Genetic Probing of the Stalk Segments Associated with M2 and M3 of the Plasma Membrane H\(^+\)-ATPase from Saccharomyces cerevisiae*

(Received for publication, April 8, 1998, and in revised form, July 13, 1998)

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The stalk region of the H\(^+\)-ATPase from Saccharomyces cerevisiae has been proposed to play a role in coupling ATP hydrolysis to proton transport. Genetic probing was used to examine the role of stalk segments S2 and S3, associated with M2 and M3, respectively. Saturation mutagenesis was used to explore the role of side group character at position Ile\(^{183}\) in S2, at which an alanine substitution was shown previously to partially uncouple the enzyme (Wang, G., Tamas, M. J., Hall, M. J., Pascaud-Ahuir, A., and Perlin, D. S. (1996) J. Biol. Chem. 271, 25438–25445). Diverse side group substitutions were tolerated at this position, although three substitutions, I183N, I183R, and I183Y required second site mutations at the C terminus of the enzyme for stabilization. Substitution of glycine and proline at Ile\(^{183}\) resulted in lethal phenotypes, suggesting that the backbone may be more important than side group at this position. Proline/glycine mutagenesis was used to study additional sites in S2 and S3. The substitution of proline at Gly\(^{186}\) resulted in a lethal phenotype, whereas substitutions in S3 of proline or serine at Gly\(^{276}\) and proline or glycine at Thr\(^{287}\) resulted in viable mutants. Mutations G270P and T287P resulted in mutant enzymes that produced pronounced growth defects and ATP hydrolysis rates that were 35% and 60% lower than wild type enzyme, respectively. The mutant enzymes transported protons at rates consistent with their ATPase activity, suggesting that the growth defects observed were due to a reduced rate of ATP hydrolysis and not to uncoupling of proton transport. The prominent growth phenotypes produced by mutations G270P and T287P permitted the isolation of suppressor mutations, which restored wild type growth. Most of the suppressors either replaced the primary site mutation with alanine or restored the wild type residue by ectopic recombination with PMA2, both of which restore \(\alpha\)-helical tendency. This study suggests that maintaining \(\alpha\)-helical character is essential to S2 and may play an important role in S3.

The plasma membrane H\(^+\)-ATPase from Saccharomyces cerevisiae is an electrogenic proton pump that is essential to cell survival. It plays a critical role in fungal cell physiology by helping to regulate intracellular pH and by generating the large electrochemical proton gradient necessary for nutrient uptake (1). The enzyme is a member of the P-type family of ion translocating ATPases that includes diverse members from plants, animals, and bacteria (2, 3). P-type ATPases generically consist of a membrane transport domain with 10 transmembrane segments, a large cytoplasmic ATP hydrolysis domain, and a narrow stalk domain that links the two larger domains. These enzymes couple ATP hydrolysis in the cytoplasmic domain to ion transport in the membrane-embedded domain forming an acylphosphate intermediate during catalysis (4, 5). The yeast H\(^+\)-ATPases couples energy with one proton transported per ATP hydrolyzed (6). There is ample evidence that dynamic changes in protein structure occur during catalysis (3). 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 (7).

Cryoelectron microscopy studies of the Ca\(^{2+}\)-ATPase (8, 9) and of the fungal H\(^+\)-ATPase (10) predict that the stalk domain can accommodate four \(\alpha\)-helical segments, most likely S2, S3, S4, and S5, the cytoplasmic extensions from M2, M3, M4, and M5 in typical topology maps. S2 and S3 are found in the LOOP1 region, which is predicted to consist of the two \(\alpha\)-helical stalk segments of approximately 25 amino acids flanking a \(\beta\)-strand domain (11, 12). Various studies have yielded evidence that the LOOP1 portion of the stalk domain undergoes conformational changes during catalysis and may play a role in coupling. In the H\(^+\)-ATPase, mutations at I183A in S2 (13) and H285Q in S3 (14) induce partial uncoupling, whereas other mutations in S2 resulted in defective enzymes (13, 15). In addition, two mutations in S2 of the Ca\(^{2+}\)-ATPase resulted in the reduction of Ca\(^{2+}\) transport (16). This region of the Na\(^+\),K\(^+\)-ATPase has been shown to undergo both differential tryptic (17) and differential iron-catalyzed cleavage during the reaction cycle (18). In this study, we probed by saturation mutagenesis position Ile\(^{183}\) and additional sites in stalk segments S2 and S3 to further analyze properties of the stalk domain in LOOP1 that are important for yeast H\(^+\)-ATPase function.

EXPERIMENTAL PROCEDURES

Yeast Strains and Cell Culture—All yeast strains were isogenic derivatives of Y55 (HO gal3 MAL1 SUC1) (19). All primary site mutations generated were transferred into yeast strain SH122 (HO ade6-1 trp5-1 leu2-1 his3-1 ura3-1 pma1::LEU2/PMA1) (20). Some suppressors were isolated from primary site pma1 mutations introduced into strain SH90 (ho::LEU2 ade6-1 trp5-1 arg4-1 leu2-1 ura3-1 mate), as indicated. Yeast cultures were grown at 30 °C in YPD medium (1% yeast extract, 2% peptone, 2% dextrose, pH 5.7) to mid-log phase (A\(_{600}\) = ~3–4).

Mutagenesis—Site-directed mutants were constructed utilizing uracil-containing phagemid vector pGW201 (13). Saturation mutagenesis was performed using an oligonucleotide that was degenerate for the first and second bases of the Ile\(^{183}\) codon. All mutations were identified by DNA sequence analysis of the purified plasmids using the Sequenase 2.0 kit (Amersham Pharmacia Biotech). The HindIII fragment containing a desired pma1 mutation linked to URA3 was excised from the...
plasmid and transplanted into SH122 using the Alkali-Cation Yeast Transformation kit (Bio 101). Isogenic pma1 mutants were isolated as described by Harris et al. (20).

pma1 mutants were grown in a 3-ml YPD culture for 18–20 h, and chromosomal DNA was isolated using the Wizard Genomic DNA Puri-

TABLE I

| Primary mutant | Second site | Growth | Low pH | Km | Vmax |
|----------------|-------------|--------|--------|----|------|
|                 |             | Higy B |        |    |      |
| GW201 (wild type) | –          | ++     | ++     | 1.9 ± 0.1 | 4.2 ± 1.0 |
| pma1 I183A     | ++         | ++     | +      | 1.2 ± 0.4 | 2.7 ± 0.2 |
| pma1 I183C     | ++         | ++     | ++     | 1.6 ± 0.3 | 3.3 ± 0.1 |
| pma1 I183N     | I863T      | ++     | ++     | 1.8 ± 0.2 | 3.2 ± 0.4 |
| pma1 I183R     | F903L      | ++     | ++     | 1.0 ± 0.1 | 1.6 ± 0.1 |
| pma1 I183S     | ++         | ++     | ++     | 0.6 ± 0.1 | 0.5 ± 0.1  |
| pma1 I183V     | ++         | ++     | +      | 0.9 ± 0.1 | 0.6 ± 0.1  |
| pma1 I183Y     | F882L      | +      | +      | 0.7 ± 0.1 | 3.0 ± 0.1  |
| pma1 I183G     | lethal      |        |        |    |      |
| pma1 I183P     | lethal      |        |        |    |      |
| pma1 1183P,184P| lethal      |        |        |    |      |

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

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

c Values represent the standard error.

d Values represent the activity of a single membrane isolation.

acidification was monitored at 590 nm over 30 min (with a data point taken every 30 s) in a microtiter plate reader (Tecan SLT Instruments).

Probing the Stalk Segments of Yeast H+-ATPase

Proton Pumping by Reconstituted Vesicles—Plasma membranes were isolated as described previously (23), and the H+-ATPase was reconstitut-
in into asolectin vesicles by a modification of the procedure of Seto-
young et al. (24). Membranes (175 μg) were resuspended (in ice) in 400 μl of extraction buffer (10 mM Hepes-Tris, pH 7.0, 0.1 M KCl, 45% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol) containing 10 mg/ml asolectin and 1 mg/ml phosphatidylserine. A 10% (w/v) stock of deoxy-
cholate was added dropwise with stirring to a final concentration of 0.5% (w/v). The mixture was left on ice for 5 min and then rapidly diluted into 10 ml of ice-cold dilution buffer (10 mM Hepes-KOH, pH 7.6, 100 mM KCl, 1 mM dithiothreitol). The vesicles were recovered by centrifugation at 250,000 × g for 1.5 h in a Beckman 50Ti rotor. The pellets were washed by resuspension in 10 ml of ice-cold dilution buffer and centrifuged, as before. The pelleted vesicles were resuspended in 200 μl of cold dilution buffer. Protein transport measurements were made as described previously (24).

Other Procedures—Protein concentrations were determined by a modification of the Lowry method (25). SDS gel electrophoresis was performed using pre-cast 10% minigels (Bio-Rad). Relative protein abundance measurements were made by gel electrophoresis and West-
ern blot analysis, as described previously (22).

RESULTS

Saturation Mutagenesis at Ile183—It was previously shown that a substitution of alanine at Ile183 in S2 resulted in prom-
inent growth phenotypes and a defective enzyme that was found to be partially uncoupled from proton transport (13). To explore side group character at position 183, saturation muta-
genesis was used to introduce a variety of amino acid substi-
tutions (Table I). Viable mutant strains were characterized for growth in the presence of hygromycin B or at low pH, phenotypes reflecting deficient proton transport by the H+-ATPase (26, 27). Most of the Ile183 mutants were resistant to hygromycin B, suggesting that enzyme function was abnormal as would be expected for substitutions at this position. However, muta-
tions I183R, I183S, and I183Y were only obtained with the concomitant introduction of a secondary site mutation in M10 or the extreme C terminus, suggesting that these primary mutations may be more destabilizing. This result further sug-
gests a potential interaction of S2 with the C terminus of the enzyme. Plasma membranes were purified from viable pma1 mutants to determine the biochemical properties of the mutant H+-ATPase enzymes. All pma1 mutants showed levels of asso-
cembled enzyme in the plasma membrane comparable with wild type, except for I183Y, which showed ~50% of wild type. Mutant enzymes 1183R, 1183S, and 1183Y exhibited ATP hy-
drolysis rates significantly lower than wild type enzyme consis-
tent with their effects on phenotype. Interestingly, 1183V, which showed a wild type growth phenotype, hydrolyzed ATP...
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TABLE II
Growth phenotypes and kinetic properties of glycine/proline pma1 mutants

| Mutant   | Location | Viability | Hyg B* | Doubling time K<sub>m</sub> | V<sub>max</sub> | Vanadate (IC<sub>50</sub>) |
|----------|----------|-----------|--------|-----------------------------|--------------|--------------------------|
| GW201 (wild type) | 2 lethal | – | 103 ± 7 | 1.7 ± 0.2<sup>a</sup> | 4.6 ± 0.3 | 0.8 ± 0.1 |
| pma1 I183G | 3 viable | ++ | 108 ± 7 | 0.8 ± 0.1 | 3.0 ± 0.4 | 0.5 ± 0.1 |
| pma1 I183P | 3 viable | ++ | 103 ± 5 | 1.9 ± 0.3 | 5.4 ± 1.0 | 6.2 ± 1.9 |
| pma1 G186P | 3 viable | ++ | 102 ± 9 | 2.0 ± 0.4 | 4.8 ± 0.1 | 1.3 ± 0.3 |
| pma1 G270P | 3 viable | ++ | 132 ± 9 | 0.7 ± 0.2 | 1.8 ± 0.5 | 4.2 ± 0.9 |
| pma1 G270S | 3 viable | ++ | 132 ± 9 | 0.7 ± 0.2 | 1.8 ± 0.5 | 4.2 ± 0.9 |
| pma1 T287G | 3 viable | ++ | 132 ± 9 | 0.7 ± 0.2 | 1.8 ± 0.5 | 4.2 ± 0.9 |
| pma1 T287P | 3 viable | ++ | 132 ± 9 | 0.7 ± 0.2 | 1.8 ± 0.5 | 4.2 ± 0.9 |

<sup>a</sup> Hygromycin B resistance (Hyg B) was assessed on YPD agar with 200 μg/ml hygromycin.
<sup>b</sup> Values represent the standard error.

TABLE II (continued)

at only 14% of the wild type, suggesting that it was destabilized upon isolation. K<sub>m</sub> values were largely unaffected in all the mutant enzymes. All the Ile<sup>183</sup> mutants exhibited proton pumping in a whole cell medium acidification assay (22) that was consistent with the level of ATP hydrolysis observed, except for I183V. Despite the fact that a large variety of side groups could be introduced at Ile<sup>183</sup>, substitution of proline or glycine at this position, as well as the double substitution of proline at positions 183 and 184, were not tolerated yielding lethal phenotypes indicative of a defective H^+-ATPase. These results suggested the possibility that the α-helical character of the stalk segments is important for enzymatic function. A proline/glycine mutagenesis approach was thus taken to further explore the role of helical character in stalk segments S2 and S3.

Proline/Glycine Mutagenesis of Stalk Segments S2 and S3—Proline/glycine mutagenesis was performed in stalk segments S2 and S3 to either replace glycine with proline or to replace a nonconserved residue near the membrane with both proline and glycine. Table II shows the location of the mutations in S2 and S3 and the effects these mutations have on cell viability and growth on hygromycin B. As discussed above, the introduction of either proline or glycine in S2 at Ile<sup>183</sup> resulted in a lethal phenotype. In addition, the replacement of glycine at position 186 with proline was lethal. Proline and glycine substitutions were better tolerated in S3 than S2. Viable mutants resulted from proline or serine substitutions at position Gly<sup>270</sup> or proline or glycine substitutions at position Thr<sup>287</sup>. Mutations G270P and T287P resulted in strong hygromycin B resistance, whereas G270S showed a weaker phenotype. The mutation T287G resulted in only a slight resistance to hygromycin B. All mutants exhibited growth rates comparable with that of wild type except for T287P, which had a doubling time that was ~30% slower (Table II). The effect of medium pH on the growth of the pma1 mutants was determined over a range of pH from 2.5 to 8.0. Cells were inoculated into YPD medium at the indicated pH and incubated with shaking at 30 °C. The OD<sub>590 nm</sub> was determined after 21 h of growth.

Suppressor Mutations of pma1 G270P and pma1 T287P—

FIG. 1. pH-dependent cell growth of Pro/Gly pma1 mutants. The effect of medium pH on the growth of wild type and pma1 mutants was determined in YPD medium with 20 mM acetate adjusted to pH 2.5–8.0. Cells were inoculated into YPD medium at the indicated pH and incubated with shaking at 30 °C. The OD<sub>590 nm</sub> was determined after 21 h of growth.

Suppressor Mutations of pma1 G270P and pma1 T287P—

Characterization of Enzymatic Properties of pma1 Mutants—Plasma membranes were purified from pma1 mutants to determine the biochemical properties of the mutant H^+-ATPase enzymes. All pma1 mutants showed levels of assembled enzyme in the plasma membrane comparable with wild type. K<sub>m</sub> values (Table II) were not significantly altered for any of the mutants. G270S and T287G had ATP hydrolysis rates comparable with wild type, whereas G270P and T287P had rates about 35 and 60% lower, respectively, than wild type enzyme. The reduced rate of ATP hydrolysis under V<sub>max</sub> conditions correlates with the growth phenotypes exhibited by mutant T287P. None of the mutants exhibited dramatic changes in vanadate sensitivity (Table II) with IC<sub>50</sub> values for vanadate ranging from 0.5 to 6.2 μM.

The pH dependence of ATP hydrolysis is often altered in pma1 mutants (22). ATP hydrolysis by wild type and pma1 mutants was measured at pH from 4.5 to 8.5, and the ATP hydrolysis rates at each pH were expressed as a function of the activity at pH 6.5. Mutant enzymes from G270S and T287G exhibited a pH-dependent ATPase profile identical to that of wild type enzyme, whereas G270P showed a slight enhancement of activity at pH 6.0. T287P, however, showed a prominent acid shift with maximal activity occurring at pH 5.5 (data not shown).

Evaluating Transport Properties of pma1 Mutants—To assess relative proton pumping by the H^+-ATPase in pma1 mutants, medium acidification by carbon-starved cells in response to glucose was monitored (22). Fig. 2 shows that T287G and G270S were equally efficient in medium acidification as wild type. T287P had the slowest initial rate consistent with a highly perturbed enzyme. G270P was intermediate between wild type and T287P. To determine whether the decreased efficiency in medium acidification of G270P and T287P was due to an uncoupled enzyme, ATP-induced proton transport in reconstituted vesicles was examined. The H^+-ATPase was reconstituted into asolectin liposomes, and the ATP-induced pH gradient formation was followed by acridine orange fluorescence quenching. To directly compare the rates for each mutant enzyme, vesicles were added to the quenching reaction mixture at a fixed level of total ATP hydrolysis. All the mutant enzymes formed pH gradients comparable with that of wild type enzyme (data not shown). This indicates that the decreased efficiency of medium acidification in the pma1 mutants was most likely related to their reduced rate of ATP hydrolysis and not due to uncoupling.
The prominent growth characteristics of mutants pma1 G270P and pma1 T287P permitted the isolation of suppressor mutations. Suppressor mutations were obtained either spontaneously, by UV treatment of yeast carrying the primary mutation, or by plasmid mutagenesis in a bacterial mutator strain (see “Experimental Procedures”). Suppressors of G270P were either primary site conversions or ectopic recombinations with PMA2. Three independent isolates each replaced the proline at position 270 with an alanine. The remaining suppressors restored the primary site by recombination with PMA2 (Fig. 3A). The size of the recombination fragments ranged from 42 to 243 amino acids of PMA1 replaced with PMA2 sequence. The replacement of PMA1 amino acids 261–302 with PMA2 sequence was identified in five independent isolates. In this recombination, five of 42 amino acids replaced were not conserved. Two other recombinations of 100 and 243 amino acids replacing PMA1 amino acids 185–284 and 222–464, respectively, were each identified once. In these, 17 (for 185–284) and 15 (for 222–464) amino acids differ between PMA1 and PMA2. Suppressors of T287P resulted from either second site mutations or restoration of the primary site by recombination with PMA2. A single recombination that replaced PMA1 amino acids 175 to 417 with PMA2 was found in six isolates (Fig. 3B). This resulted in 20 nonconserved residues replacing PMA1 amino acids. The perturbing nature of proline at positions 270 and 287 and their removal by either primary site conversion or recombination, while allowing many nonconserved substitutions, suggests that backbone structure may be more important than specific side chains in stalk segment S3. The two second site suppressors isolated for T287P resulted from the mutations T263I or E466G along with the replacement of proline 74 near the membrane-embedded transport domain. However, the stalk region has been viewed as a likely candidate to mediate such coupling (7).

Several mutations have been identified in the stalk region that either partially or fully uncouple ion transport from ATP hydrolysis. These include I183A (13) and H285Q (14) in S2 and S3, respectively, in the yeast H−-ATPase, and Y763G at the interface between M5 and S5 (32) in the Ca2+-ATPase. Whether these segments participate directly in the coupling process or form part of a core unit that acts in concert remains to be determined. The N-terminal portion of M5, including S5, has been proposed to mediate coupling by influencing the ion-binding properties associated with M5/M6 and perhaps M4 (7). The association of the stalks segments into an organized structure could help account for prominent conformational interactions observed between the membrane and cytoplasmic domains. Proteolysis studies of the Na+,K+-ATPase where the cytoplasmic loop region between M2 and M3 undergoes structural rearrangements upon ion binding suggest a coupling between this domain and M4-M6 (17, 18).

An I183A mutation in the yeast H−-ATPase, predicted to reside on the α-helical stalk segment S2 of the LOOPI domain, resulted in yeast with prominent growth defects and a partially uncoupled enzyme (13). To assess the importance of side group character at position Ile183, a saturation mutagenesis approach was taken. This approach showed that diverse amino acid substitutions were tolerated at this position. However, three of these substitutions, I183R, I183N, and I183Y were found to contain secondary site mutations in M10 or at the extreme C terminus of the H−-ATPase (Table I and Fig. 4), which appeared to stabilize the primary site mutation. It is not clear whether the stabilizing interactions between S2 and the C terminus are long range or local. However, the second site suppressors clustered at the C terminus suggest an enzyme organization in which S2, and its membrane counterpart M2 is in close proximity to M10. Additional support for this model comes from other suppressor studies of the yeast H−-ATPase in which a second site suppressor to G158D, which lies near the interface of S2 and M2, was identified in the C terminus of the enzyme (33), and suppressers to the C-terminal mutation S911AT912A were found in stalk segments S2 and S4 (29). Even though diverse side group substitutions could be made at position Ile183 in stalk segment S2, the substitution of proline or glycine at this position proved to be lethal (Table I). This suggests either that a small side group at this position is destabilizing, perhaps due to changes in packing density, or that the continuity of the α-helical backbone is compromised by the introduction proline or glycine at position Ile183.

The replacement of proline with alanine and glycine in the transmembrane domain of the Ca2+-ATPase has been shown to have a pronounced effect on enzyme function possibly by affecting α-helix stability (34). The possibility that the α-helical character of the stalk segments is important for enzymatic function of the H−-ATPase was further studied by proline and glycine mutagenesis. Introduction of either proline or glycine in S2 at Ile183 or a proline at Gly186 resulted in highly perturbed enzymes yielding lethal phenotypes, whereas alanine substitution at these positions, which would promote the α-helical character of the stalk, resulted in perturbed but viable enzymes (13). Other mutations in S2 including L275VNK to I275EGR near the membrane (15) and D185A near the β-strand region of LOOPI (13) were tolerated. Finally, in the Ca2+-ATPase, many alanine substitutions were made in S2 with little effect on enzyme function (16). These results taken together suggest that maintaining an α-helical character is essential to S2.

Proline substitutions were better tolerated in S3, both near the β-strand domain (G270), and close to the membrane interface (T287). However, G270P and T287P induced prominent growth defects and reduced ATP hydrolysis activity in mutant enzymes, indicating that these enzymes were significantly perturbed. These growth defects permitted the isolation of suppressor mutations to G270P and T287P. The suppressors isolated were of three types, primary site suppressors, second site suppressors, and ectopic recombinations with PMA2. Suppressors of G270P either replaced the proline with alanine or re-
stored the primary site by recombination with PMA2 (Fig. 3A). Suppressors of T287P resulted from either second site mutations or recombination with PMA2 (Fig. 3B). The perturbing nature of the G270P and T287P mutations led to their removal by primary site suppressors or recombination with PMA2, both of which restore α-helical tendency. Interestingly, the recombinations substituted many amino acids that were not conserved between PMA1 and PMA2. The restoration of the helical tendency by the removal of proline from positions 270 and 287, while allowing many nonconservative substitutions, suggests that backbone structure may be more important than specific side chains in stalk segment S3. The two second site suppressors isolated for T287P each replaced Pro74 near the N terminus with a leucine. This result suggests a potential interaction
of S3 with the N terminus, which is summarized in Fig. 4.

Cryoelectron microscopy of the Ca\(^{2+}\)-ATPase (8, 9) and of the fungal H\(^{+}\)-ATPase (10) predicts that the stalk domain consists of four \(\alpha\)-helical segments. Helix-helix interactions in the bundle would stabilize the stalk and allow it to transmit conformational changes efficiently between the ATP hydrolysis and proton translocating domains. It could also explain why selective cleavage of the backbone in S3 via introduction of a factor Xa site has no effect on ATP hydrolysis or ATP-driven proton transport (15). The application of proline and glycine mutagenesis to examine helical character of the stalk provides additional support for this model.

Acknowledgments—We thank Dr. Donna Seto-Young for helpful advice and Anne-Caroline Schmitt for technical assistance.

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