Site-directed Mutagenesis of the Cysteine Residues in the Neurospora crassa Plasma Membrane H\(^{+}\)-ATPase*

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A high-yield yeast expression system for site-directed mutagenesis of the Neurospora crassa plasma membrane H\(^{+}\)-ATPase has recently been reported (Mahanty, S. K., Rao, U. S., Nicholas, R. A., and Scarborough, G. A. (1994) J. Biol. Chem. 269, 17705-17712). Using this system, each of the eight cysteine residues in the ATPase was changed to a serine or an alanine residue, producing strains C148S and C148A, C376S and C376A, C409S and C409A, C472S and C472A, C532S and C532A, C545S and C545A, C840S and C840A, and C869S and C869A, respectively. With the exception of C376S and C532S, all of the mutant ATPases are able to support the growth of yeast cells to different extents, indicating that they are functional. The C376S and C532S enzymes appear to be non-functional. After solubilization of the functional mutant ATPase molecules from isolated membranes with laurylthiol, all behaved similar to the native enzyme when subjected to glycerol density gradient centrifugation, indicating that they fold in a natural manner. The kinetic properties of these mutant enzymes were also similar to the native ATPase with the exception of C409A, which has a substantially higher \( K_m \). These results clearly indicate that none of the eight cysteine residues in the H\(^{+}\)-ATPase molecule are essential for ATPase activity, but that Cys\(^{376}\), Cys\(^{409}\), and Cys\(^{545}\) may be in or near important sites. They also demonstrate that the previously described disulfide bridge between Cys\(^{409}\) and Cys\(^{840}\) or Cys\(^{869}\) plays no obvious role in the structure or function of this membrane transport enzyme.

The plasma membrane H\(^{+}\)-ATPase of Neurospora crassa is an electrogenic (1) proton pump (2), that belongs to the P-type family of ion translocating ATPases that form an aspartyl phosphate intermediate in their reaction cycle (3, 4). This ATPase is closely related to the yeast and plant plasma membrane H\(^{+}\)-ATPases (5–9). Many studies involving chemical modification of the cysteine residues in the Neurospora H\(^{+}\)-ATPase have been reported (10–17). The sulfhydryl reagent, N-ethylmaleimide was originally shown to have no effect on the H\(^{+}\)-ATPase activity (10), but subsequent studies reported inhibition by N-ethylmaleimide when treatments were carried out at elevated pH values (11–16). More recent studies of the effect of N-ethylmaleimide on the ATPase have been interpreted to indicate that Cys\(^{332}\) and Cys\(^{845}\) residues are near important sites (15, 16), but similar studies with methylmethanethiosulfonate have shown that neither of these cysteines nor cysteines at positions 376, 409, and 472 play a covalent role in the reaction cycle of the enzyme. In another study (17), the chemical states of the eight cysteine residues in the H\(^{+}\)-ATPase molecule were determined, establishing the presence of a disulfide bridge between Cys\(^{148}\) and either Cys\(^{840}\) or Cys\(^{869}\). These studies provided valuable information as to the structure of the ATPase molecule but could not address the possible function of the disulfide bridge.

Site-directed mutagenesis is a powerful tool for exploring structure-function relationships in the H\(^{+}\)-ATPase by identifying individual amino acid residues that are important for its structure and activity. We have recently developed a yeast expression system for such studies of the Neurospora H\(^{+}\)-ATPase (18), and in this article, this system is used to further probe the roles of the eight cysteine residues in its structure and molecular mechanism.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Plasmid Constructions—For mutagenesis, we began with the plasmid pSKMHA2 (18), which contains the full-length N. crassa plasma membrane H\(^{+}\)-ATPase cDNA. The NotI site at the 3′ end of the H\(^{+}\)-ATPase cDNA in pSKMHA2 was removed by partially digesting with NotI, filling in the ends with DNA polymerase “Klenow” fragment, and religating with a SpeI linker (GAC-TAGTC), which resulted in plasmid pSKMHA9. pSKMHA9 was digested with either NotI and EcoRI, EcoRI and XbaI, XbaI and Smal, Smal and Sad, or Sad and SpeI, and each of the resulting ATPase cDNA cassettes was subcloned either into pBluescript or M13 mp18/19 vectors. Single stranded “U”-DNA was isolated from Escherichia coli CJ 236 (dut-), and amino acid substitutions were made using the oligonucleotide-directed site-specific in vitro mutagenesis technique of Kunkel (19). The mutagenic oligonucleotides were designed to singly substitute each Cys (TGC) codon with a Ser (AGC) or an Ala (GCC) codon or the active site Asp (GAC) codon to an Ala (GCC) codon. The mutated cassettes were completely sequenced to verify that only the planned changes had occurred. The cassettes were then ligated into the N. crassa H\(^{+}\)-ATPase cDNA in place of the wild type cassette in pSKMHA9. In all cases, mutagenesis was confirmed again by sequencing the mutated region prior to yeast transformation. Quaaprec plasmid spin kits (Quazen Inc., Chatsworth, CA) were routinely used for plasmid isolation from E. coli. Sequence analysis of single-stranded and double-stranded DNA was performed by the dideoxynucleotide chain termination method (20) using version 2.0 sequencing kit from U. S. Biochemical Corp.

Strains and Media—E. coli strains MC1061 and HB101 were used for normal cloning, and strain JM83 was used for transformation with Bluescript plasmids. Strains J M103 and CJ 236 were used for mutagenesis and for making single-stranded uracilated DNA templates, respectively. Saccharomyces cerevisiae strain RS-72 was transformed with the dideoxynucleotide chain termination method (20) using version 2.0 sequencing kit from U. S. Biochemical Corp.

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Transformation with the plasmid containing the ATPase insert with the codon for Cys148 changed to an Ala codon generated strain C148A, and all other strains were similarly named. Yeast transformants were selected and propagated on galactose medium and then transferred to glucose medium in order to express only the mutant Neurospora ATPases (18). The cells were maintained on agar plates at 4°C and were subcultured every 2 weeks or kept at -80°C in 15% (w/v) glycerol for 6–8 months. The growth media and cell culture procedures for membrane isolation were essentially as described (18). All cultures used for membrane preparations were tested for revertants by plating roughly 10^6 cells on glucose medium and inspecting for large colonies after 24 h. Essentially no revertants were seen.

Transformations—Transformations of E. coli cells were routinely done using standard protocols (21). Transformation of the yeast cells was carried out according to the procedure of Ito et al. (22) as described previously (18).

Growth Tests—Drop tests for growth were carried out for the different mutants and control strains as described (18). To ascertain the growth rates of the various strains in liquid glucose medium, the transformants were grown for 48 h in galactose medium (A_{600nm} approximately 3.0) at 30°C, and the cells were then transferred to glucose medium at a density of about 5 × 10^5 cells/ml, after which the growth was scored every 2–6 h as the A_{600nm}.

Isolation of Membranes and Purification of the Expressed H^+-ATPase Membrane fractions from different yeast transformants were prepared essentially as described (18), except that cell homogenization was carried out in a medium bead beater chamber and all of the solutions contained 2 μg/ml chymostatin. For membrane preparations from transformants that do not grow on glucose medium (strains C376S, C532S, and D378A), cells transferred from a master galactose plate were grown for 48 h at 30°C in 50 ml of galactose medium, diluted 1:10 in galactose medium, and grown for another 24 h, harvested aseptically, and transferred into an equal volume of double strength glucose medium (all components present in concentrations twice that previously described (18)) and grown for an additional 24 h as above. In certain experiments the total membrane preparations were fractionated by sucrose density gradient centrifugation as described by Villalba et al. (23). Solubilization of the membrane proteins with lysoseltin and purification of the various expressed H^+-ATPase molecules by glycerol density gradient centrifugation were carried out as described (18) in 12-mI 20–40% (w/w) linear glycerol gradients.

H^+-ATPase Assay and Protein Estimation—The plasma membrane ATPase activity was measured as described (18). For kinetic studies of the H^+-ATPase activity, equimolar concentrations of MgSO_4: Na_2ATP in the range of 0.25–14 mM were added and the assay mixture contained 2 mM of extra Mg_2+ (as MgSO_4) to compensate for the 2 mM EDTA present to complex traces of vanadate. For the vanadate sensitivity of the ATPase activities, a range of vanadate concentrations from 0.1 to 10 μM was added to the assay tubes and the EDTA was omitted. The specific activity, K_v, and V_{max} values were calculated by linear regression analyses of Lineweaver-Burk double reciprocal plots. The protein contents of the membranes and gradient fractions were determined by the Lowry procedure as modified by Bensadoun and Weinstein (24).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis, Staining, In-gels blotting, and Densitometric Analysis—SDS-PAGE was performed essentially as described by Laemmli (25) with minor modifications (26). Disaggregation of membrane suspensions or glycerol gradient fractions and electrophoresis of the samples was done as described (18) except that only high resolution gels were used. Silver staining and immunoblotting were performed as described (18).

For densitometric analysis of the amounts of mutant Neurospora ATPases present in the glycerol gradient fractions, aliquots of the pooled peak activity fractions (numbers 9, 10, 11, and 12 from the top) were first subjected to SDS-PAGE as described above, and the gels were silver-stained and photographed. The photographs were then scanned using a Bio-Rad Image Densitometer (Model GS-670) and the ATPase region was integrated using the Molecular Analyst TM/PC Software Analysis Software Version 1.1.1). The amounts of the mutant ATPases were calculated by comparison with 5, 10, and 20 ng samples of purified native Neurospora H^+-ATPase (27) present in the same gel.

Materials—Spot linker (phosphorylated 10-mer) was obtained from New England Biolabs. Disodium ATP, lysoseltin, and chymostatin were from Sigma. The sources of all other reagents were as described (18).

RESULTS

As described previously (18), in the yeast expression system used for these studies, both the wild type yeast H^+-ATPase and the plasmid-encoded Neurospora H^+-ATPase are produced when the cells are grown in medium containing galactose as the carbon source. When the cells are transferred to glucose medium, synthesis of the yeast ATPase ceases, which limits growth to only a few doublings unless a functional Neurospora H^+-ATPase is present. Fig. 1 shows these features with several control yeast strains used in this investigation. Growth of the parent strain SKM9 with an unmutated Neurospora H^+-ATPase is shown along with the growth of strain SKM3, which contains the expression plasmid with no ATPase cDNA, and strain D378A, which contains a cDNA in which the codon for the active site Asp^{178} of the Neurospora enzyme has been changed to an Ala codon. The parent strain grows well, whereas both of the other control strains grow for only a few doublings and then stop growing as expected in glucose medium. Growth in glucose medium is thus a convenient screening assay for the functionality of the Neurospora H^+-ATPase mutants we produce.

Fig. 1. Growth of several control yeast strains. Shown are the growth characteristics of several control yeast transformants in glucose medium assessed as described under "Experimental Procedures." Unmutated Neurospora H^+-ATPase producing strain, SKM9 (○); no ATPase, plasmid control strain SKM3 (□); active site aspartate ATPase mutant D378A (△).

Table I shows the growth rates of the control strains and the various cysteine mutant ATPase strains calculated from growth curves similar to those shown in Fig. 1. Both strain C148S and strain C148A grow at about the same rate as the unmutated ATPase control strain. Strain C376S does not grow any more than the negative control strains and strain C376A grows only very slowly. Strain C409S grows about the same as the unmutated ATPase strain, and significantly better than strain C409A. The other hand, strain C4725 grows substantially slower than strain C472A. Interestingly, strain C532S does not grow any more than the negative controls, whereas strain C532A grows well. Strains C545S and C840S both grow well whereas both strains C545A and C840A grow somewhat more slowly than their serine counterparts. Strain C869S grows at an intermediate rate whereas strain C869A grows at a rate about the same as that of the wild type strain SKM9.

To ascertain the amounts of the ATPase synthesized in the various strains, membranes were isolated from each of the mutants grown in glucose medium and analyzed by SDS-PAGE.
The levels of mutant Neurospora ATPase present in the membranes of these three strains were substantially lower, which precluded further analysis of these enzymes.

When solubilized with the detergent, lysolecithin, the native Neurospora H+-ATPase is a hexamer (28) that migrates to the bottom third of a 20–40% glycerol gradient (29). This sedimentation behavior provides a simple but effective assay for proper folding of the recombinant Neurospora H+-ATPase molecules produced in yeast. To assess the extent to which the various cysteine mutants fold properly, each of the mutant ATPases was solubilized from the membranes with lysolecithin and the solubilized extract was subjected to glycerol density gradient centrifugation as described (18). The results indicated that all Cys → Ala and the six remaining Cys → Ser and Cys → Ala mutant H+-ATPase molecules migrates quantitatively to a position identical to that of the SKM9 recombinant ATPase and the native enzyme (data not shown), providing strong evidence that each of these mutant ATPases folds correctly in theoretical yields.

In addition to providing a simple folding assay, lysolecithin solubilization and glycerol density gradient centrifugation produces substantially purified preparations of the mutant ATPases useful for further analysis. Fig. 2 shows the H+-ATPase region of SDS-PAGE analyses of the pooled peak glycerol gradient fractions from two different membrane preparations from each of the various Cys → Ser and Cys → Ala mutants and the control strains SKM9 and SKM3. In the region between the 97.4 and 116.2 kDa standards, three bands are routinely seen. The uppermost of these bands is the yeast H+-ATPase, the middle one is the recombinant Neurospora H+-ATPase, and the lower one is an unidentified band of no interest in these studies. The yeast ATPase band represents residual amounts of this enzyme present when the cells are transferred to glucose medium. The analyses in Fig. 2 show that the amounts of the Neurospora ATPase synthesized are generally reproducible for each strain. To quantitate the amount of each mutant H+-ATPase present in the pooled peak glycerol gradient fractions, the gels of Fig. 2 were subjected to densitometric analysis as described under “Experimental Procedures.” The amounts of the mutant ATPases produced in the various strains are listed in Table I. The amounts were variable from strain to strain in the range of roughly half to 1.6 times that of strain SKM9. The analyses also showed that none of the partially purified ATPase preparations contained more residual yeast H+-ATPase than that of the control strain SKM3, which contained only the yeast ATPase (Fig. 2, lanes 2).

With this information, the kinetic characteristics of the various partially purified Cys → Ser and Cys → Ala mutant H+-ATPases could be determined. Importantly, the pooled gradient fractions from the control strain SKM3 showed no ATPase activity in the assay system used. Therefore, since none of the other preparations contained more yeast ATPase than strain SKM3, the ATPase activities measured reflect only that of the Neurospora enzymes. Table I shows the kinetic constants determined for the ATPases from all of the viable mutant and control strains. For all mutant ATPases analyzed, the \( K_m \) and \( V_{max} \) values are not substantially altered except in the case of mutant C409A, which shows a greater than 10-fold increase in its \( V_{max} \) with no difference in \( K_m \). Although not shown, the vanadate sensitivities of all of the mutant enzymes were comparable to that in the wild type ATPase strain SKM9 (1.5-fold, about 1 \( \mu \)M).

\[ \text{TABLE I} \]

| Name of transformant | Growth rate* |
|---------------------|--------------|
| SKM9               | 19           |
| SKM3               | 0            |
| C376A              | 2            |
| C148S              | 21           |
| C376S              | 7            |
| C409S              | 17           |
| C409A              | 10           |
| C472S              | 11           |
| C532S              | 16           |
| C532A              | 0            |
| C545S              | 16           |
| C545A              | 12           |
| C689S              | 12           |
| C689A              | 19           |

* Growth was measured in liquid glucose medium as described under “Experimental Procedures.” The growth rates were calculated from the data of Fig. 1 and similar data from experiments with the cysteine mutant strains as the slope of the plots between 30 and 40 h × 1000.

**Fig. 2.** SDS-PAGE analysis of the pooled peak glycerol gradient fractions from strains SKM9, SKM3, and the various Cys → Ser and Cys → Ala H+-ATPase mutants. Panels A and B show silver-stained SDS-PAGE gel analyses of the pooled peak glycerol gradient fractions from two different experiments carried out on different days. Three mg of membrane protein from each strain was solubilized with lysolecithin (5 mg of lysolecithin/mg of membrane protein) and the solubilized extracts were layered on the top of 20–40% (w/v) linear glycerol gradients and centrifuged overnight as described under “Experimental Procedures.” One-mL fractions were collected and aliquots were assayed for ATPase activity. The peak fractions (9–12 from the top) were pooled and analyzed by SDS-PAGE. Lane 1 contained 10 \( \mu \)L of SKM9 pooled peak glycerol gradient fractions, lane 2 contained 10 \( \mu \)L of SKM3 pooled glycerol gradient fractions 9–12, and lanes 3–16 contained 10 \( \mu \)L of the pooled peak glycerol gradient fractions from mutants C148S, C409S, C472S, C545S, C840S, C869S, C148A, C376A, C409A, C472A, C532A, C545A, C840A, and C869A, respectively. Lanes 17–19 contained 5, 10, and 20 ng of purified native N. crassa H+-ATPase, respectively. The numbers to the left of panels A and B indicate the molecular masses of the SDS-PAGE standards used in kDa. Only the approximate 100-kDa H+-ATPase regions of the gels are shown.

**DISCUSSION**

The results obtained in these studies allow several important conclusions regarding the functions of the eight cysteine residues in the Neurospora H+-ATPase molecule. First, it is clear that none of the cysteines is essential for the catalytic mechanism of the enzyme. Replacement of each cysteine with an alanine residue produces functional ATPase molecules in strains C148A, C376A, C409A, C472A, C532A, C545A, C840A, and C869A, since all of these mutant ATPases are able to support the growth of yeast cells in glucose medium to some degree. Moreover, except for the C409A enzyme all of these mutant ATPases exhibit kinetic properties similar to that of the wild type strain SKM9.

Second, although none of the cysteines is essential, several appear to be in or near important sites of action in the H+-
glucose medium (Table I). However, mutant C532A supported cell growth and displayed normal kinetic characteristics. Cys\textsuperscript{532} has been reported to be protected against reaction with N-ethylmaleimide by MgADP and may thus be in or near the nucleotide binding site (15). If so, our results suggest that some aspect of the substrate binding and phosphoryl transfer reactions occurs less efficiently when the cysteine -SH is replaced with an -OH by conversion to serine than when it is removed entirely by replacement with an alanine.

The third conclusion that can be drawn from these experiments is that the previously detected disulfide bridge linking Cys\textsuperscript{148} and either Cys\textsuperscript{150} or Cys\textsuperscript{569} (17) plays no obvious role in the structure or function of the H\textsuperscript{+}-ATPase molecule. Replacement of Cys\textsuperscript{148}, Cys\textsuperscript{340} or Cys\textsuperscript{569} with a serine or an alanine residue does not affect the ability of the ATPase to support cell growth in any major way, and the folding and kinetic properties of these mutant ATPases are normal. These results preclude an important structural role for the disulfide bridge in the Neurospora H\textsuperscript{+}-ATPase as made in yeast and assayed in these experiments.

In our original description of the yeast expression system used in these studies, it was pointed out that in certain cases measuring the activities of expressed Neurospora H\textsuperscript{+}-ATPase molecules in the presence of the endogenous yeast H\textsuperscript{+}-ATPase may be problematic. However, from the studies reported here, it is clear that the yeast H\textsuperscript{+}-ATPase is inactive in the ATPase assay procedure employed, because no ATPase activity could be detected in the appropriate glycerol gradient fractions produced with the plasmid control strain SKM3. Thus, contamination by the endogenous yeast H\textsuperscript{+}-ATPase does not appear to be a problem for future mutagenesis studies of the Neurospora H\textsuperscript{+}-ATPase using this expression system. However, as can be seen from our results with mutants C376S and C532S, a problem does remain for mutants that are totally unable to support cell growth. Whereas a negative growth test by a particular mutant is a strong indication that an important residue has been altered, the low amounts of the mutant ATPases produced makes further analysis of such interesting mutants more difficult than those that support growth. Encouragingly, preliminary results of membrane fractionation studies with mutants D378A, C376S, and C532S indicate that these ATPases are enriched in a light membrane fraction distinct from the plasma membrane. Thus, although more work will be required to study such ATPase mutants, detailed analyses of their activities and partial reactions should nevertheless be possible.

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