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Assessing hydrophobic regions of the plasma membrane H^+-ATPase from *Saccharomyces cerevisiae*

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The hydrophobic, photoactivatable probe TID {3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine} was used to label the plasma membrane H^+-ATPase from *Saccharomyces cerevisiae*. The H^+-ATPase accounted for 43% of the total label associated with plasma membrane protein and incorporated 0.3 mol of [^{125}I]TID per mol of 100 kDa polypeptide. The H^+-ATPase was purified by octyl glucoside extraction and glycerol gradient centrifugation, and was cleaved by either cyanogen bromide digestion or limited tryptic proteolysis to isolate labeled fragments. Cyanogen bromide digestion resulted in numerous labeled fragments of mass < 21 kDa. Seven fragments suitable for microsequence analysis were obtained by electrotransfer to poly(vinylidene difluoride) membranes. Five different regions of amino-acid sequence were identified, including fragments predicted to encompass both membrane-spanning and cytoplasmic protein structure domains. Most of the labeling of the cytoplasmic domain was concentrated in a region comprising amino acids 347 to 529. This catalytic region contains the site of phosphorylation and was previously suggested to be hydrophobic in character (Goffeau, A. and De Meis, L. (1990) J. Biol. 265, 15503–15505). Complementary labeling information was obtained from an analysis of limited tryptic fragments enriched for hydrophobic character. Six principal labeled fragments, of 29.6, 20.6, 16, 13.1, 11.4 and 9.7 kDa, were obtained. These fragments were found to comprise most of the putative transmembrane region and a portion of the cytoplasmic region that overlapped with the highly labeled active site-containing cyanogen bromide fragment. Overall, the extensive labeling of protein structure domains known to lie outside the bilayer suggests that [^{125}I]TID labeling patterns cannot be unambiguously interpreted for the purpose of discerning membrane-embedded protein structure domains. It is proposed that caution should be applied in the interpretation of [^{125}I]TID labeling patterns of the yeast plasma membrane H^+-ATPase and that new and diverse approaches should be developed to provide a more definitive topology model.

Introduction

The yeast plasma membrane H^+-ATPase is an electrogenic proton pump within the P-type class of ion translocating ATPases that maintains intracellular pH and a large electrochemical proton gradient [1]. The yeast H^+-ATPase consists of a single 100 kDa subunit that forms a phosphorylated intermediate, exists in at least two principal conformational states, and is sensitive to inhibition by vanadate [2]. The gene encoding the *Saccharomyces cerevisiae* H^+-ATPase, *PMA1*, is essential for growth and a high degree of amino-acid sequence similarity is observed between four fungal ATPases [3–6]. The predicted transmembrane topology of the yeast H^+-ATPase is very similar to that of the other P-type enzymes, including the Na^+/K^+-ATPase and Ca^{2+}-ATPase, although the most direct amino-acid sequence homology between the various ATPases is confined to the central cytoplasmic catalytic domain [7]. The high degree of similarity observed for hydropathy profiles and secondary structures among the P-type class of ATPases suggests a structural conservation within these enzymes.

A fundamental understanding of the physical structure and topology of the H^+-ATPase is necessary before a mechanism for ATP driven H^+-transport by this enzyme can be reasonably discerned. In the absence of X-ray crystallographic data, topological models for membrane proteins have been developed from hy-

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Abbreviations: [^{125}I]-TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophyenyl)diazirine; CNBr, cyanogen bromide; PVDF, poly(vinylidene difluoride) membrane; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

dropathy analyses, hydrophobic labeling, epitope mapping and protease accessibility studies [8]. Presently, there is little direct information about the topology of the yeast plasma membrane H^+-ATPase. A recent hydrophobic labeling study on the closely related *Neurospora* H^+-ATPase by Scarborough and colleagues has provided important information about potential membrane-embedded protein structure [9]. From this and other recent studies, a minimum consensus topological model for the fungal H^+-ATPase can be developed: The membrane-embedded portion of the enzyme appears to contain 8–12 membrane-spanning segments [9–12], the N- and C-termini are cytoplasmically-located along with another 3–5 hydrophilic loop regions [13–16], and one hydrophilic loop domain containing the site of phosphorylation (D378, *S. cerevisiae*) may comprise up to 40% of the total protein of the ATPase [11].

The consensus model provides a good working model for genetic probing of enzyme structure and function. However, it is a crude model and provides no information about hydrophobic regions of protein structure which may lie within the cytoplasmic domain. Model refinement and clarification is an important goal, especially for all hydrophobic domains of protein structure. These regions are important, not only because the membrane-embedded structures participate in and determine the specificity of ion translocation, but also because they are proposed to influence ATP hydrolysis in the active site [17,18]. The photoactivatable hydrophobic probe [^{125}I]TID {3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophyenyl)diazirine} offers the opportunity to identify potential hydrophobic sites. [^{125}I]TID is a small hydrophobic molecule that readily partitions into the lipid phase of membranes with a partition coefficient of $\sim 4.1 \cdot 10^4$ between the membrane bilayer and H_2O [19]. It is converted by illumination to a reactive carbene intermediate that forms covalent derivatives with neighboring molecules. It has been used to label a wide variety of membrane proteins, including other ATPases [9,19–21], ion channels [22] and receptor proteins [23]. In addition, its small size allows it to be absorbed into hydrophobic pockets of water soluble proteins such as calmodulin [24] and bovine serum albumin [25]. Thus, it can label hydrophobic sites in diverse environments.

In this investigation, [^{125}I]TID was used to label the H^+-ATPase in the native plasma membrane of yeast. The enzyme was purified and cleaved with either CNBr or trypsin, and a detailed analysis of label incorporated into hydrophobic fragments and specific amino acids is reported.

Materials and Methods

Materials. All culture media supplies were from Difco. Trypsin was obtained from Promega and CNBr was from Aldrich. [^{125}I]TID (10 Ci/mmol) was supplied by Amersham Corp. PVDF membranes (ProBlott, lot No. AOC001) were obtained from Applied Biosystem Inc.

Yeast strains and cell culture. The *Saccharomyces cerevisiae* strain Y55 (*HO gal3 MAL1 SUC1*) was used in this study [26]. Cells were grown in 10 liter batches of YPD medium at 22°C until mid-log phase and then harvested by centrifugation. The cells were washed in 0.5 M sucrose buffer and stored at −80°C, as described by Perlin and Brown [27].

Plasma membrane isolation. A microsomal membrane fraction was prepared by the method of Perlin and Brown [27] and plasma membranes were isolated from this fraction by a modification of the sucrose gradient centrifugation procedure of Serrano [28]. Microsomal membranes (10 mg/ml) were suspended in a buffer consisting of 10 mM Tris (pH 7), 20% (v/v) glycerol, 1 mM EGTA, 1 mM EDTA, 1 mM DTT and 0.5 mM PMSF (membrane wash buffer) and 5 ml of this suspension were layered over a sucrose step gradient consisting of 4 ml of 43.5% (w/w) and 1.5 ml of 53.5% sucrose containing 10 mM Tris (pH 7), 1 mM EDTA and 1 mM DTT. Samples were centrifuged in a SW 41 Ti rotor at $150000 \times g$ for 3 h. The interface between the 43.5% and 53.5% sucrose layer was collected. Plasma membranes were resuspended in membrane wash buffer (5 ml per mg protein) and then centrifuged at $300000 \times g$ for 1 h. For large-scale photolabeling experiments, sucrose gradient-purified plasma membranes were extracted with 0.5% deoxycholate in a buffer containing 10 mM Hepes (pH 7), 45% glycerol, 100 mM KCl, 1 mM EDTA and 1 mM DTT (glycerol extraction buffer). Deoxycholate-extracted membranes were recovered by centrifugation at $300000 \times g$ for 1 h and resuspended at 10 mg/ml in membrane wash buffer. The membranes were then centrifuged, as decribed above, resuspended in membrane wash buffer at 20 mg/ml and stored at −80°C.

Photolabeling. [^{125}I]TID (100 μCi) was added in the dark to 1 mg of sucrose gradient-purified or deoxycholate-extracted plasma membranes resuspended in 1 ml of 10 mM Tris (pH 7), 1 mM EDTA and 1 mM DTT. The sample in a shallow well of a porcelain plate (Coors Porcelain Inc.) was photolyzed by exposure to ultraviolet light (Mineralight, Ultra-Violet Prod.) at a 2 cm distance for 30 min. Unlabeled membranes (9 mg) in 25 ml of glycerol extraction buffer were added to the photolyzed samples. The suspension was centrifuged at $300000 \times g$ for 1 h. The pellet was resuspended in membrane wash buffer (25 ml) and centrifuged, as above. The labeled membrane pellet was used for futher ATPase purification. The [^{125}I]TID was specifically incorporated into H^+-ATPase on illumination. Labeling of membrane proteins was not observed in the absence of photoillumination.

Purification of ATPase. The H^+-ATPase was solubilized by the addition of 1% octyl glucoside to membranes in membrane wash buffer. The suspension was centrifuged at $150000 \times g$ for 30 min and 5 ml of the supernatant were layered onto a glycerol step gradient consisting of 1.5 ml of 70%, 2 ml of 50% and 2 ml of 35% glycerol in a buffer containing 10 mM Tris (pH 7), 1 mM EDTA and 2 mM DTT. The gradient was centrifuged at $200000 \times g$ for 16 h and then fractionated into 1 ml portions. Purified H^+-ATPase was identified by assessment of the 100 kDa protein on SDS-polyacrylamide gels [29].

CNBr digestion. A glycerol gradient fraction containing purified H^+-ATPase (1 mg in 1 ml) was extracted with 2 ml of ice cold acetone for 1 h and then centrifuged at $10000 \times g$ for 10 min. The pellet was washed with 2 ml of acetone, centrifuged as above and vacuum dried for 5 min in a Speedvac concentrator. Lipid-depleted H^+-ATPase was dissolved in 500 μl of 70% trifluoroacetic acid (TFA) or 70% formic acid and 10–30 μl of 5M CNBr in acetonitrile were added. Approximately 42- to 125-fold molar excess of CNBr over total methionine residues in the H^+-ATPase was used per digestion. Samples were digested for 24–48 h at 22°C under nitrogen and in the dark, diluted 2-fold with water and dried in a Speedvac concentrator. Samples were washed with 2 ml of water and dried, as above. Although the CNBr digestion in TFA gave greater quantities (4-fold more) of peptide fragments suitable for microsequence analysis and while fragments digested in formic acid appeared better resolved in SDS polyacrylamide gels, the peptide profiles from both procedures were very similar and the fragments of identical mobility gave equivalent amino-acid sequence.

Tryptic digestion. Partial tryptic digestion of purified H^+-ATPase was performed according to Monk et al. [4]. Glycerol gradient-purified enzyme was dialyzed in a 2 liter buffer containing 50 mM ammonium bicarbonate (pH 7.8) and 1 mM PMSF. The dialyzed enzyme was concentrated in a Speedvac to 10 mg/ml and then partially digested with trypsin (50 μg H^+-ATPase per 1 μg trypsin) for 30 min at 37°C. Tryptic peptides were extracted with ammonium bicarbonate and pelleted, as previously described [4]. The pellet was gently rinsed with 1 ml of water to remove excess salts, and then dried with a Speedvac for 5 min. The tryptic peptides recovered accounted for about 1% of the original protein.

Electrophoresis and electroblotting. Analysis of intact H^+-ATPase was performed according to Perlin et al. [29]. CNBr or tryptic peptides were resolved by gradient gel electrophoresis (16–20% acrylamide; 32:1 acrylamide:bis) using a low molecular weight resolving multiphasic buffer system [30]. Electroblotting of peptides to PVDF membranes was performed by semi-dry transfer using the method of LeGendre and Matsudaira [31] with a transfer buffer consisting of 10 mM CAPS (pH 11) and 10% methanol.

Protein microsequence analysis. Microsequence analysis of the labeled peptides was performed on a Porton Instruments gas phase microsequencer (Model PI 2090E). The amino-acid residues generated during sequence analysis were split-off and collected. Radioactivity incorporated into individual amino acids was determined by γ-counting on a LKB Gamma Counter (LKB model No. 1275).

Other procedures. Intact H^+-ATPase, CNBr or trypsin-digested peptides separated by SDS-polyacrylamide gel electrophoresis were stained with Coomassie blue R250 and the amount of radioactivity incorporated into particular bands was measured by γ-counting of excised bands. Labeled H^+-ATPase or digested peptides were visualized by autoradiography of vacuum-dried gels on Fuji RX-50 film using a Cronex HI-Plus intensifying screen. The quantities of whole enzyme or digested peptides were determined by scanning densitometry [29]. Protein was determined by the amido black method [32].

Results

[^{125}I]TID labeling of H^+-ATPase

Sucrose gradient-purified plasma membranes were photolabeled with [^{125}I]TID and washed to remove free [^{125}I]TID as described in Materials and Methods. The incorporation of covalent label into the H^+-ATPase was determined after separation of the enzyme from other membrane components by SDS-polyacrylamide gel electrophoresis. Fig. 1(A) shows that the H^+-ATPase is a predominant membrane protein comprising approximately 20% of the total membrane protein. The H^+-ATPase was the most highly labeled membrane protein and contained 43% of the total [^{125}I]TID incorporated into protein (Fig. 1(B)). However, the H^+-ATPase accounted for only 0.62% of the total label incorporated into membranes (protein and lipid). This level of label incorporation was increased to 1.2% by the use of deoxycholate-extracted membranes. The extraction, which removes about 50% of non-ATPase membrane proteins and some lipids, converts membrane vesicles into non-vesicular membrane fragments (Perlin and Mohraz, unpublished) and permits complete recovery of fully functional H^+-ATPase [33]. The H^+-ATPase from either sucrose gradient-purified plasma membranes or deoxycholate-extracted membranes was essentially identical, since limited tryptic and CNBr-digestions yielded characteristic product patterns regardless of the membrane source. SDS-polyacrylamide gel electrophoresis showed that most of the [^{125}I]TID was associated with a fast migrating lipid- and/or proteolipid-containing region. The amount of

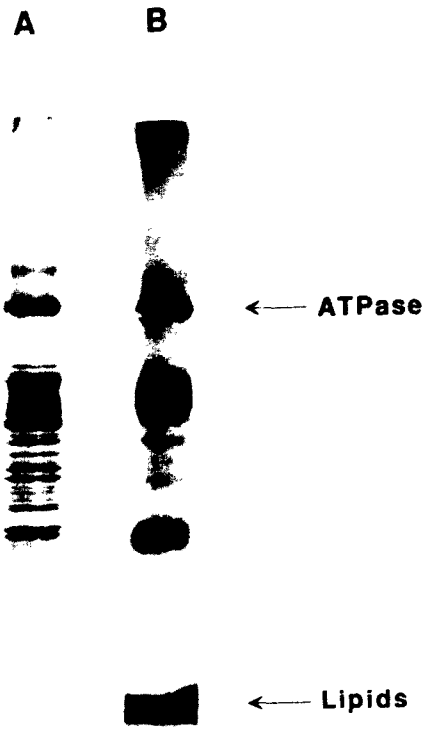

Fig. 1. SDS-polyacrylamide gel of [^{125}I]TID-labeled plasma membrane proteins. (A): Sucrose gradient-purified plasma membranes were prepared as described under Materials and Methods and 20 μg of membrane protein were applied to a SDS-polyacrylamide gel and stained with coomassie blue R-250. (B): Autoradiogram of gel profile in Lane A.

[^{125}I]TID incorporated into the 100 kDa protein was about 0.3 mol [^{125}I]TID per mol 100 kDa band. This sub-stoichiometric level of incorporation was desirable since it should minimally perturb the structural properties of the enzyme.

Characterization of CNBr-digested fragments

Membrane-associated [^{125}I]TID-labeled H^+-ATPase was purified by octyl glucoside extraction and glycerol gradient centrifugation. The glycerol gradient-purified H^+-ATPase was precipitated with acetone to remove a majority of radioactive associated phospholipids. The largely delipidated H^+-ATPase was digested with CNBr in the presence of 70% formic acid or TFA as described in Materials and Methods. The H^+-ATPase was extensively digested as indicated by the finding that the intact 100 kDa polypeptide band disappeared and all polypeptide fragments detected on SDS-polyacrylamide gels showed apparent molecular weights of less than 21 kDa (Fig. 2). There was extensive labeling of most fragments and seven major resolved fragments were suitable for microsequence analysis following electrotransfer onto PVDF membranes. The amino acid sequences derived from these peptides, as well as labeled amino acids recovered after each cycle, are shown in Table I. Five different regions of amino-acid sequence (starting with residues 153, 347, 406, 593 and 632) were obtained. According to most models of H^+-ATPase topology [9–11,15], peptide fragments A, B, C and F should lie within the cytoplasmic domain whereas peptide fragment D (D$^\wedge$) should span both transmembrane and cytoplasmic domains. Peptide fragments in E contained a mixture of fragments from putative transmembrane and cytoplasmic regions. The ratio of label (cpm) per unit mass of peptide was higher for fragments expected to contain a significant proportion of peptide as transmembrane protein (peptide D) relative to those obtained from the cytoplasmic regions

TABLE I

Characteristics of CNBr fragments

Fragment	Molecular mass (kDa)	Cpm/mass ratio [a]	Amino-acid sequence	Expected amino-acid positions [b]
A	21.7	4234	AVGAAYL	347 to 555
B	18.9	5061	LTA(C?)LAA AVGAAYLA	406 to 347 to 529
C	15.3	4355	LTA(C?)LAA(S?)RKKKGLDAIDKA	406 to 529
D(D$^\wedge$)	10.7	7079	LNAGVGFVQEFQAG(S?)IVDELKKTLA	153 to 257
E	9.5	8014	LNAGVGFVQ AVGAAYLAKK	153 to 347 to 404
F	8.5	5505	PG(S?)ELAD [c]	593–631

[a] Cpm per mass ratio was determined by γ-counting of excised bands of the Coomassie blue stained SDS-gel and dividing the total cpm by the fractional protein (mass) quantities of each band, as determined by laser densitometry.

[b] Represents the expected amino acid starting and ending position which was determined from the estimated molecular mass of the fragments.

[c] In a separate experiment, the band corresponding to this fragment contained a mixture of peptides consisting of equal amounts of the following sequences PG(S?)ELAD (593–630), TGDGVN (632–687), LNAGVGF (153–?) and AVGAAYL (347–?).

[d] Represents fragment D$^\wedge$ (11.6 kDa) as indicated in Fig. 2. This fragment has the same N-terminal amino acid sequence as fragment D but its quantity was about 5 fold less.

[e] Represents labeled amino acids.

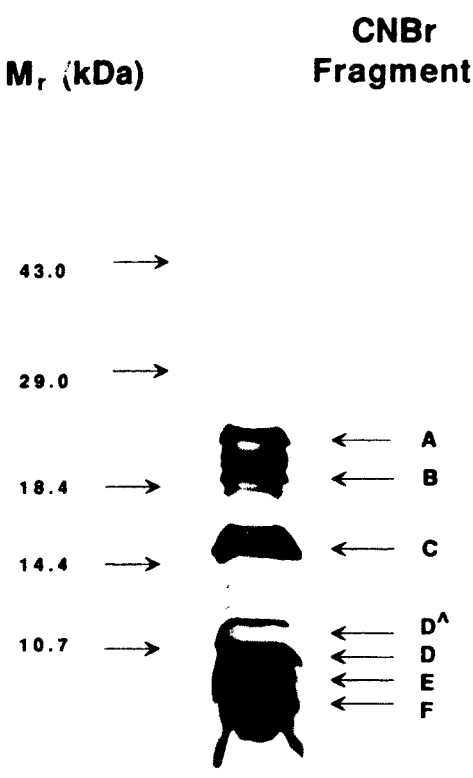

Fig. 2. SDS-polyacrylamide gel of CNBr-digested fragments. Purified enzyme was digested with CNBr in the presence of 70% formic acid as described under Materials and Methods. 100 μg of peptides were separated on a low molecular weight resolving gel system. The molecular mass of gel electrophoresis standards and designation of CNBr-digested fragments are as indicated.

(peptides A, B, C and F). This result appears to confirm that the [^{125}I]TID prefers the hydrophobic bilayer region. The incorporation of significant amounts of label into the second hydrophilic loop suggests that this region either provides a hydrophobic pocket within a hydrophilic environment or that the labeled residues are located within the membrane bilayer.

Tryptic digestion

We previously reported the successful application of an ammonium bicarbonate extraction procedure [9] to enrich for hydrophobic tryptic fragments from the *Candida* H^+-ATPase [4]. Hydrophobic tryptic fragments from the *S. cerevisiae* H^+-ATPase were analyzed by SDS-polyacrylamide gel electrophoresis and transfered to PVDF membranes for microsequencing. Six principal fragments of 29.6, 20.6, 16, 13.1, 11.4 and 9.7 kDa were obtained (data not shown). All of these fragments were significantly labeled with [^{125}I]TID (Table II). Two major fragments, 11.4 and 9.7 kDa, were analyzed by microsequencing. The 11.4 kDa fragment showed two amino-acid sequences, a primary sequence starting from AAA(-)VN (residues 272–379) and secondary sequence starting from V(-)(-)AAGG (residues 276–379). This minor sequence appears to be an atypical tryptic digestion product, which may have resulted from γ-trypsin activity, a known autolysis product of trypsin [34,35]. The 9.7 kDa fragment contained a mixture of sequences. Two sequences, starting from either AAALVN or RAAALV (residues 279–362), contained 50% of the total mass. The apparent incomplete cleavage of arginyl or lysyl peptide bonds observed here is consistent with results obtained by Rao et al. [9]. Two minor sequences, starting from YGLNQ (residues 100–174) and TVEE (residues 483–548), each contain approximately 25% of total mass. Based on the study of Monk et al. [4], it is expected that the sequences of peptide fragments B (20.6 kDa), C (16 kDa) and D (13.1 kDa) will be SAADIVFLAPGLSAIIDALKT (residues 660–857), YGLNQMADEKESLVVKFVMFFVGP (residues 100–252) and AAALVNKA (residues 272–387), respectively.

TABLE II

Characteristics of tryptic fragments

Fragments	Molecular mass (kDa)	Cpm	Amino-acid sequence	Expected amino-acid position [a]
A	29.6	2160	n.d.	
B	20.6	2732	SAADIVFLAPGLSAIIDALKT [b]	660–857
C	16.0	2759	YGLNQMADEKESLVVKFVMFFVGP [b]	100–252
D	13.1	3474	AAALVNKA [b]	272–387
E	11.4	2374	AAA(-)VN	272–379
			V(-)(-)AAGG	276–379 [c]
F	9.7	4171	mixed peptides [d]	

[a] The amino acid starting and ending positions were determined from a consideration of the molecular weight of each fragment.

[b] Data as reported by Monk et al. [4].

[c] This fragment was an atypical tryptic digestion product, as described in the text.

[d] This fragment contained a mixture of peptides consisting of the following sequences, either AAALVN or RAAALV (272–362, containing 50% of the total mass), YGLNQ (100–173, containing 25% of the total mass) and TVEE (483–548, containing 25% of the mass).

Discussion

Hydrophobic character of active site region

In this study, hydrophobic regions of the yeast plasma membrane H^+-ATPase were identified using the reactive photoactivatable probe [^{125}I]TID. It was demonstrated that extensive labeling of the H^+-ATPase occurred in plasma membranes or in enzyme-enriched membrane fragments (Fig. 1) and label was distributed throughout the enzyme (Tables I and II). One interesting finding was that peptides comprising residues 153–257 and 347–555 (Table I) show strong [^{125}I]TID labeling. These regions are believed to comprise most of the active site for ATP hydrolysis and are located in the cytoplasmic domain [15]. Residues 153–257 are expected to extend beyond transmembrane segment 2 into the first cytoplasmic loop domain, whereas residues 347–555, which cover the site of phosphorylation (D378), are located within the large central cytoplasmic loop domain. [^{125}I]TID labeling within the active site region involved in ATP hydrolysis suggests a strong hydrophobic character to this region of the enzyme. Kinetic evidence suggesting that the active site for ATP hydrolysis has hydrophobic character was obtained by Goffeau and De Meis [17]. They reported two *PMA1* mutations, K250T and G268D, in *Schizosaccharomyces pombe* which display different hydrophobic character and modify the sensitivity of the H^+-ATPase to phosphate anion, vanadate, dimethyl sulfoxide and the hydrophobic drug trifluoperazine. It was suggested that residues K250 and G268 lie within the cytoplasmic loop domain between transmembrane segments 2 and 3 [7,17] and that this region interacts with the central cytoplasmic loop domain (bounded by transmembrane segments 4 and 5 in a 10 transmembrane segment model) to form the active site. The notion that the catalytic domain is comprised of interacting protein structure regions is supported by a genetic study on the yeast H^+-ATPase in which second site suppressor mutations lying within the membrane sector and the first cytoplasmic loop domain were found to alter the vanadate and pH sensitivity of a F368 mutant enzyme lying near the site of phosphorylation [18]. These studies suggest that the active site region possesses strong hydrophobic character that is essential for normal catalytic function.

Topology of yeast plasma membrane H^+-ATPase

Limited information presently exists on the topology of the fungal plasma membrane H^+-ATPase. The assignment of specific membrane-embedded protein structure has, for the most part, relied on extrapolation from hydropathy analyses [7,11,12] and comparisons with other P-type ATPases [7] where this information is better established. Scarborough and colleagues provided direct evidence for a 10 and possibly a 12 transmembrane segment model for the closely related *Neurospora* H^+-ATPase from analyses of [^{125}I]TID-labeled protein structure remaining in H^+-ATPase-reconstituted proteoliposomes following exhaustive tryptic digestion and hydrophilic cytoplasmic segments obtained by proteolytic cleavage [9,15]. The data presented in Tables I and II are consistent with the labeling studies described for the *Neurospora* H^+-ATPase [9,15]. The [^{125}I]TID-labeled cyanogen bromide and tryptic fragments presented in this study support most features of the putative 10 or 12 transmembrane segment models. The highly-labeled 10.7 kDa cyanogen bromide fragment, spanning amino-acid residue 153–257 (Table I), is expected to start in the middle of transmembrane segment 2, extend throughout the first cytoplasmic loop and end near the start of transmembrane segment 3. Nearly all models show specifically-labeled amino acids, L153, N154, A155, E162, F163 within or near the membrane interface of transmembrane segment 2. Other labeled amino acids, A347 and V348, identified in a 21.7 kDa fragment spanning residues 347–555 would be expected to lie near the end of transmembrane segment 4 (in a 10 transmembrane segment model [7]) or segment 6 (in a 12 transmembrane segment model [9]). A 13.1 kDa tryptic fragment spanning residues 272–387 is highly labeled and is consistent with this region being highly hydrophobic. However, it is not possible to assess whether this region represents two transmembrane segments, as predicted by Serrano [11] or four transmembrane segments, as inferred by Rao et al. (segments 3–6 [9]). Only a single large tryptic fragment (20.6 kDa) which spans residues 660–857 was identified which supported label incorporation into the remaining C-terminal transmembrane fragments.

The results in this study clearly indicate that the yeast plasma membrane H^+-ATPase is extensively labeled in regions of protein predicted to lie both inside and outside the membrane bilayer. While it is not surprising that these regions display hydrophobic character, the extensive labeling pattern presents a dilemma in that it is not possible to distinguish regions of protein structure that are unambiguously bilayer-embedded. The [^{125}I]TID labeling appears to be specific for hydrophobic environments since not all fragments are labeled and the specific activity of labeling is greatest in peptides predicted on the basis of amino-acid sequence to be highly hydrophobic. Thus, it is likely that [^{125}I]TID labeling reliably identifies regions of hydrophobic character as predicted. However, distinct limitations must be imposed on the interpretation of labeling patterns for the express purpose of discerning membrane-embedded protein structure because hydrophobic domains within hydrophilic protein structure regions are also labeled. This conclusion is supported by the labeling of soluble proteins camodulin [24] and

bovine serum albumin [25]. It is apparent that labeling data alone is insufficient to develop a definitive topology model and a combinatorial approach, such as that developed by Scarborough and colleagues for the *Neurospora* H^+-ATPase, as well as new approaches will be needed to develop more reliable models.

In summary, our results suggest that fragments containing amino acid residues predicted to lie within the bilayer were labeled with the hydrophobic probe [^{125}I]TID. In addition, stretches of sequence within the second cytoplasmic loop domain were also labeled suggesting a hydrophobic environment within the catalytic ATP hydrolysis domain. The high reactivity of [^{125}I]TID within hydrophobic domains of bilayer and non-bilayer associated protein structure suggests that successful topological analysis of the H^+-ATPase will require additional approaches and more specific probes in order to discriminate between and improve existing topological model.

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