Helical Stalk Segments S4 and S5 of the Plasma Membrane H⁺-ATPase from Saccharomyces cerevisiae Are Optimized to Impact Catalytic Site Environment*

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The stalk segments of P-type ion-transporting enzymes are presumed to play important roles in energy coupling. In this work, stalk segments S4 and S5 of the yeast H⁺-ATPase were examined for helical character, optimal length, and segment orientation by a combination of proline substitution, insertion/deletion mutagenesis, and second-site suppressor analyses. The substitution of various residues for helix-disrupting proline in both S4 (L353P, L353G; A354P; and G371P) and S5 (D676P and I684P) resulted in highly defective or inactive enzymes supporting the importance of helical character and/or the maintenance of essential interactions. The contiguous helical nature of transmembrane segment M5 and stalk element S5 was explored and found to be favorable, although not essential. The deletion or addition of one or more amino acids at positions Ala354 in S4 and Asp276 in S5, which were intended to either rotate helical faces or extend/reduce the length of helical segments, resulted in enzyme destabilization that abolished most enzyme assembly. Second-site suppressor mutations were obtained to primary site mutations G371A (S4) and D676G (S5) and were analyzed with a molecular structure model of the H⁺-ATPase. Primary site mutations were predicted to alter the site of phosphorylation either directly or indirectly. The suppressor mutations either directly changed packing around the primary site or altered the environment of the site of phosphorylation. Overall, these data support the view that stalk segments S4 and S5 of the H⁺-ATPase are helical elements that are optimized for length and interactions with other stalk elements and can influence the phosphorylation domain.

The plasma membrane H⁺-ATPase of yeast is an essential enzyme that actively pumps protons across the cellular membrane in order to maintain intracellular pH and the electrochemical proton gradient necessary for growth and development. It belongs to the superfamily of P-type ion-translocating ATPases for which there are more than 150 members known (1). The fungal H⁺-ATPase is a class II, non-heavy metal-transporting enzyme that includes the plant H⁺-ATPase and the animal Na⁺,K⁺-ATPase, Ca²⁺-ATPase, and H⁺,K⁺-ATPase (2). It couples ATP hydrolysis in the cytoplasmic domain to ion transport in the membrane-embedded domain forming an acyl-phosphate intermediate during catalysis. Recently, the molecular structure of the Ca²⁺-ATPase of skeletal muscle sarcoplasmic reticulum (SERCA1a) was solved at 2.6-Å resolution (3). As expected, it confirmed a membrane transport domain with 10 transmembrane segments, a large cytoplasmic ATP hydrolysis domain, and a narrow stalk domain that links the cytoplasmic and membrane domains. The cytoplasmic region consists of three well separated domains, with the phosphorylation site in the central catalytic domain assuming a fold similar to halocid dehalogenase and the adenosine-binding site formed 25 Å away on another domain (3). These essential features are conserved among P-type enzymes despite wide ranges in amino acid conservation between family members. A detailed comparison of 8 Å structures (4) of the H⁺-ATPase from Neurospora (5) and the Ca²⁺-ATPase from sarcoplasmic reticulum (6) confirmed that important structural properties are highly conserved.

There is extensive evidence that dynamic changes in protein structure occur during catalysis (7–9). However, it is not clear how conformational changes in the catalytic region are transmitted to the transmembrane domain. It has been suggested that positional changes in the stalk segments could play a role in mediating the coupling process (10, 11). In the Ca²⁺-ATPase, the stalk region is ~24 Å long and is divided into four largely helical, rod-like structures (3, 6, 12). The stalk densities are not as apparent in the 8-Å Neurospora H⁺-ATPase structure, which likely reflects an open conformation being used to derive structural information (5). Nonetheless, the helical stalk region is expected to provide a direct physical linkage between functional domains involved in ATP hydrolysis and ion transport.

The H⁺-ATPase stalk segments, designated S2 (Gln164–Val189), S3 (Gly276–Asn291), S4 (Val348–Val372), and S5 (Gly570–Tyr589), are the cytoplasmic extensions from transmembrane segments M2, M3, M4 and M5, respectively. Segments M4–6 have been implicated in ion binding/release (13). S4 and S5 are presumed to play a pivotal role in coupling because they are contiguous with these transmembrane segments and are directly linked to the nucleotide binding-phosphorylation domain (2, 11). S4 is within 10 residues or 2.5 helix turns from the site of phosphorylation, Asp376. A mutation in the Ca²⁺-ATPase, Y763G, near the membrane interface of S5 uncouples ion transport from ATP hydrolysis (14). In the Ca²⁺-ATPase structure, M5–S5 is a long, centrally oriented, contiguous helical segment that forms a "mast-like" element extending from the outer membrane surface to the center of the cytosolic domain (3).

We previously demonstrated that the helical properties of S2 and S3 are important to enzyme function (15). In this report,

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† The abbreviations used are: SERCA, skeletal muscle sarcoplasmic reticulum; PCR, polymerase chain reaction; SSSM, second site suppressor mutations.
we have extended that analysis to examine the importance of helical character in S4 and S5, which are more directly linked to coupling of ion transport. In addition, we have examined spatial interactions of S4 and S5 by identifying second site suppressor mutations that reverse the defective phenotypes observed from point mutations within the stalk segments. Our results strongly support the view that the helical properties of stalk segments S4 and S5 are important for \( \text{H}^+\)-ATPase function, and that the length of these segments as well as the juxtaposition of helical faces are optimized to interact with the phosphorylation domain.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**—Primary site mutations generated in *vitr* were transformed into yeast strain SH122 (H0 ade6-1 trp-1 leu2-1 lys2-1 ura3-1 pma1::LEU2/PMA1) (16) or SYA (MATa ura3-52 leu2-112 his4-619 GAL1::URA3 s6-4ts GAL2 PMA1) (17). Some suppressor mutations were isolated in the strain YAK2 (MATa ade2-101 leu2-21 his3-Δ200, ura3-52 trp1Δ63 lys2-801 pma1α·HIS3 pma2α·TRP1) (18), as described previously (23).

**Site-directed Mutagenesis**—Site-directed mutants were created in plasmids pGW201 (19), pRS201ΔURA3, and Ycg2HSE-PMA1 (17) using the QuickChange Site-directed Mutagenesis Kit (Stratagene). For chromosomal integration, a 6.1-kilobase pair HindIII fragment containing a desired *pma1* mutation linked to URA3 was transplanted into yeast strain SH122. Isogenic *pma1* mutants were isolated as described by Harris et al. (16). All transformations were performed using the Alkaline Yeast Transformation Kit (Bio 101).

**pma1** mutants were grown in a 3-mL YPD (1% yeast extract, 2% peptone, 2% dextrose, pH 5.7) culture for 18–20 h, and chromosomal mutations generated by treating vector pRS201ΔURA3 (18). All transformations were performed using the Alkaline Yeast Transformation Kit (Bio 101). Additional suppressor mutations were generated by passing the plasmid through an XL1-Red mutator *Escherichia coli* strain (Stratagene). The mutated genes were used to transform the strain YAK2 (18), as described above. The transformed YAK2 were cured of their primary plasmid by plating on media containing 0.1% 5-Fluoroorotic acid, 0.67% (w/v) yeast nitrogen base without amino acids, 0.2% (w/v) CSM-URA, 2% (w/v) dextrose, 50 μg/ml uracil, 0.1% 5-fluoroorotic acid, 2% agar). After 5 days of growth, colonies were isolated and suppressors selected on the basis of full or partial reversion to wild type growth. Plasmid DNA was isolated using the Wizard Plasmid Purification Kit (Promega), and suppressor mutations were located by DNA sequence analysis of the entire gene.

**TABLE I**

| Mutant Location | Growth Low pH | Hyg B | Doubling \( K_m \) \( V_{max} \) Vanadate |
|-----------------|---------------|-------|-----------------|-----------------|------------------|
| GW201           | + + +         | –     | 72 ± 1          | 1.6 ± 0.1       | 3.7 ± 0.3        | 0.9 ± 0.1        |
| pma1 L353G      | 4              | Lethal|                 |                 |                  |                  |
| pma1 L353I      | 4              | Lethal|                 |                 |                  |                  |
| pma1 L353P      | 4              | Lethal|                 |                 |                  |                  |
| pma1 A355G      | 4              | Lethal|                 |                 |                  |                  |
| pma1 A355P      | 4              | Lethal|                 |                 |                  |                  |
| pma1 S368P      | –              | +     | 72 ± 1          | 0.6 ± 0.1       | 3.4 ± 0.4        | 0.6 ± 0.1        |
| pma1 G371A      | 4              | +     | 136 ± 5         | 0.8 ± 0.3       | 1.8 ± 0.4        | 0.4 ± 0.1        |
| pma1 G371P      | 4              | +     | 69 ± 1          | 1.2 ± 0.2       | 4.2 ± 1.1        | 8.0 ± 1.0        |
| pma1 P669A      | 5              | +     | 70 ± 1          | 1.0 ± 0.1       | 2.3 ± 0.3        | 1.5 ± 0.1        |
| pma1 P669G      | 5              | +     | 73 ± 2          | 1.0 ± 0.3       | 2.5 ± 0.5        | 1.5 ± 0.1        |
| pma1 D676A      | 5              | +     | 72 ± 2          | 0.8 ± 0.1       | 1.7 ± 0.2        | 1.5 ± 0.1        |
| pma1 D676G      | 5              | –     | 82 ± 1          | 1.4 ± 0.2       | 2.4 ± 0.7        | 2.1 ± 0.4        |
| pma1 D676P      | 5              | Lethal|                 |                 |                  |                  |
| pma1 D676R      | 5              | –     | 94 ± 1          | 1.4 ± 0.4       | 2.3 ± 0.5        | 1.6 ± 0.1        |
| pma1 M648A      | 5              | +     | 72 ± 1          | 1.7 ± 0.2       | 4.8 ± 0.2        | 1.3 ± 0.3        |
| pma1 M648G      | 5              | –     | 96 ± 1          | 1.9 ± 0.3       | 3.5 ± 0.9        | 0.9 ± 0.2        |

a Growth at low pH was determined in YPD at pH 3.0 with 20 mM acetate.

b Hygromycin B resistance (Hyg B) was assessed on YPD agar with 200 μg/ml hygromycin.

c Values represent the S.E.

**RESULTS**

**Targeted Proline Mutagenesis of S4**—Scanning proline mutagenesis has been used to probe the helical properties of stalk segments S2 and S3 (13). This approach was extended to assess the importance of helical backbone structure in S4 and S5 by making strategic substitutions in largely non-conserved residues near the ends of the two stalk segments. Table I shows the positions of these mutations and their effects on cell viability, \( \text{H}^+\)-ATPase-dependent growth phenotypes, hygromycin B resistance, low pH sensitivity (20, 23), enzyme kinetics, and sensitivity to orthovanadate. The substitution of proline in S4 for residues Leu1563 and Ala3354 near the membrane interface of M4 and at Gly371 resulted in defective enzymes incapable of...
supporting growth. A L353G mutation was also highly disruptive. In each case, the introduction of other amino acid substitutions produced active enzymes, which demonstrated that the disruptive propensity of proline, and in some cases glycine, was most likely due to its affect on backbone structure and not on amino side group character. An S368P substitution was viable, although the cells grew slowly and the enzyme displayed less than 50% of normal catalytic activity (Table I). Several mutations (e.g. S368F) at this position are known to induce 500-fold decreases in vanadate sensitivity (16), although the viable S368P mutant was fully vanadate-sensitive (Table I). However, a G371A mutation did show ~10-fold less vanadate sensitivity consistent with a recent study of this region (24). The viable mutant enzymes, L353I, A354G, G371A, and S386P, showed normal $K_m$ values and sensitivity to vanadate and, other than S368P, showed $V_{max}$ levels that were $\approx78\%$ of wild type enzyme.

The prominent growth defects induced by the S368P mutation were most pronounced when assessing the proton transport properties of this mutant. Fig. 1 shows a detailed profile for pH-dependent growth in which the S368P mutant was most sensitive to acidic medium conditions. This enzyme appeared less competent in regulating intracellular pH via the $\text{H}^+$-$\text{ATPase}$. The kinetic defect in this enzyme was apparent when examining whole-cell proton efflux mediated by the $\text{H}^+$-$\text{ATPase}$ from carbon-starved cells (Fig. 2). In this case, S368P pumped protons routinely at a rate 55–65% ($n = 3$) that of wild type enzyme or other mutants, which is consistent with its reduced $V_{max}$ in vitro. Mutant enzymes A354G and G371A were somewhat less efficient (15–20%) than wild type (Fig. 2). Interestingly, both of these enzymes showed rates of ATP hydrolysis that were $>92\%$ of wild type (Table I), suggesting that they may be partially uncoupled. Most of the other mutants showed wild type-like rates of $\text{H}^+$-$\text{ATPase}$-mediated proton transport.

**Targeted Proline Mutagenesis of S5**—In the Ca$^{2+}$-$\text{ATPase}$, M5 and S5 form a contiguous, long central helical element (3) that has been proposed to play a critical role in the coupling reaction (25). In contrast to the Ca$^{2+}$-$\text{ATPase}$ and related higher eukaryotic enzymes, the fungal $\text{H}^+$-$\text{ATPase}$ has a naturally occurring proline, Pro$^{669}$, as well as a Gly$^{670}$, near the M5 interface, which should disrupt helical continuity in this region and create hinge-like flexibility. The conversion of Pro$^{669}$ to glycine and alanine had modest effects on enzyme function (Table I), suggesting that backbone flexibility is not important at this position.

In contrast, the introduction of proline at both Asp$^{676}$ and Ile$^{684}$ at opposite ends of S5 resulted in highly defective enzymes incapable of supporting growth. As observed with S4 mutations, the introduction of either glycine or alanine at these

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**Fig. 1.** pH-dependent growth of pma1 mutants. Mutant cells (10$^3$) were inoculated into rich YPD medium containing 20 mM potassium acetate and were grown for 24 h at 30 °C. Each point represents the average of cells grown in triplicate.

**Fig. 2.** $\text{H}^+$-$\text{ATPase}$-mediated medium acidification. Carbon-starved cells were incubated in weakly buffered medium, and $\text{H}^+$-$\text{ATPase}$-mediated proton efflux was assessed by following medium acidification with the pH indicator dye bromphenol blue. Changes in the initial linear rate of acid efflux relative to wild type (GW201) were taken as differences in the rate of $\text{H}^+$-$\text{ATPase}$-mediated proton efflux. Each point is the average of three measurements.
positions was well tolerated by the enzyme (Table I). These results suggest that helical character of S5 is important for function. Mutations D676R and D676G, but not D676A, produced enzymes with low rates of ATP hydrolysis, hygromycin B resistance, and low pH sensitivity indicative of a highly defective enzyme (Table I; Fig. 1). Whole-cell proton transport assays also confirmed that these mutant enzymes were less efficient in H+-ATPase-directed proton transport (Fig. 2). It is likely that side group mass at this position is more important than charge.

Suppressor Mutations of G371A and D676G—The prominent growth characteristics of mutants G371A in S4 and D676G in S5 permitted the isolation of second-site suppressor mutations to evaluate short and long range protein-protein interactions. Spontaneous suppressor mutants were readily isolated but generally resulted in gene replacement events that restored the wild type PMA1 gene. To promote the generation of second site suppressor mutations, plasmid pRS201 containing a URA3 gene. To promote the generation of second site suppressor mutations, plasmid pRS201/URA3 was used to transform the cells. These mutants were highly reminiscent of assembly defects encountered with mutations of the phosphorylation site, Asp378 close to S4 (26). A single alanine addition was tolerated resulting in a largely assembled enzyme (65% of wild type) with only 35% of wild type enzyme activity.

**DISCUSSION**

The P-type ATPase stalk region is widely viewed as a mediator of energy coupling between the catalytic ATP binding/hydrolysis and ion translocation domain. Genetic evidence from several systems support this view by demonstrating that mutations in several stalk elements either partially or fully uncouple ion transport from ATP hydrolysis. These mutations include I183A in S2 (19), G158D near the S2/M2 interface (27), H285Q in S3 (28) of the yeast H+-ATPase, and Y763G in the S5 (14) of the Ca2+-ATPase. The Y763G mutation in S5 has been proposed to directly modulate coupling via an affect on M5/M6, which has been implicated in ion binding (11, 29). Furthermore, a K758I mutation in the Ca2+-ATPase leads to changes in the rates of dephosphorylation and Ca2+ binding (30), and perturbations in S3 produced by either mutation or the binding of the inhibitor thapsigargin interfere with energy coupling (31).

In this work, the helical character and length of stalk segments S4 and S5 of the yeast H+-ATPase were shown to be important for enzyme function and stability. The substitution of various residues for helix-disrupting proline (or in some cases glycine) in both S4 (L353P, L353G; A354P; and G371P) and S5 (D676P and I684P) resulted in highly defective or inactive enzymes (Table I). In each case, substitutions with residues other than proline restored normal or near-normal enzymatic function (Table I). Deletion or addition of one or more amino acids at positions Ala354 in S4 and Asp675 in S5, intended to either rotate helical faces or extend/reduce the length of helical segments, were in most cases deleterious resulting in enzyme destabilization that significantly decreased enzyme assembly (Fig. 3). The assembly defects observed were comparable to those observed by mutation of Asp378, the site of phosphorylation, and neighboring residues (26, 32). These results indicate that S4 and S5 play more than a simple structural role bridging the catalytic phosphorylation and nucleotide binding domains to the membrane-embedded ion transport domain. Rather, the helical elements are juxtaposed or packed in an optimal manner that is not highly flexible.

Scanning alanine mutagenesis of S4 by Ambesi et al. (24) supports the notion that side group interactions are essential in this segment. A biased preference was observed for inactivating mutations from Lys to Asp, which may define potential interaction sites. These authors further demonstrated that various mutations in consecutive residues Ile663 through Gly671 produced enzymes with significantly reduced sensitivity to orthovanadate. Similarly, G371A in this study showed a 10-fold reduced vanadate sensitivity. This behavior is linked to a shift in $E_1-E_2$ equilibrium toward the vanadate-insensitive $E_1$ conformation. It is supported by previous studies on vanadate insensitivity (16, 33), by related studies on yeast H+-ATPase M4 (34) and S4 (24), as well as S4 in the Ca2+-ATPase. In the latter study, mutations in the S4 region altered $E_1-E_2$ and/or Ca2+-E2P → Ca2+→E2P transitions suggesting that this segment links phosphorylation and Ca2+ binding (35). Collectively, these results suggest that perturbations in S4 are linked directly to changes in the disposition of the site of phosphorylation Asp378.
In the 2.6-Å resolution structure of the Ca\textsuperscript{2+}-ATPase, S5 and M5 form a long continuous \(\alpha\)-helical structure of \(\sim 60\) Å that spans the membrane domain and a significant portion of the cytoplasmic domain. It is worth noting that a naturally occurring proline, Pro\textsuperscript{669}, occurs at the interface of S5 and M5 in the yeast H\textsuperscript{1}-ATPase. This proline could disrupt the continuity of the extended M5-S5 helical segment observed in the Ca\textsuperscript{2+}-ATPase. However, it is likely that the distortion imposed by the naturally occurring proline is minimal since Pro\textsuperscript{669} can be converted to alanine thereby restoring helical integrity with little affect on enzyme behavior (Table I). This behavior may reflect the fact that Pro\textsuperscript{669} lies at the interface of helical elements constrained by different dielectric environments and/or through close interactions with other stalk and membrane elements.

The directed proline mutagenesis, helix lengthening, and shortening and twisting of stalk segment S5 in this work supports the importance of an optimized helical element. This likely results from the location of specific residues on one or more faces of the helix to form critical interactions. In fact, saturation mutagenesis of S5\textsuperscript{2} revealed two faces of the helix that tolerate mutation poorly and may be potential regions of interaction. Assorted S5 mutations in the Ca\textsuperscript{2+}-ATPase alter the rate of the reaction sequence H\textsubscript{1}E\textsubscript{2} \rightarrow Ca\textsubscript{2}E\textsubscript{1} associated with Ca\textsuperscript{2+} binding on sites at the cytoplasmic face sites and on the rate of the dephosphorylation of the ADP-insensitive phosphoenzyme, H\textsubscript{n}E\textsubscript{2}P \rightarrow H\textsubscript{n}E\textsubscript{2} (25). S5 is likely to play a pivotal role in mediating communication between the Ca\textsuperscript{2+}-binding pocket and the catalytic domain. In addition, a critically conserved residue, Arg\textsuperscript{751}, appears important for both structural and functional integrity of the enzyme (25). S5 is linked directly to the transmembrane ion binding domain composed of M4–M6, and M8, through its connection with M5.

The intramolecular rearrangement of stalk segments during catalysis remains obscure. The analysis of second site suppressor mutations (SSSM) enables short and long range interactions within a protein to be explored. In the current context, the presumptive behavior of primary and secondary site mutations were analyzed by superposition of the yeast H\textsuperscript{1}-ATPase primary sequence on conserved portions of the molecular coordinates of the SERCA Ca\textsuperscript{2+}-ATPase (Fig. 4). Primary site mutation G371A lies on the edge of stalk 4, seven residues upstream of the site of phosphorylation Asp\textsuperscript{378} (Fig. 4). This mutation introduces a slightly bulkier side group at this position suggesting some steric crowding. Given its proximity to Asp\textsuperscript{378} and the finding that mutations in this region influence the phosphorylation state of the enzyme (32), it is likely that the G371A mutation alters the environment around Asp\textsuperscript{378} via main chain interactions involving twisting or other distortions. The introduction of proline at this position was highly destabilizing resulting in a non-viable enzyme (Table I). Second site mutations appear to have both short and long range effects that in each case stabilize the main chain containing Asp\textsuperscript{378} via main chain interactions involving twisting or other distortions. The introduction of proline at this position was highly destabilizing resulting in a non-viable enzyme (Table I).

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primary site mutation. SSSM P535L lies within the highly conserved sequence D534PRR, which has been linked to coordination of Mg$^{2+}$ ions (36). This side group and backbone change may compensate for the primary site effect by directly impacting the environment around Asp$^{378}$, perhaps by making it more favorable for phosphate transfer through a decreased polarity. SSSM M405I lies 27 residues downstream of the phosphorylation site. In the Ca$^{2+}$-ATPase structure, this residue lies at the end of an elongated chain that turns abruptly back toward the membrane. It may exert its effects via main chain twisting, but there is no indication that the chain is sufficiently structured to facilitate such a molecular distortion. Rather, in the H$^+$-ATPase, it appears more likely that M405I actually lies closer to Asp$^{378}$ because the next 62 of 82 residues, including the immediately following 34 residues of the SERCA pump, are absent in the yeast enzyme (Fig. 4). This gap is predicted to bring M405I much closer to Asp$^{378}$ in which the isoleucine side group can more directly impact the environment of the site of phosphorylation. Overall, it is suggested the primary impact of the second site suppressor mutations is to restore a more normal environment either by changing the immediate environment or altering the main chain distortion.

Primary site mutation D676G on stalk 5 is complemented by SSSM V748I, G888S, and E288K, V562I. Asp$^{378}$ is upstream of and the role of the second site suppressor mutations is to impact the environment around Asp$^{378}$, and the immediately following 34 residues of the SERCA pump, are absent in the yeast enzyme (Fig. 4). This gap is predicted to bring M405I much closer to Asp$^{378}$ in which the isoleucine side group can more directly impact the environment of the site of phosphorylation. Overall, it is suggested the primary impact of the second site suppressor mutations is to restore a more normal environment either by changing the immediate environment or altering the main chain distortion.

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