Reactive Cysteines of the Yeast Plasma-Membrane H\textsuperscript+-ATPase (PMA1)

MAPPING THE SITES OF INACTIVATION BY N-ETHYLMALEIMIDE*

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We have taken advantage of cysteine mutants described previously (Petrov, V. V., and Slayman, C. W. (1995) J. Biol. Chem. 270, 28535-28540) to map the sites at which N-ethylmaleimide (NEM) reacts with the plasma-membrane H\textsuperscript+-ATPase (PMA1) of Saccharomyces cerevisiae. When membrane vesicles containing the ATPase were incubated with NEM, six of nine mutants with single cysteine substitutions showed sensitivity similar to the wild-type enzyme. By contrast, C221A and C532A were inactivated more slowly than the wild-type control, and the C221, 532A double mutant was completely resistant, indicating that Cys-221 and Cys-532 are NEM-reactive residues. In the presence of 10 mM MgADP, the wild-type ATPase was partially protected against NEM; parallel experiments with the C221A and C532A mutants showed that the protection occurred at Cys-532, located in or near the nucleotide-binding site.

Unexpectedly, the inactivation of the C409A ATPase was 4-fold more rapid than in the case of the wild-type enzyme. Experiments with double mutants made it clear that this resulted from an acidic shift in pK\textsubscript{a} and a consequent acceleration of the reaction rate at Cys-532. One simple interpretation is that substitution of Cys-409 leads to a local conformational change within the central hydrophilic domain. Consistent with this idea, the reaction of fluorescein 5'-isothiocyanate at Lys-474 was also stimulated 3.5-fold by the C409A mutation. Taken together, the results of this study provide new information about the reactivity of individual Cys residues within the ATPase and pave the way to tag specific sites for structural and functional studies of the enzyme.

P-ATPases, named because they split ATP via a covalent phosphorylated reaction intermediate, are found throughout prokaryotic and eukaryotic cells, where they transport cations as diverse as H\textsuperscript{+}, Na\textsuperscript{+}, K\textsuperscript{+}, Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, Cu\textsuperscript{2+}, and Cd\textsuperscript{2+} (1, 2). Hallmarks of the group include a 100-kDa catalytic polypeptide, embedded in the membrane by hydrophobic segments at the N- and C-terminal ends (3, 4), conserved sequences for ATP binding and formation of the β-aspartyl phosphate intermediate (3, 5–7), and sensitivity to micromolar concentrations of inorganic orthovanadate (8).

Over the past 2 decades, studies on the structure and reaction mechanism of the P-ATPases have drawn upon a variety of group-specific reagents, including sulfhydryl reagents such as N-ethylmaleimide (NEM). Efforts focused initially on measuring the number of NEM-reactive sites and classifying their properties. Of the >20 sulfhydryl groups now known to be present in Na\textsuperscript{+}, K\textsuperscript{+}-ATPase and the Ca\textsuperscript{2+}-ATPase of sarcoplasmic reticulum, very few proved to be readily accessible to NEM (9–11). In the former case, two "highly reactive" sites could be modified by NEM without loss of ATPase activity (11), and one of the two was protected by ATP, suggesting that it may lie in or near the catalytic site of the enzyme (12). In the latter case, there were also two highly reactive Cys residues, now known to flank the phosphorylation site (Cys-344... Asp-351... Cys-364); interestingly, either of the two sites could be modified by NEM without effect on ATPase activity, but when the second site was also modified, the enzyme was inactivated (13, 14). More recently, studies with NEM have been followed by studies with fluorescent and spin-label sulfhydryl reagents aimed at tagging the Na\textsuperscript{+}, K\textsuperscript{+} - and Ca\textsuperscript{2+}-ATPases for structural and mechanistic investigations (e.g. Refs. 15–22).

In parallel with the work on mammalian ATPases, the PMA1 H\textsuperscript{+}-ATPase of the fungal plasma membrane has offered a somewhat simpler experimental system in which to explore the properties of Cys residues. The Neurospora crassa enzyme, for example, contains only eight cysteines (23, 24). Brooker and Slayman (25–27) demonstrated that the ATPase was inactivated by NEM and used measurements of [\textsuperscript{14}C]NEM incorporation to define two sites on the enzyme. The first, later named the "fast" site by Davenport and Slayman (28) and identified as Cys-545 by Pardo and Slayman (29), could be labeled by NEM in several minutes at pH 8.0 and 0 °C, with no detectable effect on ATPase activity. By contrast, the second ("slow") site, later found to be Cys-532 (29), required tens of minutes to react fully with NEM, and its modification led to loss of activity. The slow site was protected by MgATP or MgADP, with dissociation constants close to the K\textsubscript{m} and K\textsubscript{V} values for those nucleotides.

Davenport and Slayman (28) used a wide array of sulfhydryl reagents to characterize the reactivity of the two sites in greater detail, and Chang and Slayman (30) found by means of limited trypsinolysis and competitive enzyme-linked immunoabsorbent assays that the reaction of the slow site with NEM...
Mapping NEM Sites on the Yeast PMA1 ATPase

### Table I
**Effect of multiple cysteine replacements on PMA1 ATPase**

| Mutation     | Expressiona | ATPase (corrected)b | K_m | K_l | pH optimum | H⁻ transportc (corrected) |
|--------------|-------------|---------------------|-----|-----|------------|-------------------------|
| Wild type    | 100     | 100                | 0.6 | 1.8 | 5.7       | 100                     |
| C221,409A    | 106     | 58                 | 0.8 | 2.8 | 5.7       | 73                      |
| C221,532A    | 96      | 107                | 0.4 | 2.5 | 5.8       | 104                     |
| C409,532A    | 97      | 92                 | 0.5 | 2.5 | 6.1       | 107                     |
| C221,409,532A| 106    | 94                 | 0.4 | 2.6 | 5.9       | 99                      |

a The specific expression of 100-kDa ATPase protein was calculated by quantitative immunoblotting (31).

b ATPase activity was measured as described previously (51). The series of experiments included nine preparations of wild-type vesicles with an average ATPase activity of 4.01 ± 0.20 μmol P/mg · min. Data for mutant ATPases are the average of 3–4 determinations except in the case of C221,409,532A, for which there were two determinations; each mutant value was corrected for expression relative to a wild-type control run in parallel on the same day.

c Fluorescence quenching of acridine orange was used to monitor proton pumping as described in Ref. 31. Freshly prepared secretory vesicles (50 μg) were suspended in buffer containing 5 mM ATP, and after stabilization of baseline fluorescence, proton pumping was initiated by the addition of 5 mM MgCl₂. At the steady state, acridine orange quenching by the wild-type enzyme averaged 515 ± 65% quenching/mg; quenching for each mutant is expressed as a percent of wild-type control run on the same day.

led to a pronounced change in tertiary conformation.

In the present study, we have turned to the closely related PMA1 ATPase of *Saccharomyces cerevisiae*, taking advantage of a recently constructed panel of site-directed mutants in which single or multiple Cys residues were replaced by Ala or Ser (31). By examining the response of these mutants to NEM, it was possible to locate two reactive sites in the yeast enzyme, one protectable by ADP (Cys-532) and the other not (Cys-221). Unexpectedly, mutation at a third site (Cys-409) enhanced the rate of inactivation by NEM compared to the wild-type enzyme (31). This series of experiments showed that dimethyl sulfide (0.02% reagent in the assay mixture) did not affect ATPase activity.

**Protein Assay**—Protein concentrations were determined by the method of Lowry et al. (34) and/or Bensadoun and Weinstein (35) using bovine serum albumin as standard.

### RESULTS

**Effect of NEM on the Wild-type Yeast ATPase**—Initially, experiments were carried out to determine the effect of NEM on the wild-type PMA1 ATPase from *S. cerevisiae*. Secretory vesicles were preincubated with NEM at pH 7.0 and 30 °C for 0 to 4 min (see “Experimental Procedures”). At each time point, the reaction was stopped by 100-fold dilution into assay mixture at pH 5.7, and the remaining ATPase activity was measured.

**NEM Inactivation of Mutant ATPases Lacking Individual Cys Residues**—The next step was to compare a series of mutant ATPases, each carrying a substitution of a single Cys by Ala. As shown in Table II, when vesicles containing the various mutant enzymes were treated with 2 mM NEM for 0 to 4 min, three different patterns of inactivation were seen. For six of the nine mutants (C148A, C312A, C376A, C472A, C569A, and C867A; rate constant = 0.290 to 0.389 min⁻¹), there was little or no change from the wild-type ATPase (rate constant = 0.307 min⁻¹). In two other mutants (C221A and C532A; rate constants = 0.169 and 0.138 min⁻¹), inactivation was significantly slower. And finally, one alanine substitution (C409A) led to a surprising increase in the rate constant; experiments addressing the mechanism of the Cys-409 effect will be described in a later section.

The simplest interpretation of the results for Cys-221 and Cys-532 is that NEM can react at either of the two sites to inhibit ATPase activity. If so, a double mutant lacking both cysteines should be virtually insensitive to NEM, as indeed observed in the experiment of Fig. 2. The rate constant for inactivation of the C221,532A double mutant was 0.022 min⁻¹, similar to that for a C221,409,532A triple mutant (0.025
Rate constants of inhibition of wild-type and mutant ATPases by NEM and FITC

TABLE II

| NEM (mM) | FITC |
|----------|------|
| Wild type | 0.307 ± 0.020 | 0.278 ± 0.014 |
| A. Single cysteine mutants | | |
| C148A | 0.389 ± 0.013 | 0.274 ± 0.031 |
| C221A | 0.169 ± 0.001 | 0.301 ± 0.017 |
| C312A | 0.299 ± 0.004 | 0.261 ± 0.042 |
| C376A | 0.319 ± 0.013 | 0.254 ± 0.039 |
| C409A | 1.350 ± 0.090 | 0.944 ± 0.119 |
| C409S | 0.732 ± 0.023 | 0.610 ± 0.031 |
| C472A | 0.324 ± 0.026 | 0.210 ± 0.056 |
| C532A | 0.138 ± 0.002 | 0.198 ± 0.035 |
| C569A | 0.290 ± 0.016 | 0.265 ± 0.069 |
| C867A | 0.309 ± 0.014 | 0.231 ± 0.066 |
| B. Multiple cysteine mutants | | |
| C221,532A | 0.022 ± 0.008 | – |
| C221,409A | 1.706 ± 0.237 | – |
| C409,532A | 0.141 ± 0.032 | – |
| C221,409,532A | 0.026 ± 0.005 | – |
| “One-cysteine” (Cys-409) | 0.015 ± 0.006 | – |

min⁻¹ and a multiple mutant containing only Cys-409 (0.015 min⁻¹; Table II).

ADP Protection—As discussed earlier, NEM inactivation of the Neurospora PMA1 ATPase at Cys-532 can be almost completely prevented by MgADP or MgATP (25–29). Fig. 3A illustrates that the situation was slightly different for S. cerevisiae, where MgADP protected the wild-type enzyme only partially. Once again, however, the effect could be traced to Cys-532, this time through experiments with mutant ATPases. Protection was nearly complete in the case of the C221A mutant (containing Cys-532; Fig. 3B) but was virtually absent for the C532A mutant (containing Cys-221; Fig. 3C).

NEM Sensitivity of Mutants Lacking Cys-409—As described above, replacement of Cys-409 by Ala had an unexpected effect on the yeast ATPase, producing a 4.4-fold increase in the NEM rate constant (from 0.307 to 1.350 min⁻¹; Table II). Replacement by Ser had a similar although smaller effect, raising the rate constant 2.4-fold to 0.732 min⁻¹.

Here again, it was possible to gain insight into the mechanism of the effect by comparing a set of mutants. As shown in Fig. 4, the C221,409A double mutant (containing Cys-532) displayed a markedly faster rate constant of inactivation than the C221A single mutant (1.706 min⁻¹ versus 0.169 min⁻¹; Table II). By contrast, the C409,532A double mutant and the C532A single mutant, both containing Cys-221, were virtually identical (0.141 min⁻¹ versus 0.138 min⁻¹; Table II; Fig. 4C). Thus, it seems clear that amino acid substitution at Cys-409 somehow enhances the reactivity of Cys-532.

To explore further the effect of the C409A mutation on the properties of Cys-532, the pH dependence of NEM inactivation was examined. Wild-type and mutant vesicles were exposed to 0.025 to 2 mM NEM for 0–4 min over a range of pH values, from pH 7 to 10, and rate constants for inactivation were calculated. As shown in Fig. 5, there was a similar pH dependence for the wild-type and C221A ATPases, suggesting that Cys-221 and Cys-532 have nearly identical pKₐ values. The C409A mutation had no detectable effect on Cys-221 (in the C409,532A double mutant) but caused a sizable acidic shift in the pH dependence of Cys-532 (in the C221,409A double mutant), consistent with a decrease in pKₐ from −9.9 to −8.9.

Trypsinolysis Experiments—To ask whether the replacement of Cys-409 might lead to a structural abnormality in the ATPase, limited proteolysis was used to probe the accessibility of Arg and Lys residues to trypsin in the wild-type and C409A enzymes. The results gave no indication of a major structural
change; both enzymes displayed essentially identical rates and patterns of degradation when incubated at a trypsin:protein ratio of 1:15 for 0–7.5 min. Likewise, there was no difference in the ability of vanadate (1–100 \( \mu M \)) or MgADP (2.5–10 mM) to protect the wild-type and mutant enzymes against trypsin (not shown).

Role of Cys-409 in Sensitivity to FITC—Evidence for a more subtle change, possibly involving only a small region of the polypeptide, came from experiments with fluorescein 5'-isothiocyanate (FITC). This inhibitor has been shown to react quite specifically with Lys-474 of the Neurospora PMA1 ATPase (36), a residue located mid-way between Cys-409 and Cys-532 in the linear sequence. As expected, the \( \text{S. cerevisiae} \) ATPase was also sensitive to FITC (0.2 mM), with a rate constant of 0.278 min\(^{-1}\) (Table II; Fig. 6). Very similar values were measured for six of the single mutants (C148A, C221A, C312A, C376A, C569A, C867A; 0.231 to 0.301 min\(^{-1}\)), whereas two mutants were marginally less sensitive (C472A, C532A; 0.210 and 0.198 min\(^{-1}\), respectively). In C409A, however, the rate constant increased 3.4-fold to 0.944 min\(^{-1}\), giving evidence for a marked enhancement of reactivity with FITC. Once again, replacement by Ser had a smaller but still significant effect, raising the rate constant 2.2-fold to 0.610 min\(^{-1}\).

DISCUSSION

Conservation and Function of Cysteine Residues—As pointed out above, an advantage of working with the fungal PMA1 H\(^+-\)ATPases is that they contain relatively few cysteines, allowing the properties of individual residues to be dissected with relative ease. Six of the cysteines found in the \( \text{S. cerevisiae} \) enzyme (Cys-148 in membrane segment 2; Cys-376, Cys-409, Cys-472, and Cys-532 in the central catalytic region; and Cys-
867 in membrane segment 10) have been completely conserved throughout the fungal PMA enzymes for which sequences are currently available (2, 37). The remaining three (Cys-221 in the small hydrophilic loop between segments 2 and 3; Cys-312 in segment 3; and Cys-569 toward the end of the central catalytic region) have varied, and additional Cys residues have appeared at as many as four additional sites (in the ATPase from *H. capsulatum*). Thus, useful hints at function can come from sequence comparisons, supplemented by evidence from site-directed mutagenesis (e.g. Refs. 31, 38, 39).

Because of their reactivity, cysteines can serve as specific points for covalent labeling by radioactive, fluorescent, and spin-labeled SH-reacting compounds, which can then be used to probe three-dimensional structures and to detect intramolecular conformational changes during ATP-dependent cation transport. With this long-range aim in mind, the current study has focused on mapping the sites at which NEM combines with the *S. cerevisiae* PMA1 ATPase. The results have shown three of the nine cysteines to be important for NEM reactivity.

**Role of Cys-221 and Cys-532**—Based on the reduction of NEM sensitivity when Cys-221 and Cys-532 are replaced by Ala, it is clear that NEM reacts at both sites to inactivate the enzyme (Fig. 7). Like its counterpart in the closely related *Neurospora* PMA1 ATPase (29), Cys-532 of the *S. cerevisiae* enzyme is protected by ADP, a reasonable finding in view of its closeness to the highly conserved nucleotide-binding region (5, 40). Recent biochemical data suggest that Cys-532 of the *Kluyveromyces lactis* PMA1 ATPase is also an ADP-protectable NEM site (41). By contrast, Cys-221 in *S. cerevisiae* and *K. lactis* is not protected by ADP nor is Cys-545 in *Neurospora* (26–29).

**Role of Cys-409**—A particularly interesting finding in the present study concerns Cys-409, located between the phosphorylation site and the ATP-binding domain. Substitution of Cys-409 by Ala sharply enhanced both the reactivity of Cys-532 toward NEM and the reactivity of Lys-474 toward FITC; substitution by Ser had similar, although less pronounced, effects.

There is no reason *a priori* to expect that Cys-409 should play a particularly important structural or functional role, given that it lies in a poorly conserved region of the overall sequence. Even though a cysteine residue is found in this position in all of the known fungal H^+^-ATPases and in the Ca^2+^-ATPase of sarcoplasmic reticulum, other P-ATPases have widely varying residues, ranging from Ser and Ala to Val, Ile, Leu, Met, and even Arg (1). Structurally, however, Cys-409 lies in a putative α-helical stretch designated by Green (5) as the C-terminal portion of the phosphorylation domain. Also significant may be its location just upstream of tryptic cleavage sites that are believed to demarcate the phosphorylation and nucleotide-binding domains of the Na^+^,K^+^- and Ca^2+^-ATPases (5, 42).

Given the effect of the C409A mutation on the reactivity of two downstream sites, Lys-474 and Cys-532, it seems reasonable to suggest that the mutation leads to a conformational change in this portion of the protein. Further evidence for interactions over the same region comes from a recent study by Maldonado and Portillo (43), who selected second-site suppressor mutations based on their ability to restore growth of a defective K474R mutant. Of the seven suppressors examined, one (V396I) mapped only 13 residues away from Cys-409, and a second (P536L) was even closer to Cys-532. (Three others, V484I, V484I/E485K, and E485K/E486K, were immediately
adjacent to Lys-474, whereas the remaining two, A165V and V169I/D170N, were in more distant parts of the ATPase.) The exact nature of the conformational change brought about by the C409A mutation can only be guessed at, but a useful clue comes from the apparent decrease in $K_m$ of Cys-532 from 9.9 to 8.9. The decrease could be accounted for at least qualitatively by the movement of Cys-532 from a hydrophobic to a hydrophilic environment, particularly if it is brought into a region of strong positive potential (in fact, an RKKK motif lies only four residues from Cys-409 in all fungal PMA ATPases). Similar changes in the $K_m$ of individual amino acid residues have been deliberately brought about in other proteins in this way, either by site-directed mutagenesis of a nearby residue as in bacterial subtilisin (44) or by denaturation, as in barley chymotrypsin inhibitor 2 (45), and have been modeled quantitatively in terms of electrostatic effects.

Worth mentioning is the fact that, although the substitution of Cys-409 by Ala has clear-cut consequences as far away as Lys-474 and Cys-532, the enzymatic properties of the C409A mutant ATPase are nearly normal, with a specific activity for ATP hydrolysis that is only slightly reduced (to 74% of the wild-type value) and a $K_m$ for vanadate (2.8 $\mu$M versus 1.2 $\mu$M in wild type) that are only slightly elevated (31). Likewise, the C409A mutation can only be guessed at, but a useful clue comes from the apparent decrease in $pK_a$ of Cys-532 from 9.9 to 8.9. The decrease could be accounted for at least qualitatively by the movement of Cys-532 from a hydrophobic to a hydrophilic environment, particularly if it is brought into a region of strong positive potential (in fact, an RKKK motif lies only four residues from Cys-409 in all fungal PMA ATPases). Similar changes in the $K_m$ of individual amino acid residues have been deliberately brought about in other proteins in this way, either by site-directed mutagenesis of a nearby residue as in bacterial subtilisin (44) or by denaturation, as in barley chymotrypsin inhibitor 2 (45), and have been modeled quantitatively in terms of electrostatic effects.

As soon as a high resolution structure becomes available for the N. crassa or S. cerevisiae enzyme, it will be interesting to see whether Cys-409 does in fact lie close to Lys-474 and/or Cys-532. In the meantime, it may be possible to exploit the C409A mutation to stimulate labeling at these two positions for structural and functional studies.

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