Functional Consequences of Alterations to Hydrophobic Amino Acids Located in the M₄ Transmembrane Sector of the Ca²⁺-ATPase of Sarcoplasmic Reticulum*

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Those hydrophobic residues between Ile²⁰⁸ and Ile²¹⁰ in transmembrane segment M₄ of the Ca²⁺-ATPase of sarcoplasmic reticulum, not previously mutated, were mutated systematically in ways that would alter their size or polarity, and functional consequences were measured. Fourteen residues in this sequence are organized as juxtapositions of large, hydrophobic (Val, Leu, Ile) and small (Ala, Gly) residues, and these were altered so that large residues were substituted for small and vice versa. Several mutants exhibited diminished Ca²⁺ transport, but mutants A305V and A306V lost all Ca²⁺ transport function. In both cases, the mutants were phosphorylated with ATP in the presence of Ca²⁺ and inorganic phosphate only in the absence of Ca²⁺, indicating that the Ca²⁺-binding sites were intact. Reduced Ca²⁺ affinity, as measured by Ca²⁺ dependence of phosphorylation from ATP, was observed for mutant A305V. In both mutants, the ADP-insensitive phosphoenzyme intermediate (E₂P) decayed slowly relative to the wild-type enzyme, suggesting that the E₂P to E₄ conformational transition was impaired, slowing the rate of the phosphatase reaction. Double mutants which reversed the order of Val²⁰⁴ and Ala²⁰⁸ and Ala²⁰⁸ and Ala²⁰⁶ and Ile²⁰⁷, resulted in the same phenotype as the single Ala mutations. These results, combined with our previous demonstration that Glu²⁰⁹ is a Ca²⁺ binding residue, that Pro³¹² is involved in E₄P to E₃P conformational changes, and that Gly³¹⁰ is involved in E₃P to E₂P conformational changes, support the hypothesis that transmembrane segment M₄ plays a key role in the Ca²⁺ transport function of the Ca²⁺-ATPase through its involvement in both the binding of Ca²⁺ and the subsequent conformational changes which bring about the translocation of Ca²⁺ to the lumen of the membrane.

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The Ca²⁺-ATPase of sarcoplasmic reticulum pumps Ca²⁺ from the sarcoplasm to the lumen at the expense of ATP hydrolysis. The molecule has been the focus of extensive structural and functional studies (MacLennan et al., 1970; Inesi, 1985; Inesi et al., 1990; Inesi and de Meis, 1985; Jorgensen and Andersen, 1988). The enzyme is a 110-kDa amphipathic protein with a cytoplasmic headpiece and stalk protruding from a globular basepiece, which forms part of the sarcoplasmic reticulum membrane structure. In the course of Ca²⁺ transport, Ca²⁺ and ATP are bound to the Ca²⁺-ATPase with high affinity and ATP is hydrolyzed, forming a phosphoenzyme intermediate. Ensuing conformational transitions cause vectorial displacement of calcium from its binding sites so that Ca²⁺ initially bound from the cytosolic surface, is released to the lumen.

The cloning of Ca²⁺-ATPase cDNA (MacLennan et al., 1985; Brandl et al., 1986) and the functional expression of full-length cDNA in COS-1 cells (Maruyama and MacLennan, 1988) have made it feasible to study structure-function relationships in the protein by studies of the functional consequences of site-specific mutagenesis. Using this approach, we have identified a number of amino acids which, when mutated, disrupt Ca²⁺ transport function. By analyzing the partial reactions of the transport-defective mutants, we have identified amino acids involved in the ATP binding site (Maruyama and MacLennan, 1988; Maruyama et al., 1988; Clarke et al., 1990c), in Ca²⁺ binding (Clarke et al., 1989a) and in conformational changes (Andersen et al., 1989; Vilsen et al., 1989; Clarke et al., 1990b; Andersen et al., 1992).

An important concept emerging from these studies is that six residues, Glu²⁰⁹, Glu²¹², Asn²⁰⁶, Thr²⁰⁹, Asp³¹², and Glu³¹⁰, which reside in transmembrane helices M₄, M₅, M₆, and M₇, are associated in formation of the Ca²⁺-binding sites. We have suggested that the four helices interact to form all or part of the pathway through which Ca²⁺ is actively transported. Conformational changes in one or more of these transmembrane segments may play a central role in causing the bound Ca²⁺ ions to become accessible to the membrane lumen. In support of this hypothesis, it has been found that some mutations to Glu³¹⁰ (Clarke et al., 1990a) and Pro³¹² (Vilsen et al., 1989), predicted to be located within transmembrane segment M₄, block the conformational transition between E₄P and E₃P, while mutation of Gly³¹⁰ blocks the conformational transition between E₃P and E₂P.

In this report, we describe the functional consequences of

1. The abbreviations used are: E₃P, ADP-sensitive phosphoenzyme intermediate; E₄P, ADP-insensitive phosphoenzyme intermediate; MOPS, 3-(N-morpholino)propanesulfonic acid.
mutations to nonpolar residues which are, for the most part, juxtaposed in a series of alternating large and small size in transmembrane segment M₄. These residues are contiguous with the putative Ca²⁺ binding ligand Glu³⁰⁹. We found that changes to hydrophobic residues predicted to form one surface of predicted transmembrane segment M₄ usually resulted in mutants with reduced Ca²⁺ transport activity, but that mutants A305V and A306V on the opposite predicted surface lacked any detectable Ca²⁺ transport function. These mutants were blocked in the E₂P to E₅ transition. Modelling suggests that Ala³⁰⁵, Ala³⁰⁶, Gly³¹⁰, and Pro³¹² form an active site occupying part of one surface of transmembrane helix M₄, while the large hydrophobic residues occupy the opposite surface.

EXPERIMENTAL PROCEDURES

Oligonucleotide-directed Mutagenesis and cDNA Expression in COS-1 Cells—The methods employed have been described in detail (Maruyama and MacLennan, 1988; Clarke et al., 1989b). A summary is as follows. Mutagenesis using synthetic oligonucleotides was performed in fragments of the rabbit fast-twitch skeletal muscle Ca²⁺-ATPase cDNA by the method of Kunkel (1985). The fragment containing the desired mutation was subcloned back into its original position, and the entire cDNA was cloned into the EcoRI site of vector p Baker (Wong et al., 1985) for expression in COS-1 cells (Gluzman, 1981). Microsomes were prepared from the transfected cells and suspended in a solution containing 0.25 M sucrose, 0.15 M KCl, 3 mM 2-mercaptoethanol, 20 μM CaCl₂, and 10 mM Tris-HCl, pH 7.5. Ca²⁺ transport activity was assayed in a reaction mixture containing 20 mM MOPS, pH 7.0, 100 mM KCl, 5 mM MgCl₂, 5 mM ATP, 0.45 mM CaCl₂, (containing 1 mCi/ml ⁴⁵Ca²⁺), 0.5 mM EGTA, and 5 mM potassium oxalate. For the assay of Ca²⁺ dependence of various functions, free Ca²⁺ concentrations were calculated by the computer program of Fabiato and Fabiato (1979). A sandwich, enzyme-linked immunosorbent assay using monoclonal antibody A52 (Zubrzycka-Gaarn et al., 1984) was used to quantify the amount of Ca²⁺-ATPase expressed in each microsomal preparation. Protein concentration was determined using a dye binding assay (Bradford, 1976) with bovine serum albumin as standard.

Analysis of Phosphoenzyme Intermediates—Ca²⁺-ATPase protein was phosphorylated at 0 °C to form E₅P in a solution containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl₂, 5 mM ATP, 0.45 mM CaCl₂, (containing 1 μCi/ml ⁸⁶⁶Ca²⁺), 0.5 mM EGTA, and 5 mM potassium oxalate. For the assay of Ca²⁺ dependence of various functions, free Ca²⁺ concentrations were calculated by the computer program of Fabiato and Fabiato (1979). A sandwich, enzyme-linked immunosorbent assay using monoclonal antibody A52 (Zubrzycka-Gaarn et al., 1984) was used to quantify the amount of Ca²⁺-ATPase expressed in each microsomal preparation. Protein concentration was determined using a dye binding assay (Bradford, 1976) with bovine serum albumin as standard.

RESULTS

Table I also shows the Ca²⁺ transport rates of the mutants relative to wild-type Ca²⁺-ATPase at pCa 5 in the presence of 5 mM ATP and 5 mM oxalate. Mutants I298A, A299V, A301V, A313V, and A313S all transported Ca²⁺ at maximal rates, close to those observed with the wild-type enzyme expressed and assayed under comparable conditions. The mutants V300A, V300S, L302A, A303V, V304A, V304L, V304S, A305G, A306G, I307A, I307S, G310A, L311A, L311S, V314A, V314L, and I315A displayed reduced Ca²⁺ transport function ranging between 15% and 62% of the rate obtained with wild-type enzyme. No measurable Ca²⁺ transport activity was observed for mutants A305V, A306V, or G310V.

An estimate of the Ca²⁺ affinity of the mutants retaining Ca²⁺ transport function was determined by measurement of the Ca²⁺ dependence of Ca²⁺ transport. Mutants A303V and I307S displayed significantly lower affinities (Kₐ₅ of 1.6 μM and 1.2 μM, respectively) than the wild-type enzyme (Kₐ₅ of 0.3 μM). A small decrease in Ca²⁺ affinity was observed for mutants L302A, V304L, A305G, A306G, I307A, G310A, L311A, V314A, and V314L, while a small increase in Ca²⁺

Fig. 1. Proposed structural model of transmembrane segment M₄, showing the amino acid sequence of the wild-type enzyme arranged in an α-helical net. The substitutions carried out in this study are indicated. The functional consequences of mutation of Pro³⁰⁶ and Pro³¹² (Vilsen et al., 1989), Glu³⁰⁹ (Clarke et al., 1989a), and Gly³¹⁰ (Andersen et al., 1992) have been described in earlier papers.
affinity was observed for mutants V300A, V300S, V304A, V304S, A313S, and I315A.

The finding of a complete loss of Ca\(^{2+}\) transport activity for the A305V and A306V mutants led us to examine their partial transport rates which were included as a control for each experiment. Since the binding of Ca\(^{2+}\) ions with high affinity is required to activate the transfer of ATP from Pi in the absence of Ca\(^{2+}\). On the other hand, the A306V mutant showed a higher apparent affinity for Ca\(^{2+}\) relative to that of the wild type, with maximal phosphorylation occurring at pCa 6.25–6.5.

Dephosphorylation of the high energy phosphoenzyme intermediate, E\(_p\), in the mutants A305V and A306V was examined by phosphorylating each enzyme at 0 °C in the presence of 2 \(\mu\)M ATP and 0.1 mM Ca\(^{2+}\) at pH 7.0, stopping the reaction after 10 s by the addition of EGTA, and measuring the decay of the phosphoenzyme intermediate through E\(_p\) to E\(_s\) and subsequent hydrolysis of E\(_p\) to E\(_s\). Dephosphorylation was also measured by the addition of both EGTA and ADP to the phosphoenzyme. In this case, dephosphorylation of E\(_p\) occurs through the repurification of ADP by the high energy phosphoenzyme intermediate. As shown in Fig. 4, the wild-type phosphoenzyme intermediate decayed rapidly after the addition of EGTA in the presence or absence of ADP, but, by contrast, the phosphorylated intermediates formed in mutants A305V and A306V were relatively stable under either condition. This suggests, first, that the phosphoenzymes formed in these mutants are not readily dephosphorylated and, second, that it is the E\(_p\) forms of the phosphoenzymes that are stabilized.

A phosphorylated intermediate of the Ca\(^{2+}\)-ATPase can also be formed from P\(_i\) but only in the absence of Ca\(^{2+}\), since Ca\(^{2+}\) binding appears to drive the native enzyme to a conformation incompatible with phosphorylation by P\(_i\) (Masuda and de Meis, 1973; de Meis and Masuda, 1974). As shown in Fig. 2, both of the mutant proteins formed phosphoenzyme intermediates from P\(_i\) in the absence of Ca\(^{2+}\). On the other hand, phosphorylation of the wild-type and both mutant enzymes by P\(_i\) was inhibited in the presence of 0.1 mM Ca\(^{2+}\).
FIG. 4. Dephosphorylation of E2P phosphoenzyme intermediates of wild-type and mutants, Ala306 → Val, and Ala306 → Val. Ca2+-ATPases were phosphorylated with [γ-32P]ATP in the presence of Ca2+ for 10 s as described in the legend to Fig. 2. Dephosphorylation was then initiated by the addition of EGTA to 1 mM for 30 s (lane 3) prior to acid quenching. ADP sensitivity of the phosphoenzyme intermediate was tested by the addition of ADP and EGTA to 1 mM 7 s prior to acid quenching (lane 2). A control sample (lane 1) was quenched 7 s after initiation of phosphorylation. Samples were analyzed by electrophoresis followed by autoradiography as described in the legend to Fig. 2.

Dephosphorylation of the E2P phosphoenzyme intermediates was also examined by first phosphorylating the enzyme with [32P]P, at 25 °C in the presence of 2 mM EGTA and 20% (v/v) dimethyl sulfoxide at pH 6.4 and then diluting the samples with 10 volumes of 50 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl2, 1 mM nonradioactive Pi, and 0.1 mM CaCl2 at 0 °C to terminate the phosphorylation reaction and allow dephosphorylation to proceed. As shown in Fig. 5, decay of the E2P intermediates of both mutants occurred at rates slower than that observed with the wild-type enzyme. The E2P intermediate of the wild-type enzyme was virtually complete within 7 s, whereas a substantial level of E2P was observed for each of the mutants 7 s after the initiation of dephosphorylation.

The loss of function induced by mutation of Ala306 or Ala306 to larger residues could have been caused by increased bulk in the transmembrane sequence. To test this possibility, we carried out double mutations by replacing Val304-Ala306 with Ala-Val and Ala306-Ile307 with Ile-Ala. The double mutants were also devoid of Ca2+-transport activity (Table I). We then examined their partial reactions. Both double mutants were phosphorylated by ATP in the presence of Ca2+ and by Pi in the absence of Ca2+ (Fig. 6). As a control, we included G310V, whose properties were reported previously (Andersen et al., 1992). Measurement of the Ca2+ dependence of phosphorylation from ATP revealed that the VA305AV mutant had a lower affinity for Ca2+ than wild-type, while the AI307IA had a Ca2+ affinity very comparable to that of wild-type (Fig. 7).

When dephosphorylation of the mutants was examined, their properties were found to be similar to those of the single A305V, A306V, and G310V mutants. The phosphorylated intermediates formed from ATP were relatively stable, both in the presence of ADP and EGTA or of EGTA alone (Fig. 8) and appeared to be blocked in the E2P to E transition. This view was supported by the experiments shown in Fig. 9, in which these mutant proteins were phosphorylated by Pi and then transferred to a buffer favoring rapid dephosphorylation of the wild-type protein. Under these conditions, the mutants were seen to dephosphorylate slowly in comparison with the wild-type enzyme.
within the membrane that affected function. Further analysis to Fig. 5. Acid quenching was carried out  at 0
Ala3ffi to Gly resulted in a mutant with 62% activity. Thus, tioning of the protein. It is also of interest that mutations of the precise size of these 2 residues is critical to proper func-
35% of control values (Table I). Clearly, the precise alignment of these residues in this section of the transmembrane se-
sequence is critical for full function.

Hydrophobic interactions are clearly important within transmembrane sequences. We therefore tested the functional consequences of mutation to Ser of a series of 4 hydrophobic residues, VaP, Va1304, Ile307, and Leu311, predicted to form a long a-helix. In earlier studies (Vilsen et al., 1989) mutations, a decrease in Ca2+ affinity of 3- to 5-fold was observed. We suggest that maintenance of this pre-

DISCUSSION

In previous studies, we demonstrated that 3 residues, Gly309, Gly310, and Pro312, located in transmembrane segment M4, play critical roles in Ca2+ transport by the Ca2+-ATPase. Changes to these residues had profound effects on either Ca2+ binding or subsequent conformational changes which accompany translocation of Ca2+ ions across the membrane. In the present study, we investigated the functional consequences of perturbing juxtaposed nonpolar residues of alternating large and small size in the transmembrane segment M4. Although most of the changes would result in an alteration in the bulk of the side chain or a decrease in hydrophobicity, none of the mutations actually caused a measurable decrease in structural integrity, since all mutant proteins were stably incorporated into the endoplasmic reticulum membrane. In previous studies of changes made to some 250 residues in the Ca2+-ATPase, very few have resulted in noticeable disruption of structural integrity of the expressed protein.

In Fig. 1, we have illustrated the results of the present study and integrated them with results from earlier investigations. The transmembrane segment, M4, and the stalk segment, S4, are predicted to form a long a-helix. In earlier studies (Vilsen et al., 1991), we attempted to define the border between S3 and M4. The triple mutant Ile-Thr-Thr, Arg-Asp-Arg was stably incorporated into the membrane, but was functionally inactive. This was considered to be consistent with the location of these residues at the S3/M4 boundary in at least one conformation of the enzyme. Lack of penetration of these residues into the membrane during transition to other conformations may, however, have been a factor in the loss of function of this triple mutant.

In the present study, we examined the functional consequences of mutations of the small Ala and Gly residues and large, hydrophobic Leu, Ile, and Val residues in M4. It is of interest that 7 of the 8 large hydrophobic residues in this sequence are juxtaposed with a small residue. Examination of Fig. 1 reveals three functional regional domains within M4. The first, consisting of Ile306, Ala307, and Val301, are relatively unaffected by mutation. We can assume that this face of the transmembrane helix, near the luminal boundary, is of little functional significance.

A second group of residues, consisting of Ala305, Ala306, Gly310, Pro312, and Ala313, create a second functional domain occupying a face of the helix in the middle of the transmembrane sequence. This is an essential surface for the Ca2+ translocation site. Mutations of these 5 residues invariably resulted in dramatic reductions in Ca2+ transport. For the Ala305 or Ala306 to Gly mutations, a small decrease in size, or for the Gly310 to Ala mutation, a small increase in size led to loss of 40-70% of Ca2+ transport activity. Mutation of these residues to Val led to total loss of activity and here the effect was a block of conformational changes which, through long range interactions, permit dephosphorylation of Asp351 in the cytoplasmic domain. This was also true for the mutation of Gly310 to Asp (Clarke et al., 1990a). The mutation of Pro312 to Ala or Gly also led to loss of Ca2+ transport activity, but here the block was in the transition of the E2P conformation to the E1P conformation, again through long range interactions (Vilsen et al., 1989).

Mutation of each of these residues also altered Ca2+ affinity. The Gly309 to Gln mutation provided the most striking loss of Ca2+ affinity, since Ca2+, even at 100 μM, could not prevent phosphorylation of the Ca2+-ATPase by P, at acid pH (Clarke et al., 1989a). Mutation of Gly310 to Ala (Andersen et al., 1992) or of Ala305 to Val also led to large reductions in Ca2+ affinity. By contrast, mutation of Pro312 to Ala or Gly enhanced Ca2+ affinity by 3-fold (Vilsen et al., 1989), and this was also true of the mutation of Ala306 to Val (Fig. 3). Thus, each of these residues appears to be intimately involved with Ca2+ binding and with the conformational changes that precede and accompany the translocation of Ca2+ across the membrane. It is no wonder, then, that they should be so sensitive to substitutions that alter their spatial environment by even small amounts.

A third group of residues consisting of Val304, Leu305, Ala306, Val304, Ile307, Pro308, Leu311, Val314, and Ile315 can be envisioned, in three dimensions, as forming a hydrophobic surface on the opposite side of the transmembrane helix from the surface containing the active site residues Ala305, Ala306, Glu308, Gly310, and Pro312 (Fig. 1). Mutations to residues on this hydrophobic surface, leading to reduction in size or polarity, led to considerable, but not complete, loss of function (Table I). In the case of the Ala306 to Val, Ile307 to Ser, and Pro308 to Ala (Vilsen et al., 1989) mutations, a decrease in Ca2+ affinity of 3- to 5-fold was observed. We suggest that maintenance of this pre-
cise hydrophobic surface is important for the efficient functioning of the Ca\(^{2+}\) pump. This point is emphasized by the fact that mutations of Val\(^{304}\) and Val\(^{314}\) to Leu, involving a relatively small change in size and hydrophobicity, led to reduction of Ca\(^{2+}\) transport function to about 35% of control values (Table I).

An important question in designing the present experiments was whether there is significance in the alteration of the small amino acids Ala or Gly, with the large bulky residues Val, Leu, or Ile in the transmembrane segment. Table I illustrates that there was relatively little significance to the large and small juxtaposition at the transmembrane boundaries for the mutations I298A, A299V, A301V, A311V, or A313S. By contrast, replacement of Ala\(^{305}\) or Ala\(^{306}\) with Vel led to complete loss of function. The inverse double mutations of residues 304 and 305 or of residues 306 and 307, resulting in a restoration of the original bulk, but in different juxtaposition, did not lead to restoration of function. The resulting proteins had no activity and were similar in character to the proteins had no activity and were similar in character to the corresponding to Gly\(^{233}\) in the Ca\(^{2+}\)-ATPase appeared to block phosphatase activity. Later studies (Andersen et al., 1989) showed that it was the E,P form of the enzyme, rather than the E,P form, that was stabilized. Accordingly, Gly\(^{233}\) is not involved in phosphatase function. The question of whether the \(\beta\)-strand domain contains phosphatase activity is, however, still not answered.

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In earlier studies, Serrano (1988) proposed that a phosphatase activity might reside in the \(\beta\)-strand domain, since mutation of a Gly in the \(\beta\)-strand domain of the H\(^{+}\)-ATPase corresponding to Gly\(^{233}\) in the Ca\(^{2+}\)-ATPase appeared to block phosphatase activity. Later studies (Andersen et al., 1989) showed that it was the E,P form of the enzyme, rather than the E,P form, that was stabilized. Accordingly, Gly\(^{233}\) is not involved in phosphatase function. The question of whether the \(\beta\)-strand domain contains phosphatase activity is, however, still not answered.