Lys297 to Gly (no expression), to Phe (low expression and low activity), to Met (low activity), and to Arg or Glu (nearly normal ATPase and slight inhibition of Ca\(^{2+}\) uptake).

**Formation and Turnover of Phosphoenzyme Intermediate in**

| Sample   | Helix | Side chain orientation | Ca\(^{2+}\) transport | ATPase activity | EP (ATP) | Decay | EP (P\(_i\)) | Decay |
|----------|------|------------------------|-----------------------|-----------------|---------|-------|-------------|-------|
| WT       |      |                        |                       |                 | +       | Fast  | +           | Fast  |
| Control  |      |                        |                       |                 | 0       | 0     | 0           | 0     |
| Lys297 → Glu | M4   | Lumen                  | 60.5 ± 12.7 (4)       | 98.7 ± 14.2 (3) | +       | Fast  | +           | Fast  |
| Lys297 → Arg | M4   | Lumen                  | 67.6 ± 4.3 (3)        | 83.0 ± 17.9 (5) | +       | Medium| +           | Fast  |
| Ile315 → Ala | M4   | Exterior                | 48.0 ± 19.4 (4)       | 88.6 ± 25.5 (5) | +       | Fast  | +           | Fast  |
| Leu311 → Ala | M4   | Exterior                | 75.0 ± 25.1 (3)       | 98.7 ± 27.5 (3) | +       | Fast  | +           | Fast  |
| Val794 → Ala | M4   | Exterior                | 19.9 ± 10.0 (6)       | 80.1 ± 29.8 (6) | +       | Medium| +           | Fast  |
| Ile315 → Ala | M4   | Side                    | 39.2 ± 6.2 (4)        | 104.0 ± 39.1 (5) | +       | Medium| +           | Fast  |
| Val793 → Ala | M5   | Exterior                | 69.2 ± 16.4 (5)       | 150.8 ± 46.6 (4) | +       | Fast  | +           | Fast  |
| Phe786 → Ala | M5   | Exterior                | 95.3 ± 6.6 (3)        | 102.0 ± 16.7 (4) | +       | Fast  | +           | Medium|
| Leu777 → Ala | M5   | Exterior                | 79.0 ± 11.7 (3)       | 93.6 ± 28.3 (3) | +       | Fast  | +           | Fast  |
| Trp794 → Ala | M6   | Exterior                | 38.6 ± 11.8 (7)       | 109.6 ± 17.5 (5) | +       | Fast  | +           | Fast  |
| Val795 → Ala | M6   | Side                    | 25.8 ± 4.9 (8)        | 75.0 ± 8.8 (3)  | +       | Fast  | +           | Medium|
| Leu802 → Ala | M6   | Exterior                | 37.7 ± 9.5 (6)        | 91.6 ± 11.2 (5) | +       | Fast  | +           | Fast  |
| Glu908 → Gln | M8   | Lumen                  | 105.2 ± 26.8 (4)      | 132.5 ± 24.2 (4) | +       | Fast  | +           | Fast  |
the presence of ATP and Ca$^{2+}$—We performed several types of experiments to uncover whether specific partial reactions of the ATPase cycle (Fig. 4) were affected by mutations. As reported previously by Clarke et al. (1), we found (Fig. 5 and Table III) that the Glu$^{309}$ → Gin, Glu$^{771}$ → Gin, Asn$^{796}$ → Ala, Thr$^{799}$ → Ala, Asp$^{800}$ → Asn, and Glu$^{908}$ → Ala mutants do not produce significant levels of phosphoenzyme intermediate when incubated with ATP and Ca$^{2+}$ (reaction 2 in Fig. 4). The phosphoenzyme levels obtained with all other mutants were within the range obtained with wild type enzyme. Minor variations of phosphoenzyme levels on the gels were not related meaningfully to the ATPase activities of the corresponding mutants (Fig. 5 and Tables I and II).

We then conducted isotopic chase experiments to evaluate the effect of mutations on the decay of the phosphorylated enzyme intermediate (reactions 3 and 4 in Fig. 4). We found

\[\text{Ca}^{2+} \text{transport} \rightarrow \text{ATPase activity} \rightarrow \text{EP (ATP)} \rightarrow \text{EP (Pi)} \rightarrow \text{Decay}\]

The original characterization is extended to evaluate the equilibrium levels of phosphoenzyme obtained by utilization of Pi, the Ca$^{2+}$ concentrations required to produce a half-maximal inhibition of phosphorylation under the original conditions, and the phosphoenzyme decay rates. WT, wild type.

**Table II**

| Sample | Helix | Side chain orientation | Ca$^{2+}$ transport | ATPase activity | EP (ATP) | Decay | EP (Pi) | [Ca$^{2+}$] | Decay |
|--------|-------|------------------------|---------------------|----------------|----------|-------|---------|-----------|-------|
| WT     |       |                        | 100                 | 100            | ++       | Fast  | ++      | 20 μM     | Fast  |
| Control|       |                        | 0                   | 0              | 0        | 0     | 0       | 0         | 0     |
| Lys$^{307}$ → Gly | M4 | Lumen | No expression | No expression | ++ | Slow | ++ | Slowing | Slow |
| Lys$^{307}$ → Met | M4 | Lumen | 10.2 ± 3.9 (10) | 40.7 ± 5.5 (4) | ++ | Slow | ++ | Slow | Slow |
| Lys$^{307}$ → Phe | M4 | Lumen | 18.5 ± 1.4 (2) | 44.0 ± 11.9 (3) | ++ | Slow | ++ | Slow | Slow |
| Lys$^{307}$ → Ala | M4 | Side | 23.7 ± 6.4 (6) | 48.0 ± 7.6 (5) | ++ | Medium | ++ | Medium | Medium |
| Val$^{300}$ → Ala | M4 | Exterior | 13.1 ± 3.8 (8) | 38.5 ± 4.6 (4) | ++ | Medium | +++ | Slow | Slow |
| Val$^{304}$ → Ala | M4 | Side | 13.5 ± 4.8 (5) | 45.1 ± 9.2 (4) | ++ | Medium | ++ | Medium | Medium |
| Pro$^{312}$ → Ala | M4 | Side | 9.7 ± 2.9 (3) | 40.8 ± 4.7 (4) | ++ | Slow | ++ | Fast | Fast |
| Leu$^{319}$ → Ala | M4 | Lumen | 12.8 ± 5.1 (9) | 55.2 ± 10.7 (6) | ++ | Medium | ++ | Fast | Fast |
| Tyr$^{313}$ → Ala | M5 | Side | 5.6 ± 2.7 (10) | 35.7 ± 6.1 (3) | ++ | Medium | ++ | Medium | Medium |
| Val$^{372}$ → Ala | M5 | Exterior | 11.3 ± 3.7 (4) | 48.8 ± 4.3 (4) | ++ | Medium | ++ | Medium | Medium |
| Cys$^{374}$ → Ala | M5 | Side | 8.4 ± 1.8 (6) | 40.0 ± 16.6 (5) | ++ | Slow | ++ | Slow | Slow |
| Ile$^{376}$ → Ala | M3 | Side | 6.9 ± 2.0 (3) | 24.8 ± 12.1 (5) | ++ | Slow | ++ | Slow | Slow |
| Leu$^{383}$ → Ala | M6 | Side | 11.7 ± 5.6 (3) | 57.0 ± 11.6 (4) | ++ | Medium | ++ | Medium | Medium |

**Fig. 4. Minimal reaction scheme for the Ca$^{2+}$ ATPase.** Only the four partial reactions that can be measured by chemical methods (Ca$^{2+}$ binding, formation of phosphorylated intermediate by utilization of ATP, vectorial translocation of bound Ca$^{2+}$, and hydrolytic cleavage of P$_i$) are listed for convenience of easy reference in the text. As implied under "Discussion," isomeric transitions (such as, or in addition to the E1 to E2 transition postulated by De Meis and Vianna (12)) occur in parallel with the four reactions outlined in the diagram.

**Fig. 5. Examples of phosphoenzyme steady-state levels formed in the presence of Ca$^{2+}$ and ATP.** The phosphoenzyme was obtained as described under "Experimental Procedures" and determined by autoradiography. Note the absence or very low levels of phosphoenzyme when the Glu$^{309}$ → Gin, Glu$^{771}$ → Gin, Asn$^{796}$ → Ala, Thr$^{799}$ → Ala, Asp$^{800}$ → Asn, and Glu$^{908}$ → Ala mutants are used. Characterization of these and all the other mutants is reported in the tables. WT, wild type.
that mutants sustaining ATPase activity comparable to that of the wild type enzyme exhibited turnover that was similar to that of the wild type enzyme. On the other hand, the rate of phosphoenzyme decay was reduced in mutants exhibiting inhibition of steady-state ATPase activity (e.g. see Lys297 → Met, Cys774 → Ala, and Ile775 → Ala in Fig. 6 and Table II).

Observations of mutational effects on phosphoenzyme turnover are very important for two reasons. (a) They confirm the inhibitory effects of mutations under conditions that are not dependent on the enzyme concentration (since phosphoenzyme decay is a first order phenomenon); this dispels doubts on whether observed inhibitions of steady-state ATPase activity (which is dependent on enzyme concentration) may in fact be related to inaccuracies in the immunological determination of expressed ATPase. (b) They indicate that the mutational effect is on ATPase partial reactions that follow formation of the phosphorylated intermediate (step 3 and/or step 4 in Fig. 4), and these reactions are rate-limiting for completion of the catalytic and transport cycle.

Formation of Phosphoenzyme by Utilization of Pi—In addition to enzyme phosphorylation by utilization of ATP in the presence of Ca$^{2+}$, phosphoenzyme can be formed in the reverse direction of the ATPase cycle by utilization of Pi in the absence of Ca$^{2+}$ (see Ref. 13). We studied this reaction by equilibrating the enzyme with Pi in the absence of Ca$^{2+}$, at mildly acid pH, and in the presence of 20% (v/v) dimethyl sulfoxide to facilitate phosphorylation, as was also done in the experiments by Clarke et al. (1).

When we used samples of wild type enzyme reacted separately with P$_i$, under these conditions, we obtained identical phosphoenzyme levels, indicating that our technique of detection was highly reproducible (Fig. 7). On the other hand, the equilibrium levels of phosphoenzyme obtained with various mutants were quite different. These differences were not related to whether the mutants exhibited or did not exhibit inhibition of steady-state ATPase activity and/or enzyme phosphorylation with ATP. In some cases (Fig. 7 and Tables I-III), high levels of phosphoenzyme were obtained through the P$_i$ reaction with mutants yielding low ATPase activity (Val300 → Ala, Cys774 → Ala, Ile775 → Ala), or no ATPase activity and no phosphoenzyme in the presence of ATP and Ca$^{2+}$ (Glu309 → Gln). On the other hand, hardly detectable levels of phosphoenzyme were obtained through the P$_i$ reaction with some mutants (Val314 → Ala and Leu719 → Ala, for instance) retaining relatively high ATPase activity and enzyme phosphorylation with ATP (Fig. 7 and Table I).

As opposed to the steady-state levels (resulting from all four steps of the diagram in Fig. 4) obtained in the presence of ATP and Ca$^{2+}$, the P$_i$ reaction (reverse of step 4 of the diagram in Fig. 4) may be considered to occur as

$$E + P_i \rightarrow E-P_i \rightarrow E-P$$

where E is the enzyme equilibrated with P$_i$. We then explored the possibility that the observed variations of E-P may be due to altered affinity of the mutants for P$_i$. We found, however, that the P$_i$ concentration used in our experiments was saturating in all cases (not shown). Therefore, the observed variations of phosphoenzyme levels were likely due to mutational effects.
ported that the Ca\(^{2+}\) sensitivity of the Glu\(^{309} \rightarrow \text{Gln}\) mutant is in the 10 \(\mu\text{M}\) range as opposed to the mw range observed in our experiments (Fig. 9). It is likely that this difference is due to the higher pH used by Andersen and Vilsen (16), favoring ionization of Ca\(^{2+}\) binding acidic functions with pK near neutrality (18), thereby increasing the affinity of the enzyme for Ca\(^{2+}\) and obscuring the effect of single mutations.

Finally, we note that mutation of Glu\(^{308} \rightarrow \text{Ala}\) interferes with inhibition of the P\(_i\) reaction by Ca\(^{2+}\), whereas mutation of Glu \rightarrow Gln leaves the enzyme perfectly functional (also noted by Clarke et al. (1)). This is of specific interest, considering that mutation Glu\(^{309} \rightarrow \text{Gln}\), Glu\(^{771} \rightarrow \text{Gln}\), or Asp\(^{800} \rightarrow \text{Asn}\) interferes strongly with inhibition of the P\(_i\) reaction by Ca\(^{2+}\) (Tables I and III and Fig. 9). The different effect of the Glu\(^{308}\) mutations to Gln or Ala suggests that although the Glu\(^{309}\), Glu\(^{771}\), and Asp\(^{800}\) contributions to Ca\(^{2+}\) complexation depend on both side chain oxygens, Glu\(^{308}\) contributes only one side chain oxygen, which is still present following mutation to Gln, but not to Ala. As the acidic function is lost by the Gln mutant, we conclude that the carbonyl oxygen is able to participate in coordination of Ca\(^{2+}\).

**DISCUSSION**

Critical Evaluation of a Putative Ca\(^{2+}\) Binding Domain by Molecular Modeling—We found it helpful to evaluate by molecular modeling whether the residues pointed out by mutational analysis can in fact generate a Ca\(^{2+}\) binding domain. Accordingly, a model was constructed by clustering transmembrane helices M4, M5, M6, and M8 and thereby forming a channel that can admit two Ca\(^{2+}\) in single file (19). Rotation of the helices for optimal positioning of acidic side chains within the lumen of the channel is favored by the amphiphilic character of the helices.

An important question is whether it is possible to have two closely spaced calcium ions bound within the same domain (in spite of possible charge repulsion). Crystallographic resolution of several Ca\(^{2+}\) binding structures (for instance, the duplex Ca\(^{2+}\) binding site of thermolysin; Ref. 20) indicates that two Ca\(^{2+}\) can in fact reside in close proximity and yields information regarding the appropriate distances between binding oxygens and Ca\(^{2+}\) and between the two bound Ca\(^{2+}\).

In previous attempts to model all six residues suggested by Clarke et al. (1), we encountered difficulty in orienting the diverging side chains of the Asn\(^{796}\), Thr\(^{799}\), and Asp\(^{800}\) (which originate from the same helix M6) for simultaneous participation in Ca\(^{2+}\) binding. For this reason we proposed exclusion of Thr\(^{799}\) (2). Our present experimental observations, however, indicate clearly that Asn\(^{796}\) is the residue to be excluded; and this solves the problem. However, we do not exclude that Asn\(^{796}\) may participate in Ca\(^{2+}\) binding by the enzyme in some specific conformational state that we do not see under our conditions. Andersen and Vilsen suggested (16) that Asn\(^{796}\) may participate in Ca\(^{2+}\) binding by the enzyme in some specific conformational state that we do not see under our conditions. Andersen and Vilsen suggested (16) that Asn\(^{796}\) may participate in Ca\(^{2+}\) binding by the enzyme in some specific conformational state that we do not see under our conditions. Andersen and Vilsen suggested (16) that Asn\(^{796}\) may participate in Ca\(^{2+}\) binding by the enzyme in some specific conformational state that we do not see under our conditions.
chain oxygens with the proximal and distal Ca\(^{2+}\), thereby contributing binding cooperativity. Finally, we place one of the Glu908 oxygens near the proximal Ca\(^{2+}\) since the different effects of mutation of this residue to Gln or Ala indicate that only one oxygen participates in Ca\(^{2+}\) binding (see “Results”).

Model 1 in Fig. 10 is not the only one that is consistent with the experimental results. For instance, it is possible to reposition the helices longitudinally so that Glu309 could bind the distal Ca\(^{2+}\) and Glu771 the proximal Ca\(^{2+}\), everything else remaining the same (model 2 in Fig. 10).

To accommodate the five residues while excluding Asn796, we found it sterically convenient to alternate the four transmembrane helices as shown in Fig. 10. In the models the five binding residues were positioned to approximate their oxygen ligands within 2.6 Å of one or both calcium ions. Additional oxygens may be contributed by coordinated water.

Although the arrangements in Fig. 10 are speculative, the important question that they answer is whether the oxygen functions singled out by mutational analysis can generate discrete areas of high binding potential for Ca\(^{2+}\). The two areas are shown in Fig. 10, A-D, as cluster of dots. No other site of high valence is present throughout the four clustered helices, consistent with the results of extensive mutational work on the four helices (23, 24).

Short Range Functions of Amino Acid Side Chains within the Transmembrane Region—The experimental observations discussed above indicate that five amino acids in the transmembrane region sustain short range functions as they participate directly in Ca\(^{2+}\) binding. In addition, several mutations within the four transmembrane helices M4, M5, M6, and M8 result in inhibition of Ca\(^{2+}\) uptake with little or no inhibition of hydrolytic activity. A similar uncoupling effect was first reported by Andersen (25) regarding the mutation Tyr763 → Gly. We found that the effect of the Tyr763 → Gly mutation is not unique but is also produced by several other mutations in the transmembrane region (Tables I and II). Inspection of Tables I and II, in the light of the structural model, indicates that pronounced uncoupling is produced by mutations of amino acids with side chains exiting helices M4 or M5 from their outer and lateral surfaces, thereby interacting with the lipid bilayer and/or other components of the transmembrane cluster. Therefore, the uncoupling effects of these mutations are likely to be produced by local interference with proper packing of the helices and opti-
mal flux of Ca$^{2+}$ during active transport. These observations provide a cogent argument for the functional role of the transmembrane channel resulting from the four clustered helices and explain the effects of various hydrophobic compounds on Ca$^{2+}$ fluxes through the ATPase (26).

Of special interest are the mutations of Lys$^{297}$ including Lys$^{297}$ → Gly, which interferes with ATPase assembly and expression, Lys$^{297}$ → Met and Lys$^{297}$ → Phe producing strong functional inhibition, and Lys$^{297}$ → Arg and Lys$^{297}$ → Glu producing little or no inhibition (Tables I and II). In the model, Lys$^{297}$ places its charge at the distal end of the M4 segment, facing the lumenal end of the channel. In fact, Lys$^{297}$ provides the only positive charge within the channel. It is apparent then that the presence of a highly polar moiety in this position is required to stabilize the channel structure. The important role of this stabilization is demonstrated by the interference with ATPase expression produced by replacement of Lys$^{297}$ with the much less restrictive Gly, which may break propagation of the M4 helix during protein assembly (27). Furthermore, the functional inhibition produced by Lys$^{297}$ replacement with Met or Phe and the lack of inhibition by Lys$^{297}$ replacement with Arg or even with Glu indicate that intrusion of a net charge (positive or negative) at the distal end of the channel seals the lumen and prevents flux of Ca$^{2+}$. This suggests to us that the M4 helix may rotate (and possibly be displaced distally so that Lys$^{297}$ can reach the membrane interface with water) in synchrony with ATPase phosphorylation to allow exit of transported Ca$^{2+}$ into the lumen.

Long Range Functional Linkages of Amino Acids within or near the Ca$^{2+}$ Binding Domain—In previous studies (1, 14, 15, 19, 28, 29) it was shown that structural perturbations in the transmembrane region interfere with Ca$^{2+}$ binding and prevent Ca$^{2+}$ activation of the enzyme reaction with ATP, as well as Ca$^{2+}$ inhibition of the enzyme reaction with Pi. These findings suggested a long range functional linkage between the Ca$^{2+}$ binding site within the transmembrane region and the catalytic site in the extramembranous region (30). We now find the following.

(a) Mutations of residues that are normally involved in Ca$^{2+}$ binding affect the catalytic site (i.e. the P$_1$ reaction) even in the absence of Ca$^{2+}$. In fact, mutations Glu$^{209}$ → Gin, Glu$^{771}$ → Gin, and Asp$^{290}$ → Asn increase the equilibrium level of the phosphoenzyme obtained with P$_1$ (in the absence of Ca$^{2+}$) through a drastic reduction of its breakdown kinetics (Fig. 8 and Table III). An analogous finding was reported by Andersen (25) regarding mutation of Glu$^{771}$ to Ala or Gly. Therefore, specific mutations in the transmembrane region produce long range conformational effects favoring phosphorylation of the catalytic site with P$_1$. This suggests that the acidic functions of these residues sustain a very important structural role possibly through hydrogen bonding with neighboring residues in the absence of Ca$^{2+}$, in addition to participating in cooperative coordination in the presence of Ca$^{2+}$. Note that Glu$^{290}$ does not sustain this function since its carboxyl oxygen, but not its carboxyl function, is required for Ca$^{2+}$ coordination.

(b) Mutations of neighboring residues that are not involved in Ca$^{2+}$ binding interfere with catalytic activity both in the presence and in the absence of Ca$^{2+}$. A specific case, in this regard, is Asn$^{396}$ whose mutation to Ala produced total inhibition of Ca$^{2+}$ uptake, ATPase activity, and phosphoenzyme formation by utilization of ATP in the presence of Ca$^{2+}$, as well as slow decay of the phosphoenzyme obtained by utilization of Pi, in the absence of Ca$^{2+}$ (Table III and Fig. 8). It is then apparent that Asn$^{396}$ sustains a very important role in stabilization of the native enzyme conformation, possibly through involvement of its side chain oxygen or amino group in hydrogen bonding.

In several other mutants (Table II), an inhibitory effect is primarily manifested with a slow decay of the phosphorylated intermediate formed by utilization of ATP in the presence of Ca$^{2+}$. Furthermore, in the absence of Ca$^{2+}$, the inhibition manifests itself with a slow decay of the phosphoenzyme formed by utilization of P$_1$ (e.g. Val$^{200}$ → Ala, Cys$^{274}$ → Ala, Ile$^{275}$ → Ala; Table I).

These experimental observations demonstrate that the long range linkage between the Ca$^{2+}$ binding domain and the catalytic site is not necessarily dependent on Ca$^{2+}$ but is rather an intrinsic feature of the protein structure. It is clear that both the energy transduction mechanism and kinetic regulation of the Ca$^{2+}$ ATPase are not only related to the organic chemistry of the catalytic site, but are strongly dependent on extended features of protein conformation.

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