The purified plasma membrane H⁺-ATPase of Schizosaccharomyces pombe and Saccharomyces cerevisiae display, in addition to the catalytic subunit of 100 kDa, a highly mobile component, soluble in chloroform/methanol. Chloroform/methanol extraction of S. cerevisiae plasma membranes led to isolation of a low molecular weight proteolipid identical to that present in purified H⁺-ATPase. NH₂-terminal amino acid sequence revealed a 38-residue polypeptide with a calculated molecular mass of 4250 Da. The polypeptide lacks the first two NH₂-terminal amino acids as compared with the deduced sequence of the PMP1 gene (for plasma membrane proteolipid) isolated by hybridization with an oligonucleotide probe corresponding to an internal amino acid sequence of the proteolipid. The polypeptide is predicted to contain an NH₂-terminal transmembrane segment followed by a very basic hydrophilic domain.

The plasma membrane of fungi contains a well characterized ATPase that pumps protons out of the cell, energizing the membrane for nutrient uptake. The fungal H⁺-ATPase is structurally and functionally related to the Na⁺/K⁺-, Ca²⁺-, and H⁺/K⁺-ATPases of animal cell membranes as well as to the H⁺-ATPase of plant-cell plasma membranes. It contains a 100-kDa catalytic subunit whose organization within the membrane is similar to that of the other members of the cation-translocating ATPase family. Particularly striking is the presence of conserved stretches which include regions involved in ATP hydrolysis and formation of the β-aspartyl-phosphate intermediate (see recent reviews by Serrano (1988), Nakamoto and Slayman (1989), and Goffeau and Green (1990)).

All the biochemical and genetic work carried out so far has been based on the implicit assumption that the fungal H⁺-ATPase contains no additional subunit. Upon classical Laemml gel electrophoresis, the purified enzyme displays a highly enriched Coomassie-stained band with a molecular mass of 100 kDa. It has been reported, however, that the Schizosaccharomyces pombe preparation contains a component moving slightly ahead of the tracking dye. This highly mobile component fails to stain with Coomassie Blue but develops a red color with periodic acid-Schiff stain, suggesting the presence of either glycoproteic or proteolipidic material (Dufour and Goffeau, 1978). The existence of a ninhydrin-positive component in the lipidic chloroform/methanol extract of the purified S. pombe H⁺-ATPase was later reported, but no indication was given as to the nature of this material and its relation to the fast moving compound observed during electrophoresis (Dufour and Goffeau, 1980).

In the present study, the possibility that the fungal H⁺-ATPase might possess a proteolipid component of high electrophoretic mobility was reexamined using novel electrophoresis conditions adapted to separating low molecular weight proteins (Schägger and von Jagow, 1987). Indeed, the purified H⁺-ATPase of S. pombe and Saccharomyces cerevisiae showed two low molecular weight, Coomassie-stained bands after electrophoresis on a Tricine-SDS-polyacrylamide gel. Chloroform/methanol extraction of these polypeptides from S. cerevisiae plasma membranes established the lipid-like properties of these highly mobile compounds. Microsequencing of the purified proteins revealed the existence of a small, single 38-residue polypeptide in both bands. This polypeptide was extracted from purified H⁺-ATPase as well as from plasma membranes. The corresponding gene was isolated by hybridization with an oligonucleotide probe derived from a portion of the amino acid sequence. Gene sequencing confirmed the amino acid sequence with the exception of two additional amino-terminal residues.

EXPERIMENTAL PROCEDURES

Materials—BNPS-skatole was purchased from Pierce Chemical Co.; carboxypeptidase Y from Boehringer Mannheim; L-α-phosphatidylinositol. 1-monooeyl-rac-glycerol, stearic acid, tripalmitine, and cholesterol linolate from Sigma; and cholesterol from Aldrich. Light and heavy molecular mass markers (range 2,512-16,949 Da and 14,400-94,000 Da, respectively) were from Pharmacia LKB Biotechnology Inc., trifluoroacetic acid from Janssen, and precoated TLC plates (silica gel 60) from Merck. All other reagents were analytical grade.

Extraction of Proteolipid by Chloroform/Methanol and Precipitation in Ether—The procedure of Cattell et al. (1971) was followed to purify proteolipid from plasma membranes isolated from S. cerevisiae as described by Goffeau and Dufour (1988). Plasma membranes (10 mg/

1The abbreviations used are: Tricine, N-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine; PTH, phenylthiohydantoin; BNPS-skatole, 2-((2'-nitrophenylsulfonyl)-3-methyl-3-bromoindolenine; SDS, sodium dodecyl sulfate.
ml protein) were suspended in 25 volumes of a mixture of chloroform/methanol (2:1) for 2 h at 4 °C. Undissolved material was removed by filtration and the extract was washed with 0.2 volume of water. After centrifugation of the milky emulsion at 2,000 × g for 30 min, the lower organic phase was concentrated to dryness under reduced pressure in a flash evaporator. The final residue was dissolved in a minimal volume of chloroform. Four volumes of diethyl ether were added to the chloroform extract, and the mixture was stored for 1 h at −20 °C. Precipitated proteins were collected by centrifugation.

Protein concentrations were determined by the method of Lowry et al. (1951) after dilution of the samples in 0.1% sodium deoxycholate. In order to detect very small polypeptides, Tricine-SDS-polyacrylamide gel electrophoresis was carried out as described by Schägger and von Jagow (1987). Proteins were fixed by a solution containing formaldehyde (Bürk et al., 1983) and stained with 0.025% Serva blue G in 10% acetic acid.

Peptide Sequencing—Ether-precipitated proteolipid (60 μg) was dissolved in 25 μl of trifluoroacetic acid and diluted to 100 μl with water. After centrifugation at 13,000 rpm for 5 min, the supernatant was directly loaded on a precycled Polybrene-coated glass filter. The final residue was dissolved in a minimal volume of chloroform. Four volumes of diethyl ether were added to the chloroform extract, and the mixture was stored for 1 h lower organic phase was concentrated to dryness under reduced vapor. The NH₂ terminus (Fearnley et al., 1990) was found near the COOH terminus, as well as free lipids including phospholipids, monoglycerides, and 2, heavy and light molecular weight markers; lane 3, S. cerevisiae plasma membranes (10 μg); lane 4, chloroform extract from S. cerevisiae plasma membranes (10 μg); lane 5, ether-insoluble fraction of 34-μg chloroform extract from S. cerevisiae plasma membranes; lane 5, ether-soluble fraction of 34-μg chloroform extract from S. cerevisiae plasma membranes.

Electrophoretical Patterns of Purified H⁺-ATPase and Chloroform/Methanol Plasma Membrane Extract—S. cerevisiae and S. pombe plasma membrane H⁺-ATPase, solubilized by lysolecithin and purified by centrifugation through a sucrose gradient (Goffeau and Dufour, 1988; Dufour et al., 1988), were subjected to a Tricine-SDS-gel electrophoresis (Fig. 1A, lanes 3 and 4). Both preparations display, in addition to the catalytic subunit of 100 kDa, two diffuse bands with apparent molecular mass lower than 10 kDa. Purified H⁺-ATPase from S. cerevisiae was treated with chloroform/methanol. This treatment extracted specifically the two highly mobile compounds which amounted to about 10% of total ATPase protein.

A more convenient extraction of the low molecular weight component was obtained from Saccharomyces cerevisiae plasma membranes which yielded about 1.5% of total membrane protein by chloroform/methanol. The chloroform/methanol-soluble fraction was subjected to a Tricine-SDS-gel electrophoresis (Fig. 1B, lane 4). This chloroform/methanol extract contains the two diffuse bands contaminating the purified H⁺-ATPase; their apparent molecular weights were of 7,500 and 4,000. Addition of diethyl ether to the chloroform extract solubilized most of the 4-kDa band (Fig. 1B, lane 6), as well as free lipids including phospholipids, monoglycerides, triglycerides, sterols, fatty acids, and sterol esters (Fig. 2B). Consistently, the lower part of the 4-kDa band comigrates with the plasma membrane proteolipid.

RESULTS

Electrophoretical Patterns of Purified H⁺-ATPase and Chloroform/Methanol Plasma Membrane Extract—S. cerevisiae and S. pombe plasma membrane H⁺-ATPase, solubilized by lyssolecithin and purified by centrifugation through a sucrose gradient (Goffeau and Dufour, 1988; Dufour et al., 1988), were subjected to a Tricine-SDS-gel electrophoresis (Fig. 1A, lanes 3 and 4). Both preparations display, in addition to the catalytic subunit of 100 kDa, two diffuse bands with apparent molecular mass lower than 10 kDa. Purified H⁺-ATPase from S. cerevisiae was treated with chloroform/methanol. This treatment extracted specifically the two highly mobile compounds which amounted to about 10% of total ATPase protein.

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with pure lecithin (data not shown). In contrast, the 7.5-kDa band was precipitated with ether (Fig. 1B, lane 5). No free lipids were detected in this ether-insoluble fraction (Fig. 2C).

Amino Acid Sequence of the Purified Proteolipid—NH₂-terminal amino acid sequencing was carried out on the ether-precipitated fraction which is completely devoid of free lipids. A 38-residue sequence was unambiguously determined except for the residue at position 16. This residue, which was suspected to be a serine since breakdown products of serine-phenylthiohydantoin were detected, is produced in markedly low yield (Fig. 3C). The low yield of tryptophan 28 (Fig. 3C) is typical of this residue. The progressively decreasing yields from glutamine 35 to phenylalanine 38 (Fig. 3C) suggest that the COOH end of the polypeptide was reached. The protein was treated with phenyl isocyanate to block the NH₂ end and subsequently cleaved with BNPS-skatole, which cleaves after tryptophan residues (Fontana, 1972). Only one peptide was generated, the sequence of which corresponds exactly to that expected from cleavage after tryptophan 28 (Fig. 3A). As for the intact polypeptide, no further residue was obtained by Edman degradation after residue 38. Carboxypeptidase Y liberated only phenylalanine within the first 10 min, which is consistent with the assignment of phenylalanine 38 as the COOH-terminal residue. It therefore seems likely that the complete amino acid sequence of the polypeptide was determined.

Ether-soluble and -insoluble fractions of the chloroform extract from S. cerevisiae plasma membranes were found to be identical in NH₂-terminal amino acid sequence (Fig. 3, A and B). These results indicate that both fractions contain the same polypeptide. It therefore seems likely that the 7.5-kDa compound is a dimeric form of the 4-kDa polypeptide. The dimer would be precipitated with ether because of spontaneous association and mutual protection of the hydrophobic portions of the polypeptides while their hydrophilic portions are exposed to the outside. The size discrepancy between the two bands probably reflects the presence of lipids as well as anomalies in migration of low molecular weight peptides as previously reported (Huang and Mathews, 1990).

Furthermore, diethyl ether was added to the chloroform extract of purified H⁺-ATPase from S. cerevisiae. The insoluble fraction was subjected to Edman degradation. The first 15 amino acid residues of the NH₂ terminus correspond exactly with those determined for the proteolipid extracted from plasma membrane (data not shown).

The corresponding gene referred to as PMP1 (for plasma membrane proteolipid) was isolated and sequenced. As compared with the deduced amino acid sequence, the purified polypeptide lacks the first two residues, found to be methionine and threonine (Fig. 4). A cysteine residue was found at position 18 of the nascent polypeptide. The presence of cysteine, undetectable by Edman degradation without pretreatment, easily explains the very low observed yield obtained during amino acid microsequencing for position 16 of the mature polypeptide, which was supposed to be a serine. Searches for homologous proteins in current protein data banks, using the FASTA algorithm (Pearson and Lipman, 1988) failed to uncover any significant primary structural similarity to known proteins.

Hydropathy Analysis of the Mature Proteolipid—A hydropathy plot was derived for the 38-residue polypeptide according to Kyte and Doolittle (1982) (Fig. 5). Two structural domains

Fig. 3. Amino acid sequence of the proteolipid from Saccharomyces cerevisiae. A, amino acid sequence of the ether-insoluble proteolipid. The 10-residue fragment generated by digestion with BNPS-skatole is underlined. B, NH₂-terminal sequence of the ether-insoluble proteolipid. C, yield of PTH-derivatives (pmol) recovered after each cycle of the Edman reaction. The one-letter notation has been used for amino acids.

Fig. 5. Hydrophobicity profile of the proteolipid. Hydropathy analysis was carried out using the algorithm of Kyte and Doolittle (1982) with a window size of 7 residues. A 20-residue region with a hydrophobicity index ≥1.6 is predicted to span the membrane.
are clearly defined. The NH₂ terminus (residues 1–24) is predicted to span the membrane. In contrast, the hydrophilic COOH end (residues 25–38) forms a very basic domain with 1 lysine and 4 arginine residues.

**DISCUSSION**

We have purified a small protein from the *S. cerevisiae* plasma membrane as well as from the purified H⁺-ATPase. This protein can be classified as a proteolipid since it is extracted by a mixture of chloroform/methanol and thus has lipid-like properties (Folch and Lees, 1951). When electrophoresed on Tricine-SDS-polyacrylamide gels, this proteolipid displays two bands. The molecular weights of the corresponding proteins are 7,500 and 4,000. The same NH₂-terminal sequence (94% homogeneous) was determined by Edman degradation during microsequencing of these two bands, suggesting that the extracted proteolipid exists as a dimer and a monomer.

The proteolipid is firmly bound to the H⁺-ATPase of the *S. cerevisiae* plasma membrane and cannot be removed by any of the attempted purification procedures preserving ATPase enzyme activity. It takes a denaturing chloroform/methanol extraction to dissociate the proteolipid from the major 100-kDa subunit of the H⁺-ATPase. As pointed out in the introduction, the H⁺-ATPase purified from the fission yeast *S. pombe* seems to contain a low molecular weight compound with a mobility and lipid-like properties similar to those described here for *S. cerevisiae* (Dufour and Goffeau, 1978). Moreover, previous quantitative studies on the subunit composition of the Neurospora crassa membrane H⁺-ATPase detected a very small oligopeptide that migrates in the tracking dye region (Scarborough and Addison, 1984). These authors could not exclude the possibility that this compound is a stoichiometric subunit of the H⁺-ATPase. Thus, the 4-kDa proteolipid might be a true component of the fungal H⁺-ATPase. It has also been reported that the mammalian Na⁺/K⁺ ATPase displays two bands. The molecular weights of the corresponding proteins are 7,500 and 4,000. The same NH₂-terminal sequence (94% homogeneous) was determined by Edman degradation during microsequencing of these two bands, suggesting that the extracted proteolipid exists as a dimer and a monomer.

The yeast plasma membrane proteolipid undergoes some posttranslational modifications; as compared with the deduced sequence of the gene, the proteolipid lacks the first two NH₂-terminal residues, suggesting that integration into the plasma membrane is achieved with minimal cleavage. Moreover, during NH₂-terminal amino acid sequencing of the mature polypeptide, alanine 21 and threonine 22 were found to occur with a lower yield than most of the other residues. This raises the possibility that threonine might be modified, which could disturb the Edman degradation of the two neighboring amino acids. It is conceivable that a fatty acid binds threonine through an ester linkage, as reported for the myelin proteolipid (Stoffel et al., 1983). Alternatively, a palmityl might be covalently bound to the proteolipid via the cysteine in position 16 (in the mature polypeptide) as is generally the case for fatty acid-acylated proteins (see review by Olson (1988)). Such a covalent modification would probably account for the solubility of the small proteolipid in chloroform/methanol.

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