Biochimica et Biophysica Acta 1108 (1992) 153–158

BBAMEM 75701

Cytoplasmic location of amino acids 359–440 of the *Neurospora crassa* plasma membrane H^+-ATPase

U. Subrahmanyeswara Rao, Delbert D. Bauzon and Gene A. Scarborough

Department of Pharmacology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC (USA)

(Received 14 February 1992)

Key words: ATPase, H^+-; Proteoliposome; Transmembrane protein topography; (*N. crassa*)

The topographic location of the region comprising amino acids 359–440 of the *Neurospora crassa* plasma membrane H^+-ATPase has been elucidated using reconstituted proteoliposomes and protein chemical techniques. Proteoliposomes containing H^+-ATPase molecules oriented predominantly with their cytoplasmic surface facing outward were cleaved with trypsin and the resulting digest was subjected to centrifugation on a glycerol step gradient to separate the released and liposome-bound peptides. The released peptides were recovered in the upper regions of the step gradient, whereas the liposome-bound peptides were recovered near the 40% glycerol interface. The released peptides present in the upper fractions were reduced, ^{14}C-carboxymethylated, and then separated by high performance liquid chromatography. Two radioactive cysteine-containing peptides with retention times of about 162 and 182 min were identified as H^+-ATPase peptides comprising residues Leu^{363}–Lys^{378} and Leu^{388}–Arg^{414}, respectively, by comparison to standards prepared from the purified ATPase. This information thus establishes a cytoplasmic location for residues 359–418 in the H^+-ATPase polypeptide chain. It also infers a cytoplasmic location for residues 419–440, since this stretch of amino acids is too short to cross the membrane and return between regions known to be cytoplasmically located. These results and the results of other recent experiments establish the topographical location of nearly all of the 919 residues in the H^+-ATPase molecule.

The primary objective of this laboratory is an understanding of the molecular mechanism by which the *Neurospora crassa* plasma membrane H^+-ATPase catalyzes ATP hydrolysis-driven electrogenic proton translocation. This enzyme belongs to the aspartyl phosphate intermediate family of transport ATPases which includes the Na^+/K^+-, H^+/K^+-, and Ca^{2+}-ATPases of animal cell plasma membranes, the Ca^{2+}-ATPase of sarcoplasmic reticulum, the *Escherichia coli* plasma membrane K^+-ATPase, and the H^+-ATPases of fungal and plant plasma membranes [1–6]. In the last few years, the genes for these enzymes have been cloned and sequenced, and analyses of the deduced amino acid sequences have revealed that their overall sequence similarity is substantial and that their predicted structures are similar with respect to the positions of the putative transmembrane regions and the residues involved in ATP binding and phosphorylation [7–13,19]. In this laboratory, we have been employing reconstituted H^+-ATPase proteoliposomes [14] and protein chemistry methodology [15] for identifying the regions of the *Neurospora* plasma membrane H^+-ATPase that are cytoplasmically located or membrane-embedded [16–18]. The results obtained from these studies have established directly or indirectly the topography of the majority of the H^+-ATPase molecule. However, the location of a stretch of about 80 amino acids beginning at Lys^{356} could not be established since known tryptic peptides expected from this region of the ATPase molecule were clearly missing from the analyses. This region is of significant interest because it contains three blocks of highly conserved residues around the phosphorylated aspartate [19] and is expected to be present on the cytoplasmic side of the membrane [17], but the absence of peptides from this region in our cytoplasmic peptide collection raised the possibility that it might be located elsewhere in the molecule. For this reason, we have further investigated the location of this previously unidentified region using a modified procedure for isolation of the peptides released from the ATPase-proteoliposomes after tryptic digestion. The results of this study demonstrate that

Correspondence to: G.A. Scarborough, Department of Pharmacology, CB No. 7365, FLOB 231H, University of North Carolina, Chapel Hill, NC 27599-7365, USA.

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

residues 359–440 are indeed present on the cytoplasmic side of the membrane, and taken together with the information obtained from our earlier studies, establish the topographic location of nearly all of the 919 amino acids present in the H^+-ATPase molecule.

Experimental Procedures

Purification and reconstitution of the H^+-ATPase. Purification of the *Neurospora* plasma membrane H^+-ATPase and freeze-thaw reconstitution into asolectin liposomes were carried out as described previously [16–18]. The proteoliposome preparations were essentially identical to those described earlier with respect to protein concentration, stimulation by nigericin, and ATPase sidedness as indicated by the tryptic degradation profile [16–18].

Tryptic digestion and separation of the released and liposome-bound peptides. Tryptic digestion of the proteoliposomes was carried out as described previously [16–18]. The digest was diluted with an equal volume of 10 mM Mes, 50 mM potassium acetate buffer, pH 6.8 with KOH, to a volume of 22 ml and then underlaid with enough 40% (w/v) aqueous glycerol solution to fill a 40 ml ultracentrifuge tube. The step gradient was then centrifuged at 60000 rpm in a Beckman 70 Ti rotor for 4 h at 4°C (262000 × *g* at $r_{average}$) to separate the released and liposome-bound peptides. After centrifugation, the solution above the glycerol shelf was fractionated into 18 approximately 1.3-ml aliquots and used for further analyses as described below.

Extraction, reduction and ^{14}C-carboxymethylation of hydrophilic constituents from the lower gradient fractions. The hydrophilic constituents in the lower fractions containing the liposomes were isolated by the procedure of Bligh and Dyer as modified by Gibson et al. [20]. Fractions 15–17 containing the liposomes were pooled and 2.2 ml were mixed with 5.5 ml of methanol and 2.75 ml of chloroform, and the mixture was incubated at room temperature for 20 min followed by centrifugation at 3000 × *g* for 5 min. The liquid phase was collected and mixed with 2.75 ml of chloroform and 2.75 ml of 1% (w/v) KCl, and the mixture incubated for 20 min at room temperature. This was then centrifuged as above and the upper phase containing the hydrophilic constituents was collected and concentrated by lyophilization, and the volume was then brought to 0.9 ml by the addition of water. Reduction with DTT and carboxymethylation with ^{14}C-iodoacetate were then carried out essentially as described [21]. The sample was degassed in vacuo for 20 min after which 0.1 ml of 2 M Tris containing 20 mM EDTA and 20 mM DTT (pH 8.3 with HCl) was added. Recrystallized guanidine hydrochloride was then added to a final concentration of 5.2 M and the mixture was flushed with nitrogen on the surface for 25 min. The tube containing the mixture was sealed with parafilm and left at room temperature for 3 h. 0.32 ml of 13.125 mM iodo[2-^{14}C]acetic acid (pH 7.0 with NaOH) (specific radioactivity, 3276 cpm/nmol) was then added and the mixture incubated for 25 min in the dark. HPLC separation of the material present in the resulting solution was carried out as described below.

Reduction, ^{14}C-carboxymethylation and HPLC analysis of the upper gradient fractions. Fractions 1–14 of the step gradient, which were free of liposomes and liposome-bound peptides, were pooled and 5 ml was concentrated by lyophilization. The volume was then brought to 0.9 ml by the addition of water, and the mixture subjected to reduction and ^{14}C-carboxymethylation as described above. In order to remove the large amounts of excess radioactive material present in the sample containing the small peptides and thus not amenable to dialysis, our standard HPLC procedure was slightly modified. While flushing with 1% (v/v) acetonitrile in 0.1% (v/v) aqueous TFA at a flow rate of 1 ml/min, the carboxymethylated peptide solution containing released peptides from 1 nmol of the ATPase was applied to a Bio-Rad RP-318 HPLC column (4.6 × 250 mm) as 0.5-ml aliquots at time intervals of 3–5 min until all of the sample was injected. Washing of the column was then continued for 60 min under the above conditions. Control experiments suggested that little or no peptide material is washed off of the column in this procedure, while most, but not all, of the excess radioactive material is. Elution of the peptides with a gradient of 1–100% (v/v) acetonitrile in 0.1% (v/v) aqueous TFA was then carried out as described previously [16,17].

Fractions (1 ml) containing the two peptides of interest with retention times of about 162 and 182 min in the initial HPLC separation were collected and 0.4 ml of the fractions were dried by lyophilization and then subjected to further purification. The dried peptide fractions were dissolved in 100 μl of 30% (v/v) acetonitrile containing 0.1% TFA, and the resulting solution was injected onto the Bio-Rad RP-318 column. The chromatogram was developed using a 60 min linear gradient of 30–38% (v/v) acetonitrile containing 0.1% (v/v) TFA, at a flow rate of 0.5 ml/min. 0.5-ml fractions were collected and the radioactivity in the fractions was determined by scintillation counting in the mixture of Patterson and Greene [22].

Materials. The sources of the materials used have been described previously [16–18,21].

Results and Discussion

As established previously [16,17], H^+-ATPase molecules present in reconstituted asolectin proteoliposomes prepared by our freeze-thaw procedure are fully functional and oriented predominantly with their

cytoplasmic surfaces facing outward. Thus, any peptides released from such proteoliposomes after tryptic cleavage of the ATPase are defined as cytoplasmically located, whereas fragments remaining associated with the liposomes are candidates for membrane-embedded and/or exocytoplasmic portions of the molecule. By purifying and determining the NH_2-terminal amino acid sequences of numerous peptides in each of these distinct classes, we have been able to establish the transmembrane topography of the majority of the ATPase molecule. However, one stretch of residues between Lys^{356} and Leu^{440} could never be accounted for. In all of these studies, Sepharose CL-6B chromatography on a relatively large column was used to separate the released and liposome-bound peptides in the tryptic digest. Although numerous released ATPase peptides could be recovered from this procedure in high yield, it seemed possible that marginally hydrophobic or otherwise reactive peptides might be lost by surface adsorption on such a column. We therefore devised an alternative, glycerol step gradient procedure for separation of the released and liposome-bound peptides with a minimum number of steps and minimal surface exposure, in order to search for peptides from the missing region.

The ATPase proteoliposome preparation was treated with trypsin, diluted, and the mixture subjected to centrifugation on a glycerol step gradient, and the gradient fractionated as described under Experimental Procedures. Turbidometric analysis of the resulting fractions indicated that the liposomes migrate to the 40% glycerol interface in this procedure (not shown). As expected, the membrane-embedded H^+-ATPase tryptic peptides are also found in this region as indicated by SDS-PAGE analysis by our recently developed procedure [23] (not shown). In order to determine whether or not any of the missing peptides were present in the liposomal fraction, the hydrophilic constituents present in this fraction were isolated by solvent extraction, reduced, ^{14}C-carboxymethylated and then analyzed by HPLC as described under Experimental Procedures. The sample was reduced and ^{14}C-carboxymethylated because there are two cysteine residues present in the missing region between residues 359 and 440. The results of this experiment are shown in the lower trace of Fig. 1. Few, if any, hydrophilic peptides were found in the liposomal fraction as indicated by the $A_{214\ nm}$; there were also no ^{14}C-carboxymethylated peptides present in the eluate.

We next analyzed the upper gradient fractions for the missing peptides. Fractions 1–14 were pooled, reduced, carboxymethylated with ^{14}C-iodoacetate, and then analyzed by reversed-phase HPLC as described as above. The results of this experiment are shown in the upper trace of Fig. 1. The profile obtained is for the most part comparable to that of our earlier experiments with respect to the various peptides and their amounts [17]. The recovery of the previously identified released peptides in the present study compared to the corresponding peptides in a standard tryptic digest [17], was on average about 91%, indicating that the step gradient procedure is as good or better than the Sepharose CL-6B column procedure [17] for isolating the released peptides. Moreover, at least three additional peaks in the 160–200 min range could be discerned when the released peptides are separated in this way. This is shown clearly in panels A and B of Fig. 2 which compare the 160–200 min region of HPLC eluates of released peptides isolated by our previous procedure (panel A) and the step gradient procedure used in the present study (panel B). The arrows in panel B indicate the additional peaks.

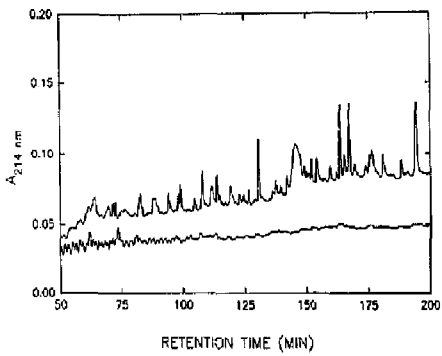

Fig. 1. HPLC separation of hydrophilic peptides in the upper and lower fractions obtained from the glycerol step gradient of the ATPase proteoliposome tryptic digest. Released peptides in the upper fractions of the step gradient were reduced and ^{14}C-carboxymethylated as described under Experimental Procedures. Peptides released from 1 nmol ATPase were separated by HPLC and the profile obtained is shown in the upper trace. Hydrophilic constituents in the lower fractions of the glycerol step gradient were extracted, reduced and ^{14}C-carboxymethylated as described under Experimental Procedures. A portion representing 1 nmol of the ATPase was analyzed by HPLC and the results are shown in the lower trace.

Two of these peaks with retention times of about 162 and 182 min were considered likely to represent cysteine-containing peptides, as their retention times were essentially identical to that of previously identified cysteine peptides comprising residues 363–379 and 388–414, respectively [21]. However, because the reduced, ^{14}C-carboxymethylated peptide mixture could not be dialyzed without losses, the radioactivity profile in this experiment contained a substantial amount of background radioactivity from the ^{14}C-iodoacetate, which partially obscured the peptide-associated label. Therefore, to find out whether the peaks at 162 and 182 min contain cysteine peptides, the material in

these peaks was collected, repurified by HPLC as described under Experimental Procedures, and the radioactivity determined. Panel C and D show the A_{214} nm and labeling profile obtained upon rechromatography of the materials in 162 and 182 min peaks,

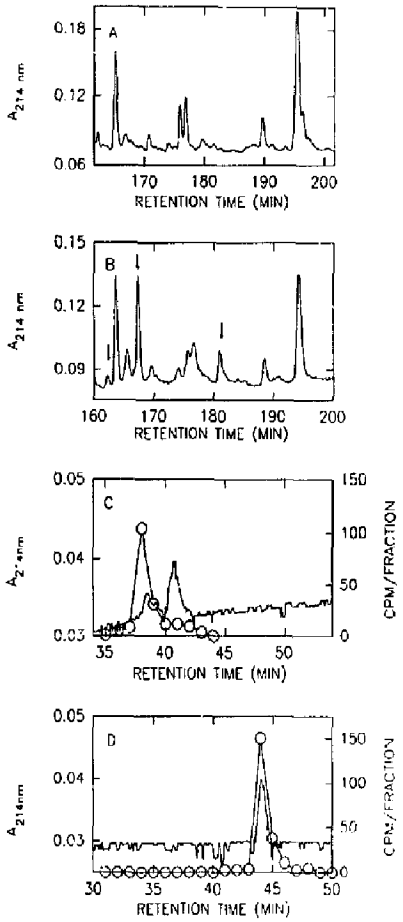

Fig. 2. Comparison of the 160–200 min region of HPLC eluates of released peptides isolated by Sepharose column chromatography (panel A) or glycerol step gradient centrifugation (panel B). The 160–200 min region of the HPLC profile shown in the upper trace of Fig. 1 is enlarged in panel B. For comparison, a similar region of a chromatogram obtained from an HPLC separation of the released peptides isolated by Sepharose CL-6B chromatography [17] is shown in panel A. The peaks marked with arrows in panel B are peaks found only when the glycerol step gradient procedure is used. The material in the peaks eluting at about 162 and 182 min was further purified by HPLC as described under Experimental Procedures. The respective ^{14}C-radioactivity and $A_{214\ nm}$ profiles are shown in panels C and D.

respectively. ^{14}C-Carboxymethylated peptides from the 162 and 182 min peaks eluted at 38 and 44 min, respectively. Under the conditions of the second HPLC run, a standard ^{14}C-carboxymethylated peptide comprising residues 363–379 [21] eluted at 38 min and a standard comprising residues 388–414 [21] eluted at 44 min. These results clearly establish the presence of peptides comprising residues 363–379 and 388–414 in the released peptide fraction.

As described in detail previously [16,17], peptides released from the proteoliposomes and isolated with recoveries at least half that of their counterpart peptides in a standard tryptic digest of the H^+-ATPase, are defined as located on the cytoplasmic side of the membrane. The peptides comprising residues 363–379 and 388–414 were recovered in amounts well above 100 per cent the amount of the same peptides in a standard run that included reduction and carboxymethylation of the hydrophilic peptide fraction. These peptides are therefore clearly cytoplasmically located. Also by our earlier convention [17], 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, at least four residues in each direction from tryptic cleavage sites are also assumed to be cytoplasmically located. Therefore, the information present in these two released peptides establishes the location of residues 359–418 on the cytoplasmic side of the membrane. Moreover, since our earlier experiments have established a cytoplasmic location for residue 441, the location of the residues 419–440 on the cytoplasmic side of the membrane can be deduced with reasonable certainty because this stretch of amino acids is too short to form transmembrane helices that cross the membrane and return [17].

The topographic model shown in Fig. 3 summarizes the information obtained in the present study together with that obtained in our earlier studies of the H^+-ATPase topography [16–18]. The results of the previous studies directly established residues 21–28, 33–40, 70–99, 186–219, 238–256, 268–271, 441–460, 471–512, 545–559, 567–663 and 897–915 as located on the cytoplasmic side of the membrane. This information identified several additional flanking sequences, including residues 29–32, 41–69, 174–185, 220–237, 257–267, 461–470, 513–544, and 560–566 as also likely to be cytoplasmically located by virtue of the fact that they are too short to cross the membrane and return. Residues 916–920 are also cytoplasmically located as this stretch is too short to cross the membrane. Residues 2–20 are also probably cytoplasmically located, but a single span to the exocytoplasmic side of the membrane can not be excluded by the available data. Three large regions comprising residues 100–173, 272–355 and 660–891 constitute the membrane-em-

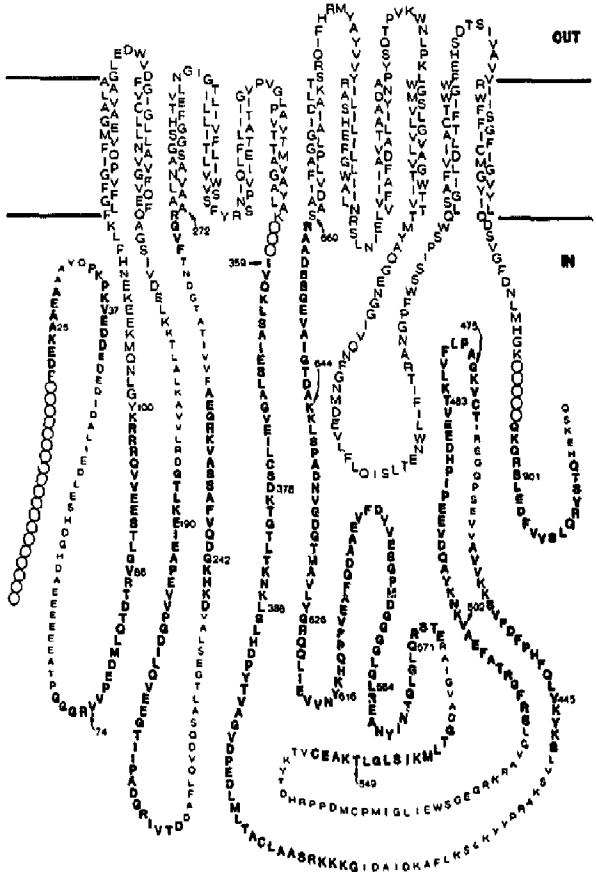

Fig. 3. Model for the transmembrane topography of the H^+-ATPase. The upper case large bold letters indicate amino acid residues that are defined as cytoplasmically located by NH_2-terminal sequence or amino acid analysis of purified peptides released from the proteoliposomes by trypsin [16,17]. The smaller letters indicate residues deduced to be cytoplasmically located as explained before [16,17]. The upper case large letters indicate amino acid residues present in peptides demonstrated to be membrane-embedded [18]. The numbers indicate the beginning of peptides directly established to be cytoplasmically located and membrane-embedded, except in the case of Asp-378 which is included as a point of reference. OUT and IN indicate points of reference outside and inside an intact cell.

bedded regions of the H^+-ATPase molecule. Finally, in the present study, the identification of two cysteine-containing peptides comprising residues 363–379 and 388–414 establishes a cytoplasmic location for residues 359–418 and infers a similar location for residues 419–440. Collectively, these results establish the transmembrane topography of nearly all of the 919 residues present in the H^+-ATPase molecule. This information should serve as a useful starting point for future efforts to understand the three-dimensional folding arrangement of the H^+-ATPase polypeptide chain and should also be valuable in comparative studies with the other transport ATPases in the aspartyl-phosphoryl-enzyme intermediate family.

Acknowledgement

This study was supported by U.S. Public Health Service National Institutes of Health Grant GM 24784.

References

1 Jørgensen, P.L. and Andersen, J.P. (1988) J. Membr. Biol. 103, 95–120.

2 Rabon, E.C. and Reuben, M.A. (1990) Ann. Rev. Physiol. 52, 321–344.
3 Andersen, J.P. and Vilsen, B. (1990) Current Opinion Cell Biol. 2, 722–730.
4 Carafoli, E. (1991) Physiol. Rev. 71, 129–153.
5 Hesse, J.E., Wieczorek, L., Altendorf, K., Reicin, A.S., Dorus, E. and Epstein, W. (1984) Proc. Natl. Acad. Sci. USA 81, 4746–4750.
6 Serrano, R. (1988) Biochim. Biophys. Acta 947, 1–28.
7 Schull, G.E., Greeb, J. and Lingrel, J.B. (1986) Biochemistry 25, 8125–8132.
8 Schull, G.E., Lane, L.K. and Lingrel, J.B. (1986) Nature 321, 429–431.
9 Schull, G.E., Schwart, Z.A. and Lingrel, J.B. (1985) Nature 316, 691–695.
10 MacLennan, D.H., Brandl, C.J., Korczak, B. and Green, N.M. (1985) Nature 316, 696–700.
11 Serrano, R., Kielland-Brandt, M.C. and Fink, G.R. (1986) Nature 319, 689–693.
12 Addison, R. (1986) J. Biol. Chem. 261, 14896–14901.
13 Hager, K.M., Mandala, S.M., Davenport, J.W., Speicher, D.W., Benz, E.J., Jr. and Slayman, C.W. (1986) Proc. Natl. Acad. Sci. USA 83, 7693–7697.
14 Scarborough, G.A. and Addison, R. (1984) J. Biol. Chem. 259, 9109–9114.
15 Rao, U.S., Hennessey, J.P., Jr. and Scarborough, G.A. (1988) Anal. Biochem. 173, 251–264.
16 Hennessey, J.P., Jr. and Scarborough, G.A. (1990) J. Biol. Chem. 265, 532–537.
17 Scarborough, G.A. and Hennessey, J.P., Jr. (1990) J. Biol. Chem. 265, 16145–16149.
18 Rao, U.S., Hennessey, J.P., Jr. and Scarborough, G.A. (1991) J. Biol. Chem. 266, 14740–14746.
19 Rao, R., Nakamoto, R.K. and Slayman, C.W. (1989) in Ion Transport, (Keeling, D. and Benham, C., eds), pp. 35–54, Academic Press, London.
20 Gibson, K.D., Wilson, J.D. and Udenfriend, S. (1961) J. Biol. Chem. 236, 673–679.
21 Rao, U.S. and Scarborough, G.A. (1990) J. Biol. Chem. 265, 7227–7235.
22 Patterson, M.S. and Greene, R.C. (1965) Anal. Chem. 37, 854–857.
23 Hennessey, J.P., Jr. and Scarborough, G.A. (1989) Anal. Biochem. 176, 284–289.