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Identification of the Major Cytoplasmic Regions of the *Neurospora crassa* Plasma Membrane H^+-ATPase Using Protein Chemical Techniques*

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The transmembrane topography of the *Neurospora crassa* plasma membrane H^+-ATPase has been investigated using purified, reconstituted components and direct protein chemical techniques. Reconstituted proteoliposomes containing H^+-ATPase molecules oriented predominantly with their cytoplasmic surface facing outward were treated with trypsin to liberate peptides present on the cytoplasmic surface of the H^+-ATPase as recently described (Hennessey, J. P., Jr., and Scarborough, G. (1990) *J. Biol. Chem.* 265, 532–537. The released peptides were then separated from the proteoliposomes by gel filtration chromatography and further purified by high performance liquid chromatography. Fourteen such peptides were identified by NH_2-terminal amino acid sequence analysis, directly defining these parts of the molecule as present on the cytoplasmic surface of the membrane. Moreover, this information identified several additional flanking stretches as likely to be cytoplasmically located by virtue of the fact that they are too short to cross the membrane and return. These results and the results of other recent experiments establish 417 residues of the 919 present in the ATPase molecule, at positions 2–100, 186–256, 441–663, and 897–920, as cytoplasmically located. Taken together with the results of our preliminary investigations of the membrane-embedded sectors of the ATPase, this information allows the formulation of a reasonably detailed model for the transmembrane topography of the ATPase polypeptide chain.

The major goal of this laboratory is an understanding of the molecular mechanism by which the ATPase in the plasma membrane of *Neurospora crassa* catalyzes ATP hydrolysis-driven proton translocation. A primary focus of our current efforts, and an essential requirement for achieving this goal, is the generation of a detailed structural model of the H^+-ATPase as it exists in the lipid bilayer. As one approach to this end, we have recently established a reconstituted H^+-ATPase-bearing proteoliposome preparation as an excellent model system for exploring certain aspects of the transmembrane topography of the ATPase, and have used this proteoliposome preparation to establish the location of the NH_2 and COOH-termini of the molecule on the cytoplasmic side of the membrane (1). In the studies reported in this paper we have extended this approach to topographic analysis of the H^+-ATPase molecule to define numerous additional portions of the ATPase polypeptide chain that are located on the cytoplasmic side of the lipid bilayer. The results of these investigations provide us with a number of well-defined structural constraints that permit the construction of a reasonably detailed model of the topography of this interesting transport enzyme.

EXPERIMENTAL PROCEDURES

Purification and Reconstitution of the H^+-ATPase—The plasma membrane H^+-ATPase of *N. crassa* was purified by the procedure of Smith and Scarborough (2, 3). Freeze-thaw reconstitution of the purified enzyme into asolectin liposomes and enrichment and concentration of the resulting proteoliposomes was carried out as described by Hennessey and Scarborough (1). The ATPase activity of the proteoliposome preparations was measured as described previously (4, 5). The proteoliposome preparations obtained were essentially identical to those described earlier (1) with respect to protein concentration, ATPase-specific activity, stimulation by nigericin, and ATPase sidedness as indicated by the tryptic degradation profile.

Tryptic Digestion of the H^+-ATPase Proteoliposomes and Isolation of the Released Peptides—Tryptic digestion of the H^+-ATPase proteoliposomes was performed essentially as described previously (1). Briefly, 15 ml of the proteoliposome preparation was brought to 1% (w/v) ammonium bicarbonate to adjust the pH to approximately 8, and trypsin was added to a final ratio of 1:10 (trypsin/H^+-ATPase, w/w). This solution was incubated at 37 °C for 7 h with occasional swirling, and then subjected to chromatography on a 2.5 × 30-cm Sepharose CL6B column equilibrated and eluted with 10 mM MES,[1] 50 mM potassium acetate, adjusted to pH 6.8 with KOH. The column was eluted at a flow rate of approximately 20 ml/h, and 15-min fractions collected. The non-turbid, post-void volume fractions that showed absorbance at 280 nm were pooled and lyophilized overnight. The resulting lyophilizate consisted of peptides released from the proteoliposomes and buffer salts dissolved in 80–90% glycerol.

Purification of the Released H^+-ATPase Peptides by HPLC—Lyophilized, released peptide samples were filtered through 0.45-μm nylon-66 filters (Rainin) and subjected to HPLC analysis as described previously (1, 6) using a Hi-Pore RP-318 reversed-phase analytical HPLC column (4.6 × 250-mm; Bio-Rad) and a Hi-Pore guard column (4.6 × 30-mm; Bio-Rad) eluted with a gradient of 1–100% acetonitrile in water containing 0.1% trifluoroacetic acid. Most of the peptides were further purified by a second HPLC run before NH_2-terminal amino acid sequence analysis. However, peptide 14 (Fig. 2 and Table I) was not, since serious losses were incurred when this peptide was subjected to rechromatography.

NH_2-terminal Amino Acid Sequence Analysis of the H^+-ATPase Peptides—NH_2-terminal sequence analyses were carried out at the Protein Chemistry Laboratory of UNCCH-NIEHS. Peptide samples were dried down on polybrene-treated, Applied Biosystems GF/C

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[1] The abbreviations used are: MES, 2-[*N*-morpholino]ethanesulfonic acid; HPLC, high performance liquid chromatography.

glass fiber filters and analyzed using an Applied Biosystems 475A sequencing system.

Other Methods—The protein content of the proteoliposomes was estimated by the procedure of Kaplan and Pederson (7) for assaying protein in the presence of lipids. The values obtained were corrected for a minor contribution of the liposomes themselves in this assay by subtracting the values obtained from identical amounts of similar liposomes prepared in the absence of the ATPase. The amount of liposomes in the preparations was determined from the phosporous content by the procedure of Bartlett (8).

Materials—The sources of most of the materials used have been described previously (1).

RESULTS AND DISCUSSION

As demonstrated in a recent paper (1), 85–90% of the H⁺-ATPase molecules in proteoliposome preparations identical to those used in the present study are functional and oriented with their cytoplasmic portion facing outward. The remaining ATPase molecules in the preparation are extremely resistant to any detectable degradation by trypsin (1) and presumably are present in the liposomes with the opposite orientation. With this information taken into account, Fig. 1 outlines the concept of our approach to defining regions of the H⁺-ATPase molecule present on the cytoplasmic side of the membrane. ATPase molecules oriented with their cytoplasmic surface facing outward are cleaved with trypsin, resulting in the formation of liposomes containing the membrane-associated and exocytoplasmic portions of the molecule, together with a collection of peptides from the cytoplasmically located regions of the molecule that are released from the liposomes. The released peptides are then separated from the liposome-bound peptides by Sepharose CL6B column chromatography and are then separated from one another by reversed-phase HPLC.

Fig. 2 shows the results obtained when the released peptide collection is fractionated by HPLC on a C_{18} column as described previously (1). More than 50 peaks with significant absorbance at 214 nm can be discerned in the sections of the chromatogram shown. Ideally, every peptide peak in such a chromatogram that can be identified by NH_2-terminal amino acid sequence analysis as originating from the H⁺-ATPase molecule by reference to the gene sequence (9, 10) represents evidence for the location of that peptide on the cytoplasmic side of the membrane. However, minor uncertainties as to the physical state of all of the ATPase molecules in the preparation warrant caution in assigning a cytoplasmic location to peptides present in very low yields. That is, although it is reasonably certain that 85–90% of the ATPase molecules in the proteoliposome preparation are functional and present with their cytoplasmic surface exposed, and that the remaining 10–15% of the ATPase molecules in the preparation are not detectably degraded by trypsin (1), it is possible that a small amount of the ATPase molecules in the preparation is present in other physical states. Moreover, it is likely that certain of the peaks present in the HPLC profile of Fig. 2 are derived from proteinaceous or non-proteinaceous contaminants of the asolectin used in these experiments and are therefore of little interest. For these reasons, it was deemed necessary to confirm the ATPase origin of the peptides present in the HPLC analysis of Fig. 2 and estimate their yields relative to the mass of the H⁺-ATPase present in the proteoliposomes at the beginning of the experiment, so that only major ATPase peptides would be chosen for NH_2-terminal sequence analysis. This was done by comparing the area in the 214-nm profile of each peak with that of the corresponding peak in the profile of a standard tryptic digest of the purified H⁺-ATPase, as described in detail previously (1). Only peptides with counterparts in the standard tryptic digest and with recoveries greater than 50% were chosen for subsequent NH_2-terminal sequencing. The peaks chosen on this basis are indicated by the *asterisks* in Fig. 2. The *asterisks with numbers* indicate the peaks that yielded unambiguous sequences.

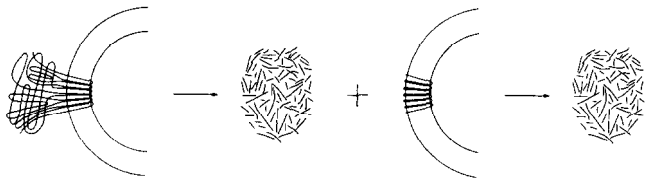

FIG. 1. **Experimental approach to defining cytoplasmic regions of the H⁺-ATPase.** See text for details.

Table I summarizes the recovery, the amino acid sequence obtained, and the position in the linear sequence of each of the H⁺-ATPase peptides numbered in Fig. 2. These data directly establish residues 74–85, 86–96, 190–215, 242–252, 445–456, 475–482, 483–499, 502–508, 549–555, 571–583, 584–615, 616–625, 626–643, and 644–659 of the ATPase molecule as present on the cytoplasmic surface of the lipid bilayer.[2]

This information provides major constraints for models of the transmembrane topography of the ATPase and together with information obtained from other recent experiments allows the formulation of a reasonably detailed and accurate model for the disposition of the ATPase polypeptide chain in the membrane. Fig. 3 presents a model that takes into account all of the available pertinent information. In the model, residues with topographic locations directly established are indicated by the *larger, bold letters* using the single-letter nomenclature for amino acid identities. Beginning at the NH_2-terminal end of the molecule, we have recently demonstrated (1) that treatment of ATPase-bearing proteoliposomes identical to those used in the present investigation with trypsin in the presence of the H⁺-ATPase ligands, Mg^{++}, ATP, and vanadate produces approximately 97-, 95-, and 88-kDa truncated forms of the H⁺-ATPase similar to those shown by Mandala and Slayman (11) to arise from cleavage of the ATPase molecule at Lys^{24}, Lys^{36}, and Arg^{73}, respectively, which provided evidence for the cytoplasmic location of these residues. The cytoplasmic location of these tryptic cleavage sites was also demonstrated recently by Mandala and Slayman (12). Because the cleft in which trypsin substrates bind during catalysis reaches several residues beyond the scissile bond in both directions along the cleaved polypeptide chain (13), at least 4 residues in each direction from the cleaved bond are presumed to be accessible in the polypeptide chain in order for trypsin to act. Therefore, in the model of Fig. 3, in addition to the residues identified by peptide purification and NH_2-terminal sequence analysis, 4 residues in each direc-

[2] As mentioned above, 10–15% of the ATPase molecules in the reconstituted proteoliposome preparation are totally resistant to degradation by trypsin. Experiments as yet unpublished indicate clearly that this population represents ATPase molecules oriented right-side-out, *i.e.* with their cytoplasmic portion facing inside the liposomes and their exocytoplasmic surface facing outward. As such, these molecules provide an important control against two potential artifacts inherent in the proteolytic cleavage approach to topographical analysis of integral membrane proteins. First, the fact that these molecules are totally resistant to tryptic cleavage establishes that the proteoliposomes are indeed impermeable to trypsin. Second, although unlikely, it is remotely possible that after removal of the major cytoplasmic portions of the ATPase, trypsin could enter the liposomes via the remaining membrane-embedded region and reach the exocytoplasmic surface of the molecule. The fact that no peptides can be removed from the exocytoplasmic surface of the ATPase, as evidenced by the resistance of the right-side-out molecules to tryptic cleavage, makes it extremely unlikely that any of the peptides present in the released peptide collection could have originated via such an artifactual mechanism.

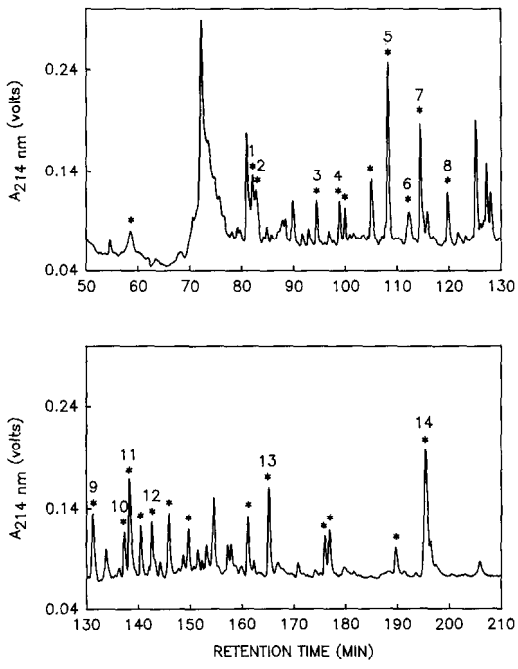

FIG. 2. **Reversed-phase HPLC purification of the individual peptides released from the proteoliposomes.** Peptides released from the proteoliposomes by trypsin were separated from the proteoliposomes by Sephadex CL6B column chromatography and then subjected to HPLC as described under "Experimental Procedures." The *asterisks* indicate the peptides subjected to subsequent NH_2-terminal sequence analysis and the *asterisks with numbers* above indicate the peptides that yielded unambiguous sequences. See text for additional details.

TABLE I

Amino acid sequences obtained from the peptides numbered 1–14 in the HPLC analysis shown in Fig. 2

See text for details.

Peptide no.[a]	Recovery[b]	Sequence obtained	Position in sequence[c]
	%		
1	63	VAEFATR	502–508
2	51	KADTGIAVEGSSDAAR	644–659
3	95	GDQVFASSAVK	242–252
4	98	VGLTSEEVVQ	86–96
5	72	TVEEDHPIPEEV	483–499
6	75	VVPEDMLQ	74–85
7	90	TLGLSIK	549–555
8	97	QLGLGTNIYNAE	571–583
9	67	GYLVAMTGDGVN	626–643
10	68	GAPLFVLK	475–482
11	72	VLQFHPFDPV	445–456
12	63	YNVVEILQQR	616–625
13	67	EIEAPEVVPGDI	190–215
14	60	LGLGGGGDMPGS	584–615

[a] From Fig. 2.

[b] Calculated from the area of the peak in the HPLC run of Fig. 2, the area of the corresponding peak in an HPLC analysis of a known amount of trypsin-digested purified H⁺-ATPase, and the amount of liposome-bound ATPase used in the experiment.

[c] Assuming COOH-terminal cleavage at the next arginine or lysine residue in the deduced amino acid sequence (9, 10).

tion from directly established tryptic cleavage sites are defined as being present on the cytoplasmic side of the membrane, and are therefore also indicated with the *larger, bold letters*. Thus, the above-mentioned studies directly establish residues 21–28, 33–40, and 70–77 as cytoplasmically located, as indicated in the model. The establishment of these residues as cytoplasmically located also allows deduction of the location of residues 2–20, 29–32, and 41–69 on the cytoplasmic side of

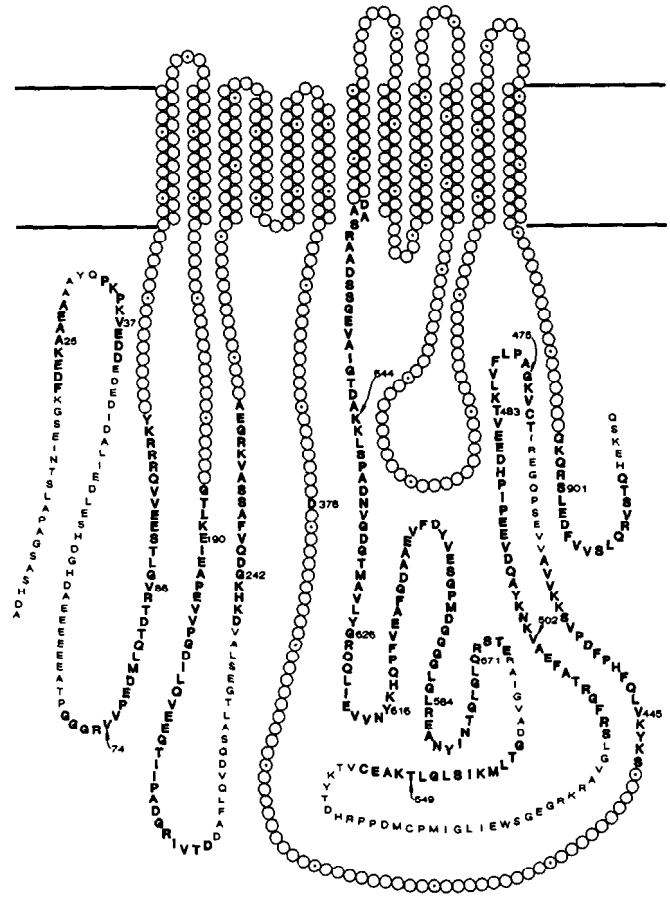

FIG. 3. **Model for the topography of the H⁺-ATPase.** The *uppercase bold letters* indicate amino acid residues that are defined as cytoplasmically located by NH_2-terminal sequence analysis of purified peptides released from the proteoliposomes by trypsin or as described previously (1) as explained in the text. An exception to this is the *D* at residue 378 which is included only to indicate the position of the phosphorylated active site aspartate. The *numbers* indicate the first sequenced residue in the peptides established to be cytoplasmically located. The *smaller letters* indicate residues deduced to be cytoplasmically located, as explained in the text. The *dots* are placed at 10-residue intervals for counting purposes. The indicated number of membrane-spanning segments was chosen on the basis of our previously reported hydropathy analysis of the H⁺ATPase molecule (6).

the membrane with reasonable certainty, because these stretches are too short to form transmembrane helices that cross the membrane and return between residues established to be on the cytoplasmic side of the membrane. Residues with such inferred locations are indicated in the model by the *smaller letters*. The caveats with such deduced stretches are, of course, that they could conceivably enter into the plane of the membrane inside the structure of the H⁺-ATPase or cross the membrane bilayer as non-helical structures. For these reasons, it is useful to assign *smaller letters* to these stretches in order to acknowledge the fact that their location is likely, but not quite as certain as the stretches established directly by NH_2-terminal amino acid sequence analysis of peptides released from the membrane. Continuing along the polypeptide chain, the identification of peptides 6 and 4 described in Fig. 2 and Table I establish residues 70–100 as located on the cytoplasmic side of the membrane, as indicated by the *bold letters* in the model.

At this point, the polypeptide chain approaches its first entry into the lipid bilayer. From experiments similar in concept to that schematized in Fig. 1, but in which the peptides remaining associated with the liposomes were ana-

lyzed by our recently developed methodology for analyzing the hydrophobic portions of the ATPase (6, 14), we have obtained evidence that the great majority of the membrane-embedded domain of the ATPase is contained in three tryptic fragments with approximate molecular masses based upon their mobilities in SDS-polyacrylamide electrophoresis gels of 7, 7.5, and 21 kDa, beginning at residues 100, 272, and 660, respectively, and with 8–12 membrane crossings (15 and unpublished data, with M_r values altered to take into account the information in Ref. 16). The model in Fig. 3 was constructed to take this information into account, but amino-acid letter assignments have not been made for these regions because the emphasis of this paper is on the cytoplasmic regions of the H⁺-ATPase molecule and because our investigation of the membrane-embedded region of the ATPase is not quite complete. The results of these latter studies will be elaborated upon in detail in a forthcoming paper. However, for the purposes of the present discussion, suffice it to say that it is reasonably certain that the ATPase polypeptide chain crosses the membrane and returns between residues 100 and 186, accounting for the ~7-kDa hydrophobic peptide beginning at residue 100 that remains associated with the membrane after tryptic cleavage.

At this point, the polypeptide chain appears again on the cytoplasmic side. The identification of peptide 13 described in Fig. 2 and Table I establishes residues 186–219 as located on this side of the membrane as shown. Likewise, the identification of peptide 3 defines residues 238–256 as also present on the cytoplasmic side, and, by deduction, indicates the presence of residues 220–237 on the same side. The chain then approaches the next membrane-embedded region and proceeds with two to four membrane crossings, accounting for the ~7.5-kDa hydrophobic peptide beginning at residue 272, found associated with the liposomes (see above).

From the information thus far available, the topographical location of the next ~80 residues (~360–440) is unclear. From our studies of the chemical state of the 8 cysteine residues in the H⁺-ATPase molecule (17), the retention times in the HPLC system used in the present investigation of two tryptic cysteine-containing peptides comprising residues 363–379 and 388–414 are well established at about 160 and 179 min, respectively. Moreover, during the course of the cysteine work, these two peptides did not exhibit any problems with respect to losses, and were recovered quantitatively. Nevertheless, repeated attempts to find these peptides in the released peptide collection have been uniformly negative. Because the first of these peptides contains the active site aspartate at position 378, it is likely that this part of the stretch in question is accessible from the cytoplasmic side of the membrane, as indicated in the model. However, the failure to find these peptides in the released peptide collection may indicate that this part of the ATPase molecule, including the active site aspartate, is embedded in the membrane, or is intimately associated with other parts of the molecule that are. It is pertinent in this regard that the Ca^{2+}-binding sites of the closely related Ca^{2+}-translocating ATPase of sarcoplasmic reticulum appear to be located close to one another and deep in the membrane-embedded region of the molecule (18–25, but also see 26), and also appear to be near (20, 23), probably within 4 Å of (27), the terminal phosphoryl group of bound ATP next to the active site aspartate, suggesting that the active site aspartate in the Ca^{2+}-ATPase may also be embedded in the membrane. Clearly, additional work will be required to elucidate the reasons for the absence of these peptides in the released peptide collection and ascertain whether or not they are present in the membrane-embedded region of the H⁺-ATPase. It might be mentioned in this context, however, that if the active site aspartate is indeed close to or embedded in the membrane in the aspartyl-phosphoryl-enzyme intermediate-type ATPases, then direct energy coupling mechanisms for these enzymes such as those proposed in detail earlier (28, 29) become entirely reasonable possibilities.

After this stretch of questionable residues, the topography of the rest of the H⁺-ATPase molecule is fairly well-established. The identification of peptides 11, 10, 5, 1, 7, 8, 14, 12, 9, and 2 (Fig. 2 and Table I) in the released peptide collection establishes the topographical location of residues 441–460, 471–486, 479–503, 498–512, 545–559, 567–587, 580–619, 612–629, 622–647, and 640–663, respectively, on the cytoplasmic side of the membrane. This information additionally infers the cytoplasmic location of residues 461–470, 513–544, and 560–566, as indicated by the small letter assignments of these residues.

The ATPase polypeptide chain then enters into the last membrane-embedded region and proceeds with four to six membrane crossings, accounting for the ~21 kDa hydrophobic peptide beginning at residue 660 that remains associated with the liposomal membrane after tryptic cleavage (see above). And finally, the identification by NH_2-terminal sequence analysis of an undecapeptide beginning at Ser^{901} in our previous investigation of the peptides released from the reconstituted proteoliposomes (1) directly established the presence of residues 897–915, and by deduction, residues 916–920 of the ATPase molecule, on the cytoplasmic side of the membrane as indicated in the model of Fig. 3.

In conclusion, in this paper we have demonstrated by direct protein chemical methods the presence of 14 peptide segments of the *N. crassa* plasma membrane H⁺-ATPase, and by inference, the presence of several additional segments of the molecule, on the cytoplasmic side of the membrane. This information, taken together with the results of previous investigations of the locations of the NH_2 and COOH-termini, has localized at least 417 of the 919 residues in the H⁺-ATPase molecule as cytoplasmically located. With this information, and additional preliminary results regarding the membrane-embedded segments of the molecule, a first generation topographical model of the H⁺-ATPase containing a substantial amount of established detail has been constructed. The model should be a useful starting point for further experimentation on the topography and other structural aspects of the H⁺-ATPase molecule and could also be useful for comparative studies with the other transport ATPases in the aspartyl-phosphoryl-enzyme intermediate family.

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