The first putative transmembrane helix of the 16 kDa proteolipid lines a pore in the V_o sector of the vacuolar H^+ -ATPase

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The 16 kDa proteolipid is the major component of the vacuolar H^+ -ATPase membrane sector, responsible for proton translocation. Expression of a related proteolipid from the arthropod *Nephrops norvegicus* in a *Saccharomyces* strain in which the *VMA3* gene for the endogenous proteolipid has been disrupted results in restored vacuolar H^+ -ATPase function. We have used this complementation system, coupled to cysteine substitution mutagenesis and protein chemistry, to investigate structural features of the proteolipid. Consecutive cysteines were introduced individually into putative transmembrane segment 1 of the proteolipid, and at selected sites in extramembranous regions and in segments 3 and 4. Analysis of restored vacuolar H^+ -ATPase function showed that segment 1 residues sensitive to mutation to cysteine were clustered on a single face, but only if the segment was helical. Only residues insensitive to mutation

could be covalently modified by the cysteine-specific reagent fluorescein 5-maleimide. A cysteine introduced into segment 3 was the only residue accessible to a relatively hydrophobic reagent, suggesting accessibility to the lipid phase. Analysis of disulphide bond formation between introduced cysteines indicates that the first transmembrane α -helices of each monomer are adjacent to each other at the centre of the proteolipid multimeric complex. The data are consistent with a model in which the fluorescein maleimide-accessible face of helix 1 lines a pore at the centre of a hexameric complex formed by the proteolipid, with the mutationally sensitive face oriented into the protein core. The implications for ion-transport function in this family of proteins are discussed in the context of this structural model.

INTRODUCTION

The hydrophobic nature of integral membrane proteins ensures that for most proteins of this class the generation of structural information continues to present special difficulties. Recent strategies employing a site-directed mutagenesis approach have, however, been successfully applied to the analysis of structure–function relationships in a number of membrane proteins. Exploiting the strong reactivity of the thiol group, these strategies involve the mutagenic introduction of cysteine residues at specific sites within a protein [1,2]. These residues are subsequently probed with cysteine-specific reagents, providing an assessment of the accessibility and location of individual sites within the protein. Similarly, analysis of disulphide cross-linking between introduced cysteines has been used to study intimate protein–protein interactions at both inter- and intra-molecular levels [3,4].

We have used this approach to study the structure and function of the 16 kDa proteolipid [5–7], which is a principal component of the V_o membrane sector of the vacuolar H^+ -ATPases (V_oV_1 -ATPase) [8]. The vacuolar H^+ -ATPases (V -ATPases) are the primary ion pumps that generate the proton motive force required for many secondary transport processes and for acidification of intracellular organelles [9,10]. They are multiprotein complexes of fundamental importance to a variety of physiological processes [11]. The V -ATPases were thought to operate exclusively as ATP-driven proton pumps, but recent reports suggest that related enzymes can transport Fe^{3+} [12] or Na^+ [13].

The V -ATPases are analogous in structure to F_oF_1 -ATPases, and both seem to have evolved from a common ancestor [14].

The 16 kDa proteolipid has limited similarity to the 8 kDa subunit c of the F_oF_1 -ATPase, from which it may have evolved by duplication of a progenitor gene [15]. Structural studies of the 8 kDa subunit in organic solvents indicate a simple hairpin-like structure of two α -helices that resembles the structure predicted for the folded protein in the membrane [16,17]. By analogy, it seems plausible that the 16 kDa proteolipid will contain two such α -helical hairpins. Both the 8 kDa subunit c and 16 kDa proteolipid are intimately involved in proton conductance, and both contain a conserved carboxyl residue that is DCCD-reactive and crucial for movement of the ion [18,19].

We have previously presented a molecular model [20,21] for a 16 kDa proteolipid from the hepatopancreas of the lobster *Nephrops norvegicus* [22–24]. This model, developed from protein chemistry, electron microscopy and Fourier-transform infrared spectroscopy [21], proposes that each 16 kDa polypeptide folds as a bundle of four α -helices arranged sequentially in an antiparallel manner. In this arrangement the polar face present on each transmembrane helix is positioned towards the centre of the bundle, resulting in a predominantly hydrophobic periphery in contact with the lipid phase. The macromolecular complex of 16 kDa proteolipids is modelled as a hexameric channel on the basis of the dimensions and features of particles from images from electron microscopy of the protein isolated in membrane sheets from the arthropod *Nephrops*. A hexamer provides the best dimensional fit and is also consistent with studies on subunit stoichiometry in the V -ATPase [8].

To test the validity of this model, we have coupled the mutagenesis approach outlined above with protein chemical analysis. An ideal expression system for such cysteine-substi-

Abbreviations used: DCCD, *N,N'*-dicyclohexylcarbodiimide; FM, fluorescein 5-maleimide; IAA, iodoacetic acid; V -ATPase, vacuolar H^+ -ATPase; V_oV_1 -ATPase, V_o membrane sector of the vacuolar H^+ -ATPases.

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tution studies is a *Saccharomyces* mutant in which the endogenous *VMA3* gene for the proteolipid has been inactivated [25]. This mutation is complemented by a cDNA for the *Nephrops* proteolipid, resulting in functional substitution of the lobster proteolipid into the yeast V-ATPase [26]. We have introduced individual cysteine residues into the *Nephrops* proteolipid and expressed each mutant form of proteolipid in the *vma3* yeast strain. Complementation of the mutation provides a means of screening the functional effects of cysteine replacements, and the isolated yeast vacuolar membrane provides material for protein chemistry studies. Associations within the proteolipid complex are determined by monitoring the propensity of each introduced cysteine to form disulphide bonds. The proximity of each cysteine to the lipid or aqueous phases is determined by monitoring accessibility to cysteine-specific reagents. In combination, these data provide a refinement and verification of our published model. In this study we have focused on the position and orientation of the putative transmembrane segment 1 of the proteolipid, primarily because our preliminary model makes definite structural predictions for this segment that are readily testable. We present information consistent with a structural model in which the first transmembrane segment of the proteolipid is helical and lines a central pore in the V_o complex that is accessible from the aqueous phase. The implications of these structural data for our appreciation of the mechanism of proton translocation and for the structural basis of V_o permeability properties are discussed.

MATERIALS AND METHODS

Strains and plasmids

Routine manipulations were carried out in *Escherichia coli* XL1-Blue with the pBluescript SK plasmid (Stratagene). Expression in *Saccharomyces* was performed with the yeast-*E. coli* shuttle vectors pLGSD5-ATG (provided by L. Guarente) and pYES2 (Invitrogen), with expression under the control of the inducible *GAL1* promoter. To obtain expression of mutant forms of 16 kDa proteolipid that could not support growth at pH 7.5, the appropriate cDNA was subcloned into a plasmid variant of pYES2 containing the strong constitutive promoter and transcription termination regions of the yeast *PMA1* gene, kindly provided as a *HindIII* fragment in the plasmid YEp351 by R. Serrano [27]. The *XhoI* cloning site separating the *PMA1* promoter and termination region was modified by standard procedures to give a unique *BamHI* cloning site, and the plasmid pCON1. Alternatively, yeast cells transformed with inducible plasmids were grown in selective yeast nitrogen-base medium at pH 5.5, supplemented with 2% (v/v) glycerol, with expression induced by 0.5% galactose. All the vectors contained the *URA3* gene for auxotrophic selection in yeast and ampicillin resistance gene for selection in *E. coli*. Expression of the 16 kDa proteolipid was performed in the haploid *Saccharomyces cerevisiae* strain W303-1B (*MAT α* , *leu2*, *his3*, *ade2*, *trp1*, *ura3*) in which the *VMA3* gene for the endogenous 16 kDa proteolipid is disrupted by the insertion of a *LEU2* gene (*LEU2::vma3*), kindly provided by N. Nelson [25]. Control investigations were made with the wild-type strain INVSC1 (Invitrogen; *MAT α* , *leu2*, *his1*, *trp1*, *ura3*). Standard yeast manipulation techniques and growth conditions were described previously [26].

Construction of cysteine-containing mutants

Cloning of the cDNA for the *Nephrops* 16 kDa proteolipid has been described previously [21,22]. The coding region of the 16 kDa proteolipid cDNA was subcloned into the *BamHI* site

of pBluescript (SK), and a cDNA encoding a cysteine-free proteolipid (Cys⁵⁴ → Ser) was constructed by a rapid site-directed mutagenesis procedure using PCR [28]. This method requires only one specific mutagenic primer and two forward and reverse universal sequencing primers of the plasmid. Universal M13 sequencing primers were extended in base composition, increasing their T_m to approx. 60 °C. The mutagenic primer was synthesized to contain a single base change in the codon for cysteine, which created a serine codon (TGC → TCC) and a T_m of 60 °C. The first PCR reaction using the 5' forward pBluescript sequencing primer and the 3' mutagenic primer generated a product that was purified from an agarose gel to prevent contamination with primers, which would otherwise lead to the generation of wild-type fragments in the second round of PCR. In the second round of PCR, the sense strand of this product served as a 5' mutagenic 'megaprimer' and the reverse pBluescript sequencing primer served as the 3' primer. The product of the second PCR reaction was digested with *BamHI* and subcloned into pBluescript for sequence analysis. Once the mutation had been confirmed, the fragment was subcloned into a yeast expression vector for further analysis.

The cDNA encoding the cysteine-free 16 kDa proteolipid was used as a template for the replacement of individual cysteine residues at selective sites using the two-stage PCR-based mutagenesis procedure outlined above. Mutagenic primers were designed to provide the change to a cysteine codon with the minimum number of base changes. Eight consecutive residues within the first putative membrane-spanning region of the *Nephrops* proteolipid (Phe²⁴, Ser²⁵, Ala²⁶, Leu²⁷, Gly²⁸, Ala²⁹, Ala³⁰ and Tyr³¹) were individually changed. In addition, substitutions within the putative third (Gly¹⁰¹, Leu¹⁰², Ser¹⁰³, Leu¹⁰⁵) and fourth (Leu¹⁴⁷, Ile¹⁴⁸) membrane-spanning helices, the N-terminal region (Ser⁶) and the extramembranous region between the first and second helices (Ser⁴⁴) were constructed. All mutational changes were verified by sequencing with the dideoxy chain-termination procedure.

Vacuolar membrane isolation and determination of ATP-hydrolysing activity

Vacuolar membrane vesicles were isolated from yeast spheroplasts as described [29]. ATPase specific activity and K_m determinations were performed as previously described [26]. Vesicles were stored in 10 mM Tris/HCl, 1 mM EDTA, pH 7.6, with 10% (v/v) glycerol at -80 °C. The catalytic V_1 sector of the V-ATPase was stripped from the membrane by the action of 100 mM KNO₃, with 5 mM Mg²⁺-ATP [30]. Inhibition with DCCD was performed as in [26].

Chemical modification of cysteine residues

Dye-maleimides were purchased from Molecular Probes. Fluorescein 5-maleimide (FM) was used to probe for cysteine residues in hydrophilic environments. The hydrophobic probe benzophenone 4-maleimide was used to identify lipid-exposed cysteine residues. Maleimides were prepared in dimethylformamide to a stock concentration of 0.25 M. Addition of the reagents gave a reaction mixture which contained not more than 2% (v/v) dimethylformamide. Membrane vesicles corresponding to 200 µg total protein were washed once with 10 mM Tris/HCl, 1 mM EDTA, pH 7.0, resuspended in the same buffer with the inclusion of 10 mM dithiothreitol and incubated under nitrogen for 15 min at 30 °C. The vesicles were then harvested by centrifugation at 13000 g for 15 min at 4 °C and the supernatant was discarded. The washed vesicles were resuspended in reaction

buffer containing 100 mM Tris/HCl, 1 mM EDTA, pH 7.0, 10 % glycerol, and 50 μ M phenylmethylsulphonyl fluoride. The final concentration of maleimide was 2.5 mM. Each reaction was incubated under nitrogen for 2 h at 30 °C, and then terminated by addition of 2-mercaptoethanol to 2 % (v/v). Protein was then precipitated with 9 volumes of acetone/ethanol (1:1) at -20 °C, and the pellets were washed once with acetone/ethanol. Protein pellets were resuspended in 10 mM Tris/HCl, 1 mM EDTA, pH 7.0, and the 16 kDa proteolipid was extracted with 5 volumes of chloroform/methanol (2:1) as previously described [18]. The vacuum-dried organic phase was resuspended in sample buffer containing 50 mM Tris/HCl, pH 6.8, 10 % glycerol, 4 % (w/v) SDS and 2.5 % 2-mercaptoethanol before SDS/PAGE analysis.

As a positive control, the 16 kDa proteolipid was denatured with SDS before incubation with the maleimide reagents. Membrane vesicles corresponding to 200 μ g total protein were washed once with 10 mM Tris/HCl, 1 mM EDTA, pH 7.0, resuspended, and the protein was precipitated with acetone/ethanol, as above. The 16 kDa proteolipid was then extracted with chloroform/methanol, as described above. The vacuum-dried organic phase was resuspended in reaction buffer including 1 % SDS and 50 μ M dithiothreitol and reacted with excess maleimide reagent as described above. The reaction was terminated by addition of 0.25 volumes of 5-fold concentrated sample buffer.

Mass shift assay for modification of proteolipids by dye-maleimides

Modification by dye-maleimides results in a mass shift of the proteolipid during SDS/PAGE. To measure the relative proportion of modified proteolipid, SDS/PAGE gels were stained with silver, dried and scanned by a laser densitometer. The relative proportions of modified and unmodified proteolipid, averaged from several experiments, were determined from the areas under the absorbance peaks. Values are expressed relative to the total proteolipid in the sample.

SDS/PAGE and Immunoblotting

SDS/PAGE was performed on 12.5 % or 15 % acrylamide gels with the buffer system of Schägger and von Jagow [31]. For immunoblotting, proteins were electrophoretically transferred to a poly(vinylidene difluoride) membrane and probed using standard procedures with polyclonal antibodies raised against the native *Nephrops* 16 kDa proteolipid [32]. The alkaline phosphatase colour development system was used to assay antibody binding. Protein assay was performed with the bicinchoninic acid method (Pierce).

Detection of intermolecular disulphide bonds

A two-dimensional SDS/PAGE procedure was used to detect dimers resulting from disulphide bond formation between adjacent proteolipids in the V-ATPase complex. For the first-dimension (non-reducing) analysis, vesicle proteins were precipitated directly with acetone/ethanol or pretreated with 1.5 mM Cu(II) phenanthroline [33] for 30 min at room temperature before precipitation. Protein pellets were dissolved in sample buffer (detailed above) in the absence of reducing agent and subjected to SDS/PAGE. Gel strips corresponding to individual lanes from non-reducing gels were excised and incubated in SDS/PAGE sample buffer, in the presence or absence of 2-mercaptoethanol, for 10 min at room temperature. These were then sealed onto second-dimension gels with a solution of molten agarose in the appropriate reducing or non-

reducing sample buffer. Monomeric and dimeric forms of the *Nephrops* proteolipid were detected by electrophoresis and immunoblotting under the conditions described above.

To investigate interpeptide disulphide bond formation *in vivo*, a yeast total-membrane fraction [34] was prepared in the presence or absence of 10 mM iodoacetic acid, which scavenges any available reduced cysteine residues during cell breakage. The total cell membrane fraction was resuspended in a buffer containing 200 mM Tris/HCl, pH 8.0, 1 mM EDTA, 2 % SDS and 10 mM iodoacetic acid and incubated under nitrogen in the dark at room temperature for 1 h. The protein was then reprecipitated and analysed for disulphide bond formation by SDS/PAGE and immunoblotting. The presence of high concentrations of iodoacetic acid throughout the isolation ensured that any disulphide-linked dimers must have formed *in vivo*.

RESULTS

Functional analysis of cysteine-substitution mutants

The *vma3* mutation in *Saccharomyces* is conditionally lethal, allowing growth at pH 5.5 but not at pH 7.5. Transformation with a cDNA encoding the *Nephrops* proteolipid results in complementation of this mutation and incorporation of the arthropod protein into a functional hybrid vacuolar H^+ -ATPase [26], exhibited as a recovery in the ability to grow at pH 7.5 [25]. The ability of the cysteine-substituted forms of proteolipid to complement the *vma3* mutation and restore growth at pH 7.5 after galactose-induced expression provides a simple assay for the functional effects of introduced mutations. The *Nephrops* form of proteolipid is particularly suitable for cysteine substitution experiments because it contains only a single cysteine residue in its native form (Cys⁵⁴). This can be readily altered by mutagenesis, yielding a cysteine-free polypeptide (Cys⁵⁴ → Ser), which is a template for subsequent cysteine substitutions. Replacement of this single cysteine residue with serine did not alter either the growth or enzymological characteristics of the yeast strain (Table 1). The effects of introducing cysteine residues at different positions in the cysteine-free mutant on ATP-hydrolysing activity *in vitro* and on growth are summarized in Table 1. Expression of mutant forms of the proteolipid was confirmed in each case by immunoblot analysis of isolated vacuolar membrane proteins (see Figure 4). Cysteine replacement of Phe²⁴, Ser²⁵ and Tyr³¹ (within the first putative transmembrane helix) resulted in slow growth at pH 7.5 and significantly higher K_m values for the hybrid V-ATPases. Mutation of Gly²⁸ to cysteine (Gly²⁸ → Cys) resulted in failure to grow at pH 7.5. However, analysis of vesicle membranes isolated from a yeast strain constitutively expressing this Gly²⁸ → Cys form of the proteolipid, using the plasmid pCON1 and growth in selective minimal medium at pH 5.5, revealed ATPase activity but with greatly increased K_m .

Cysteine substitutions in the extramembranous regions (Ser⁶ → Cys and Ser⁴⁴ → Cys) and at Leu¹⁰⁵ (Leu¹⁰⁵ → Cys) in the putative third helix did not significantly alter growth or V-ATPase functional characteristics from those of the control Cys⁵⁴ → Ser strain. Cysteine replacement of Ser¹⁰³ in helix 3 (Ser¹⁰³ → Cys) resulted in an enzyme with somewhat elevated K_m for ATP, similar to the characteristics of the Ser²⁵ → Cys strain (Table 1). Mutation of Gly¹⁰¹ in helix 3 (Gly¹⁰¹ → Cys) resulted in a hybrid V-ATPase with normal K_m for ATP hydrolysis *in vitro*, but that did not restore growth at pH 7.5. Similarly, neither cysteine replacement within helix 4 resulted in restored growth at pH 7.5, although membranes isolated from both mutants retained V-ATPase-specific ATP-hydrolysing activity (Table 1). The mutation Ile¹⁴⁸ → Cys was functionally similar to the Gly²⁸

Table 1 V-ATPase activity of *Saccharomyces* strains expressing mutant forms of the *Nephrops* 16 kDa proteolipid

Vacuolar membrane vesicles were isolated from *Saccharomyces* strains expressing mutated forms of the *Nephrops* 16 kDa proteolipid and assayed for V-ATPase-specific ATP-hydrolysing activity, as detailed in Materials and methods. *Nephrops* 16 kDa and *vma3* refer to the strains expressing the unmodified *Nephrops* proteolipid and to the untransformed *LEU2::vma3* strain W303-1B, respectively. Strains expressing mutant forms of the proteolipid are designated according to the identity and position of the changed residue. Values are expressed as means $\pm$ S.D. (*n*). Qualitative assessment of growth at pH 7.5 in buffered yeast-extract-peptone medium with 1% (w/v) galactose is shown relative to growth of wild-type cells under the same conditions: ++, growth equivalent to wild-type and non-mutated strains; +, slow growth; +/–, very weak growth; –, no growth. ND, value not determined.

Strain	Specific activity* (units per mg of protein)	K_m ATP (mM)	Growth at pH 7.5†
<i>vma3</i>	≤ 0.01 (2)	ND	–
Wild type	0.21 ± 0.20 (3)	0.20 ± 0.01	++
<i>Nephrops</i> 16kDa (Cys ⁵⁴)	0.20 ± 0.03 (6)	0.40 ± 0.11	++
Cys ⁵⁴ → Ser	0.16 ± 0.01 (4)	0.55 ± 0.04	++
Helix 1			
Phe ²⁴ → Cys	0.27 ± 0.02 (3)	1.17 ± 0.21	+ / –
Ser ²⁵ → Cys	0.30 ± 0.06 (3)	0.89 ± 0.10	+
Ala ²⁶ → Cys	0.13 ± 0.03 (4)	0.48 ± 0.10	++
Leu ²⁷ → Cys	0.12 ± 0.04 (3)	0.54 ± 0.12	++
Gly ²⁸ → Cys‡	0.17 (1)	3.00	–
Ala ²⁹ → Cys	0.14 ± 0.03 (3)	0.36 ± 0.05	++
Ala ³⁰ → Cys	0.14 ± 0.05 (3)	0.42 ± 0.12	++
Tyr ³¹ → Cys	0.25 ± 0.03 (3)	1.60 ± 0.20	+ / –
Extramembranous			
Ser ⁶ → Cys	0.11 ± 0.02 (3)	0.40 ± 0.04	++
Ser ⁴⁴ → Cys	0.12 ± 0.03 (4)	0.49 ± 0.17	++
Helix 3			
Gly ¹⁰¹ → Cys§	0.22 ± 0.02 (3)	0.31 ± 0.03	–
Ser ¹⁰³ → Cys	0.40 ± 0.08 (3)	0.80 ± 0.08	++
Leu ¹⁰⁵ → Cys	0.20 ± 0.04 (5)	0.35 ± 0.05	++
Helix 4			
Leu ¹⁴⁷ → Cys§	0.13 ± 0.02 (3)	0.27 ± 0.10	–
Ile ¹⁴⁸ → Cys§	0.97 ± 0.20 (3)	3.50 ± 0.80	–

* Units of ATPase activity are μ mol phosphate per h.

† Growth at pH 7.5 in yeast-extract-peptone medium supplemented with galactose.

‡ Expressed constitutively and grown in synthetic yeast nitrogen-base medium with glucose at pH 5.5.

§ Expression in selective yeast nitrogen-base medium with 2% (v/v) glycerol, pH 5.5, induced with 0.5% galactose.

→ Cys strain, whereas the mutation Leu¹⁴⁷ → Cys resulted (as for Gly¹⁰¹ → Cys) in an enzyme with K_m similar to that found in functionally normal strains. This pattern of mutational sensitivity is discussed below in the context of protein chemical labelling experiments. There was a broadly positive correlation between ATPase specific activity and K_m . However, specific activity will vary according to the levels of expression, whereas K_m for the hybrid enzyme will not. The mutant Gly²⁸ → Cys, for example, was expressed constitutively at lower levels and showed relatively low specific activity (Table 1).

Activity measurements in the presence of inhibitors confirm that the observed ATP hydrolysis rates arose exclusively from V-ATPase activity. Incubation of vacuolar membranes with 5 μ M DCCD resulted in the loss of 70–80% of ATP-hydrolysing activity from membranes of all cysteine-substitution mutants. Removal of V_1 by the combined effects of nitrate and cold shock in the presence of Mg-ATP resulted in complete abolition of activity in all cases.

Chemical probing with cysteine-specific reagents

Vacuolar membrane vesicles isolated from yeast strains expressing each cysteine-substituted form of the *Nephrops* proteolipid were probed with a variety of thiol reagents. The water-soluble reagent FM [2] was used to probe for residues accessible to the aqueous phase, whereas the relatively hydro-

phobic reagent benzophenone 4-maleimide was used to probe for residues exposed to the lipid bilayer.

Modification of the single cysteine residue within the *Nephrops* proteolipid by reaction with FM, which has a mass of 428 Da, causes an appreciable shift in migration of the polypeptide on SDS/PAGE. This is illustrated in Figure 1(a), which shows the electrophoretic analysis of proteolipid extracted with chloroform/methanol after reaction with FM. The mutant form of the proteolipid in which a cysteine residue has been introduced at position 29 (Ala²⁹ → Cys) shows a partial shift to a higher-mass species after reaction of intact membrane vesicles with FM. A virtually complete shift to the higher-mass species occurs when denatured protein is modified. Note that the mutant form of the proteolipid in which the sole native cysteine residue has been changed (Cys⁵⁴ → Ser) is not modified, confirming the complete absence of cysteine residues in this polypeptide and of non-specific labelling. We have previously shown that addition of a C-terminal affinity tag comprising six histidine residues (His $\times$ 6) also causes an appreciable band shift [26]. Modification of the native cysteine residue (Cys⁵⁴) in this species with FM causes an additional shift, particularly under denaturing conditions. Similar results are observed when the proteolipid is visualized by immunoblotting of total vacuolar membrane proteins (Figure 1b), indicating that the addition of the polar fluorescein molecule does not significantly alter solubility in non-polar solvents. A 30 kDa polypeptide, previously reported to be soluble in chloroform/methanol [26,35], also undergoes a characteristic

Figure 3 Modification of introduced cysteine residues with benzophenone-4-maleimide

Vacuolar membrane vesicles corresponding to 200 μ g protein, isolated from *Saccharomyces* cells expressing the Ser¹⁰³ $\rightarrow$ Cys mutant form of proteolipid, were reacted with FM (F) or benzophenone 4-maleimide (B) under native conditions, as described in the Materials and methods section. The proteolipid was extracted with chloroform/methanol, separated by SDS/PAGE on a 15% gel and visualized by silver staining. The position and mass (in kDa) of the nearest protein marker is indicated.

Figure 1 Mass shift assay of proteolipid cysteine modification by FM

Vacuolar membrane vesicles were treated with FM under native (—) or denaturing (+) conditions as described in the Materials and methods section. After incubation in the presence of FM, proteolipid was extracted with chloroform/methanol, separated by SDS/PAGE on 1% gels and visualized by silver staining (a). (b) Immunoblot analysis of FM-modified vacuolar membrane proteins. In (a), the track marked 16K contains 500 ng of proteolipid prepared from *Nephrops* hepatopancreas as described in [20]. C54S, cysteine-free form of proteolipid; His x 6, proteolipid with C-terminal polyhistidine tag [26]; Cys²⁹, Ala²⁹ $\rightarrow$ Cys mutated form of proteolipid. The positions and masses (in kDa) of protein markers are indicated.

mass shift after FM modification (Figure 1a). This species does not represent the dimer of the 16 kDa proteolipid, and its identity remains unknown.

An assay based on the mass shift on SDS/PAGE was used to characterize the accessibility of substituted cysteine residues to probes with different chemical properties under native conditions. Time-course experiments with the Ala²⁹ $\rightarrow$ Cys form of the proteolipid gave a half-time for the FM reaction of approximately 40 min (data not shown), and an incubation period of 2 h was

Figure 2 FM modification of cysteine-substituted proteolipids under non-denaturing conditions

Vacuolar membrane vesicles were reacted with FM under native conditions, extracted with chloroform/methanol and the extracted protein separated by SDS/PAGE on 15% gels, as in Figure 1. The relative proportions of FM-modified (dark shading) and unmodified (light shading) proteolipid were determined for each cysteine-replacement mutant by laser densitometry of the resulting silver-stained gels. Numbers at the top of each column indicate the numbers of replicate experiments averaged for each determination. In each case, the standard error was less than 10% of the mean value. The coding for individual cysteine-replacement mutants is as described in the text. Cys⁵⁴ represents the non-mutated form of *Nephrops* proteolipid and Cys⁵⁴-His x 6 represents the polyhistidine-tagged proteolipid, as in the legend to Figure 1.

Figure 4 Immunoblot analysis of intermolecular disulphide bond formation between mutagenically introduced cysteine residues

(a) Vacuolar membrane vesicles, isolated from *Saccharomyces* strains expressing each cysteine-substituted form of proteolipid, were diluted with acetone/ethanol and the precipitated protein recovered. Vacuolar membrane proteins (10 µg in each lane) were separated by non-reducing SDS/PAGE on a 15% gel. Monomeric (M) and dimeric (D) species of the proteolipid were detected by immunoblotting, as described in the Materials and methods section. (b) As for (a), with vacuolar membrane vesicles pretreated with 1.5 mM Cu(II) phenanthroline. C26, C6 and C44 represent the Ala²⁶ → Cys, Ser⁶ → Cys and Ser⁴⁴ → Cys mutant forms of proteolipid, respectively, and each shows disulphide cross-linked dimers, as in (a). Membranes from the mutant strains S25C (C25), L27C (C27) and C54S are shown for comparison. No other mutant strain shown in (a) displayed cross-linking; they are therefore omitted. (c) Immunoblot analysis of total membrane fractions isolated from cells expressing the S6C (C6), S44C (C44) or cysteine-free (C54S) forms of proteolipid, in the presence (+ IAA) or absence of 10 mM iodoacetic acid during cell breakage. Presence of IAA prevents formation of the disulphide-linked proteolipid dimer *in vitro* in the S44C mutant.

therefore used to ensure that maximal steady-state modification was achieved. Susceptibility of each cysteine-substituted form of the *Nephrops* proteolipid (under native conditions) to modification by FM is shown in Figure 2. Substitutions within the putative helix 1 of the proteolipid to give Ala²⁶ → Cys, Ala²⁹ → Cys and Ala³⁰ → Cys all provided residues that were accessible to FM (38%, 60% and 45% proteolipid modification, respectively). The N-terminal Ser⁶ → Cys residue was also strongly reactive with FM (37% modification). Substitutions giving Tyr³¹ → Cys and Ser⁴⁴ → Cys, which are at positions within the proteolipid predicted to be situated at the interface between bilayer and cytoplasm (Tyr³¹ → Cys) or in an extramembranous loop (Ser⁴⁴ → Cys), also gave residues accessible to FM (46% and 31% modification, respectively). The cysteine residue at position 54 in the native proteolipid (Cys⁵⁴ and Cys⁵⁴-His × 6) showed weak reactivity to FM (15% and 19% modification). Cysteine residues substituted into positions 24, 25, 27 and 28 were not reactive towards FM under non-denaturing, native conditions (Figure 2). Similarly, cysteine substitutions within the putative helices 3 or 4 gave residues that did not react with FM.

The hydrophobic probe benzophenone 4-maleimide was unable to modify any of the cysteine residues introduced into the putative helix 1, into helix 4 or into extramembranous regions under non-denaturing conditions (results not shown). However, replacement of Leu¹⁰⁵ in helix 3 by cysteine gave a residue that could be labelled by benzophenone-4-maleimide (but not FM) when the reagent was added to native vacuolar membrane vesicles (Figure 3).

We have used glycerol gradient centrifugation of detergent-solubilized vacuolar membranes [29] to preclude the possibility

that the dye maleimide-reactive proteolipid represents a free population of protein not incorporated into the V-ATPase. No such population was observed (results not shown).

Formation of intermolecular disulphide bonds

To test for the presence of homodimers of proteolipid occurring as a consequence of disulphide bond formation, vacuolar membrane proteins isolated from each strain expressing cysteine-substituted forms of proteolipid were analysed by SDS/PAGE under non-reducing conditions (Figure 4a). Only the Ser⁶ → Cys or Ser⁴⁴ → Cys mutant proteolipids were able to form covalently linked dimers in untreated membranes, observed as a tight band on SDS/PAGE. This dimer species migrates faster than the diffuse non-covalently linked dimer, which is a solubilization artefact (Figure 4a). Incubation with the oxidant Cu(II) phenanthroline resulted in the formation of disulphide-linked proteolipid dimers in membranes of the Ala²⁶ → Cys mutant and partly increased the degree of dimerization in the Ser⁶ → Cys and Ser⁴⁴ → Cys strains (Figure 4b). Significantly, this treatment did not produce proteolipid dimers in the membranes of any other mutant strain.

Iodoacetic acid will act to carboxymethylate reduced cysteine residues, and this reagent was used to discriminate between disulphide bond formation *in vivo*, and *in vitro* after membrane isolation. In the Ser⁴⁴ → Cys mutant, inclusion of iodoacetic acid during breakage of the yeast cells prevented formation of covalently linked dimers (Figure 4c). Inclusion of iodoacetic acid after membrane isolation did not prevent disulphide bond formation, suggesting that the formation of disulphide bonds

Figure 5 Analysis of disulphide-linked proteolipid dimers by two-dimensional SDS/PAGE and immunoblotting

Vacuolar membrane proteins (10 μ g) isolated from cells expressing the cysteine-free (C54S) or S6C (Cys⁶) forms of proteolipid were analysed by non-reducing SDS/PAGE, as in Figure 4. Gel strips corresponding to individual lanes were excised and incubated in SDS/PAGE sample buffer, in the presence (lower panel) or absence (upper panel) of 2-mercaptoethanol, before electrophoresis in the second dimension, as described in the Materials and methods section. The monomeric (M) and dimeric (D) forms of the proteolipid were detected by immunoblotting.

between Ser⁴⁴ $\rightarrow$ Cys proteolipids occurs *in vitro* after removal of the reducing environment of the cytoplasm. Similar analysis of the Ser⁶ $\rightarrow$ Cys mutant proteolipid showed the presence of covalently linked dimers formed *in vivo* (Figure 4c). Irrespective of the time at which dimers were formed, it should be noted that covalent linkage did not compromise the activity of the hybrid V-ATPase complex (see Table 1).

The presence of a disulphide linkage between proteolipids was confirmed by two-dimensional SDS/PAGE (Figure 5), in which gel strips corresponding to lanes from the non-reducing gels in Figure 4 were excised, incubated under non-reducing (Figure 5, upper panel) or reducing (Figure 5, lower panel) conditions and placed horizontally onto second polyacrylamide gels. Note that in the Ser⁶ $\rightarrow$ Cys mutant, a proteolipid dimer is present under non-reducing conditions but that this dimer breaks down to monomeric species only after incubation with reducing agent. Identical results were obtained for both the Ser⁴⁴ $\rightarrow$ Cys and Ala²⁶ $\rightarrow$ Cys mutant strains (not shown).

DISCUSSION

Complementation of the *vma3* mutation in *Saccharomyces* represents a useful system for probing structure and function of *vma3*-related proteolipids. Transformation with cDNAs encoding cysteine-substituted forms of the *Nephrops* proteolipid abolished the pH-sensitive phenotype characteristic of *vma* mutants and restored growth at pH 7.5, with each expressed form of proteolipid being correctly targeted to the vacuolar membrane and integrated into a hybrid V-ATPase. Resultant hybrid V-ATPase complexes showed variable functional proper-

Figure 6 Projection of helix 1 of the *Nephrops* proteolipid

(a) Helix 1 of the proteolipid is represented by a helical wheel, shown as regular in nature with 3.6 residues per turn, and is plotted using the program PERSCAN v7.0 developed by D. Donnelly [37]. The helix is shown with an anti-clockwise rotation, starting from the glycine residue at position 14. Residues 24–31 in the sequence FSALGAAY, individually mutated to cysteine, are circled. Cysteine substitutions giving rise to V-ATPase dysfunction (elevated K_m , slow or no growth at pH 7.5) are shaded. Residue positions that are accessible to FM are indicated by asterisks. The arrow indicates the most highly conserved helical face determined by the PERSCAN program. (b) Proposed position of helix 1 within the proteolipid hexamer. The diagram represents a bisected hexamer viewed along the plane of the membrane, with helices represented as cylinders. This schematic representation accommodates spatial constraints dictated by disulphide cross-linking. The relative orientation of helix 1 within the four-helical bundle of each proteolipid monomer has been determined on the basis that mutationally sensitive residues will be on internal helical faces and that FM-accessible residues Ala²⁶, Ala²⁹, Ala³⁰ and Tyr³¹ will be proximal to the central pore.

ties, with differences in K_m for ATP (Table 1), reflected as variations in growth rate at pH 7.5 among different cysteine-substitution mutants. A projection of segment 1 of the *Nephrops* proteolipid as an α -helix provides a clearer appreciation of the functional data (Figure 6a). Examination of the positions of substituted residues reveals that substitutions that result in high K_m and slow or no growth at pH 7.5 (Phe²⁴ $\rightarrow$ Cys, Ser²⁵ $\rightarrow$ Cys, Gly²⁸ $\rightarrow$ Cys and Tyr³¹ $\rightarrow$ Cys) are clustered towards one face of the putative helix. Other substitutions in putative helix 1, and those in extramembranous regions, did not significantly influence hybrid V-ATPase function.

Although the position of helix 1 within the four-helical bundle cannot be determined from the functional data alone, the general case seems to be that residues located towards the interiors of

proteins are more likely to be conserved [36,37], and less likely to tolerate mutational change. Analysis of aligned proteolipid sequences with the programme PERSCAN v7.0 [37] confirms that the face that is sensitive to cysteine substitution is the most highly conserved on this helix, and likely therefore to be internal within the folded proteolipid (Figure 6a).

When the positions of FM-accessible residues are considered, they are found to be broadly coincident with those that are unaffected by mutation (Figure 6a). FM accessibility, like functional sensitivity, shows α -helical periodicity. Accessibility of helix 1 residues to a water-soluble reagent indicates not only that they are excluded from tight protein-protein interfaces, but further suggests that they line a region in a proteolipid complex that is accessible from the aqueous phase [23,24]. Moreover, it is likely that this position is at the heart of the complex because none of the helix 1 cysteine replacements were accessible to benzophenone 4-maleimide. The accessibility of the cysteine replacing Tyr³¹ and of Cys⁵⁴ in the non-mutated *Nephrops* proteolipid can be explained because both are predicted to lie close to the polar head groups of the lipid bilayer, and are likely to be at least partly accessible from the aqueous phase. Cysteines replacing putative extramembranous residues Ser⁶ and Ser⁴⁴ were not modified by benzophenone 4-maleimide, which is consistent with locations for these residues in sites that are inaccessible from the lipid phase.

Disulphide formation can occur only between cysteine residues lying in close proximity. The cross-linking that occurs between cysteines replacing Ser⁶, Ala²⁶ or Ser⁴⁴ must indicate that these residues come into close contact with the same residue of a neighbouring proteolipid in the functional V_o complex. Any structural model for the proteolipid multimer must therefore take into account proximity between short N-terminal regions, the short helix-1-helix-2 loops of each monomer and between cysteines at position 26 within putative helix 1. The only model in which all of these spatial constraints can be accommodated, that explains the maleimide labelling data but still maintains the observed six-fold symmetry of the complex, is one in which the first helices of each proteolipid monomer are adjacent to each other at the centre of the complex, lining a central pore (Figure 6b). The minimum distance that can occur between Cys²⁶ residues in a regular hexameric complex is achieved when helix 1 is oriented such that the cysteinyl side chain points directly into the centre of the pore (Figure 6b). Such an orientation is of course consistent with the accessibility of this residue to water-soluble FM (Figure 2) and to Cu(II) phenanthroline (Figure 4b). The positioning of helix 1, on the basis of the protein chemical data, is consistent with our preliminary published model, and the presence of a central pore is consistent with studies by electron microscopy [24].

Cross-linking between Ser⁶ → Cys residues *in vivo* also offers further independent evidence that the N-terminus of the proteolipid is exposed to the non-reducing environment of the vacuole. Cysteine residues replacing Ser⁴⁴ are reduced *in vivo*, remaining accessible to the reducing environment of the cytoplasmic phase, and cannot therefore be tightly packed into the whole ATPase structure. In addition, the subsequent cross-linking between Cys⁴⁴ residues *in vitro* does not influence the activity of the V-ATPase. These observations suggest that all of the loop between helix 1 and helix 2 may not have a crucial role in the association between the proteolipid and other V-ATPase subunits.

The face of helix 1 that is sensitive to mutation could have some structural role in the pathway of H⁺ translocation. This involvement could be direct, with residues on this internal face contributing to an H⁺ pathway, or indirect, by influencing helical

packing. Looser packing of transmembrane helices within the proteolipid complex could result in partial destabilization of the V_o complex and weakening of the subunit interactions critical to V-ATPase function, with demonstrable effects on ATP affinity. Mutations introduced into helix 1 of the native *Saccharomyces* proteolipid also show the same general pattern of sensitivity [19,38], consistent with the relative orientation proposed in our model.

The 16 kDa proteolipid has been proposed to represent a tandem repeat of two-helical domains [15]. Helices 1 and 3 would occupy symmetrical positions within the four-helical bundle of the 16 kDa proteolipid, and by sequence alignment [23,24] the face of helix 3 containing Leu¹⁰⁵ would be equivalent to the pore-lining face of helix 1. However, the converse labelling patterns of these helices indicates that they cannot occupy exactly equivalent positions within the hexameric proteolipid complex. Whereas residues in helix 1 are accessible to FM, cysteine replacements in transmembrane segment 3 are not. A cysteine replacing Leu¹⁰⁵ of segment 3 is in turn uniquely accessible to benzophenone 4-maleimide, suggesting that it may lie in closer proximity to the lipid phase at the periphery of the proteolipid hexamer. It is interesting to note that cysteine replacement of Ser¹⁰³, a residue that would be on the opposite face of helix 3 to the benzophenone maleimide-accessible residue, results in a hybrid V-ATPase with elevated K_m (Table 1). This characteristic would be consistent with the orientation of Ser¹⁰³ into the four-helical bundle.

Both Ser⁶ → Cys and Ser⁴⁴ → Cys mutants can be modified by FM, implying that both are accessible from the aqueous phase. This leads to two possibilities. First, the V-ATPase would be present within a single homogeneous population of right-side-out vesicles and occur in only one orientation relative to the vesicle interior, with the N-terminus of the proteolipid on the inside. FM would reach the cysteine replacing Ser⁶ on the inside of the vesicle by diffusing across the bilayer. Alternatively, the V-ATPase could be represented in two equal populations of vesicles with opposite orientations. In this situation, all of the extramembranous cysteine residues exposed to the outside would be modified, whereas no modification would occur with those residues exposed to the vesicle interior. However, the membrane vesicles produced by the method described [29] are reported to be almost exclusively right-side-out [39]. In addition, we do not observe any additional FM labelling of cryptic residues exposed when the membrane is permeabilized with n-octyl glucoside (results not shown). The observed modification is therefore more consistent with the first possibility. Incomplete modification of the proteolipid under non-denaturing conditions would occur because of steric hindrance within the V_o complex. This ambiguity does not, however, affect our molecular model with respect to the disposition of helix 1 residues relative to the central pore.

The efficient removal of V₁ subunits by nitrate/cold shock treatment completely abolished V-ATPase activity, but did not give rise to increased accessibility to FM. The closure of V_o to protons in the absence of V₁ subunits has been reported [26,40,41], and seems to be dependent on the presence of other V_o subunits because reconstitution of proteolipid alone leads to assembly of a proton-permeable channel [42,43]. The product of the *YMA6* gene has been shown to remain tightly associated with V_o after removal of other V₁ subunits [30,44], and its association with the vacuolar membrane is dependent on the presence of the 16 kDa proteolipid. This polypeptide could therefore function to occlude the central pore of the proteolipid complex, but its precise role remains to be elucidated. It seems unlikely that a pore at the centre of V_o could contain entirely the proton translocation pathway. The DCCD-reactive Glu¹⁴⁰ residue, which is found on the putative helix 4 of the proteolipid and is essential for proton

movement [18,19], would not (according to our model) be adjacent to the central pore. Furthermore, the accessibility of this residue to a lipophilic reagent such as DCCD could imply proximity to the lipid phase. In the case of F-ATPases, nine protons may be translocated during a cycle of catalysis involving three pairs of $F_1 \alpha\beta$ subunits and three ATP molecules [45]. This implies the involvement of nine subunit c proteolipids, consistent with the observed stoichiometry in F_o [46]. If the F- and V-ATPases share a related mechanism [47], this rationale can be applied to the V-ATPase for a proton/ATP stoichiometry of 2 [48,49]. Six protons would be translocated in each cycle, implying the involvement of six 16 kDa proteolipids and six distinct proton-translocating pathways rather than a single channel. The central pore of the V_o complex may instead act as conduit for protons expelled from individual pathways (involving Glu¹⁴⁰). This would obviate the need to conduct protons across the full span of the bilayer.

It is interesting to note that although cysteine replacement of the residues Gly¹⁰¹ of helix 3 and Leu¹⁴⁷ of helix 4 resulted in loss of the ability to grow at pH 7.5, the ATP-hydrolysing properties of the recombinant enzymes were not significantly different from those found in normal functional strains (Table 1). In contrast, mutation of the neighbouring residue Ile¹⁴⁸ resulted in a V-ATPase with the elevated K_m typical of other dysfunctional mutations. The residue Leu¹⁴⁷ would be on the same helical face as the functionally critical Glu¹⁴⁰, leading to the possibility that the face of helix 4 containing both Glu¹⁴⁰ and Leu¹⁴⁷ has a specific role in proton conductance. Furthermore, it is possible that the mutagenesis approach could discriminate between changes specifically affecting the proton conductance pathway and those that have a more general effect, for example on the integrity of helix folding and packing, or on subunit interactions.

A possible functional mechanism may involve presentation of a V_1 subunit to each proteolipid in turn via a rotation in part of the structural domain connecting V_1 and V_o , brought about by the headgroup binding charge mechanism [50]. The transient interaction would then facilitate proton translocation by each proteolipid during one complete cycle. A similar mechanism, involving rotation of the γ and ϵ subunits in the F_1 domain of the F_1F_o -ATPase, has been proposed by Wilkens and Capaldi [51]. This would require that the V_1 nucleotide-binding subunits are fixed relative to the proteolipid subunits of V_o .

The ability to measure directly cysteine modification by using mass shift during electrophoresis is potentially very powerful and could see widespread application. By employing a range of reagents with various chemical characteristics it should be possible to construct a map of a membrane protein based on cysteine accessibility. When this approach is coupled to analysis of inter- and intra-molecular cross-linking between cysteines, there is potential for generating a relatively detailed three-dimensional model of the protein. We shall extend this approach and further refine the model for the 16 kDa proteolipid.

We thank Nathan Nelson for provision of the *LEU2::vma3* yeast strain, and L. Guarente and R. Serrano for making plasmids available. This work was supported by the Wellcome Trust in the form of a Prize Fellowship (P.C.J.) and by the United Kingdom Science and Engineering Research Council (J.B.C.F. and M.E.F.).

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