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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 protein folding. Furthermore, in contrast to the wild-type enzyme, the mutant ATPases could not be protected against trypsinolysis by ligands such as MgATP, MgADP, or inorganic orthovanadate. Thus, Asp-378 functions in an unexpectedly complex way during the acquisition of a mature structure 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 P_2-ATPases (2). Like other members of the group including the Na^+,K^+-, H^+,K^+-, and Ca^{2+}-ATPases of animal cells, it has a 100-kDa catalytic subunit that is anchored in the lipid bilayer by four hydrophobic segments at the N-terminal end and six hydrophobic segments at the C-terminal end (reviewed in Refs. 3–7). Overall, the P_2-ATPases share ~20% amino acid sequence homology, with the best-conserved stretches found in the central catalytic region of the protein. By contrast, the membrane segments have diverged markedly during evolution, presumably to handle the array of different cations (H^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Cu^{2+}, Cd^{2+}) that are pumped.

The catalytic mechanism of the P-ATPases has been the subject of intense study during the past several decades, starting with the pioneering work of Post, Albers, and their colleagues on the Na^+,K^+-ATPase (8–10). During the reaction cycle, the γ-phosphoryl group of ATP is transferred to an aspartyl residue embedded in a strongly conserved sequence motif (CSDKTGTLT) 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* by (16–17). Although the relevant phosphopeptide 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

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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 (*ura*3–52; *leu*2–3,112; *sec*6–4; *GAL*; *pma1::YIpGAL-PMA1*) and SY9 (*ura3–52*; *leu2–3,112*; *sec18–1*; *GAL*; *pma1::YIpGAL-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 (22). In addition, SY4 contains the temperature-sensitive *sec6–4* mutation to block fusion of the secretory vesicles with the plasma membrane, and SY9 contains the temperature-sensitive *sec18–1* mutation to disrupt membrane flow from the endoplasmic reticulum to the Golgi (23). All three strains were derived from NY605 (*MATa*; *ura3–52*; *leu2–3,112*; *GAL*), kindly supplied by Dr. Peter Novick of the Department of Cell Biology, Yale School of Medicine. Growth conditions and media were as described by Rao and Slayman (25).

Plasmids

YCp2HSE-pma1 (D378N, D378S, and D378E)—For mutagenesis of residue 378, the 495-base pair *Sty*I to *Bam*HI fragment of the *PMA1* gene (1) was subcloned into a modified Bluescript vector (Stratagene, La Jolla, CA). Single-stranded DNA was generated in *Escherichia coli* strain TG1 with the helper phage M13K07, and site-directed mutagenesis was carried out by the method of Taylor *et al.* (30). After the sequence of the mutated fragment was confirmed by double-stranded sequencing, it was moved into pPMA1.2 (22), and the 3.8-kilobase *Hin*dIII to *Sac*I fragment containing the full-length mutant gene was placed under heat shock control in the centromeric plasmid YCp2HSE (22).

YCppma1 (D378N, D378S, and D378E)—For constitutive expression, the *PMA1* promoter (31) contained within the 1.1-kilobase *Hin*dIII to *Cla*I fragment of the *PMA1* genomic clone (1) was used to replace the *Hin*dIII to *Cla*I region in pPMA1.2. The 4.7-kilobase *Hin*dIII to *Sac*I 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).

Secretory Vesicle Isolation, Gel Electrophoresis, Immunoblotting, Enzyme Assays

Methods for secretory vesicle isolation, SDS-polyacrylamide gel electrophoresis, and immunoblotting together with assays for ATPase activity and protein have been described previously (22).

Metabolic labeling and Immunoprecipitation

Synthesis of plasmid-encoded ATPase protein was assayed by ^{35}S-labeling and immunoprecipitation under conditions permitting expression of the plasmid-borne but not the chromosomal *PMA1* gene (22). SY4 cells transformed with the appropriate plasmid were grown to mid-exponential phase in minimal medium + 2% galactose at 23 °C. The cells were harvested by centrifugation and resuspended in minimal medium + 2% glucose for an additional 3 h period, still at 23 °C. The concentration of cells was then adjusted to give 25 ml of culture with A_{600} = 1.0, and the suspension was shifted to a shaking waterbath at 39 °C. After 30 min, 50 μCi of [^{35}S]methionine (Amersham Pharmacia Biotech) was added, and the incubation was continued for 1.5 h. Sodium azide was added to a final concentration of 10 mM, and the cells were harvested by centrifugation and resuspended in 0.14 ml of 10 mM NaN_3. After the addition of 0.14 ml of 2.8 M sorbitol, 10 mM NaN_3, 55 mM β-mercaptoethanol, 50 mM K_2HPO_4, 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 mM NaN_3, 50 mM K_2HPO_4, pH 7.5. The cells were collected by centrifugation, suspended in 4.0 ml of cold lysis buffer (12.5% w/v sucrose, 1 mM EDTA, 20 mM triethanolamine, adjusted to pH 7.5 with acetic acid, 1 mM 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 418 $\times g$ to remove unbroken cells and cell debris. The supernatant fraction was carefully removed and centrifuged for 45 min at 175,000 $\times g$. Finally, the total membrane pellet was resuspended by Teflon glass homogenization in 200 μl of 1 mM 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 mM NaCl, 2 mM EDTA, 10 mM Tris, pH 7.5, 0.1% SDS, 1% Triton X-100, and 1% sodium 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 mM EGTA, adjusted to pH 7.5 with Tris (no protease inhibitors). 5 μl of membrane suspension were added to 10 μl of 5 mM $MgCl_2$, 20 mM Tris, pH 7.0, including 0.1 mM sodium vanadate or 5 mM MgADP when indicated. After preincubation at 30 °C for 2 min, tosylphenylalanyl chloromethyl ketone-trypsin (Worthington Biochemical Corp., Freehold, NJ) was added to give the desired trypsin:protein ratio. At the end of the incubation, the reaction was stopped by adding 20 μl of 2 mM diisopropyl fluorophosphate. Samples were immunoprecipitated as described (34) followed by electrophoresis on 8% polyacrylamide gels. Fluorography was performed by impregnating the fixed gels with 1 M sodium salicylate (30 min at room temperature), and the gels were dried and exposed to film (Hyperfilm MP, Amersham).

RESULTS

Failure of Mutant ATPases with Substitutions of Asp-378 to Reach the Secretory Vesicles—In the immunofluorescence study by Harris *et al.* (27), the substitution of Asn for Asp-378 in the PMA1 ATPase caused a proliferation of intracellular membranes that were related to (and very likely derived from) the endoplasmic reticulum. The membranes could be labeled with antibody against an epitope-tagged D378N construct, consistent with the idea that newly synthesized mutant ATPase became arrested at this early step in the secretory pathway. Indeed, parallel work in our laboratory has provided direct evidence that the arrest is virtually complete. In the experiment of Fig. 1, D378N, D378S, and D378E mutant alleles were placed under control of the heat shock promoter in plasmid YCp2HSE and transformed into yeast strain SY4, which carries a temperature-sensitive block in the fusion of secretory

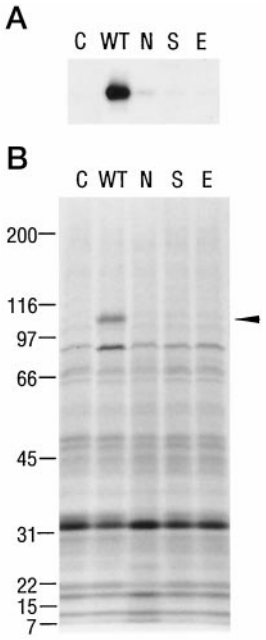

FIG. 1. **Failure of yeast H^+-ATPases carrying D378N, D378S, and D378E mutations to progress through the secretory pathway.** Yeast strain SY4 (*sec6–4*), in which the wild-type chromosomal copy of the *PMA1* gene has been placed under control of the *GAL1* promoter (22), was transformed with the following plasmids: YCp2HSE with no ATPase gene (*lanes* designated *C*); YCp2HSE-PMA1 containing the wild-type ATPase gene (*lanes* designated *WT*); and YCp2HSE-pma1(D378N), YCp2HSE-pma1 (D378S), and YCp2HSE-pma1 (D378E) (*lanes* designated *N*, *S*, and *E*, respectively). The cells were shifted from galactose medium at 23 °C to glucose medium at 39 °C and incubated for 2 h, and secretory vesicles were isolated and analyzed by SDS-polyacrylamide gel electrophoresis followed 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.

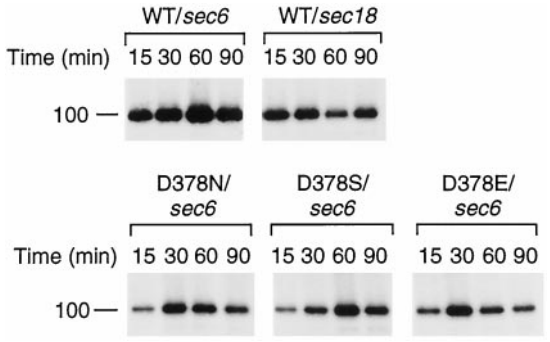

FIG. 2. **Time course of labeling of wild-type and D378N, D378S, and D378E mutant ATPases.** Cells were handled as described in the legend to Fig. 1 except that [^{35}S]methionine was added 30 min after the temperature was shifted to 39 °C. After 15, 30, 60, and 90 min of labeling, cells were harvested, lysed, and subjected to centrifugation as described under "Materials and Methods." The total membrane pellet fractions were collected and analyzed by immunoprecipitation with anti-ATPase antiserum, SDS-polyacrylamide gel electrophoresis, and fluorography.

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 Coomassie staining (Fig. 1*B*) or immunoblotting with anti-ATPase antibody (Fig. 1*A*). Little or no ATPase protein was seen in vesicles from any of the three mutants, even though a substantial amount was present in the wild-type control (12% of total secretory vesicle protein, as judged by gel scanning). There were no other appreciable differences in the protein pattern of the mutant vesicles, suggesting that the mutational block in biogenesis was specific for the PMA1 ATPase.

Synthesis and Stability of the Mutant ATPases—To ask whether there was a defect in the synthesis of the mutant ATPases or whether they were abnormally susceptible to degradation, a modified version of the same experiment was performed in which [^{35}S]methionine was added to the cell suspension 30 min after the temperature shift to 39 °C. After an additional 15-, 30-, 60-, or 90-min incubation, the cells were lysed, and total membranes were isolated and subjected to immunoprecipitation with anti-ATPase antibody. The experiment included two different controls in which plasmid containing the wild-type *PMA1* gene had been transformed into cells carrying temperature-sensitive alleles of *sec6* and *sec18*, respectively. This made it possible to compare wild-type ATPase that 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 ^{35}S-labeled membranes from the experiment of Fig. 2 were treated with reagents commonly used to strip nonintegral proteins from the membrane: 0.1 M Na_2CO_3, 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/*sec*6). 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 tryptic 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.

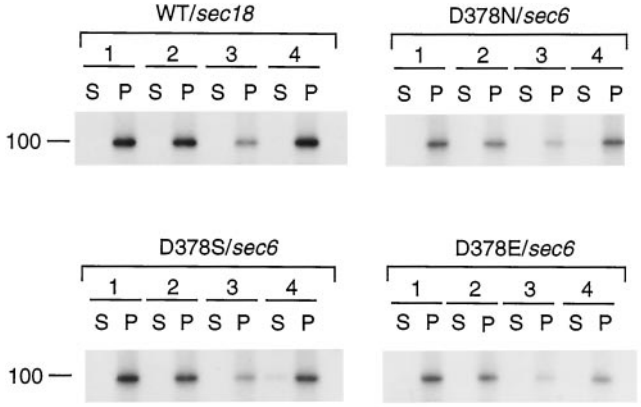

FIG. 3. **Behavior of the D378N, D378S, and D378E mutant ATPases as an integral membrane protein.** ^{35}S-labeled yeast total membranes (50 μg) were added to 1.0 ml of 50 mM MES-Tris, pH 5.7 (*lane 1*), 0.1 M sodium carbonate, pH 11 (*lane 2*), 1.0 M NaCl (*lane 3*), or 1.6 M urea (*lane 4*), each containing protease inhibitors as described under "Materials and Methods." The samples were mixed gently and centrifuged at 220,000 $\times$ *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.

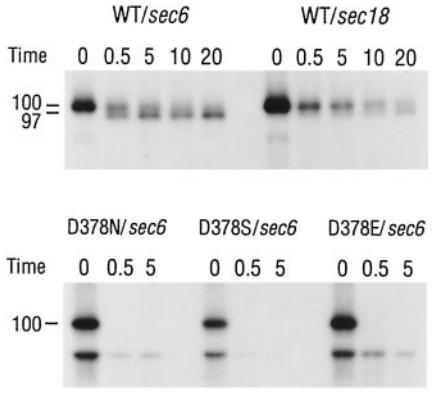

FIG. 4. **Time course of trypsinolysis of wild-type and D378N, D378S, and D378E ATPases.** ^{35}S-labeled total membranes were incubated at a trypsin:protein ratio of 1:20 for 0–20 min at 30 °C. The samples were then immunoprecipitated with anti-ATPase antiserum and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

gands, consistent with the idea that the ATPase could exist in at least two different conformational states. Chang *et al.* (36) have since shown that vanadate- and ADP-protectable proteolytic fragments are seen 5 min after the incorporation of [^{35}S]methionine and cysteine into newly synthesized H^+-ATPase, suggesting that a mature conformation is achieved quite early in biogenesis.

The first step in the present study was to compare the sensitivity of wild-type and mutant ATPases to trypsin. In the experiment of Fig. 4, ^{35}S-labeled total membranes were prepared from cells expressing plasmid-encoded ATPase, incubated at a trypsin:protein ratio of 1:20 for 0–20 min and immunoprecipitated with anti-ATPase antibody. For newly synthesized wild-type enzyme that had reached the secretory vesicles (WT/*sec6*), the 100-kDa mature protein was clipped almost immediately by trypsin to give a 97-kDa form, which remained in appreciable amounts for the rest of the experiment. Even when wild-type ATPase was kept in the endoplasmic reticulum through use of the temperature-sensitive *sec18* allele (WT/*sec18*), a similar pattern was obtained. By comparison, the three mutant ATPases (D378N/*sec6*, D378S/*sec6*, and D378E/*sec6*) were exceedingly sensitive to proteolysis. Even without trypsin, there was noticeable degradation, presumably mediated by an endogenous protease, whereas incubation at a trypsin:protein ratio of 1:20 caused the 100-kDa polypeptide to disappear completely after only 0.5 min, with no trace of the 97 kDa form.

Because vanadate ions have been shown to protect the wild-type yeast ATPase against trypsinolysis, presumably by binding to the 100-kDa polypeptide in the E_2 conformation (35), it seemed worthwhile to ask whether the mutant ATPases might be similarly protectable. As background for this experiment, wild-type and mutant enzymes were incubated for 10 min over a range of trypsin concentrations to find conditions that led to equivalent amounts of degradation; comparable results were obtained at trypsin:protein ratios of 1:4 for WT/*sec6* and WT/*sec18* and 1:50 for D378N/*sec6*, D378S/*sec6*, and D378E/*sec6* (not shown). Next, ^{35}S-labeled membranes were incubated at these trypsin:protein ratios over a range of vanadate concentrations (0–100 μM), and the digests were immunoprecipitated and analyzed. As shown in Fig. 5*A*, wild-type ATPase that had reached the secretory vesicles (WT/*sec6*) was visibly protected by vanadate at concentrations as low as 1 μM. Significant protection was also seen for wild-type enzyme arrested in the endoplasmic reticulum (WT/*sec18*). For the three mutant ATPases, however (D378N/*sec6*, D378S/*sec6*, and D378E/*sec6*), there was no detectable effect of vanadate, even at the highest concentration tested (100 μM). Thus, vanadate fails to bind to the altered phosphorylation domain of the D378N, D378S, and D378E ATPases or, if it does bind, fails to elicit the conformational change that normally protects the protein against trypsinolysis.

Fig. 5*B* 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*) or endoplasmic reticulum (WT/*sec18*), 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 noticeable 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).

Data from the present study, together with the findings of Harris *et al.* (27) and Portillo (28), point to an explanation for this puzzling result. It is now clear that mutant PMA1 ATPases carrying substitutions of Asp-378 are synthesized and inserted into the membrane but are unable to move along the secretory pathway to the cell surface. At the same time, they prevent the concurrent processing of the wild-type ATPase, as shown by experiments with epitope-tagged protein (27–28). Because a functional ATPase is required for growth (1), the result is a strong selective pressure for genetic events that eliminate the mutant form. In the study by Harris *et al.* (27), the D378N

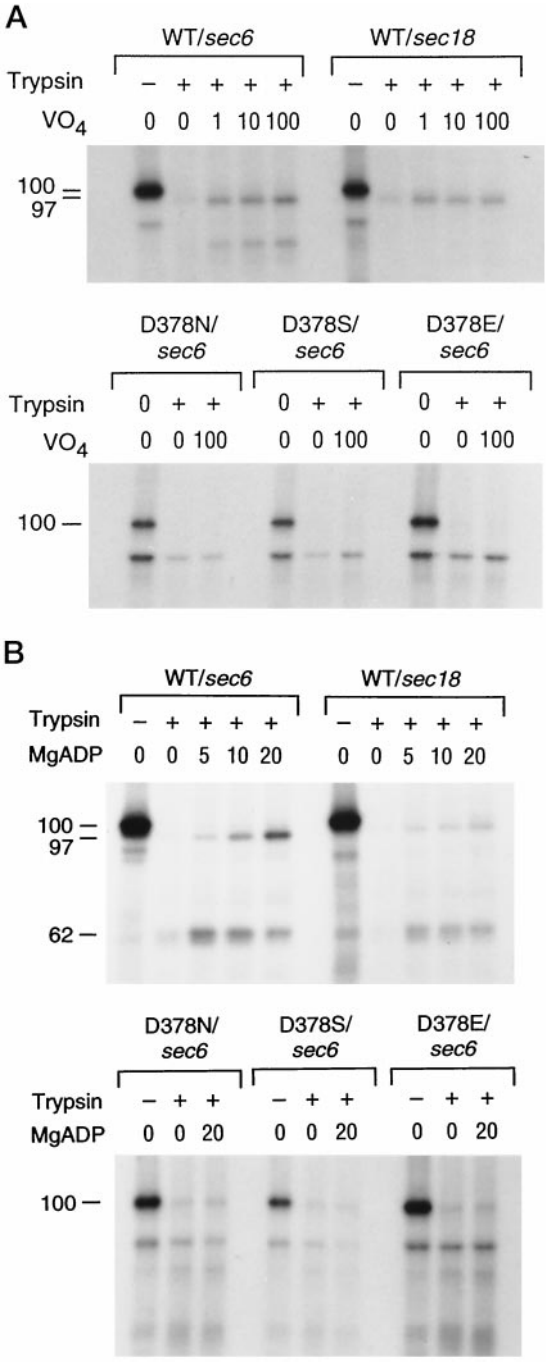

FIG. 5. *A*, effect of vanadate on trypsinolysis of wild-type and D378N, D378S, and D378E ATPases. ^{35}S-Labeled total membranes were incubated for 10 min at a trypsin:protein ratio of 1:4 (WT/*sec6*), WT/*sec18*), or 1:50 (D378N/*sec6*; D378S/*sec6*; D378E/*sec6*) over a range of vanadate concentrations from 0 to 100 μM, as indicated. The samples were then immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. *B*, effect of MgADP on trypsinolysis of wild-type and D378N, D378S, and D378E ATPases. ^{35}S-Labeled total membranes were incubated for 10 min at a trypsin:protein ratio of 1:4 (WT/*sec6*), WT/*sec18*), or 1:50 (D378N/*sec6*; D378S/*sec6*; D378E/*sec6*) over a range of MgADP concentrations from 0 to 20 mM, as indicated. The samples were then immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

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. 1*B*, 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 endo(sarco) plasmic reticulum. Furthermore, because relatively little ma-

[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 α subunits with wild-type *Torpedo* β subunit in *Xenopus* oocytes. In every case, immunoprecipitation from a crude microsomal fraction yielded roughly equal amounts of α and β, suggesting that the mutations had no appreciable effect on the insertion of α into the membrane, on α-β interactions, or on the stability of the ATPase complex. Functionally, however, the mutant proteins displayed little or no Na^+,K^+-ATPase activity. (A slight increase over background was seen, but since it also occurred in control preparations expressing *Torpedo* β subunit alone, it was thought to result from induction of the α-subunit of the host cell.) Parallel assays of [^{3}H]ouabain binding were carried out in intact oocytes expressing the mutant proteins, giving low values that were once again comparable to the β-subunit control (48). From these data the authors concluded that the mutant ATPases had lost ouabain binding ability, although they recognized the possibility that the mutant ATPases had simply failed to reach the plasma membrane.

Subsequently, work on the phosphorylation site of Na^+,K^+-ATPase was extended by Kuntzweiler *et al.* (49), who constructed and characterized an Asp-to-Asn mutation in a ouabain-sensitive form of the sheep α1 subunit. When the mutant protein was stably expressed in 3T3 cells and crude membranes were isolated, immunoblotting with specific monoclonal antibody revealed high levels of sheep α1 subunit. Interestingly, the mutant ATPase was able to bind [^{3}H]ouabain with high affinity. Binding was not affected by inorganic phosphate, consistent with the idea that phosphate exerts its stimulatory effect on the wild-type enzyme by acting at the catalytic phosphorylation site. Ouabain binding was, however, enhanced by Mg^{2+} and inhibited by Na^+, K^+, and ATP, indicating that the Asp-to-Asn mutation had not disrupted the interaction of inorganic cations and nucleotides with the enzyme. Thus, even though the subcellular location of the mutant ATPase was not established in 3T3 cells, it seemed likely that the protein had retained at least some semblance of a normal tertiary structure.

[3] G. Rosas and R. Rao, personal communication.

Of particular relevance to the present study, Pedersen and co-workers (50) have recently coexpressed the α1 and β subunits of pig 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 [^{3}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 was evidenced 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

1. Serrano, R., Kielland-Brandt, M. C. & Fink, G. R. (1986) *Nature* **319,** 689–693
2. Lutsenko, S. & Kaplan, J. H. (1995) *Biochemistry* **34,** 15607–15613
3. Serrano, R. (1988) *Biochim. Biophys. Acta* **947,** 1–28
4. Serrano, R. (1991) in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis, and Energetics* Vol. I, pp. 523–585, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
5. Wach, A., Schlesser, A. & Goffeau, A. (1992) *J. Bioenerg. Biomembr.* **24,** 309–317
6. Fagan, M. J. & Saier, M. H., Jr. (1994) *J. Mol. Evol.* **38,** 57–99
7. Rao, R. & Slayman, C. W. (1996) in *The Mycota* (Brambl, R., and Marzluf, G. A., eds) Vol. III, pp. 29–56, Springer-Verlag New York Inc., New York
8. Post, R. L., Sen, A. K. & Rosenthal, A. S. (1965) *J. Biol. Chem.* **240,** 1437–1445
9. Fahn, S., Koval, G. J. & Albers, R. W. (1968) *J. Biol. Chem.* **243,** 1993–2002
10. Post, R. L. & Kume, S. (1973) *J. Biol. Chem.* **248,** 6993–7000
11. Degani, C., Dahms, A. S. & Boyer, P. D. (1974) *Ann. N. Y. Acad. Sci.* **242,** 77–79
12. Amory, A., Foury, F. & Goffeau, A. (1980) *J. Biol. Chem.* **255,** 9353–9357
13. Amory, A. & Goffeau, A., (1982) *J. Biol. Chem.* **257,** 4723–4730
14. Dame, J. B. & Scarborough, G. A. (1980) *Biochemistry* **19,** 2931–2937
15. Dame, J. B. & Scarborough, G. A. (1981) *J. Biol. Chem.* **256,** 10724–10730
16. Malpartida, F. & Serrano, R. (1981) *Eur. J. Biochem.* **116,** 413–417
17. Foury, F., Amory, A. & Goffeau, A. (1981) *Eur. J. Biochem.* **119,** 395–400
18. Hager, K. H., Mandala, S. M., Davenport, J. W., Speicher, D. W., Benz, E. J., Jr. & Slayman, C. W. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 7693–7697
19. Addison, R. (1986) *J. Biol. Chem.* **261,** 14896–14901
20. Ghislain, M., Schlesser, A. & Goffeau, A. (1987) *J. Biol. Chem.* **262,** 17549–17555
21. Walderhaug, M. O., Post, R. L., Saccomani, G., Leonard, R. T. & Briskin, D. P. (1985) *J. Biol. Chem.* **260,** 3852–3859
22. Nakamoto, R. K., Rao, R. & Slayman, C. W. (1981) *J. Biol. Chem.* **266,** 7940–7949
23. Novick, P., Field, C. & Schekman, R. (1980) *Cell* **21,** 205–215
24. Walworth, N. C. & Novick, P. J. (1987) *J. Cell Biol.* **105,** 163–174
25. Rao, R. & Slayman, C. W. (1993) *J. Biol. Chem.* **268,** 6708–6713
26. Rao, R., Nakamoto, R. K., Verjovski-Almeida, S. & Slayman, C. W. (1992) *Ann. N. Y. Acad. Sci.* **671,** 195–203
27. Harris, S. L., Na, S., Zhu, X., Seto-Young, D., Perlin, D. S., Teem, J. H. & Haber, J. E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 10531–10535
28. Portillo, F. (1997) *FEBS Lett.* **402,** 136–140
29. Cid, A., Perona, R. & Serrano, R. (1987) *Curr. Genet.* **12,** 105–110
30. Taylor, J. W., Ott, J. & Eckstein, F. (1985) *Nucleic Acids Res.* **13,** 8765–8785
31. Capieaux, E., Vignais, M.-L., Sentenac, A. & Goffeau, A. (1989) *J. Biol. Chem.* **264,** 7437–7446
32. Geitz, R. D. & Sugino, A. (1988) *Gene* **74,** 527–534
33. Ito, H., Fukuda, Y., Murata, K. & Kimura, A. (1983) *J. Bacteriol.* **153,** 163–168
34. Chang, A. & Slayman, C. W. (1991) *J. Cell Biol.* **115,** 289–295
35. Perlin, D. S. & Brown, C. L. (1987) *J. Biol. Chem.* **262,** 6788–6794
36. Chang, A., Rose, M. D. & Slayman, C. W. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 5808–5812
37. Maruyama, K. & MacLennan, D. H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 3314–3318
38. Portillo, F. & Serrano, R. (1988) *EMBO J.* **7,** 1793–1798
39. Villalba, J. M., Palmgren, M. G., Berberian, G. E., Ferguson, C. & Serrano, R. (1992) *J. Biol. Chem.* **267,** 12341–12349
40. Supply, P., Wach, A. Thines-Sempoux, D., & Goffeau, A. (1993) *J. Biol. Chem.* **268,** 19744–19752
41. Supply, P., de Kerchove d'Exaerde, A., Roganti, T., Goffeau, A. & Foury, F. (1995) *Mol. Cell. Biol.* **15,** 5389–5395
42. Chang, A. & Fink, G. R. (1995) *J. Cell Biol.* **128,** 39–49 6815–6823
43. Na, S., Hincapie, M., McCusker, J. H. & Haber, J. E. (1995) *J. Biol. Chem.* **270,**

44. Maruyama, K., Clarke, D. M., Fujii, J., Inesi, G., Loo, T. W. & MacLennan, D. H. (1989) *J. Biol. Chem.* **264,** 13038–13042
45. Centeno, F., Deschamps, S., Lompre, A. M., Anger, M., Moutin, M. J., Dupont, Y., Gjedde, P., Villalba, J. M., Moller, J. V., Falson, P. & le Maire, M. (1994) *FEBS Lett.* **354,** 117–122
46. Reis, E. M. R., Slayman, C. W. & Verjovski-Almeida, S. (1996) *Biosci. Rep.* **16,** 107–113
47. Sorin, A., Rosas, G. & Rao, R. (1997) *J. Biol. Chem.* **272,** 9895–9901
48. Ohtsubo, M., Noguchi, S., Takeda, K., Morohashi, M. & Kawamura, A. (1990) *Biochim. Biophys. Acta* **1021,** 157–160
49. Kuntzweiler, T. A., Wallick, E. T., Johnson, C. L. & Lingrel, J. B. (1995) *J. Biol. Chem.* **270,** 16206–16212
50. Pedersen, P. A., Rasmussen, J. H. & Jorgensen, P. L. (1996) *J. Biol. Chem.* **271,** 2514–2522
51. Pedersen, P. A., Rasmussen, J. H. & Jorgensen, P. L. (1996) *Biochemistry* **35,** 16085–16093