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

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Gene A. Scarborough† and John P. Hennessey, Jr.§

From the Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599

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 NH2-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 NH2 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 trypic 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, 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 lyophilized 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 NH2-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.

NH2-terminal Amino Acid Sequence Analysis of the H+-ATPase Peptides—NH2-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
described previously (1). More than 50 peaks with significant recoveries greater than 50% were chosen for subsequent NH2-terminal sequencing. The peaks chosen on this basis are indicated by the asterisks in Fig. 2. The asterisks with numbers 1-4 indicate the peaks that yielded unambiguous sequences.

Table 1 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, 90-96, 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 NH2-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, Mg2+, 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 Lys64, Lys66, and Arg67, 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 NH2-terminal sequence analysis, 4 residues in each direct

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2As 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 liposome 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 artificial mechanism.

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

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

| Peptide no. | Recovery | Sequence obtained | Position in sequence |
|-------------|----------|-------------------|----------------------|
| 1           | 63       | VAEFATR           | 502–508              |
| 2           | 51       | KADTG1VEGSSDAAR   | 644–659              |
| 3           | 95       | GDQVFASAVK       | 242–252              |
| 4           | 98       | VGLTFSEFVQ       | 86–96               |
| 5           | 72       | TVEDRF1PPEEV     | 483–499             |
| 6           | 75       | VVIPDMQL         | 74–85               |
| 7           | 90       | T1GLSLK          | 549–555             |
| 8           | 97       | Q1GLVT1YNAE      | 571–583             |
| 9           | 67       | G1YLMAT1GQV       | 626–643             |
| 10          | 68       | G1PLFLVK         | 475–482             |
| 11          | 72       | V1QP1HP1PFV      | 445–456             |
| 12          | 63       | YV1VE11QQR       | 616–625             |
| 13          | 67       | E1EAPFV1PQDI     | 190–215             |
| 14          | 60       | L1G1GG1G1MPGS     | 584–615             |

1 From Fig. 2.

2 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.

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

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**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 NH2-terminal sequence analysis and the asterisks with numbers above indicate the peptides that yielded unambiguous sequences. See text for additional details.

**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 NH2-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 hydrophathy analysis of the H+-ATPase molecule (6).

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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 NH2-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 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 NH2-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-

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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, 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 Ca2+-binding sites of the closely related Ca2+-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 (29, 33), 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 Ca2+-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 461–471, 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 NH2-terminal sequence analysis of an undecapeptide beginning at Ser391 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, 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, together with the results of previous investigations of the locations of the NH2 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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