Substitutions of Aspartate 378 in the Phosphorylation Domain of the Yeast PMA1 H⁺-ATPase Disrupt Protein Folding and Biogenesis*

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There is strong evidence that Asp-378 of the yeast PMA1 ATPase plays an essential role in ATP hydrolysis by forming a covalent β-aspartyl phosphate reaction intermediate. In this study, Asp-378 was replaced by Asn, Ser, and Glu, and the mutant ATPases were expressed in a temperature-sensitive secretion-deficient strain (sec6–4) that allowed their properties to be examined. Although all three mutant proteins were produced at nearly normal levels and remained stable for at least 2 h at 37 °C, they failed to travel to the vesicles that serve as immediate precursors of the plasma membrane; instead, they became arrested at an earlier step of the secretory pathway. A closer look at the mutant proteins revealed that they were firmly inserted into the bilayer and were not released by washing with high salt, urea, or sodium carbonate (pH 11), treatments commonly used to strip nonintegral proteins from membranes. However, all three mutant ATPases were extremely sensitive to digestion by trypsin, pointing to a marked abnormality in their membrane topology. A more detailed examination of the mutant proteins revealed that they were trapped in secretory vesicles. The vesicles are abundant, easily isolated (24), and lend themselves well to assays of ATP hydrolysis. The vesicle trapping was found to be located in the familiar CS/TDK sequence (21). Thus, Asp-378 seems certain to play a key role in catalysis by the yeast PMA1 ATPase.

The yeast plasma membrane H⁺-ATPase, encoded by the PMA1 gene (1), belongs to a widespread and versatile family of cation transporters known as the P₂-ATPases (2). Like other P₂-ATPases, the PMA1 ATPase plays an essential role in ATP hydrolysis and ATP-driven H⁺-aspartyl phosphate reaction intermediate. In the reaction cycle, the γ-phosphoryl group of ATP is transferred to an aspartyl residue embedded in a strongly conserved sequence motif (CSDKTGGT) near the beginning of the central hydrophilic loop. The resulting β-aspartyl-phospho-intermediate can be recognized by its instability at alkaline pH, sensitivity to hydroxylamine, and borohydride-mediated reduction to homoserine (11). In the PMA1 H⁺-ATPase of the fission yeast Schizosaccharomyces pombe, a phospho-intermediate with the same properties has been detected and carefully documented (12–13). It has also been studied in the closely related H⁺-ATPase of Neurospora crassa (14–15) and in the H⁺-ATPase of Saccharomyces cerevisiae (16–17). Although the relevant phosphoprotein has not yet been isolated and directly sequenced in fungi, the fungal enzymes do contain the hallmark CSDKTGTLT motif (1, 18–20). Furthermore, peptide purification and sequencing has been accomplished for the very similar H⁺-ATPase from corn roots, where the phosphorylated Asp residue was found to be located in the familiar CS/TDK sequence (21). Thus, Asp-378 seems certain to play a key role in catalysis by the yeast H⁺-ATPase.

With the cloned PMA1 gene in hand, efforts are now under way to probe structure-function relationships of the yeast ATPase by site-directed mutagenesis. We have introduced a new experimental strategy for this purpose (22) based on a sec6 yeast strain with a temperature-sensitive block in the last step of the secretory pathway (23). The same strain carries both wild-type and mutant copies of the PMA1 gene, which have been placed under control of GAL1 and heat shock promoters, respectively. In galactose medium at 23 °C, the wild-type gene is expressed, and the normal form of the ATPase is delivered to the plasma membrane where it can support growth. In glucose medium at 37 °C, on the other hand, expression shifts to the mutant gene, and newly synthesized mutant ATPase becomes trapped in secretory vesicles. The vesicles are abundant, easily isolated (24), and lend themselves well to assays of ATP hydrolysis and ATP-driven H⁺ translocation (22). As an early application of the new expression strategy, we...
undertook recently to analyze the functional role of amino acids in the phosphorylation domain of the yeast H+ -ATPase. Mutant forms of the ATPase with substitutions in several of the conserved amino acid residues (Lys-379, Thr-380, Thr-382, and Thr-384) were expressed in secretory vesicles and characterized with respect to enzymatic, transport, and structural properties (25). Consistent with the fact that these particular residues are found in all known P-ATPases, they proved essential for activity of the yeast enzyme, and only the most conservative substitutions (Lys to Arg or Thr to Ser) could be tolerated.

However, mutations of the phosphorylated residue (Asp-378) proved to have surprisingly complex consequences. Our initial study showed that replacement of Asp-378 by Ser or even by Asn or Glu interrupted ATPase biogenesis at a step before the secretory vesicles; the same amino acid substitutions also behaved as dominant lethal mutations, preventing growth when coexpressed with a wild-type PMA1 gene (26). An important insight came when Harris et al. (27) detected an abnormal proliferation of intracellular membranes during the induction of D378N. By means of immunofluorescence staining, coexpressed wild-type ATPase was shown to accumulate in the same membranes, which also contained the endoplasmic reticulum marker, Kar2. Similar results have since been reported by Portillo (28). Thus, Asp-378 plays a critical role in ATPase biogenesis.

In the current study, we have explored the molecular basis for this behavior. Although the D378N, D378S, and D378E mutant ATPases are synthesized and inserted into the membrane at a reasonably normal rate, limited trypsinolysis has revealed a profound defect in protein folding. It seems likely that the poorly folded proteins are detected by a “quality control” mechanism within the secretory pathway, preventing their export from the endoplasmic reticulum to the Golgi and resulting in membrane proliferation.

MATERIALS AND METHODS

Yeast Strains

Two strains of S. cerevisiae were used for this project: SY4 (ura3–52; leu2–3,112; sec6–4; GAL; pma1::YlpGAL-PMA1) and SY9 (ura3–52; leu2–3,112; sec18–1; GAL; pma1::YlpGAL-PMA1). In each case, the chromosomal copy of the PMA1 gene was placed under galactose control by gene disruption (29) with the integrating plasmid, YIpGAL-PMA1. After the addition of 0.14 ml of 2.8 M sorbitol, 10 m M NaN3, 55 m M β-mercaptoethanol, 50 m M K2HPO4, pH 7.5, and zymolyase 20T (0.7 mg/ml), the suspension was incubated at 39 °C for 35 min. Digestion was then terminated by the addition of 3.0 ml of cold 1.4 M sorbitol, 10 m M NaN3, 50 m M K2HPO4, pH 7.5. The cells were collected by centrifugation, resuspended in 4.0 ml of 0.1 M EDTA, 20 m M triethanolamine, adjusted to pH 7.5 with acetic acid, 1 m M diisopropyl fluorophosphate, and a protease inhibitor mixture as described previously (Ref. 22), and lysed by homogenization with a Dounce homogenizer. The resulting homogenate was centrifuged for 3 min at 413 × g to remove unbroken cells and cell debris. The supernatant fraction was carefully removed and centrifuged for 45 min at 175,000 × g. Finally, the total membrane pellet was resuspended by Teflon glass homogenization in 200 μl of 1 M EGTA, adjusted to pH 7.5 with Tris-containing protease inhibitors as described above. Aliquots (50 μl) were frozen at −70 °C.

For immunoprecipitation, aliquots (10 μl) were diluted into 0.5 ml of 150 m M NaCl, 2 m M EDTA, 10 m M Tris, pH 7.5, 0.1% SDS, 1% Triton X-100, and 1 m M deoxycholate and treated as described by Chang and Slayman (34) with an affinity-purified polyclonal antibody raised against the closely related plasma membrane H+ -ATPase of N. crassa (18). The immunoprecipitated samples were then subjected to SDS-polyacrylamide gel electrophoresis and fluorography.

Trypsinolysis

Radiolabeled total membranes were diluted to a protein concentration of 1 mg/ml in 1 m M EGTA, adjusted to pH 7.5 with Tris (pH 7.5), and placed under control of the heat shock promoter in plasmid YCp2HSE-pma1 (D378N, D378S, and D378E)—For constitutive expression, the PMA1 promoter (31) contained within the 1.1-kilobase HindIII to SacI fragment containing the full-length mutant gene was placed under heat shock control in the centromeric plasmid YCp2HSE (29). YCpma1 (D378N, D378S, and D378E)—For constitutive expression, the PMA1 promoter (31) contained within the 1.1-kilobase HindIII to ClaI fragment of the PMA1 genomic clone (1) was used to replace the HindIII to ClaI region in pPMA1.2. The 4.7-kilobase HindIII to SacI fragment containing the reconstructed gene was then moved to the LEU2 centromeric plasmid, YCplac111 (32). Plasmids were transformed into yeast by the lithium acetate method of Ito et al. (33).
vesicles with the plasma membrane. The transformed cells were then grown in galactose medium at 23 °C and transferred to glucose medium at 39 °C. As described by Nakamoto et al. (22), this maneuver should lead to the specific appearance of newly synthesized plasmid-encoded ATPase in the vesicles that accumulate as a result of the block. Secretory vesicles were isolated, subjected to SDS-polyacrylamide gel electrophoresis, and analyzed by immunoblotting with polyclonal anti-ATPase antiserum (panel A) or Coomassie staining (panel B). The arrow in panel B indicates the position of the 100-kDa ATPase polypeptide.

To ask whether the D378N, D378S, and D378E ATPases had reached the secretory vesicles (WT/sec6)1 with that arrested in the endoplasmic reticulum (WT/sec18), the same location at which the mutant ATPases become arrested in D378N/sec6, D378S/sec6, and D378E/sec6.

As shown in Fig. 2, newly synthesized ATPase was readily detected in the total membrane fraction from both wild-type controls and from all three mutants, and although there were minor differences in the time course and extent of labeling, the mutant ATPases were indistinguishable in molecular size (100 kDa) from the wild-type polypeptide. Thus, the failure of the mutant ATPases to reach the secretory vesicles in the experiment of Fig. 1 did not reflect a problem with synthesis or stability; rather, it was caused by a defect in membrane trafficking.

Physical Association of the Mutant ATPases with the Membrane—To test whether the D378N, D378S, and D378E ATPases were properly inserted into the lipid bilayer, total 35S-labeled membranes from the experiment of Fig. 2 were treated with reagents commonly used to strip nonintegral proteins from the membrane: 0.1 M Na2CO3, pH 11, 1.0 M NaCl, or 1.6 M urea. After exposure to the various reagents, the membranes were collected by centrifugation, and supernatant and pellet fractions were subjected to immunoprecipitation with anti-ATPase antibody. As illustrated in Fig. 3, there was little or no difference between wild-type ATPase arrested in the endoplasmic reticulum (WT/sec18) and any of the three mutants (D378N/sec6, D378S/sec6, D378E/sec6). Only in the case of D378S was a visible amount of ATPase seen in the supernatant fraction after treatment with urea, and even here, quantitative densitometry indicated that more than 90% of the ATPase remained in the pellet. Thus, like their wild-type counterpart, the mutant ATPases appear to become embedded in the membrane at an early step in their biogenesis.

Limited Trypsinolysis to Probe the Conformation and Ligand-binding Ability of the Mutant ATPases—To ask whether the mutant proteins might be poorly folded, limited trypsinolysis was employed. This method, which gives information about the accessibility of Arg and Lys residues to proteolytic attack, has been widely used to probe the structure of P-ATPases and other membrane proteins. In previous studies with the yeast H+ -ATPase, for example, Perlin and Brown (35) reported that the trypsin degradation pattern of the 100-kDa polypeptide depended upon the presence or absence of physiological li-

1 The abbreviations used are: WT, wild-type; MES, 4-morpholineethanesulfonic acid.
Folding and Biogenesis of the Yeast PMA1 ATPase

![Figure 3](image1)

**Fig. 3.** Behavior of the D378N, D378S, and D378E mutant ATPases as an integral membrane protein. $	ext{^{35}}$S-labeled yeast total membranes (50 μg) were added to 1.0 ml of 50 mM MES-Tris, pH 5.7 (lane 1), 0.1 M sodium carbonate, pH 11 (lane 2), 1.0 M NaCl (lane 3), or 1.6 M urea (lane 4), each containing protease inhibitors as described under “Materials and Methods.” The samples were mixed gently and centrifuged at 220,000 × g for 1 h, and the pellets were resuspended in 1.0 ml of 50 mM MES-Tris, pH 5.7. Equal volumes of the supernatant fractions (S) and resuspended pellets (P) were analyzed as described in the legend to Fig. 2.

Figure 4 illustrates the results of a parallel experiment with a different ligand, MgADP. In the case of wild-type ATPase located in secretory vesicles (WT/sec6), the addition of 5 mM MgADP during the incubation with trypsin led to the accumulation of a 62-kDa fragment, whereas higher concentrations of MgADP (10 and 20 mM) gave no detectable protection of the larger 97-kDa form. For all three mutants, however, the highest concentration of MgADP tested (20 mM) had little or no effect on the 62-kDa fragment and no detectable effect on the 97-kDa fragment. Therefore, there is no convincing evidence that the mutant ATPases can interact with 20 mM MgADP; similar results were obtained with MgATP (not shown).

**DISCUSSION**

Asp-378 has been strongly conserved throughout the P-ATPase family, and there is abundant biochemical evidence that it reacts with the γ-phosphoryl group of ATP during the catalytic cycle. As expected, replacement of the corresponding Asp residue by Asn, Glu, Ser, Thr, His, or Ala in the sarcoplasmic reticulum Ca$^{2+}$-ATPase gave an enzyme that was no longer capable of Ca$^{2+}$-dependent phosphorylation or Ca$^{2+}$ transport (37). When Asp-378 was mutated to Asn in the yeast PMA1 ATPase, however, the enzyme surprisingly appeared able to form a phosphorylated intermediate, split ATP, and pump protons (38).
mutation was transformed into yeast cells whose wild-type PMA1 gene had been tagged with 12 silent base changes. The “normal” segregants that grew up proved to carry all 12 base changes, indicating that they had arisen by gene conversion from the wild-type copy rather than by simple reversion. To avoid the loss of dominant lethal mutations in this way, it is sufficient to place the mutant allele behind a regulated promoter and grow the cells under nonexpressing conditions (cf. Ref. 22).

Further work will be required to understand the precise way in which the D378N, D378S, and D378E mutations disrupt ATPase processing. The limited trypsinolysis experiments carried out as part of the present study provide a useful first step by revealing the unexpectedly severe effect of the mutations on protein conformation, damaging the ability of the ATPase to fold into a comparatively trypsin-resistant state. Given the inability of the abnormal mutant ATPase to proceed along the secretory pathway, folding must in turn be a prerequisite for further processing. Rather, the mutant protein accumulates in discrete cytoplasmic structures that may be related to (or derived from) the endoplasmic reticulum, since they also contain the endoplasmic reticulum protein Kar2 (27). Similar structures have been seen in yeast cells overproducing two other ATPases. In experiments aimed at expressing a heterologous Arabidopsis plasma membrane H^+-ATPase in yeast, Villalba et al. (39) transformed cells with a high copy plasmid carrying the Arabidopsis AHA1 gene under control of the yeast PMA1 promoter; functional plant ATPase was indeed produced, but it became trapped in stacks of membranes surrounding the yeast nucleus. Similarly, Supply et al. (40) set out to improve the expression of an alternative yeast H^+-ATPase (PMA2) that is normally made at exceedingly low levels. When they placed the PMA2 gene behind the PMA1 promoter and transformed yeast cells, there was a striking accumulation of intracellular membranes that stained with PMA2 antibody. Thus, as reviewed recently (41), membrane proliferation appears to be a common response of cells overproducing a yeast or plant H^+-ATPase or making an ATPase that is structurally defective (e.g. PMA1 D378N, D378S, or D378E).

The mechanism by which biogenesis of the wild-type PMA1 ATPase is blocked under these circumstances, at least by the D378N mutation (Refs. 27–28),2 will also deserve further attention. Interestingly, there does not appear to be a gross disruption of the secretory pathway, since normal amounts of secretory vesicles can be isolated when temperature-sensitive sec6–4 cells expressing D378N, D378S, or D378E are shifted to 39 °C; furthermore, Coomassie staining of the vesicles does not reveal any dramatic changes in other protein bands (Fig. 1B, this study). Thus, the block appears restricted to the H^+-ATPase, perhaps pointing to an interaction of wild type and mutant polypeptides with one another or with some ATPase-specific element of the secretory pathway. In this regard, it is perhaps pertinent that two new genes have been described whose products affect ATPase biogenesis. One is AST-1, cloned by Chang and Fink (42) by virtue of its ability to rescue a temperature-sensitive mutant of PMA1 that is targeted to the vacuole. The other is MOP-2, in which mutations interfere with the normal accumulation of PMA1 protein in the plasma membrane (43).

Finally, one may ask whether mutations of the phosphorylated Asp residue have a similar effect on the structure and biogenesis of other P-ATPases, either in mammalian cells or in yeast. In the case of the sarcoplasmic reticulum Ca^2+-ATPase, replacement of this residue (Asp-351) by Asn, Glu, Ser, Thr, His, or Ala caused no noticeable change in the transient expression of the protein in COS-1 cells (44). It should be noted, however, that the Ca^2+-ATPase is an endomembrane enzyme and that even the wild-type form remains in the endoplasmic reticulum. Furthermore, because relatively little ma-

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2 N. DeWitt and C. W. Slayman, unpublished data.
terial can be recovered in the COS-1 expression system, only functional assays were performed (Ca\(^{2+}\) transport; ATP-stimulated formation of a phosphorylated intermediate), and there was no direct examination of the structure of the mutant proteins.

More recently, the wild-type sarcoplasmic reticulum Ca\(^{2+}\)-ATPase has been expressed in yeast, where it was initially reported to reach the plasma membrane, based on subcellular fractionation and immunoblotting (45). However, a subsequent look at intact yeast cells by immunofluorescence microscopy has suggested that most (and perhaps all) of the Ca\(^{2+}\)-ATPase remains in an intracellular compartment (46). This question will need to be resolved and the work extended to mutant enzymes to learn whether or not amino acid substitutions at Asp-351 interfere with the structure and biogenesis of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase in yeast.

In the meantime, there has been parallel work on a related yeast Ca\(^{2+}\)-ATPase: PMR1, which is localized in the Golgi and pumps Ca\(^{2+}\) into that organelle (47). Here, replacement of the phosphorylated Asp residue by Asn or Glu had little or no effect on targeting to the Golgi, and preliminary trypsinolysis studies have indicated that protein folding is relatively normal.3 Mutations of the phosphorylated Asp of the Na\(^{+}\),K\(^{+}\)-ATPase have been extensively studied by three different groups, each making use of a different expression system: Xenopus oocytes (48), cultured 3T3 cells (49), and yeast (50–51). To begin with, Ohtsubo et al. (48) replaced Asp-376 of the Torpedo enzyme with Asn, Glu, or Thr and co-expressed each of the mutant effect on the wild-type enzyme by acting at the catalytic phosphoacceptor site, consistent with the idea that phosphate exerts its stimulatory effect on the wild-type enzyme, and inhibited by Na\(^{+}\),K\(^{+}\)-ATPase in yeast, using a strong, inducible CYC-GAL promoter in cells engineered to overproduce the GAL4-activating protein. Upon the addition of galactose, the ATPase was synthesized and traveled (at least in part) to the cell surface, where it could be detected by high affinity \([\text{H}]\)-ouabain binding in intact cells (51). Furthermore, two different substitutions of the phosphorylated Asp residue (D369N, D369A) failed to reduce the number of ouabain binding sites seen in the intact-cell assay, suggesting that both of the mutant ATPases equaled the wild-type enzyme in their capacity to be handled by the yeast secretory apparatus. Proper folding of the mutant proteins equaled those handled by the yeast secretory apparatus. Proper folding of the mutant proteins was validated by their ability to bind ADP and ATP as well as ouabain, even though as expected they could not be phosphorylated by ATP nor could they carry out ATP hydrolysis.

Thus, despite strong sequence conservation, mutations at the phosphorylation site of the yeast H\(^{-}\)-ATPase appear to have a much more damaging effect on structure and on biogenesis than do their counterparts in other yeast and mammalian P\(_{2}\)-type ATPases. Further work will be required to understand the basis for this intriguing difference.

REFERENCES