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Identification of the Membrane-embedded Regions of the *Neurospora crassa* Plasma Membrane H^+-ATPase*

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Reconstituted proteoliposomes containing functional *Neurospora crassa* plasma membrane H^+-ATPase molecules oriented predominantly with their cytoplasmic surface exposed were treated with trypsin and then subjected to Sepharose CL-6B column chromatography to remove the liberated peptides. The peptides remaining associated with the liposomes were then separated from the phospholipid by Sephadex LH-60 column chromatography and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Six H^+-ATPase peptides with approximate molecular masses of 7, 7.5, 8, 10, 14, and 21 kDa were found to be tightly associated with the liposomal membrane. Amino acid sequencing of the 7-, 7.5-, and 21-kDa peptides in the LH-60 eluate identified them as H^+-ATPase fragments beginning at residues 99 or 100, 272, and 660, respectively. After further purification, the approximately 10- and 14-kDa peptides were also similarly identified as beginning at residues 272 and 660. The approximately 8-kDa fragment was purified further but could not be sequenced, presumably indicating NH_2-terminal blockage. To identify which of the liposome-associated peptides are embedded in the membrane, H^+-ATPase molecules in the proteoliposomes were labeled from the hydrophobic membrane interior with 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine and cleaved with trypsin, after which the membrane-associated peptides were purified and assessed for the presence of label. The results indicate that the approximately 7-, 7.5-, and 21-kDa peptides are in contact with the lipid bilayer whereas the approximately 8-kDa peptide is not. Taken together with the results of our recent analyses of the peptides released from the proteoliposomes, this information establishes the transmembrane topography of nearly all of the 919 residues in the H^+-ATPase molecule.

The long term goal of this laboratory is an elucidation of the molecular mechanism by which the H^+-ATPase in the plasma membrane of *Neurospora crassa* transduces the chemical energy of ATP hydrolysis into a transmembrane electrochemical proton gradient. To understand the mechanism of any enzyme, including a transport enzyme such as H^+-ATPase, it is essential to understand first its structure in significant detail. As one approach in this regard we have recently reported direct protein chemical analyses of the transmembrane topography of the H^+-ATPase molecule (1, 2). In these studies, reconstituted proteoliposomes demonstrated to contain H^+-ATPase molecules oriented predominantly with their normally cytoplasmic surface facing outward were cleaved with trypsin, and the peptides released were purified and identified by NH_2-terminal amino acid sequence analyses. The results of these experiments identified 417 residues of the H^+-ATPase molecule as located on the cytoplasmic surface of the membrane, including the NH_2 and COOH termini. In the present paper this approach is elaborated further with an analysis of the parts of the H^+-ATPase molecule which remain associated with liposomes after tryptic cleavage, employing our recently developed methodology for analyzing hydrophobic peptides (3, 4). The results allow the construction of a reasonably detailed model for the transmembrane topography of this interesting transport enzyme.

EXPERIMENTAL PROCEDURES

Purification and Reconstitution of the H^+-ATPase

N. crassa plasma membrane H^+-ATPase was purified according to the procedure of Smith and Scarborough (5, 6). ^{14}C-labeled H^+-ATPase was prepared as above except that the growth medium contained 0.25% (w/v) nutrient broth and yeast extract, and the cells were grown in the presence of 0.25 mCi of ^{14}C-labeled algal protein hydrolysate. The specific radioactivity of the purified ^{14}C-labeled H^+-ATPase was 10,600 cpm/mg protein. Freeze-thaw reconstitution of the ATPase into asolectin liposomes, purification by glycerol gradient centrifugation, and concentration of the resulting proteoliposomes by step gradient centrifugation were carried out as described by Hennessey and Scarborough (1). The specific ATPase activities in the presence and absence of nigericin, protein concentration, and ATPase sidedness of the H^+-ATPase proteoliposome preparations were essentially identical to those described earlier (1, 2).

Photolabeling of H^+-ATPase Proteoliposomes with [^{125}I]TID[1]

Fifteen ml of a proteoliposome suspension containing 27 μg of ATPase protein/ml was placed in a 50-ml beaker on ice for 5 min. While stirring magnetically, 60 μCi of [^{125}I]TID in 40 μl of 75% ethanol was added slowly to the proteoliposome suspension and the mixture incubated in the dark for 15 min. The suspension was then irradiated at 366 nm using an ultraviolet lamp (model UVSL-25 Mineralight lamp, Ultraviolet Products Inc., San Gabriel, CA) placed 8 cm above the surface of the suspension for 1 h with continuous stirring on ice.

Tryptic Digestion of the H^+-ATPase Proteoliposomes and Isolation of the Liposome-bound Peptides

Tryptic digestion of the H^+-ATPase proteoliposomes and Sepharose CL-6B column chromatography of the resulting tryptic digests

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[1] The abbreviations used are: [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; FITC, fluorescein 5-isothiocyanate; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; HPLC, high pressure liquid chromatography.

were carried out as described previously (1, 2). Void volume fractions containing the liposome-bound peptides were pooled and lyophilized to dryness overnight.

Sephadex LH-60 Column Chromatography of the Liposome-bound Peptides

The lyophilized liposome-bound peptide fraction obtained from the Sepharose CL-6B column chromatography was first treated with 30 μl of neat trifluoroacetic acid/250 μg of reconstituted ATPase and then diluted with 2 ml of chloroform/methanol (1:1, v/v)/250 μg of ATPase. The resulting clear, deep yellow solution was then subjected to chromatography on a 2.5 × 90-cm Sephadex LH-60 column equilibrated in and eluted with chloroform/methanol (1:1, v/v) containing 0.1% (v/v) trifluoroacetic acid at a flow rate of approximately 10 ml/h. Fractions (4.5 ml) were collected and assayed for absorbance at 280 nm and radioactivity as described below.

Analysis of the Sephadex LH-60 Column Fractions by SDS-PAGE

Sephadex LH-60 column eluates were analyzed by SDS-PAGE using our previously described procedure for analyzing hydrophobic peptides (4) except that all the gels were of 0.075-cm thickness, the resolving gels were made with a buffer containing 0.665 M Tris, 0.086 M Tris-HCl, and 0.1% SDS, and the stacking gels and sample disaggregation solution were made with a buffer containing 0.003 M Tris, 0.122 M Tris-HCl, and 0.1% SDS.

Aliquots from the Sephadex LH-60 column fractions were placed in 1.5-ml polypropylene microcentrifuge tubes and dried under a stream of nitrogen. The residue in each tube was dissolved in 50 μl of neat trifluoroacetic acid, and the acid was then removed under a stream of nitrogen. The sample tubes were then placed on ice, and 25 μl of ice-cold sample disaggregation solution was added followed by incubation on ice for 30 min. Electrophoresis was carried out at room temperature at a constant current of 13 mA/gel until the tracking dye completely entered the resolving gel and then at 16 mA/gel until the tracking dye reached the bottom of the gel. After electrophoresis the gels were fixed with 12.5% aqueous trichloroacetic acid for 30 min and then stained with silver using the procedure of Morrissey (7).

Electroblotting of the Liposome-bound Peptides onto PVDF Membranes

Preelectrophoresis of SDS-PAGE Gels—SDS-PAGE gels to be used for electroblotting were polymerized overnight as described above and then preelectrophoresed using a resolving gel buffer that contained 0.665 M Tris, 0.086 M Tris-HCl, 1 mM cysteine, and 0.1% SDS at a constant current of 13 mA for 18 h. The upper tank buffer was then replaced with a stacking gel buffer containing 0.003 M Tris, 0.122 M Tris-HCl, and 0.1% SDS. Small amounts of sample disaggregation solution containing bromphenol blue as a tracking dye were then added to the end wells, and preelectrophoresis continued until the tracking dye migrated to the interface between the stacking and resolving gels. This preelectrophoresed gel was then used for electrophoretic separation of the hydrophobic peptides as described above.

Prelabeling of the Hydrophobic Peptides with FITC—Sephadex LH-60 column fractions containing the hydrophobic peptides of interest were pooled and divided into two portions, one containing 0.10 (portion A) and the other containing 0.9 (portion B) of the volume. The solvent in these aliquots was removed under a stream of nitrogen after which 50 μl of neat trifluoroacetic acid was added to each portion and then removed under a stream of nitrogen. Five μl of a 1 mg/ml FITC solution in acetone (freshly prepared) was added to the dried portion A, and the acetone was then removed under nitrogen. After this, 20 μl of 50 mM sodium carbonate containing 1% SDS was added and the mixture incubated in the dark at room temperature for 2 h. One μl of 100 mM lysine was then added to derivatize the excess FITC. Two hundred μl of ice-cold disaggregation solution was then added and the resulting solution mixed with its corresponding dried portion B, incubated on ice for 30 min, and distributed into nine wells and electrophoresed as described above.

Electrophoretic Transfer—After electrophoresis, the gels were washed in a transfer buffer containing 10 mM CAPS and 10% (v/v) methanol, pH adjusted to 11.0 with NaOH (three changes, 10 min each) (8). Electrophoretic transfer of peptides from the gels to the PVDF membranes was carried out in the above transfer buffer using a Bio-Rad Trans-blot cell at 500 mA constant current for 20 min with a distance of 8 cm between the electrode plates. The PVDF membrane was removed and washed in distilled water (one to three times, 5 min each) and air dried. The peptides transferred onto the membrane were detected under UV light, and the peptides of interest were cut out with a razor blade and used for NH_2-terminal amino acid sequence analysis.

Estimation of [^{125}I]TID Labeling of the Individual Liposome-bound Peptides—Sephadex LH-60 column fractions obtained from [^{125}I]TID-labeled H⁺-ATPase proteoliposomes were analyzed by SDS-PAGE as described above. The gels were then stained with silver and immediately placed between two layers of BiogelWrap (BioDesign Inc. of New York) and dried at room temperature for 2 days. The peptide bands of interest were then cut out and counted for radioactivity as described below. The appropriate background corrections for the various samples were determined by counting unstained regions in the immediate vicinity of the bands of interest.

Purification of the Approximately 8-kDa Peptide

Sephadex LH-60 column fractions enriched in the approximately 8-kDa peptide were pooled and dried under nitrogen. To the dried sample was added 100 μl of neat trifluoroacetic acid followed by 100 μl of 2-propanol and 50 μl of water, and the resulting hazy solution was centrifuged at 16,000 × *g* for 5 min. The supernatant fluid was applied to a Bio-Rad RP-304 column (4.6 × 250 mm) followed by elution with a linear gradient of 10–100% (v/v) 2-propanol in 0.1% (v/v) aqueous trifluoroacetic acid using a Waters HPLC system at a flow rate of 0.2 ml/min over a period of 200 min. The material eluting at 155–160 min contained essentially pure 8-kDa peptide as judged by SDS-PAGE analysis.

Amino Acid Analysis and NH_2-terminal Amino Acid Sequencing

Peptides electroblotted onto the PVDF membranes and peptides from the LH-60 column eluate dried down on PVDF membranes were sequenced at the UCLA Microsequencing Center under the direction of Dr. A. V. Fowler using a Porton PI 2090E gas phase sequenator. The amounts of the various peptides sequenced were as follows: the approximately 21-kDa peptide, 1,400 pmol; the approximately 7-kDa peptide, 700 pmol; the approximately 7- and 7.5-kDa peptide mixture, 730 pmol; the approximately 14- and 10-kDa electroblotted peptides, 70 and 100 pmol, respectively. The peptides were sequenced only once since each sequence obtained was readily found in the published amino acid sequence deduced from the gene sequence (24, 25).

Other Methods

^{14}C radioactivity was determined using the liquid scintillation mixture described by Patterson and Greene (9). Samples containing ^{125}I were counted directly in a Cobra Auto-Gamma counter (Packard Instruments). Other methods were as described previously (1–6).

Materials

^{14}C-Labeled algal protein hydrolysate (approximately 50 mCi/mg atom of carbon) and [^{125}I]TID (approximately 10 Ci/mmol) were from Amersham Corp. PVDF membrane was from Millipore or Bio-Rad. FITC isomer I and CAPS were obtained from Sigma. The sources of the other materials used in this study have been described previously (1–6).

RESULTS

Fig. 1 outlines the approach used in these studies for isolating the membrane-embedded regions of the ATPase molecule. Reconstituted proteoliposomes previously established to contain H⁺-ATPase molecules oriented predominantly with their cytoplasmic surface facing outward (1, 2) were cleaved with trypsin. The peptides released by this treatment were then removed by chromatography of the mixture on a column of Sephadex CL-6B, resulting in liposomes containing only

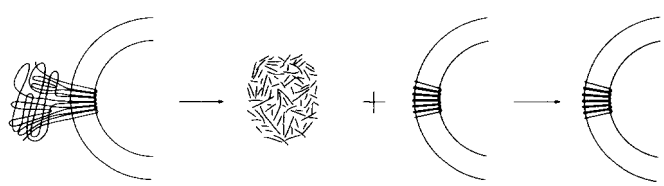

FIG. 1. **Experimental approach to defining membrane-embedded regions of the H⁺-ATPase.** See "Results" for details.

peptides tightly associated with the membrane. Fig. 2 shows the relative amounts of the H⁺-ATPase molecule which remain associated with the proteoliposomes (void volume) or are released from the proteoliposomes (later fractions) in such an experiment. For this type of experiment radioactive H⁺-ATPase molecules prepared as described under "Experimental Procedures" were used for convenience. The results of three experiments of this kind indicate that about 60% of the ATPase molecule is released from the proteoliposomes, and about 40% remains bound. This is an important experiment because if substantial amounts of the ATPase molecule were not released the significance of peptides remaining associated with the liposomes would be uncertain.

As we have shown previously (1, 2), the peptides released from the proteoliposomes in such an experiment are defined as being present on the cytoplasmic side of the membrane in the intact H⁺-ATPase molecule. Conversely, any peptides remaining associated with the liposomes are candidates for segments of the molecule which are normally embedded in the membrane. It was therefore of interest to attempt to identify the parts of the ATPase molecule remaining associated with the proteoliposomes. Unfortunately, this was not immediately a simple task because the membrane-embedded segments of integral membrane proteins are extremely difficult to manipulate by conventional techniques of protein chemistry. However, the recent development of effective procedures for purifying the H⁺-ATPase hydrophobic peptides by Sephadex LH-60 column chromatography in an organic solvent mixture (3) and for analyzing the hydrophobic peptides by SDS-PAGE (4) has now made such an analysis possible.

Fig. 3 shows a typical SDS-PAGE analysis of the individual fractions obtained upon Sephadex LH-60 column chromatography of the liposome-bound peptides by our previously reported procedures (3, 4). Peptide bands with molecular masses of about 100, 58, 42, 32, 21, 17, 14, 11, 10, 8, 7.5, and 7 kDa, by reference to molecular mass standards, are reproducibly found in reasonable amounts. Each of these bands is identified in the figure by an *arrow* with its approximate molecular mass.

Several of these peptides are of little or no significance with respect to the transmembrane topography of the H⁺-ATPase molecule. The band at approximately 100 kDa represents ATPase molecules oriented with their cytoplasmic portion

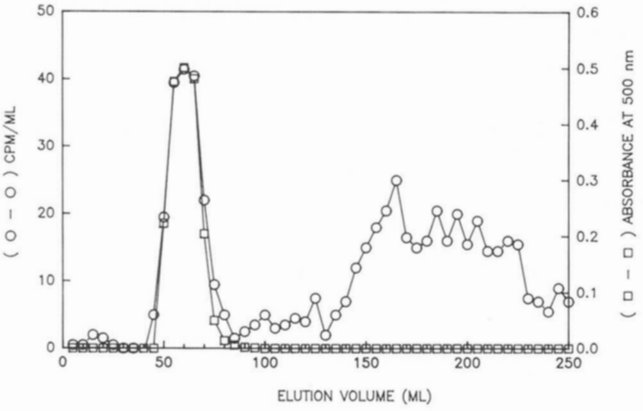

FIG. 2. **Separation of the liposome-bound and released peptides by Sepharose CL-6B column chromatography.** Reconstituted proteoliposomes containing ^{14}C-labeled H⁺-ATPase molecules were treated with trypsin and the digest fractionated by Sepharose CL-6B column chromatography as described under "Experimental Procedures." Fractions were collected and assayed for turbidity ($A_{500\,nm}$) and radioactivity. The void volume of the column was about 60 ml.

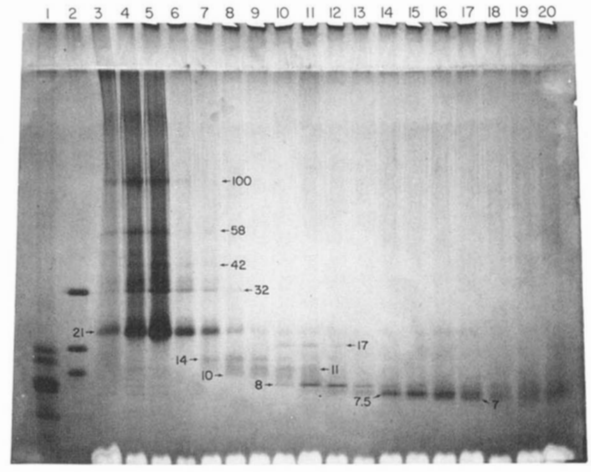

FIG. 3. **Typical SDS-PAGE analysis of the Sephadex LH-60 column fractions.** The liposome-associated peptide fraction obtained from 4 nmol of reconstituted ATPase was resolved on a Sephadex LH-60 column, and 0.1 ml of the peptide-containing fractions was analyzed by SDS-PAGE as described under "Experimental Procedures." The *uppermost numbers* refer to the gel lanes. Fractions 23–40 were analyzed in *lanes 3–20*, respectively. The approximately 100-, 58-, 42-, 32-, 21-, 17-, 14-, 11-, 10-, 8-, 7.5-, and 7-kDa peptides referred to under "Results" are indicated by the *arrows* with their approximate M_r values. *Lane 1*, myoglobin and its cyanogen bromide fragments (M_r values of 17, 14.5, 10.7, 8.2, 6.3, and 2.5 (23)), 5 μg total; *lane 2*, concanavalin A, 1 μg.

inside the liposomes and totally resistant to tryptic cleavage (2). The band at approximately 58 kDa is a minor band occasionally present in the H⁺-ATPase preparation which is also resistant to degradation by trypsin. It appears to be present in substantial quantities only because the great majority of the H⁺-ATPase molecules in the proteoliposome preparation have been degraded to smaller fragments by trypsin. The band at approximately 42 kDa is a dimer of the approximately 21-kDa hydrophobic H⁺-ATPase fragment to be discussed below (4). And, the approximately 32-, 17-, and 11-kDa bands arise from the concanavalin A used in the H⁺-ATPase isolation procedure, as can be seen by comparison with the concanavalin A standard in *lane 2* (and see Refs. 3 and 4).

On the other hand, the approximately 21-, 14-, 10-, 8-, 7.5-, and 7-kDa bands are not present in the purified H⁺-ATPase, concanavalin A, or lipid preparations used for the production of the H⁺-ATPase-bearing proteoliposomes employed in these studies, indicating that these peptides are H⁺-ATPase fragments containing potentially important information regarding the H⁺-ATPase topography.

Two of these peptides emerge from the LH-60 column in a form pure enough for direct NH_2-terminal sequence analysis. Thus, the material present in fractions similar to those analyzed in *lanes 4* and *5* of Fig. 3 containing predominantly the approximately 21-kDa peptide[2] and the fraction analyzed in *lane 19* containing the approximately 7-kDa peptide were subjected to NH_2-terminal sequence analysis without further purification. The major sequence obtained from fractions similar to those analyzed in *lanes 4* and *5* was SAADIVFLAPGLGAIIDALKTSR, identifying the approximately 21-kDa

[2] The amounts of the other peptides present in the fractions containing the approximately 21-kDa band are grossly exaggerated because of overloading of the gel in these lanes and by nonlinearity of the silver staining procedure. When smaller amounts are applied, it is clear that the approximately 21-kDa peptide present in this region of the LH-60 eluate is easily pure enough for unambiguous NH_2-terminal sequencing.

peptide as an H⁺-ATPase fragment beginning at Ser^{660}. From the size of this peptide and possible downstream tryptic cleavage sites, it probably ends near Lys^{891}, as suggested before (3). The only other sequence obtained from this material (approximately 15% of the major sequence) was from the approximately 32-kDa concanavalin A band.

The sequence information obtained from a fraction similar to the fraction analyzed in *lane 19* (Fig. 3) indicated the presence of two peptides with the NH_2-terminal sequences YGLNQMKEEKEN and KYGLNQMKEEKEN in roughly equal amounts. This information establishes the identity of the approximately 7-kDa band as a mixture of essentially identical ATPase fragments beginning at Lys^{99} and Tyr^{100} and from their size, presumably ending near Lys^{173}. There is little chance that this peptide ends before Lys^{173} because the next tryptic cleavage site upstream from this residue is nearly 60 residues away.

Because fractions analogous to that analyzed in *lane 16* contained only the approximately 7- and 7.5-kDa bands the prior identification of the approximately 7-kDa band made possible the identification of the approximately 7.5-kDa band in such fractions by difference. Therefore, the peptide mixture in a fraction similar to that analyzed in *lane 16* was sequenced. The results indicated, as expected, the presence of the YGLN..., KYGLN... pair of peptides and in addition, the presence of another major peptide with the sequence AAALVNAASGGSGHF, identifying the approximately 7.5-kDa band as a peptide beginning at Ala^{272}, and from its size, presumably ending near Lys^{355}. It is highly unlikely that this peptide is any shorter than 84 residues because the nearest tryptic cleavage site upstream from Lys^{355} is 40 residues away. Interestingly, the information obtained from this sequencing run (initial and repetitive yields) indicated the presence of roughly equal amounts of the approximately 7- and 7.5-kDa peptides in the fraction analogous to that analyzed in *lane 16* even though the silver staining intensity of the material in this fraction suggests a predominance of the approximately 7.5-kDa peptide. Thus, it would appear that the silver staining intensity of the approximately 7-kDa peptide is substantially less than that of the approximately 7.5-kDa peptide.

None of the remaining H⁺-ATPase peptides of interest emerges from the LH-60 column in a form pure enough for NH_2-terminal sequence analysis. And, unfortunately, repeated efforts to develop an HPLC system for the separation of the hydrophobic peptides have been, for the most part, unsuccessful, presumably as a result of their hydrophobic nature. However, minor modifications of our recently developed procedure for SDS-PAGE of hydrophobic peptides as described under "Experimental Procedures" allowed the use of this system as a final purification step for the approximately 10- and 14-kDa peptides. After purification by SDS-PAGE these peptides could then be electroblotted onto PVDF membrane and sequenced. Although the sequencing yields from such blots were relatively low the sequencing information nevertheless clearly identified the approximately 10- and 14-kDa peptides as H⁺-ATPase fragments beginning at Ala^{272} and Ser^{660}, respectively. From their size, these peptides presumably end near Lys^{379} and Arg^{813}. Thus, the approximately 10-kDa peptide is a precursor of the approximately 7.5-kDa peptide, and the approximately 14-kDa peptide is a cleavage product of the approximately 21-kDa peptide.

Finally, the approximately 8-kDa peptide is one of the few liposome-associated peptides that could be purified by HPLC, probably indicating a less hydrophobic character of this peptide. Unfortunately, numerous attempts to sequence this peptide failed, presumably indicating that it is NH_2-terminally blocked. Thus, additional information will be required to establish the identity of the approximately 8-kDa peptide. It should be mentioned, however, that the approximately 8-kDa peptide does not contain any major new topographical information, as the only part of the H⁺-ATPase polypeptide chain unaccounted for as membrane-embedded or cytoplasmically located in the present and previous studies (1, 2), *i.e.* an approximately 80-amino acid segment between residues 359 and 440, has recently been identified as cytoplasmically localized.[3] The approximately 8-kDa peptide therefore must be a partial tryptic cleavage product comprising parts of the ATPase molecule with an already established topographical location.

The results presented thus far establish the approximately 7-, 7.5-, 8-, 10-, 14-, and 21-kDa fragments as candidates for membrane-embedded sectors of the H⁺-ATPase molecule. However, whereas in our earlier studies the release of a peptide from the ATPase-bearing proteoliposomes established that peptide as located on the cytoplasmic side of the membrane, the failure to release a peptide does not prove that that peptide is embedded in the membrane. This is because of the distinct possibility that certain peptides might be cleaved by trypsin but nevertheless remain bound or become bound to the liposome surface or nonmembranous parts of the molecule which could conceivably remain after tryptic digestion. For this reason it was necessary to seek independent evidence as to whether or not the 7-, 7.5-, 8-, 10-, 14-, and 21-kDa peptides are embedded in the membrane. To do this the H⁺-ATPase-bearing proteoliposomes were treated with the lipophilic photolabeling reagent TID, established previously by Brunner and his colleagues to label primarily the membrane-embedded sectors of integral membrane proteins from within the bilayer (10–16). The proteoliposomes were then treated with trypsin, the released peptides removed by Sepharose CL-6B chromatography, and the liposome-bound peptides fractionated by Sephadex LH-60 chromatography as described under "Experimental Procedures."

Fig. 4*A* shows the distribution of the radioactivity from TID in the LH-60 column eluate. Reasonable amounts of label are found in the fractions containing the various peptides (fractions 23–40), but the great majority of the label is found in the later column fractions (not shown) in which the liposomal phospholipid is found[4] (17). This emphasizes an advantage of the LH-60 column step in addition to a preliminary peptide separation in experiments of this kind since the large amounts of labeled phospholipid would otherwise strongly interfere with the peptide analyses. To deconvolute this labeling profile and estimate the amount of label present in the individual peptides we once again took advantage of our recently developed SDS-PAGE procedure for analyzing hydrophobic peptides. Thus, the peptides in the individual fractions obtained were first separated by SDS-PAGE after which the resulting gel was silver stained, and the individual peptide bands in each lane were excised and counted for the presence of label from TID. *Panels B–D* of Fig. 4 show the results of part of this analysis, which has been carried out three times, with essentially the same results each time. The approximately 21-, 7.5-, and 7-kDa bands contain substantial

[3] U. S. Rao and G. A. Scarborough, manuscript in preparation.

[4] U. S. Rao, J. P. Hennessey, Jr., and G. A. Scarborough, unpublished results.

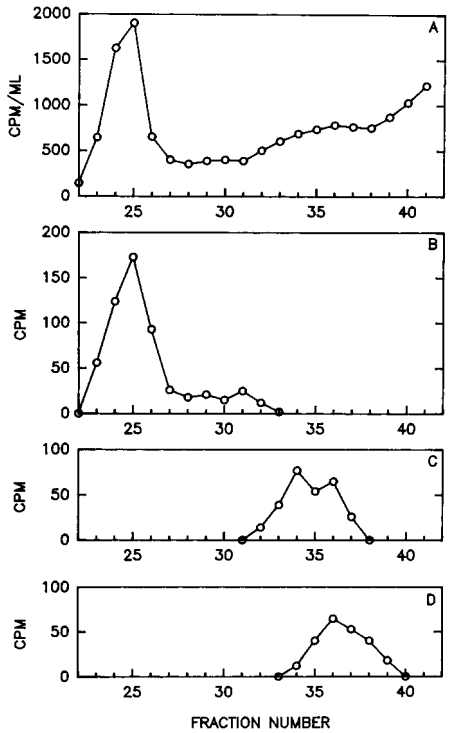

FIG. 4. **[^{125}I]TID labeling of the liposome-associated H⁺-ATPase peptides.** Proteoliposomes containing 4 nmol of the H⁺-ATPase were labeled with [^{125}I]TID, incubated with trypsin, and the liposome-bound peptides were separated from the released peptides by Sepharose CL-6B chromatography and partially separated from each other by Sephadex LH-60 chromatography as described under "Experimental Procedures." *Panel A* indicates the radioactivity distribution in the LH-60 fractions (4.5 ml). *Panels B–D*, 1 ml from each of the peptide-containing LH-60 fractions was analyzed by SDS-PAGE after which the gel was silver stained and the radioactivity associated with selected peptide bands in each fraction determined as described under "Experimental Procedures." The SDS-PAGE analysis shown in Fig. 3 was carried out with the LH-60 fractions from this experiment. Therefore, the peptide composition of the analyzed fractions can be seen in Fig. 3. *Panel B*, radioactivity associated with the approximately 21-kDa peptide; *panel C*, radioactivity associated with the approximately 7.5-kDa peptide; *panel D*, radioactivity associated with the approximately 7-kDa peptide.

amounts of the TID label,[5] indicating that they are almost certainly embedded in the membrane.[6] On the other hand, little, if any, of the TID label was found associated with the approximately 8-kDa peptide (not shown). This suggests that the approximately 8-kDa peptide does not interact with the bilayer lipids although it could be present in the membrane-embedded region of the molecule protected from labeling by contact with other parts of the polypeptide chain. Finally, the amounts of the approximately 10- and 14-kDa peptides present in these gel analyses were too low to estimate the extent of their labeling reliably.

As an additional test of the membrane-embeddedness of the liposome-associated peptides, an experiment similar in design to that described in Figs. 1–3, was carried out with an additional 150 mM KCl present during tryptic cleavage and subsequent Sepharose CL-6B column chromatography to diminish electrostatic interactions that might be the basis for peptide binding to the liposomes. The peptide profile obtained was quite similar to that shown in Fig. 3 except that the amount of the approximately 8-kDa peptide was diminished significantly (not shown). These results support the premise that the approximately 21-, 7.5-, and 7-kDa peptides are membrane-embedded whereas the approximately 8-kDa peptide is not.

DISCUSSION

The results presented indicate that after tryptic cleavage of reconstituted proteoliposomes containing *N. crassa* plasma membrane H⁺-ATPase molecules oriented predominantly with their cytoplasmic surface facing outward, four major peptides with molecular masses of about 7, 7.5, 8, and 21 kDa and two minor peptides of about 10 and 14 kDa remain associated with the liposomes. The 7-, 7.5-, and 21-kDa peptides begin at residues 99 or 100, 272, and 660 and end near residues 173, 355, and 891, respectively. The approximately 10-kDa peptide contains the approximately 7.5-kDa peptide at its NH_2 terminus and an additional 24 or so residues. The approximately 14-kDa peptide has the same NH_2 terminus as the approximately 21-kDa peptide but is shorter by about 78 residues. The identity of the approximately 8-kDa peptide is uncertain, but it is probably an incomplete cleavage product comprising parts of the molecule already accounted for as cytoplasmically located or membrane-embedded. The approximately 7-, 7.5-, and 21-kDa liposome-associated peptides each contains membrane-embedded segments whereas the approximately 8-kDa peptide does not.

A model for the transmembrane topography of the membrane-embedded regions of the H⁺-ATPase based on these results is shown Fig. 5. Because all of the ATPase molecules in the proteoliposomes which can be cleaved by trypsin are situated in the membrane with their cytoplasmic surface exposed (1, 2) and because each of the membrane-embedded peptides identified originates from the interior of the ATPase polypeptide chain, each of the membrane-embedded peptides must begin and end on the cytoplasmic side of the membrane and have an even number of membrane-spanning segments. Thus, starting from the NH_2 terminus, the first membrane-embedded peptide, beginning at residue 99, 100, must have a minimum of two membrane-spanning stretches and is probably too short to have four. Moreover, an analysis of the hydrophobicity of this peptide also suggests that it has only two membrane-spanning stretches because of a wealth of charged residues at its NH_2 and COOH termini. The positions of the two membrane-embedded stretches indicated in the model were chosen to minimize the presence of these charges, as well as three negative charges in the middle of this peptide, in the lipid bilayer.

The second membrane-embedded region, beginning at residue 272, must also have at least two membrane-spanning segments. However, it is long enough to have four, and our earlier hydropathy analysis (3) suggests that it may. On the basis of these considerations, we therefore tentatively propose that the second membrane-embedded peptide possesses four

[5] It should be pointed out that for each peptide the amount of TID label recovered in the analysis shown in Fig. 4, *B–D* was uniformly about 12% of the label present in the aliquot from the LH-60 column eluate taken for the SDS-PAGE analysis. This presumably reflects significant lability of the TID label in experiments of this kind. However, since the extent of label loss was the same for all of the fractions it is our assumption that the amount of label recovered in each peptide is roughly proportional to the amount of label associated with that peptide before the analysis was performed.

[6] The labeling of a peptide by TID does not prove that that peptide is embedded in the lipid bilayer because hydrophobic pockets in soluble proteins can also be labeled by this reagent (26). However, ATPase peptides that are labeled by TID and also remain firmly associated with the liposomes after tryptic cleavage and Sepharose CL-6B chromatography are much more likely to be membrane embedded, particularly in view of the fact that nearly two-thirds of the ATPase molecule is removed from the liposomes after such a treatment.

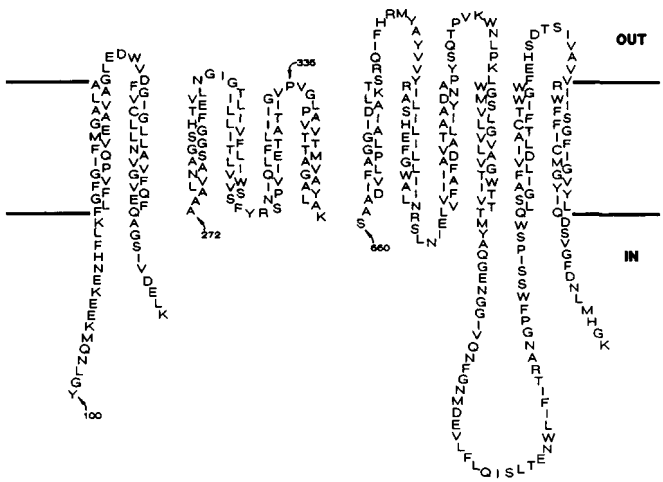

FIG. 5. **Model of the transmembrane topography of the membrane-embedded segments of the H⁺-ATPase.** The *letters* indicate amino acid residues of the H⁺-ATPase present in peptides demonstrated to be membrane-embedded, except for *OUT* and *IN*, which indicate points of reference outside and inside of an intact cell, respectively. The *numbers* indicate the first residues of the membrane-embedded peptides established by NH_2-terminal amino acid sequence analysis, except for the *335* which is included to identify Pro^{335} (see "Discussion"). The *horizontal lines* delineate the nonpolar interior of the membrane.

membrane-spanning segments, as indicated in the model, although the evidence for this is no stronger than that from any other hydropathy analysis. It is of interest in this regard that the proline residue at position 335 is conserved in most, if not all, of the ATPases in the aspartyl-phosphoryl-enzyme intermediate family (18) of ion-translocating ATPases. It may be that this proline is needed for the polypeptide chain to turn sharply enough (19) to allow four membrane-spanning stretches in this region of the molecule.

The third membrane-embedded region, beginning at residue 660, must also have at least two membrane-spanning stretches by the above criteria. Again, however, our earlier hydropathy analysis (3) suggests that this region may contain as many as six membrane-spanning segments, as indicated in the model. Moreover, the TID labeling data shown in Fig. 4 suggest that this peptide contains substantially more membrane-spanning segments than the others. We therefore tentatively propose that this membrane-embedded region of the H⁺-ATPase molecule contains six membrane-spanning segments, in agreement with recent models for the topography of the COOH-terminal region of the related sarcoplasmic reticulum Ca^{2+}-translocating ATPase (20, 21). It should be mentioned in this regard that the model of Fig. 5 does not intend to propose the precise points of entry and exit of the ATPase polypeptide chain into and out of the membrane in the membrane-embedded segments, particularly in the COOH-terminal membrane-embedded region. Alternative arrangements for the COOH-terminal region, such as one with an initial point of entry around residue 688 and an additional membrane-spanning stretch in the relatively long cytoplasmic loop indicated in the model, are almost equally feasible for this region of the molecule. The arrangement shown was chosen on the basis of our earlier hydropathy analysis and an attempt to minimize the number of charged residues in the membrane. But it should be kept in mind in all considerations of this kind for membrane transport molecules that because the membrane-embedded domain of these molecules almost certainly contains a polar region through which the transport occurs it may be incorrect to adhere too rigorously to models in which polar residues must be excluded from the membrane. This also suggests that in general, models for the transmembrane topography of membrane transport molecules based solely on hydropathy analyses may tend to underestimate the actual number of membrane-spanning stretches present in the molecule.

Fig. 6 presents a model for the overall topography of the H⁺-ATPase taking into account the information gained from these studies and information available from several of our earlier studies as well. As established before (2) the first 99 residues of the ATPase molecule are located on the cytoplasmic side of the membrane. As shown here, residues 100 to at least 173 constitute the first, approximately 7-kDa, membrane-embedded sector, with probably two membrane-spanning segments. From our previous studies the locations of residues 174–185 were uncertain. But the fact that the first membrane-embedded sector must end after residue 173 on the cytoplasmic side of the membrane establishes the locations of these residues on the cytoplasmic side of the membrane because residue 186 was previously established to be present on this side of the membrane, and residues 174–185 constitute a stretch far too short to cross the membrane and return. Thus, in keeping with our earlier notation (2) residues

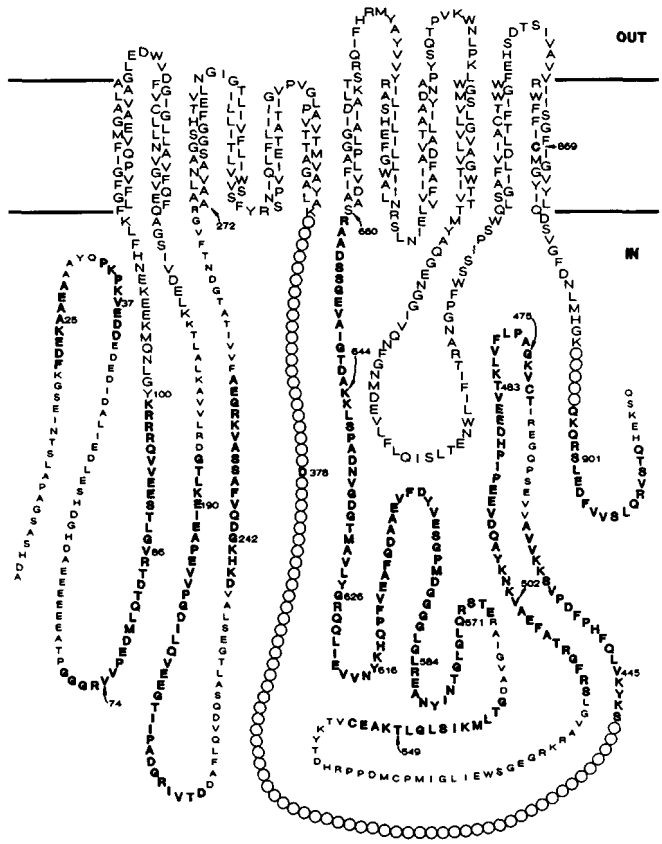

FIG. 6. **Model of the transmembrane topography of the complete H⁺-ATPase molecule.** The characteristics of the membrane-embedded region described in the legend to Fig. 5 apply to this figure as well. The *uppercase bold* amino acid designations indicate residues directly established to be located on the cytoplasmic side of the membrane in our previous studies (2) except for the active site aspartate at position 378 and the cysteine at position 869, which are included as points of reference. The *smaller letters* indicate residues deduced from the present experiments and our earlier studies to be cytoplasmically located as explained before (2) and under "Discussion." The *numbers* indicate the beginning of the peptides directly established in our previous experiments to be cytoplasmically located (2) except for those indicating the beginning of the membrane-embedded peptides and those at Asp^{378} and Cys^{869}.

174–185 with such a deduced location are indicated by the *smaller letters*, in contrast to *larger letters* that indicate a topographical location directly established by NH_2-terminal sequence analysis.

The next 71 residues were established directly or indirectly in our earlier studies to be present on the cytoplasmic side of the membrane, but the location of residues 257–271 remained uncertain. However, the establishment of Ala^{272} in these studies as a tryptic cleavage site on the cytoplasmic side of the membrane also allows the deduction of the location of residues 257–271 on the cytoplasmic side of the membrane, again because this stretch is too short to cross the membrane and return.

The next 84 residues or more constitute the second, approximately 7.5-kDa, membrane-embedded segment described in this paper. As mentioned above it is unlikely that this peptide is any shorter than 84 residues, but it is at present uncertain where this peptide emerges on the cytoplasmic side of the membrane. The location of the next nearly 80 residues on the cytoplasmic side of the membrane will be the subject of a future paper, and the next 219 residues, from Ser^{441} through Arg^{659}, were established previously to exist on the cytoplasmic side of the membrane (2).

The next 232 residues or more constitute the third, approximately 21-kDa, membrane-embedded segment of the H^+-ATPase molecule defined in the studies presented here. This peptide cannot end before Lys^{891} because this is the first tryptic cleavage site downstream from Cys^{869}, which was demonstrated in another recent study in this laboratory (22) to be present in the approximately 21-kDa peptide. However, this peptide must end before Ser^{901} because this is the first residue in a COOH-terminal peptide known to be released from the liposomes (1). The approximately 21-kDa peptide must, therefore, appear on the cytoplasmic side of the membrane before Gln^{897}. The location of residues 892–896 cannot be stated from the available data, but the remaining residues at the COOH terminus have been shown previously to be present on the cytoplasmic side of the membrane (1, 2), as indicated in the model.

In conclusion, in this article we have presented evidence establishing the parts of the *N. crassa* plasma membrane H^+-ATPase which are embedded in the membrane. Taken together with information gained from our earlier topographical studies this information allows a nearly complete description of the two-dimensional structure of the H^+-ATPase molecule. This two-dimensional model of the H^+-ATPase structure should be of considerable value in future efforts to elucidate the three-dimensional structure and mechanism of this transport enzyme and may be of value in similar studies of other transporters, particularly those in the aspartyl-phosphoryl-enzyme intermediate family of ion-translocating ATPases.

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