Direct Evidence for the Cytoplasmic Location of the NH$_2$- and COOH-terminal Ends of the Neurospora crassa Plasma Membrane H$^+$-ATPase*

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Reconstituted proteoliposomes containing Neurospora plasma membrane H$^+$-ATPase molecules oriented predominantly with their cytoplasmic portion facing outward have been used to determine the location of the NH$_2$ and COOH termini of the H$^+$-ATPase relative to the lipid bilayer. Treatment of the proteoliposomes with trypsin in the presence of the H$^+$-ATPase ligands Mg$^{2+}$, ATP, and vanadate produces approximately 97-, 95-, and 88-kDa truncated forms of the H$^+$-ATPase similar to those already known to result from cleavage at Lys$^{*}$, Lys$'$, and Arg$^{*}$ at the NH$_2$-terminal end of the molecule. These results establish that the NH$_2$-terminal end of the H$^+$-ATPase polypeptide chain is located on the cytoplasmic side of the membrane. Treatment of the same proteoliposome preparation with trypsin in the absence of ligands releases approximately 50 water-soluble peptides from the proteoliposomes.

Separation of the released peptides by high performance liquid chromatography and spectral analysis of the purified peptides identified only a few peptides with the properties expected of a COOH-terminal, tryptic undecapeptide with the sequence SLEDFVVSLQR; SDS, sodium dodecyl sulfate. PAGE, polyacrylamide gel electrophoresis; SLED... tryptic peptide with the sequence SLEDFVVSLQR.

With the sequence SLEDFVVSLQR: SDS, sodium dodecyl sulfate.

The long-term goal of this laboratory is to elucidate the molecular mechanism by which the plasma membrane H$^+$-ATPase of Neurospora crassa transduces the chemical energy of ATP hydrolysis into a transmembrane electrochemical proton gradient. Of critical importance in achieving this goal is an understanding of the molecular structure of the H$^+$-ATPase and its topography relative to the membrane in which it is embedded. Previous investigations of the topography of the H$^+$-ATPase have been for the most part limited to interpretations of hydropathy profiles generated from analyses of the gene sequence. However, recent studies in this laboratory have provided methodology for preparing reconstituted H$^+$-ATPase proteoliposomes in which the enzyme molecules are oriented predominantly with their cytoplasmic side facing outward (1), and methodology that allows for direct analyses of the protein chemistry of the H$^+$-ATPase (2). The combination of a well-defined, membrane-bound H$^+$-ATPase preparation and methodology that allows for detailed analysis of the protein chemistry of the H$^+$-ATPase provides the essential ingredients for rigorous physical and chemical investigations of the topography of the H$^+$-ATPase.

In the study described here, we present an improved procedure for preparing the reconstituted H$^+$-ATPase proteoliposomes in which the bulk of the protein-free liposomes and any unreconstituted H$^+$-ATPase molecules are removed and the proteoliposomes are concentrated, greatly simplifying subsequent analyses. We then initiate our investigation of the H$^+$-ATPase topography by establishing the location of the NH$_2$- and COOH-terminal ends of the H$^+$-ATPase relative to the lipid bilayer in these proteoliposomes. The establishment of the location of the ends of the H$^+$-ATPase polypeptide relative to the bilayer imposes simple but important constraints on subsequent topographic models of the enzyme, including whether there are an even or odd number of transmembrane segments in the H$^+$-ATPase, and, as a model of the intramembranous portion of the enzyme is developed (3), the direction in which each of the membrane-spanning segments must traverse the membrane.

EXPERIMENTAL PROCEDURES

Purification and Reconstitution of the H$^+$-ATPase—The plasma membrane H$^+$-ATPase of Neurospora crassa was purified as described previously (4, 5). The H$^+$-ATPase was reconstituted into asolectin liposomes using a modification of the methods of Scarborough and Addison (1) and Goormaghtigh et al. (6). Briefly, 2 g of asolectin, purified by the procedure of Kagawa and Racker (7) and dissolved in chloroform, was placed in a 50-ml glass culture tube, and the solvent evaporated under a stream of N$_2$ followed by overnight storage under vacuum. Eighteen ml of buffer A (10 mM MES containing 50 mM potassium acetate, pH adjusted to 6.8 with KOH), and 2 ml of 0.1 M EDTA, pH adjusted to 6.8 with KOH, were then added to the dried asolectin. The tube was then flushed with N$_2$, capped, and the mixture sonicated in a bath-type sonicator (Laboratory Supplies, Co., Inc., Hicksville, NY) until transparent (approximately 3 h). The liposome suspension was then diluted with 18 ml of buffer A and 2 ml of 0.1 M

The abbreviations used are: MES, 2-(N-morpholino)ethanesulfonic acid; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SLED... tryptic peptide with the sequence SLEDFVVSLQR; SDS, sodium dodecyl sulfate.

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Five ml aliquots of the proteoliposome preparation were overlaid on eight 35 ml linear gradients of 24-40% (w/v) glycerol in buffer A. The gradient tubes were centrifuged at 60,000 rpm in a Beckman 70 Ti rotor (602,000 × g at ωmax) for 18 h at 4 °C to separate the protein-free liposomes from those containing the reconstituted II'-ATPase (6). After centrifugation, the gradients were fractionated into 2.1 ml aliquots and assayed for turbidity and activity in the presence and absence of nigericin (1). Fractions containing the peak activity from all of the gradients were pooled (total volume approximately 84 ml), diluted to 120 ml with buffer A, and 30 ml aliquots were underlaid with 10 ml of a solution containing 60% (w/v) glycerol in buffer A. The step-gradients were then centrifuged at 60,000 rpm in a Beckman 70 Ti rotor for 2 h at 4 °C to concentrate the proteoliposomes. The band of turbidity near the density interface in each tube was isolated, pooled (total volume typically 15 ml), and the proteoliposome suspension either used immediately or stored at −20 °C and used within 3 days. When stored at −20 °C the proteoliposome suspension did not freeze, due to the high concentration of glycerol in the solution, and showed no loss of activity in the presence and absence of nigericin over the course of 1 week.

Trypsin Treatment of the H'-ATPase-bearing Liposomes in the Presence and Absence of Ligands and Analysis of the Products by SDS-PAGE—The enriched, concentrated H'-ATPase proteoliposome suspension was mixed with 0.05 volumes of a solution containing 200 mM MgSO4, and 200 mM Na2ATP, pH adjusted to 6.8 with Tris, and 0.01 volumes of 10 mM sodium orthovanadate, and preincubated at 30 °C. N-Tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (1 mg/ml in buffer A, freshly prepared) was added to a final concentration of 1:10 (trypsin/H'-ATPase, w/w), and the samples were incubated at 30 °C for 0-160 min with occasional swirling. For all samples the sum of the preincubation time plus the time of incubation with trypsin equaled 160 min. At the end of the incubation, the digests were mixed with an equal volume of acetic acid, centrifuged at 30,000 g at 4 °C for 15 min, and the resulting supernatants were collected, and immediately analyzed by SDS-PAGE (8) on an 11 X 14 X 0.15 cm gel containing 7.5% polyacrylamide in the resolving gel and 5% polyacrylamide in the stacking gel. Control samples containing no trypsin, or trypsin and no ligands were also processed and analyzed in the same way. The gels were stained with silver (9) and the staining intensities of the bands quantitated using a Bio-Rad model 620 densitometer and Bio-Rad 1D Analyst software.

Trypsin Treatment of the H'-ATPase-bearing Liposomes and Separation and Analysis of the Peptides Released—Fifteen ml of the enriched, concentrated H'-ATPase proteoliposome suspension (approximately 1.5 mg of protein) was brought to 1% (w/v) ammonium bicarbonate, mixed with a N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin solution (1 mg/ml in buffer A, freshly prepared) and the mixture incubated at 37 °C for 7 h with occasional swirling. Following the incubation, the digest was loaded onto a 2.5 x 32-cm column of Sepharose CL2B equilibrated with buffer A, eluted with the same buffer at a flow rate of approximately 20 ml/h, and 20-min fractions were collected. The fractions containing the tryptic peptides released from the proteoliposomes (i.e. non-turbid, post-void volume fractions that absorbed light at 280 nm) were collected, pooled, and lyophilized overnight. Aliquots of the resulting sample, containing the released peptides in approximately 80% (w/v) glycerol, were filtered through 0.45-μm nylon-66 filters (Rainin), and subjected to HPLC analysis. HPLC analyses were performed using a Waters HPLC instrument equipped with model 440 and 441 UV detectors and interfaced with a NEC AVC IV computer and Waters Baseline 810 Chromatography Workstation software to control the pumps and collect and analyze the data. Peptides were resolved using a Bio-Rad RP-318 reversed-phase C4 column (4.6 X 250 mm) eluted with a gradient of 0-100% acetonitrile in H2O containing 0.1% trifluoroacetic acid as described previously (2). Eluate fractions containing peptides with the appropriate 214 and 280 nm signals to be candidates for the expected COOH-terminal tryptic peptide(s) were collected, and the NH2-terminal sequences of two of these were determined (see "Results and Discussion"). After this initial screening, the located and identified COOH-terminal peptide in the HPLC profile was verified by rechromatographing the appropriate eluate fraction and subjecting the repurified peptide to NH2-terminal sequence analysis as described under "Results and Discussion."
FIG. 1. Separation of H⁺-ATPase-containing liposomes from H⁺-ATPase-free liposomes by glycerol gradient centrifugation. The H⁺-ATPase was reconstituted into sonicated asolectin liposomes and the preparation subjected to glycerol density gradient centrifugation as described under "Experimental Procedures." The gradients were fractionated and the fractions assayed for turbidity and H⁺-ATPase activity in the presence and absence of nigericin.

Additional support for these conclusions is presented in Fig. 2. As described in detail before (1), SDS-PAGE analysis of H⁺-ATPase-bearing proteoliposomes in the presence and absence of H⁺-ATPase ligands can be used to estimate the percent of H⁺-ATPase molecules in the preparation that are functional and oriented with their cytoplasmic side facing outward. Membrane-embedded H⁺-ATPase molecules oriented with their cytoplasmic side out are readily degraded by trypsin with the production of relatively small peptides. Additionally, recent data (not shown) indicates that H⁺-ATPase molecules with the reverse orientation are extremely resistant to any degradation by trypsin. Fig. 2A shows a time course of tryptic digestion of the H⁺-ATPase proteoliposome preparation described in this report in the absence of H⁺-ATPase ligands. Densitometric analyses of these gel lanes showed that approximately 80% of the mass of the H⁺-ATPase was protected as the 97-, 95-, and 88-kDa forms until the later stages of the tryptic digestion, indicating that about 80% of the H⁺-ATPase molecules in the preparation, virtually all of the cytoplasmic-side-out molecules, are responsive to the H⁺-ATPase ligands and are therefore functional. The combined results of all of these analyses thus demonstrate that 85–90% of the H⁺-ATPase molecules in the proteoliposome preparation used in these studies are oriented with their cytoplasmic surface facing outward and that the great majority of these molecules are functional.

The improved reconstituted H⁺-ATPase preparation is therefore well-defined with respect to the H⁺-ATPase orientation and functionality, enriched in the H⁺-ATPase-containing liposomes, essentially free of unreconstituted H⁺-ATPase molecules, and adequately concentrated with respect to H⁺-ATPase protein, and is thus a very useful system for studies of the H⁺-ATPase topography.

The results shown in Fig. 2 also establish the topographic location of the amino-terminal end of the H⁺-ATPase molecule. A recent study by Mandal and Slayman (15) demonstrated that conversion of the 100-kDa native polypeptide to the ~97-, 95-, and 88-kDa forms by trypsin is due to cleavage at Lys²⁴, Lys⁵⁸, and Arg⁷³, respectively. Thus, the generation of these truncated forms of the H⁺-ATPase is a measure of tryptic cleavage at the NH₂-terminus of the molecule.

Tryptic digestion of H⁺-ATPase molecules in the presence of the active site ligands Mg²⁺ plus vanadate or Mg²⁺ plus ATP plus vanadate results in truncation of the H⁺-ATPase molecule to ~97-, 95-, and 88-kDa forms, with relatively slow degradation of these forms to smaller peptide fragments (15, 16). And, as elaborated in detail earlier (1), this ligand-protection of the H⁺-ATPase against tryptic digestion is a measure of the functionality of the H⁺-ATPase molecules in the reconstituted proteoliposome preparation. Fig. 2B shows the time course of tryptic digestion of the H⁺-ATPase proteoliposome preparation described in this report in the presence of Mg²⁺, ATP, and vanadate. Densitometric analyses of these gel lanes showed that approximately 80% of the mass of the H⁺-ATPase was protected as the 97-, 95-, and 88-kDa forms until the later stages of the tryptic digestion, indicating that about 80% of the H⁺-ATPase molecules in the preparation, virtually all of the cytoplasmic-side-out molecules, are responsive to the H⁺-ATPase ligands and are therefore functional. The combined results of all of these analyses thus demonstrate that 85–90% of the H⁺-ATPase molecules in the proteoliposome preparation used in these studies are oriented with their cytoplasmic surface facing outward and that the great majority of these molecules are functional.
COOH terminus of the H⁺-ATPase in the cytoplasmic portion of the enzyme molecule was to analyze the peptides released from the above-described H⁺-ATPase proteoliposomes after trypsin treatment in the hopes of identifying a fragment from the COOH terminus of the enzyme. Two factors greatly simplified the search for such a peptide among the numerous tryptic cleavage products released from the proteoliposomes. First, hydropathy analyses of the amino acid sequence of the H⁺-ATPase (2, 17, 18) and investigations of the fragments of the H⁺-ATPase remaining associated with the liposomes after trypsin treatment (3) indicate that there are approximately 40 residues on the COOH-terminal side of the last membrane-spanning segment. This COOH-terminal portion of the enzyme contains six potential tryptic cleavage sites, with the largest tryptic fragment expected to contain between 11 and 29 residues and no tyrosine or tryptophan (Fig. 3). Thus, if one or more peptides are released from the COOH terminus of the H⁺-ATPase by trypsin treatment of the proteoliposomes, the largest of these peptides should have a substantial absorbance at 214 nm and relatively little absorbance at 280 nm. The second factor that simplified the search is the analytical capability of currently available HPLC data acquisition and analysis software. With such software, it is possible to definitively examine the absorbance characteristics of each peak in an HPLC eluate and thus pinpoint peptides of a specific type for further purification and analysis.

Fig. 4 shows the results of HPLC analysis of the peptides released from the H⁺-ATPase proteoliposomes by tryptic digestion. In this experiment, the proteoliposomes were treated with trypsin and subjected to gel filtration chromatography to separate the released peptides from the liposomes. The eluate fractions containing the released peptides were pooled, and the peptides concentrated by lyophilization and subjected to HPLC analysis. Out of a total of approximately 50 peaks in the profile, four peaks, indicated by the *arrows with the numbers*, had relatively high $A_{214}$ and distinctively high ratios of $A_{214}$ to $A_{280}$ and were thus chosen as most likely to be the COOH-terminal peptide of interest. NH₂-terminal sequence analysis of the fraction containing peak number 1, eluting at about 108 min, was determined first and yielded the sequence **TVEEDHIPPEEDVDAKY**, identifying the major peptide in this sample as residues 483-499 of the H⁺-ATPase sequence (17, 18). Sequence analysis of the fraction containing peak number 2, eluting at about 154 min, indicated the presence of the COOH-terminal peptide **SLEDFFVSLQR**, which begins at residue 901 and ends at residue 911 of the H⁺-ATPase sequence. Rechromatography of this sample on the same gradient revealed the presence of peptides eluting at 152 and 154 min. Therefore, the peptide in the peak eluting at 154 min in the second chromatogram was identified by tryptic cleavage experiments (Ref. 1, and Fig. 2), about 10-15% of the H⁺-ATPase molecules in the preparation are resistant to trypsin, presumably indicating a reverse orientation of these molecules in the plasma membrane. NH₂-terminus of the H⁺-ATPase, deduced from the gene sequence (17, 18). The spaces indicate the possible tryptic cleavage sites, and the 900 indicates residue 900.

**Fig. 3.** Amino acid sequence of the COOH-terminal end of the Neurospora plasma membrane H⁺-ATPase and possible tryptic cleavage sites. Shown are the last 36 residues of the COOH terminus of the H⁺-ATPase, deduced from the gene sequence (17, 18). The spaces indicate the possible tryptic cleavage sites, and the 900 indicates residue 900.

**Fig. 4.** HPLC analysis of the peptides released from the H⁺-ATPase proteoliposomes by tryptic digestion. Tryptic peptides released from the H⁺-ATPase proteoliposomes were prepared as described under “Experimental Procedures.” A sample (880 μl) containing the peptides released from 200 μg of reconstituted H⁺-ATPase protein was subjected to HPLC analysis. The numbered arrows indicate the peptide peaks that were considered as potential candidates for the COOH-terminal peptide as described under “Results and Discussion.” Insets, enlargements of the boxed portions of the HPLC elution profiles. The peak representing the SLED... peptide is identified by the downward pointing arrow. Panel A shows the $A_{214}$ profile, and panel B shows $A_{280}$ profile.

ATPase molecules in the preparation are resistant to trypsin, presumably indicating a reverse orientation of these molecules. In order to ensure that the SLED... peptide was released from the predominant population of cytoplasmic H⁺-ATPase molecules, rather than from the minor population of H⁺-ATPase molecules with the other orientation, it was important to quantitate the recovery of this peptide relative to the amount of H⁺-ATPase used in the experiment. To do this, the hydrophilic peptides generated from tryptic hydrolysis of the purified H⁺-ATPase were used as a calibration standard for the HPLC analysis. Previous analyses of this peptide mixture indicated that, subsequent to HPLC analysis, approximately 80% of the mass of the peptides is recovered relative to the amount expected from the starting mass of purified H⁺-ATPase (2). Thus, the areas of the peaks in the 214 nm profile from HPLC analysis of the calibration standard reflect on average approximately 80% of
the mass of each peptide expected from trypsin cleavage and HPLC analysis of a given starting amount of the purified H'-ATPase, and the areas of comparable peaks in the HPLC analysis of the peptides released by trypsin treatment of the reconstituted H'-ATPase (Fig. 4) can be used to estimate the mass, and therefore the recovery, of individual peptides relative to the starting mass of the enzyme. Based on this calibration standard, measurements of the area of the peak at 154 min from five preparations of the H'-ATPase proteoliposomes indicated that 45 ± 5% of this peptide was recovered relative to the amount expected if complete trypsin cleavage of all of the cytoplasmic-side-out H'-ATPase molecules in the proteoliposomes, and complete recovery of the peptides from the various sample processing steps, had occurred. Correcting this recovery value for the 〜20% losses that occur with the standard peptides from the purified H'-ATPase, the estimated amount of the SLED...peptide released from the proteoliposomes would be substantially higher. And, this is a minimum estimate since the manipulations involved in purifying peptides released from the proteoliposomes are much more extensive than those involved in the purification of the standard peptides, significantly increasing the probability of additional losses of the proteoliposome-derived peptides. This recovery estimate thus precludes the possibility that the SLED...peptide was derived from H'-ATPase molecules with their cytoplasmic side facing inward, as peptides generated from these molecules could result in no greater than 15% recovery of peptide mass upon complete hydrolysis and recovery. These data thus provide direct physical evidence that the SLED...peptide, and thus the COOH-terminal end of the H'-ATPase molecule, must reside in the cytoplasmic domain of the functional H'-ATPase molecule.

Prior to this study, little evidence was available regarding the location of the NH2- and COOH-terminal ends of the Neurospora H'-ATPase relative to the membrane bilayer. The results presented here provide conclusive evidence that both ends of the H'-ATPase polypeptide are present on the cytoplasmic side of the membrane. This finding is of fundamental importance for developing a topographic model of the H'-ATPase as it exists in the membrane in that the location of the ends of the polypeptide on the same side of the membrane directly implies that the H'-ATPase polypeptide must contain an even number of membrane-spanning segments. Furthermore, as a model for the membrane-embedded regions of the H'-ATPase molecule is evolved (3), these data will have important implications regarding the direction in which the membrane-spanning segments of the polypeptide pass through the lipid bilayer.

Evidence regarding the location of the terminal ends of other ATPases in the aspartyl-phosphoryl-enzyme intermediate family is limited but generally consistent with the findings reported here. Davis and Hammes' (19) examination of the reconstituted yeast plasma membrane H'-ATPase indicates the presence of an elastase cleavage site on the cytoplasmic side of the membrane approximately 45 residues away from the COOH terminus, which is in reasonable agreement with the cytoplasmic location of residues 901-911 of the Neurospora H'-ATPase reported here. However, these investigators concluded that the COOH terminus of the yeast H'-ATPase is on the exocyttoplasmic side of the membrane due to the resistance of the COOH terminus to degradation by carboxypeptidase Y in the absence of detergents. As an alternative explanation of these findings, Davis and Hammes (19) suggested that the COOH terminus might be present on the cytoplasmic side but inaccessible to carboxypeptidase Y. In view of the results presented here, we favor this latter explanation because, at least for the Neurospora enzyme, it is unlikely that the last nine relatively hydrophilic residues are buried in the bilayer. Otherwise, it must be concluded that these two enzymes, which have similar function and 75% sequence identity, have different topographies. Regarding the topography of other ATPases in this family, N-ethylmaleimide labeling of a cysteine residue near the NH2 terminus of the Ca2+-ATPase in a sarcoplasmic reticulum vesicle preparation (20), has been taken to indicate that the NH2 terminus of this enzyme is on the cytoplasmic side of the membrane. And, the localization of the calmodulin-binding region of the plasma membrane Ca2+-ATPase suggests that the COOH terminus of this enzyme is on the cytoplasmic side of the membrane (21, 22). Additionally, analyses of tryptic peptides released from isolated plasma membrane vesicles have been offered as evidence that the NH2 terminus of the α-subunit of the Na+/K+-ATPase is located on the cytoplasmic side of the membrane (23, 24). Interestingly, a recent monoclonal antibody binding study by Ovchinnikov et al. (25) concludes that the COOH terminus of the α-subunit of the Na+/K+-ATPase is on the exocyttoplasmic side of the membrane. Thus, the Na+/K+-ATPase topography may be significantly different from that of the Neurospora plasma membrane H'-ATPase and the plasma membrane Ca2+-ATPase. The apparent difference could somehow be related to the presence of the essential β-subunit of the Na+/K+-ATPase, which appears to interact with membrane-spanning segments in the COOH-terminal portion of the α-subunit (26). Alternatively, an inherent problem with antibody binding as a method for topographical analysis of integral membrane protein structure is that of cross-reactivity of antibodies with regions of the molecule other than those they were designed for. Cross-reactivity of monoclonal antibodies for segments of the acetylcholine receptor has been documented and appears to be responsible for the incongruous topographic models of this molecule that have been generated from this experimental approach (27–29). This would suggest that the results of antibody binding studies of the Na+/K+-ATPase should probably be confirmed by other analytical methods.

In conclusion, in this article we have described an improved preparation of reconstituted proteoliposomes containing the Neurospora plasma membrane H'-ATPase and have used these proteoliposomes to demonstrate that the NH2- and COOH-terminal ends of the membrane-bound form of the H'-ATPase are located on the cytoplasmic side of the molecule. These findings are an important first step toward defining the topography of this H'-ATPase and provide valuable constraints for interpreting the results of our investigations of its membrane-embedded regions (3), and for future studies of the extramembranous portions of this molecule. Additionally, these findings may be relevant to the topography of other ATPases in the aspartyl-phosphoryl-enzyme intermediate family.

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