In the P₂-type ATPases, there is growing evidence that four α-helical stalk segments connect the cytoplasmic part of the molecule, responsible for ATP binding and hydrolysis, to the membrane-embedded part that mediates cation transport. The present study has focused on stalk segment 4, which displays a significant degree of sequence conservation among P₂-ATPases. When site-directed mutants in this region of the yeast plasma membrane H⁺-ATPase were constructed and expressed in secretory vesicles, more than half of the amino acid substitutions led to a sealfold decrease in the rate of ATP hydrolysis, although they had little or no effect on the coupling between hydrolysis and transport. Strikingly, mutant ATPases bearing single substitutions of 13 consecutive residues from Ile-359 through Gly-371 were highly resistant to inorganic orthovanadate, with IC₅₀ values at least 10-fold above those seen in the wild-type enzyme. Most of the same mutants also displayed a significant reduction in the K₅ₐ for MgATP and an increase in the pH optimum for ATP hydrolysis. Taken together, these changes in kinetic behavior point to a shift in equilibrium from the E₂ conformation of the ATPase toward the E₁ conformation. The residues from Ile-359 through Gly-371 would occupy three full turns of an α-helix, suggesting that this portion of stalk segment 4 may provide a conformationally active link between catalytic sites in the cytoplasm and cation-binding sites in the membrane.

P-type ATPases, which are found throughout prokaryotic and eukaryotic cells, use the energy from ATP hydrolysis to pump inorganic cations across cell membranes. In the most abundant subfamily, designated P₂, the 100-kDa ATPase polypeptide is embedded in the lipid bilayer by four hydrophobic segments at the N-terminal end of the molecule and six at the C-terminal end (1). The central hydrophilic region protrudes into the cytoplasm and contains catalytic sites for ATP binding and formation of the characteristic β-aspartyl phosphate intermediate.

It was proposed more than a decade ago that conformational changes in the catalytic portion of the P-type ATPases are transmitted into the membrane via a stalk-like region made up of the cytoplasmic extensions from some, but not necessarily all, of the transmembrane segments (2). Recently, the stalk has been directly visualized in cryoelectron microscopic studies of the sarcoplasmic reticulum Ca²⁺-ATPase (3) and the plasma membrane H⁺-ATPase of Neurospora crassa (4), both at 8-Å resolution. In the case of the Ca²⁺-ATPase, the stalk appeared as a narrow structure connecting the compact, wedge-shaped cytoplasmic domain with the membrane-spanning segments; it contained four rod-like densities (very likely α-helices), which were tentatively identified as stalk segments 2–5 (3). The Neurospora H⁺-ATPase was strikingly similar to the Ca²⁺-ATPase in its membrane region, but the cytoplasmic portion was noticeably less compact, and instead of a single stalk, there were several apparent connections between the cytoplasmic portion and the membrane (4). As the authors pointed out (3–5), the two structures may represent different conformational states, since the Ca²⁺-ATPase was crystallized in the presence of decavanadate and the H⁺-ATPase, in the absence of added ligands. Indeed, there is compelling evidence that the reaction cycle of the P-ATPases alternates between two major conformations (E₁ and E₂), that are different enough to be distinguished from one another by a variety of biochemical and biophysical methods (6) including proteolytic digestion patterns (7–9).

Recently, Soteropoulos and Perlin (10) carried out an informative mutagenesis study of stalk segments 2 and 3 in the yeast plasma membrane H⁺-ATPase, a close relative of the Neurospora enzyme. Their approach was to replace selected amino acids in S2 (Ile-183 and Gly-186) and S3 (Gly-270 and Thr-287) with helix-breaking residues, Gly and Pro, in order to test the helical nature of S2 and S3. At both positions in S2, the mutations proved to be lethal; at the positions in S3, the cells were viable, but the Pro replacements led to a significant reduction in ATPase activity. The authors concluded that the α-helical nature of S2, and to a lesser extent that of S3, may help to stabilize the stalk and/or promote the proper conformational interaction between the cytoplasmic and membrane-embedded portions of the ATPase.

In the present study, we have performed alanine-scanning mutagenesis along the entire length of S4, again in the yeast H⁺-ATPase. S4 almost certainly contributes to the stalk structure seen by cryoelectron microscopy, and it links the Asp residue that is phosphorylated by ATP to membrane segment 4 (M₄). M₄ in turn plays a central role in cation binding and translocation, based on mutagenesis studies of the sarcoplasmic reticulum Ca²⁺-ATPase (11–16), plasma membrane Ca²⁺-ATPase (17), Na⁺,K⁺-ATPase (16, 18–20), and gastric H⁺,K⁺-ATPase (21). Consistent with this picture, the mutagenesis results to be described below provide evidence that stalk segment 4 helps to mediate the E₁/E₂ conformational change in the yeast H⁺-ATPase.
Yeast Strain—Strain SY4 of Saccharomyces cerevisiae (MATa, ura3-52, leu2-3, 112, his4-619, sec6-4 GAL) was employed throughout this study. In strain SY4, the chromosomal copy of the PMA1 gene encoding the yeast plasma membrane H+-ATPase has been placed under control of the GAL1 promoter as described previously (22). SY4 also carries the temperature-sensitive sec6-4 mutation which, upon incubation at 37 °C, blocks the fusion of secretory vesicles with the plasma membrane (23).

Mutagenesis—To introduce mutations into the S4 region of the H+-ATPase by polymerase chain reaction (24), two restriction fragments of the PMA1 gene were employed, both subcloned into a modified Blue-script plasmid (Stratagene, La Jolla, CA). Mutations from Lys-355 to Ala were introduced into a 615-base pair BstXI/EcoRI restriction fragment, whereas mutations from Gly-371 to Leu-375 were introduced into a 495-base pair Styl/BamHI fragment. After DNA sequencing to verify the mutation, the restriction fragment was moved into plasmid PMA1.2 (22). The 3.8-kilobase pair HindIII/SacI fragment containing the entire pma1-coding region was then cloned into the yeast expression vector YCpH2HSE (22), placing the mutant allele under control of two tandemly arranged heat-shock elements. Finally, the plasmids were transformed into wild-type SY4 (see above) according to the method of Ito et al. (25).

Isolation of Secretory Vesicles and Quantitation of Expression of ATPase—Transformed SY4 cells were grown to mid-exponential phase (A600, ~1) at 23 °C in supplemented minimal medium containing 2% galactose and 0.5% potassium acetate medium containing 50 mM sodium orthovanadate, tosylphenylalanyl chloromethyl ketone-treated trypsin (Tyr-325 through Ala-354; Ref. 32 and 33). With the exception of Ala-358, Ala-365, and Ala-370, which were replaced with Ser, each residue was changed to Ala. Each mutant allele was cloned into the expression vector pGAL-PMA1 (22). The 3.8-kilobase pair HindIII/SacI fragment containing the entire pma1-coding region was then cloned into the yeast expression vector YCpH2HSE (22), placing the mutant allele under control of two tandemly arranged heat-shock elements. Finally, the plasmids were transformed into wild-type SY4 (see above) according to the method of Ito et al. (25).

ATP Hydrolysis—Unless otherwise noted, ATP hydrolysis was assayed at 30 °C in 50 mM MES/Tris, pH 7.5, 5 mM MgCl2, and an ATP-regenerating system (5 mM phosphoenolpyruvate and 50 µM pyruvate kinase). The reaction was terminated after 20–40 min, and the release of inorganic phosphate from ATP was determined by the method of Fiske and Subbarow (27). ATP hydrolysis was also assayed under conditions similar to that used for quantification of proton transport (see below), as described previously (29). Briefly, secretory vesicles (5–10 µg of protein) were diluted into 200 µl of 0.6 M sorbitol, 0.1 M KCl, 20 mM HEPES/KOH, pH 6.7, Na2ATP (0.3 to 3.0 mM), and MgCl2 (5 mM excess over the ATP concentration) at 30 °C. The reaction was stopped after 20–40 min by addition of trichloroacetic acid to a final concentration of 5%, and inorganic phosphate was determined. ATP hydrolysis was also assayed under conditions similar to that used for quantification of proton transport (see below), as described previously (29). Briefly, secretory vesicles (5–10 µg of protein) were diluted into 200 µl of 0.6 M sorbitol, 0.1 M KCl, 20 mM HEPES/KOH, pH 6.7, Na2ATP (0.3 to 3.0 mM), and MgCl2 (5 mM excess over the ATP concentration) at 30 °C. The reaction was stopped after 20–40 min by addition of trichloroacetic acid to a final concentration of 5%, and inorganic phosphate was determined.

Proton Transport and Fluorescence Quenching—ATP-dependent proton transport was assayed by measuring the initial rate of acridine orange fluorescence quenching as described previously (29). The specific initial rate of quenching was adjusted for the level of ATPase expression to the level of ATPase activity. ATP-dependent fluorescence quenching was determined over a range of ATP concentrations and plotted as a function of the rate of ATP hydrolysis, assayed under similar conditions (see above).

Tryptsinolysis—Limited trypsinolysis was performed on isolated secretory vesicles as described previously (30). Vesicles were suspended at 0.5 mg/ml in 20 mM Tris-HCl, pH 7.0, and 5 mM MgCl2. Following preincubation for 5 min in the absence of trypsin, an enzyme concentration of 0.1, 1.0, and 10 µg/ml in the presence of 20 mM orthovanadate, tosylphenylalanyl chloromethyl ketone-treated trypsin was added (trypsin/protein ratio of 1:4), and the incubation was continued for 20 min. The reaction was terminated by the addition of 1 mM diisopropyl fluorophosphate, and the products were analyzed by immunoblotting with polyclonal antiserum against the ATPase.

Protein Determination—Protein concentrations were determined by the method of Lowry et al. (31) as modified by Ambesi et al. (26), with bovine serum albumin as standard.

RESULTS

Selection of Residues for Mutagenesis—The goal of the present study was to explore the functional role of amino acid residues throughout stalk segment 4 (S4), which links membrane segment 4 (M4) with the phosphorylation site (Asp-378) of the yeast plasma membrane H+-ATPase (Fig. 1). Residues from Lys-355 to Leu-375 were subjected to alanine-scanning mutagenesis, filling in the 21-amino acid stretch between previously published studies of M4 (Tyr-325 through Ala-354; Ref. 29) and the phosphorylation domain (Cys-376 through Thr-384; Refs. 32 and 33). With the exception of Ala-358, Ala-365, and Ala-370, which were replaced with Ser, each residue was changed to Ala. Each mutant allele was cloned into the expression vector YCpH2HSE, transformed into yeast strain SY4, and expressed under the control of a heat-shock promoter after turning off the wild-type PMA1 allele (22). Secretory vesicles containing newly synthesized mutant ATPase were then isolated and characterized (26).

Expression and ATP Hydrolysis—As summarized in Table I (top part), quantitative immunoblotting revealed that Ala/Ser substitutions in S4 had only a modest effect on biogenesis, with mutant H+-ATPases reaching the secretory vesicles at 35–101% of the amount seen in the wild-type control. Likewise, 19 of 21 mutant enzymes clearly retained the ability to hydrolyze ATP, with specific activities ranging from 27 to 118% after correction for the level of expression in the vesicles. Only two of the mutants showed more serious defects in ATP hydrolysis as follows: L368A (15%) and I374A (16%), and even in these cases, the uncorrected activities were 4–5-fold greater than the background values measured in the empty plasmid control. Thus, none of the residues in S4 appeared to be completely essential for ATP hydrolysis, although most of the mutations caused at least a 50% decrease in the rate of hydrolysis.

Proton Transport—Given that S4 physically links the phosphorylated Asp residue to the membrane, it was important to examine the effects of the Ala/Ser substitutions on the ability of the ATPase to transport protons. This was measured by fluorescence quenching of the pH-sensitive dye, acridine orange (Table I, top part). In four of the mutants (K356A, L369A, I374A, and L375A), ATP-dependent quenching was clearly...
**Stalk Segment 4 of the Yeast PMA1 ATPase**

TABLE I  
Effect of mutations in stalk segment 4 on expression, ATP hydrolysis, and proton transport

| Mutation          | Expression | ATP hydrolysis | Proton transport |
|-------------------|------------|----------------|------------------|
|                   |            | Uncorrected    | Corrected       | %     | Uncorrected | Corrected | %     |
|                   |            | units/mg       | units/mg        |       |            |           |       |
| Alinate scan      |            |                |                 |       |            |           |       |
| Wild type         | 100        | 4.28           | 4.28            | 100   | 761         | 761       | 100   |
| Vector            | 3          | 0.09           | ND              | ND    | 6           | ND        | ND    |
| K355A             | 57         | 1.68           | 2.95            | 69    | 215         | 377       | 50    |
| K356A             | 35         | 0.65           | 1.86            | 43    | ND          | ND        | ND    |
| Q357A             | 97         | 2.38           | 2.45            | 57    | 580         | 598       | 79    |
| A365S             | 91         | 4.61           | 5.07            | 118   | 797         | 876       | 115   |
| I359A             | 66         | 2.07           | 3.14            | 73    | 375         | 572       | 75    |
| V360A             | 84         | 1.62           | 1.93            | 45    | 312         | 371       | 49    |
| Q361A             | 97         | 1.44           | 1.48            | 35    | 265         | 276       | 36    |
| K362A             | 93         | 1.25           | 1.34            | 31    | 225         | 242       | 32    |
| L363A             | 53         | 1.52           | 2.87            | 67    | 361         | 681       | 89    |
| S364A             | 98         | 4.83           | 4.93            | 115   | 1059        | 1081      | 141   |
| A365S             | 87         | 4.30           | 4.94            | 115   | 826         | 949       | 124   |
| I366A             | 54         | 1.77           | 2.88            | 77    | 326         | 604       | 79    |
| E367A             | 59         | 1.16           | 1.97            | 48    | 374         | 624       | 82    |
| S368A             | 85         | 3.91           | 4.60            | 107   | 859         | 1010      | 133   |
| L369A             | 86         | 0.46           | 0.53            | 12    | ND          | ND        | ND    |
| A370S             | 84         | 3.79           | 4.51            | 105   | 802         | 955       | 125   |
| G371A             | 93         | 3.67           | 3.95            | 92    | 590         | 634       | 83    |
| V372A             | 101        | 1.72           | 1.70            | 40    | 228         | 226       | 30    |
| E373A             | 87         | 4.26           | 4.90            | 114   | 915         | 1055      | 138   |
| I374A             | 55         | 0.37           | 0.67            | 16    | ND          | ND        | ND    |
| L375A             | 36         | 0.42           | 1.17            | 27    | ND          | ND        | ND    |
| Additional substitutions | | | | | | | |
| I359F             | 98         | 0.25           | 0.26            | 6     | ND          | ND        | ND    |
| S364D             | 110        | 0.84           | 0.85            | 20    | 198         | 180       | 24    |
| S364E             | 113        | 2.41           | 2.13            | 50    | 414         | 366       | 48    |
| S364F             | 107        | 1.27           | 1.19            | 28    | 154         | 144       | 19    |
| S364K             | 79         | 1.85           | 2.34            | 55    | 463         | 586       | 77    |
| S364L             | 78         | 1.57           | 2.01            | 47    | 197         | 253       | 33    |
| S364F             | 75         | 1.28           | 1.71            | 40    | 332         | 443       | 58    |
| A365F             | 83         | 1.29           | 1.55            | 36    | 262         | 316       | 42    |
| A365L             | 86         | 0.72           | 0.84            | 20    | 113         | 131       | 17    |
| A370F             | 43         | 0.33           | 0.77            | 18    | ND          | ND        | ND    |
| A370L             | 49         | 0.17           | ND              | ND    | ND          | ND        | ND    |
| V372F             | 20         | 0.13           | ND              | ND    | ND          | ND        | ND    |

* The amount of mutant H⁺-ATPase in isolated secretory vesicles was determined by quantitative immunoblotting and calculated as a percent of the wild-type control (30).

* Vanadate-sensitive ATP hydrolysis was measured as described under "Experimental Procedures." One unit is defined as 1 μmol of P₈/min. Values are reported with and without correction for the amount of mutant H⁺-ATPase expressed in the secretory vesicles.

* The initial rate of fluorescence quenching (proton transport) was determined as previously described (29). One unit is defined as 1% of total fluorescence quenching/min.

* ND, not determined. Corrections for expression have not been made for mutants with measured ATPase activities below 4% of the wild-type control.

above background, but the activities were so low that quantitative determinations were not possible. In one mutant (E367A), the initial rate of proton transport (85% of wild type) appeared to exceed the rate of ATP hydrolysis (46% of wild type). Closer examination of this mutant seemed warranted, given the fact that Glu-367 is strongly conserved among P₂-type ATPases. Here, a striking result was obtained; of the 21 mutants studied, 9 showed a very significant degree of vanadate resistance, with IC₅₀ values above 10 μM (Table II, top part). The resistant mutations began at position 360 and continued (with interruptions) through position 374. Upon closer inspection of this region, it was apparent that 4 of the 6 mutants still sensitive to vanadate carried substitutions of Ser for Ala or Ala for Ser. Because a less conservative substitution at one of these positions (S368F) had previously been shown to produce a highly vanadate-resistant enzyme (34), it seemed worthwhile to make further replacements of Ser for Ala or Ala for Thr. Among the additional mutants that were tested, all but one (V372F) were expressed in secretory vesicles at 43% or more of the wild-type level, and all but two (A370L and V372F) had measurable rates of ATP hydrolysis (Table I, bottom part). Furthermore, for every mutant with sufficient activity to be assayed, there was a close correspondence between the initial rate of ATP hydrolysis and the initial rate of ATP-dependent proton transport (Table I), indicating that these substitutions (like the initial set described above) had little or no effect on the coupling between hydrolysis and transport.

Vanadate Resistance—Each of the mutant ATPases was next examined for changes in sensitivity to inorganic orthovanadate, an inhibitor that binds tightly to the E₃ conformation of P-type ATPases. Here, a striking result was obtained; of the 21 mutants studied, 9 showed a very significant degree of vanadate resistance, with IC₅₀ values above 10 μM (Table II, top part). The resistant mutations began at position 360 and continued (with interruptions) through position 374. Upon closer inspection of this region, it was apparent that 4 of the 6 mutants still sensitive to vanadate carried substitutions of Ser for Ala or Ala for Ser. Because a less conservative substitution at one of these positions (S368F) had previously been shown to produce a highly vanadate-resistant enzyme (34), it seemed worthwhile to make further replacements of Ser for Ala or Ala for Thr. Among the additional mutants that were tested, all but one (V372F) were expressed in secretory vesicles at 43% or more of the wild-type level, and all but two (A370L and V372F) had measurable rates of ATP hydrolysis (Table I, bottom part). Furthermore, for every mutant with sufficient activity to be assayed, there was a close correspondence between the initial rate of ATP hydrolysis and the initial rate of ATP-dependent proton transport (Table I), indicating that these substitutions (like the initial set described above) had little or no effect on the coupling between hydrolysis and transport.

In five of the additional mutants (I359F, S364D, A365F, A365L, and A370F), the IC₅₀ value for vanadate rose above 10 μM (Table II, top part).
mM (Table II, bottom part), confirming that substantial vanadate resistance could be observed at these positions as well. The distribution of resistance along S4 is illustrated in Fig. 3A, which demonstrates that the 13-amino acid stretch from Ile-359 through Gly-371 can undergo mutations that elevate the IC$_{50}$ more than 5-fold above the wild-type value.

Other Kinetic Properties—In previous mutagenesis studies of the yeast H$^+$-ATPase, vanadate resistance has frequently been accompanied by a reduction in the apparent $K_m$ value for MgATP and a rise in pH optimum (29, 30). This “coordinated” phenotype has been interpreted as a shift in equilibrium from the vanadate-sensitive E$_2$ conformation toward the E$_1$ conformation, where MgATP and the transported proton are expected to bind with higher affinity than in E$_2$. In the present study, 6 of the 14 vanadate-resistant mutants in the stalk 4 region (I359F, S364D, I366A, E367A, L369A, and A370F) displayed $K_m$ values of 0.5 mM or lower, representing at least a 3-fold change from the value seen in the wild-type control; six additional mutants (Q361A, K362A, L363A, A365F, A365L, and I374A) gave smaller but reproducible reductions to 0.6–0.9 mM (Table II). Thus, a strong but not complete correlation could be seen between vanadate resistance and a decreased $K_m$ for MgATP (Fig. 3B).

Not surprisingly, given the many ways in which the pH dependence of the H$^+$-ATPase might be altered, the situation was less clear-cut with regard to pH optimum. Four of the vanadate-resistant mutants (L363A, I366A, E367A, and A370F) displayed a conspicuous alkaline shift of 0.3 to 0.6 pH units.

![Fig. 2. Coupling between ATP hydrolysis and proton transport in S4 mutants.](image)

![Fig. 3. Kinetic parameters for mutants of stalk segment 4.](image)
units (Table II). The rest retained an essentially normal pH optimum, except for a few with a minor shift in the acid direction (e.g., K362A).

E₁ to E₂ Conformational Change Assessed by Limited Trypsinolysis—If the cluster of kinetic changes seen in many of the stalk 4 mutants reflects a shift in equilibrium from the vanadate-sensitive E₂ conformation toward the vanadate-insensitive E₁ conformation, it should be possible to detect the shift by limited trypsinolysis (29, 30). In the experiment of Fig. 4, secretory vesicles were incubated with trypsin for 20 min in the presence of 0, 1, 10, or 100 μM vanadate. The reaction was terminated by the addition of 1 mM diisopropyl fluorophosphate as described under “Experimental Procedures.” The fragment pattern was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting. WT, wild type.

**FIG. 4.** Trypsinolysis of I366A and E367A. Secretory vesicles were incubated for 20 min at a trypsin/protein ratio of 1:4 in the presence of 0, 1, 10, or 100 μM vanadate, and the reaction was terminated by the addition of 1 mM diisopropyl fluorophosphate as described under “Experimental Procedures.” The fragment pattern was analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting. WT, wild type.

**FIG. 5.** Location of vanadate-resistant mutations in the yeast plasma-membrane H⁺-ATPase. Mutations that confer a 5-fold or greater increase in vanadate resistance are indicated by solid circles. P, phosphorylation site (Asp-378).

DISCUSSION

This study reports an impressive stretch of 13 consecutive vanadate-resistant mutations in stalk segment 4 of the yeast PMA₁ H⁺-ATPase. While similar mutations have been described previously for the yeast ATPase (29, 30, 32, 35–37), they have been scattered throughout the 100-kDa polypeptide, with no discernible structural or functional pattern (Fig. 5). The cluster in S₄ therefore deserves special interest.

At the structural level, S₄ is customarily depicted as an α-helix, acting with three other stalk helices to connect the cytoplasmic and membrane-embedded portions of the ATPase. Evidence for the α-helical nature of S₄ came originally from the use of standard algorithms (as reviewed in Ref. 38) and has since been reinforced by cryoelectron microscopy of the sarcoplasmic reticulum Ca²⁺-ATPase, where an α-helical backbone was found to fit comfortably into each of four rod-like densities within the stalk region (5). Significantly, however, the residues represented by the vanadate-resistant mutants from Ile-359 through Gly-371 would not be restricted to one face of such a
helix; instead, they would occupy three full turns near the middle of S4.

Functionally, it seems likely that the mutations cause a shift in E1-E2 equilibrium toward the vanadate-insensitive E1 conformation, given that most of them display coordinated changes in the $K_m$ value for MgATP and the pH optimum of the H$^+$-ATPase. In an earlier paper (29), we described three similar mutants, spaced at intervals along transmembrane segment M4 (I332A, V336A, and V341A). Likewise, Blostein and co-workers (39, 40) have pointed out that a shift in E1-E2 equilibrium could explain the kinetic behavior of two Na$^+$,K$^+$-ATPase mutants: E233K, located in the M2-M3 cytoplasmic loop (39, 40), and a1M2, a deletion mutant lacking 32 amino acids at the N terminus. In both mutants, vanadate resistance was accompanied by a decrease in the $K_m$ value for MgATP and by marked K$^+$ activation of Na-ATPase activity at micromolar ATP concentrations, a condition under which the E2(K) to E1 step is normally rate-limiting.

The concentration of E1-E2 mutants in stalk segment 4 of the yeast H$^+$-ATPase suggests that S4 may provide a critical, conformational mobile link between the cytoplasmic phosphorylation site and cation-binding sites in the membrane. Independent evidence for this idea has come from mutagenesis studies by Inesi and co-workers (41) on stalk segment 4 of the sarcoplasmic reticulum Ca$^{2+}$-ATPase. Here, single substitutions of conserved S4 residues significantly slowed the rate of ATP hydrolysis and Ca$^{2+}$ uptake, even though measurable levels of phosphorylated intermediate were formed (41). Single substitutions of non-conserved residues were less damaging, but multiple substitutions of such residues interfered with the Ca$_2^+$,E$_2$P to Ca$_2^+$,E$_2$ transition (as reflected by the rate of phosphoenzyme turnover) and the E$_1$ to E$_2$ transition (as reflected by the time course of EGTA inactivation, Ref. 42). Thus, once again it seems likely that stalk 4 has a profound influence on rate-limiting conformational transitions.

To visualize the way in which S4 performs this function, high resolution structures for both the E1 and E2 conformations will be required. Reporter groups engineered into specific locations along S4 will also help to track the conformational changes; studies along these lines are presently under way in several laboratories including our own.

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