Stalk Segment 5 of the Yeast Plasma Membrane H\(^+\)-ATPase

LABELING WITH A FLUORESCENT MALEIMIDE REVEALS A CONFORMATIONAL CHANGE DURING GLUCOSE ACTIVATION* [S]

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Glucose is well known to cause a rapid, reversible activation of the yeast plasma membrane H\(^+\)-ATPase, very likely mediated by phosphorylation of two or more Ser/Thr residues near the C terminus. Recent mutagenesis studies have shown that glucose-dependent activation can be mimicked constitutively by amino acid substitutions in stalk segment 5 (S5), an \(\alpha\)-helical stretch connecting the catalytic part of the ATPase from trans-membrane segment 5 (Miranda, M., Allen, K. E., Pardo, J. P., and Slayman, C. W. (2001) J. Biol. Chem. 276, 22485-22490). In the present work, the fluorescent maleimide Alexa-488 has served as a probe for glucose-dependent changes in the conformation of S5. Experiments were carried out in a “3C” version of the ATPase, from which six of nine native cysteines had been removed by site-directed mutagenesis to eliminate background labeling by Alexa-488. In this construct, three of twelve cysteines introduced at various positions along S5 (A668C, S672C, and D676C) reacted with the Alexa dye in a glucose-independent manner, as shown by fluorescent labeling of the 100 kDa Pma1 polypeptide and by isolation and identification of the corresponding tryptic peptides. Especially significant was the fact that three additional cysteines reacted with Alexa-488 more rapidly (Y689C) or only (V665C and L678C) in plasma membranes from glucose-metabolizing cells. The results support a model in which the S5 \(\alpha\)-helix undergoes a significant change in conformation to expose positions 665, 678, and 689 during glucose-dependent activation of the ATPase.

\(\alpha\)-type ATPases are a widespread family of cation pumps found throughout prokaryotic and eukaryotic cells (1). Actively studied members of the family include the plasma membrane H\(^+\)-ATPases of yeast, \textit{Neurospora}, and higher plants, as well as the Na\(^+\)-K\(^+\)-H\(^+\)-K\(^+\)- and Ca\(^{2+}\)-ATPases of animal cells. All of these examples are abundant membrane proteins, and in many cases they hydrolyze 25% or more of total cellular ATP (e.g. 2). It is thus not surprising that regulatory mechanisms have evolved to adjust ATPase activity to the physiological needs of the cell.

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The yeast plasma membrane H\(^+\)-ATPase, which is encoded by the \textit{PMA1} gene (3), has been known for almost two decades to be strongly regulated by glucose (4). When yeast cells are placed in carbon-free medium, there is a rapid, 5- to 10-fold decrease in Pma1 ATPase activity. When glucose is added back, activity rebounds completely in less than 5 min. Although the mechanism is not yet fully understood, there is growing evidence to implicate the C terminus of the ATPase, acting as an autoinhibitory domain (reviewed in Ref. 5). Mutations at two potential phosphorylation sites within this region, Ser-899 and Thr-912, affect the ability of glucose to stimulate ATPase activity (6), and thermolysin digests of the 100-kDa H\(^+\)-ATPase polypeptide have revealed two (as yet unidentified) phosphopeptides that decrease in amount during carbon starvation and increase again upon glucose addition (7). Thus, it has been proposed that the C terminus becomes dephosphorylated during carbon starvation, allowing it to interact in an inhibitory way with one or more catalytically important parts of the ATPase; upon addition of glucose, the C terminus is rephosphorylated, and the inhibition is released (5, 8).

Recently, genetic evidence has suggested that stalk segment 5 (S5) of the H\(^+\)-ATPase polypeptide may take part in this regulatory interaction. S5, which stretches from Pro-669 to Tyr-689, is one of several \(\alpha\)-helical segments that connect the cytoplasmic domains of the ATPase to the membrane-embedded domain. The overall shape of the stalk was first seen by cryoelectron microscopy of the \textit{Neurospora} plasma membrane H\(^+\)-ATPase and the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (9, 10); a 2.6 Å x-ray crystallographic structure of the latter enzyme has since provided a high-resolution view (11). Remarkably, S5 and its related transthemembrane segment (M5) appear as a continuous \(\alpha\)-helix, 60 Å long, around which the rest of the polypeptide is organized. It was thus of considerable interest when scanning mutagenesis of S5 identified seven periodically spaced positions at which single amino acid substitutions led to strong, constitutive activation of the yeast H\(^+\)-ATPase (Phe-666, Leu-671, Ile-674, Ala-677, Ile-684, Arg-687, Tyr-689, Ref. 12). At each of these positions, which lie along one face of the \(\alpha\)-helix, replacement by a Cys residue increased ATPase activity in starved cells to levels usually seen only in glucose-metabolizing cells. Furthermore, at two additional positions on the same face of S5, Portillo and co-workers (13) have described mutations that suppress the effect of amino acid substitutions at Ser-899 and Thr-912 in the C terminus.

With this information as background, the present study was designed to look directly at S5 of the yeast Pma1 H\(^+\)-ATPase, using a fluorescent maleimide (Alexa-488) to probe the previ-
secretory vesicles containing wild-type (WT) pma1::YIpGAL-PMA1 and the lines were drawn by least-squares analysis.

For the present study, the BglII-SacII restriction fragment carrying each mutation was moved into a modified version of plasmid pPMA1.2 (15) or plasmid pGW201 (17). Both plasmids were designed to add a 10-histidine tag to the N terminus of the ATPase and to replace all but three of the nine original Cys residues (Cys-376, Cys-409, and Cys-472) with Ala. To express the mutant ATPase in secretory vesicles, the 3.8-kb HindIII-SacI fragment from PMA1.2-3C, which contains the entire pma1 coding region, was cloned into the yeast expression vector YCP2HSE (15), placing the mutant allele under control of two tandemly arranged heat-shock elements. Plasmids were then transformed into yeast according to the method of Ito et al. (18). To integrate the Cys mutations into the chromosomal copy of the PMA1 gene, the 6.1-kb HindIII fragment from pGW201-3C, containing the mutant allele linked to URA3, was excised from the plasmid and transspliced into strain NY13 using the Alkali-Cation Yeast transformation kit (Bio 101). In both cases, the presence of the desired mutation was confirmed by DNA sequencing.

Isolation of Secretory Vesicles and Plasma Membranes—Secretory vesicles were prepared from SY4-derived strains by the method of Ambesi et al. (19) and suspended in 0.8 M sorbitol, 1 mM EDTA, 10 mM TEA/acetic acid, pH 7.2. To isolate plasma membranes from glucose-starved and glucose-metabolizing cells, NY13-derived strains were grown to mid-exponential phase in supplemented minimal medium containing 4% glucose, washed, and incubated with or without glucose as described by Miranda et al. (12). A microsomal membrane fraction was then prepared by the method of Perlin et al. (20), washed with 1 mM EDTA/Tris, and resuspended in a small volume of the same buffer. All procedures were carried out at 0–4°C.

ATP Hydrolysis—Unless otherwise noted, ATP hydrolysis was assayed at 30°C in 0.5 ml of 50 mM MES/Tris, pH 5.7 or 6.25, 5 mM KN3, 5 mM Na2ATP, 10 mM MgCl2, and an ATP regenerating system (5 mM phosphoenolpyruvate and 50 μg/ml pyruvate kinase). The reaction was terminated after 20–40 min with 50 μl of 30% trichloroacetic acid, and the release of inorganic phosphate from ATP was determined by the method of Fiske and Subbarow (21). Specific activity was calculated as the difference between ATP hydrolysis in the absence and in the presence of 100 μM sodium orthovanadate, a potent inhibitor of P-type ATPases.

Isoform analysis—To examine the reactivity of the introduced Cys residues, Alexa-488 (Alexa-Fluor-488 C5 maleimide sodium salt; Molecular Probes) was used as a probe. Secretory vesicles were suspended at a protein concentration of 1 mg/ml in 0.8 M sorbitol, 1 mM EDTA, 10 mM TEA, pH 7.2; plasma membranes were suspended at a protein concentration of 0.5 mg/ml in 50 mM HEPES, pH 7.0. In both cases, Alexa-488 was added from a fresh stock solution to a final concentration of 1 mM, and the suspension was incubated at 30°C. Aliquots were removed at intervals and diluted 100-fold into ATPase reaction mixture containing 1 mM β-mercaptoethanol. Inorganic phos-
Fig. 3. HPLC separation of peptides labeled with Alexa-488. To label all Cys residues completely, secretory vesicles expressing 3C ATPase (panel A) or D676C ATPase (panel C) were incubated with 1 mM Alexa-488 in the presence of 1% SDS for 1 h at 30 °C. To label only Cys residues that are accessible in the native state, vesicles with 3C ATPase (panel B) or D676C ATPase (panel D) were exposed to Alexa-488 for 8 min in the absence of SDS. In all cases, the reaction was stopped by the addition of 20 mM β-mercaptoethanol, and the ATPase was purified and digested with trypsin as described under “Experimental Procedures.” Peptides were separated by HPLC, and labeling with Alexa-488 was detected at 493 nm.

**TABLE I**

| Peak fraction | Major spectral peak | Calculated mass | N-terminal sequence | Cys residue |
|---------------|---------------------|----------------|-------------------|-------------|
| 1a            | m/z 1260.7          | 1259.4         | 470IVCVK474        | Cys-472     |
| 1b            | m/z 1260.9          | 1259.4         | 470IVCVK474        | Cys-472     |
| 1c            | m/z 1260.3          | 1259.4         | 470IVCVK474        | Cys-472     |
| 1d            | m/z 1260.7          | 1259.4         | 470IVCVK474        | Cys-472     |
| 2             | NA                  | 2446.7         | 366LSAIESLAGVE...K379 | Cys-376     |
| 3             | NA                  | 3587.9         | 366LSLHEPYVEGVS...R414 | Cys-409     |
| 4             | NA                  | 2686.7         | 666SAADICFLAPGL...K679 | Cys-665     |
| 4             | NA                  | 2714.8         | 666SAADIVFLCPGLS...K679 | Cys-668     |
| 4             | NA                  | 2698.8         | 666SAADIVFLAPG...K679 | Cys-672     |
| 4             | NA                  | 2670.8         | 666SAADIVFLAPGLS...K679 | Cys-676     |
| 4             | NA                  | 2672.7         | 666SAADIVFLAPGLS...K679 | Cys-678     |

*a* The calculated mass was obtained by addition of the protonated mass of the peptide and the mass for Alexa-488 C5 maleimide (697.67).

*b* Not analyzed by MALDI-MS.

Phosphate was determined as described above (21). The rate constants for inactivation were calculated by fitting the data to Equations 1 and 2, when Alexa-488 brought about a monophasic or biphasic, complete inactivation of the ATPase, or to Equation 3, when inactivation was not complete. $A_0$, $k$, and $F$ correspond to the...
activity at zero time, the pseudo-first order rate constant of inactivation, and the final residual activity, respectively.

Labeling of the ATPase with Alexa-488 was carried out under native and denatured conditions. To label the native enzyme, secretory vesicles and plasma membranes were suspended as described above. In both cases, Alexa-488 was added to a final concentration of 1 mM, and the suspension was incubated for either 5 or 20 min at 30°. The reaction was stopped by 30-fold dilution into 5 mM HEPES, 10% glycerol, 20 mM β-mercaptoethanol, pH 7.5. The membranes were centrifuged at 100,000 × g for 35 min and resuspended in 1 ml of 20 mM Tris, 300 mM NaCl, 5 mM β-mercaptoethanol, pH 8.0. The ATPase was then solubilized by the addition of 0.5% SDS and purified by Ni-NTA affinity chromatography as described below.

For complete labeling of sulphydryl groups in the ATPase, secretory vesicles (1 mg/ml) or plasma membranes (0.5 mg/ml) were first denatured with 1% SDS and then exposed to 1 mM Alexa-488 for 60 min at 30°C. The reaction was stopped by the addition of 20 mM β-mercaptoethanol, and the ATPase was purified by Ni-NTA affinity chromatography.

**Purification of His-tagged ATPase by Ni-NTA Affinity Chromatography**—To purify the ATPase, the SDS-solubilized membrane fraction was mixed with 0.5 ml of Ni-NTA previously equilibrated with 20 mM Tris, pH 8.0, 300 mM NaCl, and 5 mM β-mercaptoethanol, and incubated at room temperature for 1 h with continuous shaking. The mixture was then transferred to a disposable column (Poly-Prep Chromatography columns, Bio-Rad), the flow-through was discarded, and the column was washed with 20 ml of 10 mM Tris, 25 mM Na2HPO4, 20 mM imidazole, 200 mM NaCl, 0.1% SDS, pH 8.0. Finally, the His-tagged ATPase was eluted with 0.3–0.5 ml of 10 mM Tris, 25 mM Na2HPO4, 250 mM imidazole, 200 mM NaCl, 0.1% SDS, pH 6.3.

**Trypsinolysis and Peptide Isolation**—Purified ATPase was concentrated to 50–100 μl with a Biomax-30K filter (Millipore) and precipitated with 4 volumes of cold acetone. The protein was collected by centrifugation, and the pellet was suspended in 0.15 ml with a Biomax-30K filter (Millipore) and precipitated with 4 volumes of cold acetone. The protein was collected by centrifugation, and the pellet was suspended in 0.15 ml of 250 mM 100 mM NH4HCO3, pH 8.0. The ATPase was then digested with trypsin (2.5 μg) for 4 h at 30°C and for a further 2 h after a second addition of trypsin (2.5 μg). At the end of the incubation, trypsinolysis was stopped by the addition of 1 mM DFP.

To separate labeled peptides by HPLC, the tryptic digest was injected into a Vydac C18 column and eluted with a gradient of 0–80% acetonitrile in 0.005% trifluoroacetic acid as described previously (22). The absorbance of the eluate was measured at 210 nm to detect all peptides and at 483 nm to detect Alexa-labeled peptides. Peptides were purified using either a C18 aquapore column with the same acetonitrile gradient or the original C18 Vydac column with a different gradient. N-terminal amino acid sequences of the purified fluorescent peptides were determined by MALDI-MS and/or Edman degradation. HPLC, mass spectrometry, and peptide sequencing were performed in Yale University’s Keck Foundation Biotechnology Resource Laboratory.

**Protein Determination**—Protein concentrations were determined by the method of Lowry et al. (23) as modified by Ambesi et al. (19), with bovine serum albumin as standard.

**RESULTS**

**Design of Experiments**—As described in the Introduction, the initial goal of this study was to ask whether Alexa-488 can react with any of the Cys residues previously introduced into stalk segment 5. For this purpose, we began with a well-characterized expression system that uses a temperature-sensitive mutation in the SEC6 gene to arrest newly synthesized H⁺-ATPase in secretory vesicles, just prior to fusion with the plasma membrane (15). Because the vesicles are oriented inside-out, the impermeant Alexa dye should in principle have access to cytoplasmically exposed regions of the H⁺-ATPase, including S5 (Fig. 1).

In an early control experiment, wild-type ATPase was tagged with 10 histidine residues at the N terminus to allow for subsequent purification (24); it was then expressed in secretory vesicles and exposed to 1 mM Alexa-488 for 0–10 min. The ATPase was rapidly inactivated under these conditions (Fig. 2A), indicating that one or more of its 9 native cysteines must be reactive with Alexa-488. We therefore turned to the set of mutant strains constructed by Petrov and Slayman (25), in which varying numbers of native Cys residues were replaced by Ala or Ser. In that study, a totally Cys-free H⁺-ATPase was found to possess little or no enzymatic activity in secretory vesicles, and versions containing one or two cysteines were markedly less active than the wild-type control. However, a mutant with three cysteines (Cys-376, Cys-409, and Cys-472; see Fig. 1) was able to split ATP and pump protons at close to 80% of the wild-type rate. Furthermore, in the experiment of Fig. 2A, the His-tagged version of this 3C construct proved to be virtually insensitive to Alexa-488. It was thus well suited for the present study.

As a critical control, secretory vesicles containing His-tagged, 3C H⁺-ATPase were incubated with Alexa-488 for 8 min. The ATPase was then solubilized, purified by Ni-NTA affinity chromatography, digested with trypsin, and analyzed by HPLC. None of the three cysteines was significantly labeled under these conditions (Fig. 3, panel B), even though labeled peptides corresponding to all three could be readily identified.

\[ \text{Inhibition} = \frac{\text{Activity}_{\text{control}} - \text{Activity}_{\text{sample}}}{\text{Activity}_{\text{control}}} \times 100 \]

\(^a\) V. Petrov, unpublished experiments.
TABLE III
Effect of cysteine substitutions on ATPase expression, activity, and inhibition by Alexa-488: studies on plasma membranes from glucose-starved and glucose-metabolizing cells

| Mutation | Expression\(^a\) | ATPase activity\(^b\) | Inhibition\(^c\) |
|----------|-----------------|---------------------|-----------------|
|          | GS*             | GM                  | GS              | GM              |
| Wild-type| 100             | 100                 | 2.04            | 8.44            |
| 3C       | 96              | 85                  | 3.00            | 9.08            |
| V665C    | 93              | 82                  | 1.78            | 5.68            |
| A668C    | 117             | 108                 | 3.62            | 8.85            |
| S672C    | 103             | 100                 | 2.92            | 9.92            |
| A673C    | 87              | 81                  | 7.72            | 11.20           |
| D676C    | 109             | 104                 | 2.00            | 8.36            |
| L678C    | 118             | 104                 | 1.68            | 8.65            |
| K679C    | 74              | 77                  | 2.90            | 6.30            |
| T680C    | 112             | 105                 | 4.50            | 9.21            |
| Q683C    | 124             | 117                 | 4.40            | 11.10           |
| H686C    | 112             | 120                 | 1.95            | 6.94            |
| M688C    | 109             | 109                 | 2.71            | 10.88           |
| Y689C    | 125             | 132                 | 14.60           | 14.46           |

* The amount of H\(^+\)-ATPase in isolated plasma membranes was determined by quantitative immunoblotting and calculated as a percent of the wild-type control.

* Vanadate-sensitive ATPase activity was measured at pH 6.25 as described under “Experimental Procedures.” One unit is defined as 1 μmol of Pi/min.

* Values for plasma membranes obtained from glucose-starved (GS) or glucose-metabolizing cells (GM).

* Inhibition of ATPase activity by Alexa-488 C\(_2\) maleimide in glucose-metabolizing and glucose-starved cells. Values are the mean for at least three different membrane preparations with an average S.E. less than 1%.

![Graph](image)

**Fig. 5.** Labeling of the 100-kDa H\(^+\)-ATPase by Alexa-488 in plasma membranes. Plasma membranes were isolated from glucose-starved or glucose-metabolizing 3C, V665C, A668C, S672C, D676C, L678C, or Y689C cells, and incubated with 1 mM Alexa-488 under native conditions for 8 min (lanes 3 and 4, 13–16) or 20 min (lanes 5–12, 17 and 18), as described under “Experimental Procedures.” To allow quantitation of labeling, 3C membranes were also incubated with 1 mM Alexa-488 for 1 h under denaturing conditions (lanes 1 and 2). Labeled ATPase was then purified and analyzed by SDS-PAGE. A, fluorescence of the 100-kDa band as a measure of labeling by Alexa-488; B, Coomassie-stained 100-kDa band; C, stoichiometry of Alexa-488 labeling (mol/mol 100-kDa polypeptide), calculated as described in the text.

in ATPase that had been denatured with SDS before exposure to the Alexa dye (Fig. 3, panel A and Table I). Thus, Cys-376, Cys-409, and Cys-472 do not react appreciably with Alexa-488 in the native, fully folded ATPase, and the His-tagged 3C construct could be adopted as background for all of the experiments to be described.

**Reactivity of A668C, S672C, and D676C with Alexa-488 in Secretory Vesicles**—The recent study by Miranda et al. (12) yielded a complete set of mutant H\(^+\)-ATPases with Cys substitutions at individual positions along stalk segment 5. When these mutations were placed one at a time into the His-tagged 3C background and the mutant ATPases were expressed in secretory vesicles, 12 of them had sufficient activity to be studied further: V665C, A668C, S672C, A673C, D676C, L678C, K679C, T680C, Q683C, H686C, M688C, and Y689C. Fortunately, these 12 cysteines were scattered throughout the length of S5, where in principle they could give useful information about structure and reactivity.

To look directly at the reactivity of the S5 cysteines, secretory vesicle preparations were incubated with 1 mM Alexa-488 for 8 or 20 min. The mutant ATPases were then purified and subjected to SDS-polyacrylamide gel electrophoresis, and the 100-kDa ATPase band was analyzed for fluorescence (Fig. 4). Consistent with the results described above, native 3C ATPase was essentially unreactive under these conditions. When its labeling was compared with that of denatured 3C enzyme, which was assumed to contain 3.0 mol of Alexa dye per mol of 100-kDa polypeptide (lane 1), the corresponding stoichiometry was only 0.1 for native 3C ATPase after 8 min of incubation (lane 5) and 0.2 after 20 min of incubation (lane 7).

Most reactive among the cysteines introduced into stalk segment 5 was D676C. This residue could be completely labeled to a stoichiometry of 1.1 in only 8 min (lane 6). Two other cysteines, A668C and S672C, reacted more slowly, reaching stoichiometries of 1.0 and 1.1 after 20 min (lanes 8 and 9). The remaining nine cysteines displayed little if any reactivity with Alexa-488 (not shown). As expected, denaturation of the mutant ATPases before exposure to Alexa-488 gave stoichiometries corresponding to 4 Cys residues per mol of 100-kDa polypeptide (lanes 2–4).

To confirm the identity of the labeled residues, the mutant ATPases were purified, digested with trypsin, and analyzed by HPLC. As shown in Fig. 3D, a new fluorescent peptide (peak 4) was seen in the digest of D676C ATPase that had been exposed to Alexa-488 under native conditions; the same peptide was also labeled under denaturing conditions (Fig. 3C). This peptide was identified by N-terminal sequence analysis as Ser-660–Lys-679 (Table I, peptide 4). Corresponding results were obtained for A668C and S672C (not shown).

A parallel set of experiments was carried out to examine the effect of Alexa-488 on ATPase activity. As illustrated in Fig. 2D and Table II, D676C ATPase was rapidly inhibited by the Alexa dye, with a rate constant of 0.757 min\(^{-1}\). Interestingly, the inhibition was not complete; ~40% residual activity remained even after 8 min of exposure. As expected based on the time course of labeling, the A668C and S672C ATPases were inhibited much more slowly, with rate constants of 0.028 and 0.063 min\(^{-1}\), respectively (Fig. 2, B and C and Table II). The inhibition of S672C approached completion at the later time points, while ~70% residual activity remained in A668C. The remaining nine mutants had negligible rate constants of inactivation (0.002–0.013 min\(^{-1}\), similar to the 3C control (0.007 min\(^{-1}\)). Thus, in the secretory vesicle form of the H\(^+\)-ATPase, three
of the Cys residues introduced into stalk segment 5 could clearly react with Alexa-488, and modification led to partial (A668C, D676C) or complete (S672C) inhibition of ATPase activity.

Glucose-dependent Reactivity of V665C, L678C, and Y689C at the Plasma Membrane—The next step was to ask whether Cys-668, Cys-672, Cys-676, or any of the other Cys residues placed along stalk segment 5 might undergo glucose-dependent changes in reactivity with Alexa-488. For this purpose, it was necessary to express the mutant ATPases at the plasma membrane, where glucose regulation is known to have its full effect. Accordingly, the chromosomal copy of the PMA1 coding sequence was replaced with each of the His-tagged, 3C mutant constructs described above. Gene replacement was carried out in cells possessing a normal copy of the SEC6 gene, so when the mutant ATPase was expressed constitutively under control of the PMA1 promoter, it could move uninterruptedly from its site of synthesis in the endoplasmic reticulum to the plasma membrane. As expected, all 12 mutant ATPases, which had specific activities in secretory vesicles ranging from 28 to 72% of the wild-type level (Table II), were able to support normal or near normal growth.

In the experiment of Table III, plasma membranes were prepared from glucose-starved and glucose-metabolizing cells expressing each of the His-tagged, 3C-mutant alleles, and assayed for ATPase activity. As found previously for untagged, 9C-based constructs (12), Y689C was constitutively activated at the plasma membrane, hydrolyzing ATP equally well under glucose-starved and glucose-metabolizing conditions (14.60 and 14.46 units/mg). All but one of the other mutants gave activation ratios between 2.0 and 5.1, and thus were clearly regulated by glucose. A673C was intermediate in its behavior, with an activation ratio of 1.4.

With this background information in hand, the next step was to examine the reactivity of S5 Cys residues with Alexa-488 in plasma membranes from glucose-starved and glucose-metabolizing cells (Fig. 5). Not surprisingly, the three cysteines already found to react with Alexa-488 in secretory vesicles (A668C, S672C, D676C; see above) gave comparable results in plasma membranes, and the stoichiometry of labeling under glucose-starved conditions (1.0–1.2; see lanes 9, 11, and 13) was very close to that under glucose-metabolizing conditions (1.4–1.5; see lanes 10, 12, and 14). Strikingly, however, three additional mutant ATPases became labeled by Alexa-488 in plasma membranes, but only when the membranes were prepared from glucose-metabolizing cells. They were V665C, L678C, and Y689C, which reached stoichiometries of 1.2, 0.8, and 1.4, respectively, in glucose-metabolizing preparations (Fig. 5, lanes 8, 16, and 18). By contrast, the values in glucose-starved preparations were only 0.1–0.3 (lanes 7, 15, and 17), similar to those seen in the 3C control (0.1; lanes 3 and 5).

To verify the identity of the Alexa-reactive Cys residues in these three mutants, labeled ATPase was digested with trypsin and analyzed by HPLC. In two cases (V665C and L678C), a new fluorescent peptide was seen and identified by Edman degradation as Ser-660–Lys-679 (Table I). No fluorescent peak was found for Y689C, presumably because the corresponding peptide (Met-668–Arg-695) was too hydrophobic to be isolated under the experimental conditions that were used.

Once again, parallel measurements were carried out to ask whether hydrolytic activity was affected by modification with Alexa-488. As already seen in secretory vesicles, the A668C, S672C, and D676C ATPases were clearly inactivated by Alexa dye at the plasma membrane (Table III). Worth noting is the fact that all three mutants displayed higher rate constants of inactivation under glucose-starved conditions than under glucose-metabolizing conditions. Because a similar effect was seen in the wild-type and 3C controls and in all but two of the other mutants, the most likely explanation is a conformational change affecting one or more of the native cysteines.

The most significant result in Table III is that glucose-dependent modification of Cys-665, Cys-678, and Cys-689 by Alexa-488 had virtually no effect on ATPase activity. The rate constants of inactivation were very low in both glucose-starved and glucose-metabolizing samples: 0.029 and 0.014 min⁻¹ for V665C, 0.035 and 0.025 min⁻¹ for L678C, and 0.001 and 0.003 min⁻¹ for Y689C, similar to the values seen in the 3C control (0.025 and 0.013 min⁻¹) (Table III). Thus, unlike the situation for positions 668, 672, and 676, the addition of the bulky Alexa group at positions 665, 678, and 689 had little or no effect on the catalytic properties of the H⁺-ATPase.

**DISCUSSION**

In this study, Alexa-488 has been used as a fluorescent probe to examine the role of stalk segment 5 in the glucose-dependent activation of yeast Pma1 H⁺-ATPase. Several conclusions can be drawn from the results. First, because Alexa-488 is known to be membrane-impermeant (26), its ability to reach Cys residues at positions 668, 672, and 676 in inside-out secretory vesicles confirms that, as expected, stalk segment 5 is located on the cytoplasmic side of the membrane. More significant is the fact that these three cysteines were modified by Alexa dye under all of the experimental conditions tested: before and after the ATPase had reached the plasma membrane, and before and after it had been fully activated by glucose. In a helical-wheel diagram, A668C, S672C, and D676C lie on one face of S5 (Fig. 6, open rectangles) which, because it is accessible to Alexa-488, is presumably exposed to the aqueous medium. Indeed, when the Pma1 H⁺-ATPase was aligned with SERCA1 Ca²⁺-ATPase and modeled using the SERCA structure (11) as a template, Ala-668, Ser-672, and Asp-676 all appeared at the protein surface (Fig. 7A).

By contrast, the opposite face of the S5 helix is defined by residues previously shown to be involved in the activation of Pma1 ATPase by glucose. These residues include Phe-666, Leu-671, Ile-674, Ala-677, Ile-684, Arg-687, and Tyr-689 (12) and Pro-669 and Gly-670 (13), marked by gray rectangles in the helical-wheel diagram of Fig. 6. The first seven are positions at
which substitution by Cys led to constitutive activation, suggesting that the mutations had somehow interfered with the ability of the starvation signal (presumably, dephosphorylation of the C terminus, Ref. 5) to down-regulate enzyme turnover. The other two positions (Pro-669 and Gly-670) are sites of suppressor mutations that also result in constitutive activation (13). In the three-dimensional structural model of the Pma1 ATPase, all nine residues are located in the interior of the protein (Fig. 7D). Six of them are closely associated with the mechanistically essential P (phosphorylation) domain, while the other three lie at the interface of S5 with the membrane and interact with stalk segment 4 (S4).

In sharp contrast to A668C, S672C, and D676C are three other cysteines, V665C, L678C, and Y689C, which had little or no ability to react with Alexa-488 in secretory vesicles or in plasma membranes from glucose-starved cells, but gained the ability to react in plasma membranes from glucose-metabolizing cells. One obvious possibility is that the autoinhibitory C terminus shields all three residues under the former conditions, but becomes phosphorylated and moves aside under the latter conditions. Given the complete absence of homology between the C-terminal domains of the Pma1 and SERCA ATPases, there is not yet a way to visualize the location of this part of Pma1. It is worth noting, however, that the model of Fig. 7 places V665 and L678 on one side of the protein while Tyr-689 lies at the base of a cavity on the opposite side. Thus, the dephosphorylated C terminus may shield one (Y689C) or two (V665C and L678C) of these residues directly, but it would have difficulty shielding all three. At least part (and possibly all) of the glucose-dependent labeling by Alexa-488 may therefore result from a primary conformational change elsewhere that indirectly exposes V665C, L678C, and Y689C to the aqueous medium.

There is a further reason to be interested in one of these residues: Y689C, which lies at the interface of stalk segment 5 with the membrane. Although the Y689C mutant ATPase is labeled by Alexa-488 in a glucose-dependent way (see above) it is constitutively active even in the absence of glucose (12). The labeling result indicates that the mutant enzyme can still respond conformationally to glucose; for example, the C terminus may restrict the access of Alexa-488 to Y689C when it is dephosphorylated (in the absence of glucose) but may shift position to allow access when it is phosphorylated (in the presence of glucose). At the same time, the constitutive nature of the Y689C mutation means that the starvation-induced conformational change is no longer able to down-regulate ATPase activity, perhaps because the mutated version of S5 can no longer interact in an inhibitory way with the P-domain. Tyr-689 corresponds to Lys-758 in the SERCA Ca$^{2+}$-ATPase, where Sorensen and Andersen (27) found that substitution by Ile speeds the dephosphorylation of the enzyme from E$_2$P to E$_2$ while simultaneously slowing the rate of Ca$^{2+}$ binding from E$_2$ to Ca$_2$E$_1$. Indeed, the same authors have since described complex kinetic changes resulting from mutations at 14 other positions in S5 of the SERCA ATPase, pointing to a more general role of S5 in communicating between catalytic and transport sites (28).
A recent paper by Soteropoulos et al. (29) provides independent evidence for an important functional role of stalk segment 5 of the yeast Pma1 ATPase. In that study, the introduction of a helix-disrupting proline at either of two positions (D676P or I684P) destroyed the ability of the ATPase to support growth, as did the deletion of Asp-676 or the insertion of one, two, three, or four Ala residues following Asp-676. Cells bearing a glycine at position 676 were viable, but they were unable to grow in acetate-containing medium at low pH. This conditional phenotype allowed the authors to select second-site suppressors that could restore the growth of D676G under acidic conditions. One of the resulting mutations (V562I) again pointed to an interaction between S5 and the phosphorylation domain, while others gave evidence of interactions with stalk segment 3 (E288K), the small cytoplasmic loop between membrane segments 6 and 7 (V748I), and a region close to the putative C-terminal phosphorylation sites (G888S).

In summary, the present study adds to knowledge of structure-function relationships within the yeast Pma1 ATPase by revealing a glucose-dependent change in stalk segment 5. The next step will be to understand how this change is triggered (most likely by phosphorylation/dephosphorylation near the C terminus), and in turn, how it is transmitted to the catalytic region and into the membrane. The introduction of Cys residues along S5 and the ability to label six of them specifically with Alexa-488 will provide useful tools for future work along these lines.

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