Alanine-scanning Mutagenesis along Membrane Segment 4 of the Yeast Plasma Membrane H\(^+\)-ATPase

EFFECTS ON STRUCTURE AND FUNCTION*

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Membrane segment 4 of P-type cation pumps has been suggested to play a critical role in the coupling of ATP hydrolysis to ion translocation. In this study, structure-function relationships in M4 of the yeast (Saccharomyces cerevisiae) plasma membrane H\(^+\)-ATPase have been explored by alanine-scanning mutagenesis. Mutant enzymes were expressed behind an inducible heat-shock promoter in yeast secretory vesicles, as described previously (Nakamoto, R. K., Rao, R., and Slayman, C. W. (1991) J. Biol. Chem. 266, 7940–7949). One substitution (I329A) led to arrest of the enzyme at an early stage of biogenesis, and three others (G333A, L338A, G349A) reduced ATP hydrolysis to near-background levels. The remaining 26 mutants were expressed well enough in secretory vesicles (44–121% of wild type) and had sufficient ATPase activity (16–123% of wild type) to be characterized in detail. When acridine orange fluorescence quenching was used to measure rates of ATP-dependent proton pumping over a range of ATP concentrations, only minor changes were seen. In kinetic studies, however, seven of the mutant enzymes (I331A, I332A, V334A, V336A, V341A, V342A, and M346A) were resistant to vanadate inhibition, and three of them (I332A, V336A, and V341A) also had a decreased \(K_{\text{m}}\) and increased pH optimum for ATP hydrolysis. Limited trypsinolysis was used to probe the structure of two different Val-336 substitutions, V336A, described above, and V336R, which displayed little or no ATPase activity. Both were cleaved at a relatively normal rate to give a pattern of fragments essentially identical to that seen with the wild-type enzyme. However, while vanadate, ADP, and ATP were able to protect the wild-type and V336A enzymes against trypsinolysis, the V336R ATPase was protected only by ADP and ATP. Taken together, the data suggest that key residues in the M4 segment may help to communicate the \(E_1\)-\(E_2\) conformational change to ion-binding sites in the membrane.

The plasma membrane H\(^+\)-ATPase of Saccharomyces cerevisiae belongs to a large, physiologically important family of cation pumps known as the P-type ATPases, which are widely distributed in bacterial, fungal, plant, and animal cells (for reviews, see Fagan and Saier, 1994; Rao and Slayman, 1996). Like other members of the group, the yeast H\(^+\)-ATPase has a single catalytic polypeptide of \(M_r \sim 100,000\) that alternates between two major conformational states \((E_1\) and \(E_2\)) with the transient formation of a covalent β-aspartyl phosphate intermediate (Dame and Scarborough, 1981; Amory and Goffeau, 1982). It is encoded by the PMA1 gene and, because it generates the proton-motive force required for nutrient uptake, is essential for growth (Serrano et al., 1986).

Hydropathy analysis has revealed a common topology for the P-ATPases in which a central hydrophilic region is flanked by four well-defined hydrophobic segments on the N-terminal side and four to six hydrophobic segments on the C-terminal side (Serrano, 1988; Nakamoto et al., 1989). The central region protrudes into the cytoplasm (Toyoshima et al., 1993) and, consistent with the notion that it is the catalytic portion of the protein, contains consensus sequences for ATP binding and phosphorylation (Serrano, 1988; Taylor and Green, 1989). Special attention has been paid to the 8–10 hydrophobic segments, which are presumed to be membrane-spanning α-helices that assemble together to form the ion translocation pathway. Although high resolution structures are not yet available for any of the P-ATPases, cryoelectron microscopy of unstained, frozen-hydrated crystals of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase has provided a clear image of three distinct transmembrane lobes into which 10 helices can be readily fitted (Stokes et al., 1994). Similar images have been obtained for the plasma membrane H\(^+\)-ATPase of Neurospora (Cyrklaff et al., 1995). Experimental support for the role of specific transmembrane segments in cation pumping has come from site-directed mutagenesis of the sarcoplasmic reticulum ATPase, where residues required for high affinity Ca\(^{2+}\) binding have been identified in segments M4, M5, M6, and M8 (Clarke et al., 1989a; Clarke et al., 1989b; Clarke et al., 1990; Andersen and Vilsen, 1992a). Similarly, Ser-775 in M5 of the Na\(^+\),K\(^+\)-ATPase has been suggested to play a role in K\(^+\) binding (Arguello and Lingrel, 1995). One can therefore imagine that ion translocation is driven by conformational changes associated with ATP hydrolysis in the cytoplasm, which in turn cause cation-binding sites in the membrane to cycle between high affinity and low affinity states.

Within the membrane-embedded portion of the P-ATPases, there is reason to be particularly interested in the fourth hydrophobic segment (M4). This is the only transmembrane segment that has been relatively well conserved throughout evolution. As illustrated in Fig. 1, a proline residue is located near the middle of M4 of every known P-ATPase and lies just 43 residues upstream of the aspartate that is phosphorylated by ATP. Immediately adjacent to the conserved proline is a glutamic acid residue that has been implicated in high affinity
Ca\(^{2+}\) binding by the sarcoplasmic reticulum ATPase (see above). Strikingly, this glutamate is very well conserved among fungal, plant, and animal H\(^{-}\)-ATPases and has been reported to stabilize a K\(^{-}\)-ATPase (Kuntzweiler et al., 1995). A cysteine residue appears in the homologous position of Cu\(^{2+}\) and Cd\(^{2+}\)-ATPases, where it may also be involved in cation binding (Fu et al., 1995). Site-directed mutagenesis of other residues within the M4 segment of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase has led to mutants which, while able to form the phosphorylated intermediate, are defective in the conformational changes necessary for ion transport (Vilsen et al., 1989; Vilsen et al., 1991; Andersen and Vilsen, 1992b; Andersen et al., 1992; Clarke et al., 1993). Taken together, these observations point to a critical role of M4 in the translocation pathway.

In the present study, we have used alanine-scanning mutagenesis to examine the functional role of amino acid residues in membrane segment 4 of the yeast plasma membrane H\(^{-}\)-ATPase. Mutant ATPases were expressed under the control of a heat-shock inducible promoter in a previously engineered yeast strain known as SY4 (Nakamoto et al., 1991). This strain contains a temperature-sensitive mutation in the SEC6 gene, which governs the last step of plasma membrane biogenesis, newly synthesized mutant ATPase becomes arrested in the secretory vesicles that accumulate at the restrictive temperature. The secretory vesicles are isolated by differential centrifugation and size fractionation (Walworth and Novick, 1987) and, owing to the fact that they are well sealed and inside-out, can readily be characterized with respect to ATP hydrolysis and ATP-dependent proton transport (Nakamoto et al., 1991).

**EXPERIMENTAL PROCEDURES**

**Yeast Strain**—Strain SY4 of Saccharomyces cerevisiae (MATa; ura3–52; leu2–3, 112; his4–619; sec6–4; GAL pma1::YIpGAL-PMA1) was used throughout this work. In SY4, which has been described in detail (Nakamoto et al., 1991), the wild-type PMA1 gene encoding the plasma membrane H\(^{-}\)-ATPase has been placed under control of the GAL1 promoter by gene disruption (Cid et al., 1987). SY4 also carries the sec6–4 mutation that, on incubation at 37°C, blocks the fusion of secretory vesicles with the plasma membrane (Schkeman and Novick, 1982).

**Mutagenesis**—Mutations were introduced into a 615-base pair BstEII to EcoRI fragment of the PMA1 gene (Serrano et al., 1986), previously subcloned into a modified Bluescript plasmid (Stratagene, La Jolla, CA), either by the Amesher's kit for oligonucleotide-directed mutagenesis (Amersham Corp.) or by polymerase chain reaction (Sarkar and Sommer, 1990). Mutations were verified by sequencing between the BstEII site and an internal Sty1 site. The 373-base pair BstEII to Sty1 fragment carrying the mutation was then moved into pGEM3Zf(+) (Life Technologies, Gaithersburg, MD) at the EcoRI site, generating plasmids harboring the desired mutations of the PMA1 coding region. Plasmids were transformed into strain SY4 by the method of Ito et al. (1983).

**Isolation of Secretory Vesicles**—In a typical expression experiment, transformed SY4 cells were grown to mid-exponential phase (A500 = 1) at 25°C in supplemented minimal medium containing 2% galactose (chromosomal PMA1 gene on, plasmid pMA1.2 (Nakamoto et al., 1991). Finally, the 3770-base pair HindIII to SacI restriction fragment carrying the entire ATPase coding sequence was cloned into vector YCp2HSE (Nakamoto et al., 1991), placing the mutant allele under heat-shock control. Plasmids were transformed into strain SY4 by the method of Ito et al. (1983).
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KOH, pH 6.7, 2 mM phosphoenolpyruvate and 50 μM pyruvate kinase). The specific activity of each secretory vesicle preparation was measured as the difference between hydrolysis in the absence and presence of 100 μM orthovanadate, a potent inhibitor of P-ATPases. The liberation of inorganic phosphate was assayed by the method of Fiske and Subbarow (1925). For determination of $K_m$ values, the same assay mixture was used except that Na₂ATP was varied between 0.15 and 5 mM with MgCl₂ always in excess of ATP by 5 mM. Actual MgATP concentrations were calculated by the method of Fabiato and Fabiato (1979). To determine the effects of pH on hydrolytic activity, the pH of the assay mixture was adjusted at 30°C with Tris base from 5.0 to 8.0. ATP hydrolysis was also assayed under fluorescence quenching conditions (see below). Secretory vesicles (25–50 μg of protein/ml) were diluted to 200 μl of 0.6 M sorbitol, 0.1 M KCl, 20 mM HEPES/KOH, pH 6.7, Na₂ATP (0.3 mM to 3.0 mM), and MgCl₂ (5 mM excess over the ATP concentration) at room temperature (23°C). After an appropriate time (20–40 min), the reaction was stopped by addition of chloroacetic acid to a final concentration of 1%, and the liberation of inorganic phosphate was determined as described above.

**Fluorescence Quenching—** ATP-dependent proton pumping was monitored by fluorescence quenching of the pH-sensitive dye, acridine orange. Briefly, freshly prepared secretory vesicles were suspended (25–50 μg of protein/ml) in 0.6 M sorbitol, 0.1 M KCl, 20 mM HEPES/KOH, pH 6.7, 2 μM acridine orange, and varying amounts of Na₂ATP (0.3–3.0 mM) at room temperature. After stabilization of the baseline fluorescence, proton pumping was initiated by the addition of MgCl₂ (5 mM excess over the ATP concentration). Fluorescence quenching was monitored on a Hitachi F2000 fluorescence spectrophotometer (excitation, 430 nm; emission, 530 nm) equipped with F2000 Intracellular Cation Measurement System software (Hitachi). Fluorescence intensity values collected at 0.5-s intervals for 5–20 s following initiation of proton pumping were subjected to least-squares analysis to determine the rate of fluorescence quenching ($\Delta F/\Delta t$). The specific initial rate of quenching was then calculated as follows: specific initial rate = $100(\Delta F/\Delta t)R_{tot}$-1 mg protein−1 where $\Delta F/\Delta t$ is defined above and $R_{tot}$ is the total fluorescence intensity at the time proton pumping was initiated. The specific initial rate is expressed as the percent of quenching observed per min per mg of secretory protein (% Q/min/mg).

**Trypsinolysis—** Secretory vesicles were washed by dilution and centrifugation (100,000 × g, 35 min) with 1 mM EDTA/Tris, pH 7.5, and 350, 351, and 354 were replaced by serine. In every case, plasmid YCP2HSE carrying the mutated gene was transformed into yeast strain SY4 and expressed under control of the heat-shock promoter, and secretory vesicles were isolated and characterized.

Because some mutations could prevent proper folding or insertion of the ATPase polypeptide into the membrane or could lead to instability, it was important to begin by determining the relative amount of mutant protein in the secretory vesicles. In fact, only one of the mutations (I329A) completely blocked the ability of the ATPase to reach the vesicles (Table I). In this case, immunoprecipitation of 35S-labeled yeast total membranes revealed that the mutant polypeptide was present at approximately 50% of the wild-type level but that it was arrested at an earlier stage of biogenesis, very likely in the endoplasmic reticulum (data not shown); the I329A mutant was not studied further. One of the remaining mutant ATPases reached the secretory vesicles at levels ranging from 37% (G349A) to 121% (T330A) of the wild-type control.

The next step was to determine the ability of the mutant

### RESULTS

**Expression and ATP Hydrolysis—** In this study, membrane segment 4 of the yeast PMA1 H⁺-ATPase was subjected to alanine-scanning mutagenesis. Residues 325 through 354 were included, based on hydropathy analysis that defined a maximal length for the segment (Nakamoto et al., 1989). Each residue was substituted in turn by alanine, except that pre-existing alanines at positions 347, 350, 351, and 354 were replaced by serine. In every case, plasmid YCP2HSE carrying the mutated gene was transformed into yeast strain SY4 and expressed under control of the heat-shock promoter, and secretory vesicles were isolated and characterized.

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| Mutation | Expression | ATPase activity | Proton transport |
|----------|------------|-----------------|-----------------|
|          |            | Uncorrected     | Corrected %     | Coupling ratio % |
| Wild-type | 100        | 3.00            | 3.00            | 5.91            | 100             |
| Vector   | 7          | 0.09            | 0.14            | 38              | /               |
| V325A    | 44         | 0.50            | 1.14            | 38              | /               |
| T326A    | 56         | 1.12            | 2.00            | 67              | 3.35            |
| L327A    | 74         | 0.63            | 0.85            | 28              | /               |
| G328A    | 72         | 1.55            | 2.15            | 72              | 4.50            |
| I329A    | 14         | 0.16            | 0.38            | 38              | /               |
| T330A    | 121        | 1.70            | 1.71            | 57              | 4.48            |
| I331A    | 91         | 0.75            | 0.82            | 27              | 6.59            |
| I332A    | 71         | 0.69            | 0.97            | 32              | 5.46            |
| G333A    | 43         | 0.11            | 0.26            | 9               | *               |
| V334A    | 96         | 0.77            | 0.80            | 27              | 4.66            |
| F335A    | 80         | 0.52            | 0.65            | 22              | /               |
| V336A    | 108        | 1.02            | 0.94            | 31              | 5.66            |
| G337A    | 88         | 1.27            | 1.44            | 48              | 5.84            |
| L338A    | 71         | 0.37            | 0.52            | 17              | /               |
| P339A    | 73         | 1.54            | 1.69            | 44              | 5.72            |
| A340F    | 91         | 0.49            | 0.49            | 16              | 5.25            |
| V341A    | 92         | 1.44            | 1.27            | 42              | 6.06            |
| V342A    | 113        | 2.48            | 2.76            | 92              | 4.75            |
| T343A    | 90         | 2.09            | 2.09            | 70              | 6.53            |
| T344A    | 110        | 3.10            | 2.95            | 98              | 4.89            |
| T345A    | 105        | 3.10            | 2.95            | 98              | 4.89            |
| M346A    | 96         | 1.59            | 1.66            | 55              | 5.52            |
| A347S    | 93         | 2.80            | 3.01            | 100             | 5.78            |
| V348A    | 90         | 1.78            | 1.98            | 66              | 5.15            |
| G349A    | 38         | 0.68            | 0.68            | 23              | /               |
| A350S    | 85         | 0.75            | 0.82            | 27              | 6.59            |
| V351S    | 69         | 1.50            | 1.77            | 72              | 4.50            |
| L352A    | 116        | 1.55            | 1.35            | 45              | 5.42            |
| L353A    | 44         | 0.47            | 0.47            | 36              | /               |
| A354S    | 107        | 2.50            | 2.34            | 78              | 6.45            |
| V356L    | 98         | 1.58            | 1.61            | 54              | 5.77            |
| V356E    | 93         | 1.56            | 1.73            | 58              | 5.80            |
| V356R    | 75         | 0.09            | 0.12            | 4               | /               |
| G337V    | 42         | 0.13            | 0.31            | 10              | /               |
| G337R    | 78         | 0.14            | 0.18            | 6               | /               |
enzymes to hydrolyze ATP. The resulting values, before and after correction for the relative amount of mutant ATPase detected in the secretory vesicles, are listed in Table I. In general, mutations in the N-terminal half of M4 (Tyr-325 to Val-341) were more deleterious, with corrected ATPase activities averaging only 34% of the wild-type value; mutations in the C-terminal half (Val-342 to Ala-354) were less harmful, with activities averaging 69% of the control (Fig. 2). The most significant effect came from mutations at five positions, where corrected ATPase activities ranged from 23% (G349A) downward to 22% (P335A), 17% (L338A), 16% (V341A), and 9% (G333A).

Additional substitutions were made at two positions that deserved special attention based on homology with the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase. The first, Val-336, aligns with Glu-309 of the rabbit sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, a residue required for the high affinity binding of Ca\(^{2+}\) and for Ca\(^{2+}\)-dependent phosphorylation (Clarke et al., 1989a; Andersen and Vilsen, 1992a). In the case of the yeast H\(^{+}\)-ATPase, replacement of Val-336 with glutamate and leucine caused only a minor reduction in ATPase activity (58 and 54%, respectively), whereas introduction of a positive charge (V336R) abolished hydrolysis altogether (Table I). The second site, Gly-337, corresponds to Gly-310 of the rabbit sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, where valine and proline substitutions blocked the E\(_{2}\)- to E\(_{2}\) conversion (Andersen et al., 1992). Valine and arginine substitutions at this position in the yeast H\(^{+}\)-ATPase completely inactivated the enzyme (Table I).

**ATP-dependent Proton Transport**—Given the proposed role of membrane segment 4 in coupling cation transport to ATP hydrolysis, it was important to examine the M4 mutants for possible coupling defects. The experimental approach for making such measurements was based on the topology of the secretory vesicles, in which the catalytic domain of the H\(^{+}\)-ATPase faces the medium. Thus, as has been shown previously (Nakamoto et al., 1991), the addition of ATP leads to proton translocation into the vesicles, creating an interior-acid pH gradient that can readily be monitored by means of the pH-sensitive fluorescent dye, acridine orange. A control experiment was carried out with vesicles expressing wild-type H\(^{+}\)-ATPase to explore the quantitative relationship between ATP hydrolysis and the fluorescence response (Fig. 3A). Both the initial rate and the extent of quenching increased when the ATP concentration was raised from 0.3 to 3.0 mM, and as expected, quenching was abolished by the proton ionophore, carbonyl cyanide p-trifluoromethoxyphenylhydrazone. Significantly, when ATP hydrolysis was assayed under the same conditions and the initial rate of quenching was plotted as a function of the rate of hydrolysis, the linear relationship between the two gave evidence of constant coupling between hydrolysis and pumping (Fig. 3B).

Similar experiments were carried out with each of the M4 mutants to see whether coupling was altered by any of the amino acid replacements (Table I). For four of the alanine substitutions (G333A, L338A, G349A, and L353A) as well as three of the other mutations (V336R, G337R, and G337V), ATPase activity under pumping conditions was too low (≤16% of the wild-type rate) to give a measurable signal in the quenching assay. Four additional mutants (Y325A, L327A, P335A, and P339A) with ATPase activities (uncorrected) between 17 and 21% were clearly capable of ATP-dependent pumping, although the slope (coupling ratio) of the quenching versus ATP hydrolysis plot could not be determined quantitatively. The coupling ratio for 22 of the remaining 23 mutants ranged from 76 to 112% of the wild-type value, indicating that alanine substitutions throughout membrane segment 4 had little or no effect on the coupling between ATP hydrolysis and proton pumping (see Fig. 4 for examples). The T326A mutant gave a ratio (57%) that was further from wild-type value, raising the possibility of partial uncoupling, although even in this case proton transport...
changes in the apparent Km V336A, ("Experimental Procedures"). The data were subjected to least-squares sensitive ATPase activity measured under the same conditions relatively high resulting in a relatively low pH optimum (pH 5.7; Table II) and ties for protons and ATP at the inner surface of the membrane, of this step might be expected to decrease the apparent affinity—

and Zuckier, 1989). In the wild-type PMA1 ATPase, the speed of this step might be expected to decrease the apparent affinities for protons and ATP at the inner surface of the membrane, resulting in a relatively low pH optimum (pH 5.7; Table II) and relatively high Km for MgATP (0.8 mM; Table II). A rapid E1P

H+ in

\[
E_1 \overset{ATP}{\rightarrow} E_1P \overset{E_2P}{\rightarrow} E_2 \overset{H+ out}{\rightarrow} E_1
\]

\text{Reaction 1}

The E1P to E2P step appears to be exceedingly rapid in the fungal H+ -ATPases, based both on measurements of 18O exchange (9.200/s; Amory et al., 1982) and on the analysis of current voltage data (3,000–60,000/s; reviewed by Slayman and Zuckier, 1989). In the wild-type PMA1 ATPase, the speed of this step might be expected to decrease the apparent affinities for protons and ATP at the inner surface of the membrane, resulting in a relatively low pH optimum (pH 5.7; Table II) and relatively high Km for MgATP (0.8 mM; Table II). A rapid E1P to E2P transition would presumably also mean that a substantial amount of enzyme is in the vanadate-sensitive E2 conformation during steady-state ATP hydrolysis, leading to a high apparent affinity (low Km) for vanadate (1.4 µM; Table II). If this is the case, it follows that a mutational defect in the E1P to E2P transition would tend to increase the pH optimum for ATP hydrolysis, lower the apparent Km for MgATP, and raise the apparent Km for vanadate, exactly the changes seen in I332A,
V336A, and V341A. Related changes (increased vanadate resistance and pH optimum) have been reported for a mutation, S368F, between membrane segment 4 and the phosphorylation site (Perlin et al., 1992).

For the fungal H^+ -ATPases, the most direct way to distinguish between E1 and E2 conformations is by limited trypsinolysis (Dame and Scarborough, 1981; Mandala and Slayman, 1988; Perlin and Brown, 1987). Typically, the ATPase is exposed to trypsin in the presence of high concentrations of ATP or ADP (to favor the E1 state) or vanadate (to favor the E2 state), and the products of proteolysis are examined by immunoblotting. In the experiment of Fig. 7, this kind of analysis was carried out to look for possible effects of two different Val-336 mutations, V336A, which (as described above) displays kinetic abnormalities consistent with a relative slowing of the E1 to E2 transition, and V336R, which has little or no ATPase activity (Table I) and could conceivably be completely blocked at the E1 to E2 step. In the experiment of Fig. 7A, secretory vesicles expressing wild-type, V336A, or V336R ATPase were incubated at a trypsin:protein ratio of 1:4 for 0–20 min. At each time point, the reaction was stopped by the addition of diisopropyl fluorophosphate (1 mM), and the digestion products were separated by SDS-polyacrylamide gel electrophoresis and blotted with anti-ATPase antibody. Under the conditions of the experiment, the 100-kDa wild-type ATPase was rapidly cleaved (within 0.5 min) to a 97-kDa form and then to 62-, 58-, 48-, and 46-kDa fragments. Similar trypsin patterns were observed for both the V336A and V336R enzymes, a result that suggests relatively normal protein folding. When digestion was carried out for 20 min at the same trypsin:protein ratio in the presence of 100 μM vanadate, 10 mM ADP, or 10 mM ATP, however, a clearcut difference was seen (Fig. 7B). For the wild-type and V336A enzymes, all three ligands protected against proteolysis, with patterns that could be attributed to the E1 conformation (ADP or ATP protection, 97, 62, 58, and 46-kDa fragments) or the E2 conformation (vanadate protection, 97 and 80-kDa fragments). By contrast, the V336R enzyme was protected against proteolysis by ADP or ATP but not by vanadate, consistent with the idea that this particular mutant form had difficulty reaching the vanadate-sensitive E2 conformational state.

**DISCUSSION**

For the sake of discussion, the M4 mutants described in this study can be divided into four groups as follows.

Group 1 includes mutant enzymes defective in biogenesis. The most severely affected was I329A, which was synthesized but did not reach the secretory vesicles. Five additional mutants were expressed at reduced levels in secretory vesicles, including Y325A (44%), G333A (43%), G337R (42%), G349A (37%), and L353A (44%). It is tempting to suggest that the residues defined by these mutations play a structural role, perhaps by stabilizing interactions between M4 and adjacent transmembrane helices or between M4 and the lipid bilayer.

Group 2 consists of mutant enzymes that reached the secretory vesicles but had less than 30% of normal ATPase activity. After correction for expression, alanine replacement at eight positions (Leu-327, Ile-331, Gly-333, Val-334, Pro-335, Leu-338, Pro-339, and Gly-349) gave specific activities that ranged from 9 to 28% of the wild-type value, with no qualitative change in kinetic properties. Substitution by alanine at a ninth position, Val-341, reduced ATPase activity to 16% but also led to shifts in the K_m, K_i, and pH optimum; for this reason, the V341A mutant has been placed in Group 3 (see below). Interestingly, seven of the eight Group 2 residues (Leu-327 to Pro-339) cluster around the well conserved PVGLP motif (Fig. 1), defining a region that is clearly important for function. A
Group 2 residue that deserves special mention is Pro-335, found in all known P-ATPases. In spite of the ubiquitous presence of this proline, its replacement does not completely interfere with the biogenesis or function of the yeast H\(^{+}\)-ATPase (see also Serrano and Portillo, 1990); similar results have been obtained for the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (Vilsen et al., 1989).

Group 3 mutants include I332A, V336A, V341A, and M346A and are of particular interest, given the fact that their kinetic defects are consistent with a slowing of the transport-linked E\(_{2}\)P to E\(_{1}\)P step in the reaction cycle. A plausible case can be made that at least one of these residues, Val-336, actually lines the transport pathway, since it corresponds to Glu-309 of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (Vilsen et al., 1989a; see Fig. 1). As described in an earlier section, the same Glu is present in the Na\(^{+}\),K\(^{-}\), H\(^{+}\), and plasma membrane Ca\(^{2+}\)-ATPases of animal cells and the Mg\(^{2+}\)-ATPase of *Salmonella typhimurium*, and a cysteine occupies the equivalent position in the heavy metal-transporting ATPases (see Fig. 1). Taken together, these considerations make it reasonable to think that Val-336 lies on the inner face of the M4 segment. It is perhaps significant that the other Group 3 residues (Ile-332, Val-341, and Met-346) are spaced at regular intervals from Val-336, and that ranged from 36% (L353A) to 123% (A350S) of the wild-type value. These residues can be assumed to be relatively unimportant for ATPase structure and function.

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**REFERENCES**

Amory, A., and Geoffea, A. (1982) *J. Biol. Chem.* 257, 4723–4730

Amory, A., Geoffea, A., McIntosh, D. B., and Boyer, P. D. (1982) *J. Biol. Chem.* 257, 12509–12516

Andersen, J. P., and Vilen, B. (1992a) *J. Biol. Chem.* 267, 19383–19387

Andersen, J. P., and Vilen, B. (1992b) *Acta Physiol. Scand.* 146, 151–159

Andersen, J. P., and Vilen, B., and MacLennan, D. H. (1992) *J. Biol. Chem.* 267, 2767–2774

Arguello, J. M., and Lingrel, J. B. (1995) *J. Biol. Chem.* 270, 22764–22771

Cid, A., Perona, R., and Serrano, R. (1987) *Curr. Genet.* 12, 105–110

Clarke, D. M., Loo, T. W., Inesi, G., and MacLennan, D. H. (1989a) *Nature* 339, 476–478

Clarke, D. M., Maruyama, K., Loo, T. W., Leberer, E., Inesi, G., and MacLennan, D. H. (1989b) *J. Biol. Chem.* 264, 11246–11251

Clarke, D. M., Loo, T. W., and MacLennan, D. H. (1990) *J. Biol. Chem.* 265, 6262–6267

Clarke, D. M., Loo, T. W., Rice, W. J., Andersen, J. P., Vilen, B., and MacLennan, D. H. (1993) *J. Biol. Chem.* 268, 18359–18364

Cyrlaff, M., Auer, M., Kuhlbrandt, W., and Scarborough, G. A. (1995) *EMBO J.* 14, 1854–1857

Dame, J. B., and Scarborough, G. A. (1981) *J. Biol. Chem.* 256, 10724–10730

Fabio, A., and Fabio, F. (1979) *J. Physiol.* (Paris) 75, 463–565

Fagan, M. J., and Saier, M. H. Jr. (1994) *J. Mol. Evol.* 38, 57–99

Fische, C. H., and Subbarow, Y. (1925) *J. Biol. Chem.* 66, 375–400

Fro, D., Beeler, T. J., and Dunn, T. M. (1995) *Year* 253–292

Hager, K. M., Mandal, S. M., Davenport, J. W., Speicher, D. W., Benz, E. J., Jr., and Slayman, C. W. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 7693–7697

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* 153, 163–168

Josephson, L., and Cantley, L. C., Jr. (1977) *Biochemistry* 16, 4572–4578

Kuntzweiler, T. A., Walllicks, E. T., Johnson, C. L., and Lingrel, J. B. (1995) *J. Biol. Chem.* 270, 2993–3000

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* 183, 265–275

Mandal, S. S., and Slayman, C. W. (1988) *J. Biol. Chem.* 263, 15122–15128

Nakamoto, R. K., Rao, R., and Slayman, C. W. (1989) *Ann. N. Y. Acad. Sci.* 574, 165–179

Nakamoto, R. K., Rao, R., and Slayman, C. W. (1991) *J. Biol. Chem.* 266, 7940–7949

Perlin, D. S., and Brown, C. L. (1987) *J. Biol. Chem.* 262, 6788–6794

Perlin, D. S., San Francisco, M. J. D., Slayman, C. W., and Rosen, B. P. (1986) *Arch. Biochem. Biophys.* 248, 53–61

Perlin, D. S., Harris, S. L., Monk, B. C., Seto-Young, D., Na, S., Anand, S., and Haber, J. E. (1990) *Acta Physiol. Scand.* 146, 183–192

Pick, U. (1982) *J. Biol. Chem.* 257, 6111–6119

Rao, R., and Slayman, C. W. (1993) *J. Biol. Chem.* 268, 6708–6713

Rao, R., and Slayman, C. W. (1996) in *The Yeast Saccharomyces: Metabolism and Gene Expression* (Strathern, J. N., Jones, E. A., eds) Vol. 3, pp. 29–56, Springer-Verlag, Berlin

Sarkar, G., and Sommer, S. S. (1990) *BioTechniques* 8, 404–407

Schekman, R., and Novick, P. J. (1982) in *The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression* (Strathern, J. N., Jones, E. A., Broach, J. R., eds) pp. 361–398, Cold Spring Harbor laboratory, Cold Spring Harbor, NY

Serrano, R. (1988) *Biochim. Biophys. Acta* 947, 1–28

Serrano, R., and Portillo, F. (1990) *Biochim. Biophys. Acta* 1018, 195–199

Serrano, R., Kielland-Brandt, M. C., and Fink, G. R. (1986) *Nature* 321, 689–693

Slayman, C. W., and Zuckier, G. R. (1989) *J. Biol. Chem.* 264, 21024–21030

Walworth, N. C., and Novick, P. J. (1987) *J. Cell Biol.* 105, 163–174