Helical Interactions and Membrane Disposition of the 16-kDa Proteolipid Subunit of the Vacular H^+-ATPase Analyzed by Cysteine Replacement Mutagenesis*

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Theoretical mechanisms of proton translocation by the vacular H^+-ATPase require that a transmembrane acidic residue of the multicycopy 16-kDa proteolipid subunit be exposed at the exterior surface of the membrane sector of the enzyme, contacting the lipid phase. However, structural support for this theoretical mechanism is lacking. To address this, we have used cysteine mutagenesis to produce a molecular model of the 16-kDa proteolipid complex. Transmembrane helical contacts were determined using oxidative cysteine cross-linking, and accessibility of cysteines to the lipid phase was determined by their reactivity to the lipid-soluble probe N-(1-pyrenyl)maleimide. A single model for organization of the four helices of each monomeric proteolipid was the best fit to the experimental data, with helix 1 lining a central pore and helix 2 and helix 3 immediately external to it and forming the principal intermolecular contacts. Helix 4, containing the crucial acidic residue, is peripheral to the complex. The model is consistent not only with theoretical proton transport mechanisms, but has structural similarity to the dodecameric ring complex formed by the related 8-kDa proteolipid of the F_1 F_0-ATPase. This suggests some commonality between the proton translocating mechanisms of the vacular and F_1 F_0-ATPases.

In recent years, a number of experimental strategies that exploit the reactivity of cysteine sulfhydryl groups have been successfully applied to the analysis of membrane protein structure. In particular, site-specific labeling of mutagenically introduced cysteines with selective probes has been used to study the environment of individual residues (1, 2), and cross-linking between cysteines has proved valuable in mapping helical contacts (3–5). In this study, we have used these approaches to analyze the membrane disposition and organization of the 16-kDa proteolipid, a highly conserved integral membrane subunit of the vacular H^+-ATPase (V-ATPase) (6, 7). These en...
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linking approach and the map of lipid-accessible positions obtained by site-specific labeling combine to produce a detailed structural model of the proteolipid complex.

EXPERIMENTAL PROCEDURES

Cysteine Replacement Mutagenesis—Individual cysteine replacements were introduced into the cDNA for the Nephrops 16-kDa proteolipid by the PCR-based method of Landt et al. (24), using Pfu polymerase (Stratagene). Initially, the wild type Cys was mutated to serine (20), and the resulting cDNA subcloned between HindIII (5′) and XbaI (3′) sites of pSK-Bluescript (Stratagene) to provide a template for subsequent mutations. Cysteine replacement cDNAs were subcloned between HindIII (5′) and XbaI (3′) sites of pYES2 (Invitrogen), and the mutations confirmed by automated sequencing from overlapping internal primers using the ABI Prism system.

Double cysteine replacements on helix 1 and helix 2 of the proteolipid were constructed by excising a 350-base pair Eco52I-XbaI fragment from pYES2 plasmids bearing cDNAs for helix 1 single cysteine mutants and replacing it with the corresponding fragment from helix 2 cysteine mutants. The Eco52I site is located in the region of the proteolipid cDNA corresponding to the cytoplasmic loop between helix 1 and helix 2. Double cysteine replacements on helix 2 and helix 4 were constructed by introducing a 380-base pair PvuII-PvuII fragment from pYES2 plasmids bearing cysteine mutants, replacing it with the same fragment extracted from plasmids for helix 2 single cysteine mutants, and checking the orientation of the insert in the reconstructed cDNA. PvuII sites are located at the extreme 5′ end of the pYES2 multiple cloning site, and at a position in the proteolipid cDNA corresponding to the mid-helix 3 region. Helix 3-helix 4 and helix 2-helix 3 double cysteine mutants were constructed by superimposing the sequence mutation onto single cysteine mutant template cDNAs, using the PCR method described above. In the case of the helix 1 double cysteine replacement at positions 25 and 27, both mutations were incorporated into a single, naturally occurring cysteine in the proteolipid.

Expression of 16-kDa Proteolipids—For constitutive expression in S. cerevisiae, proteolipid cDNAs were subcloned into the 3 μg·plate−1 vector pPMA1 (23) to provide cDNAs for cysteine mutants, replacing it with the corresponding fragment from helix 2 single cysteine mutants, and checking the orientation of the insert in the reconstructed cDNA. PvuII sites are located at the extreme 5′ end of the pYES2 multiple cloning site, and at a position in the proteolipid cDNA corresponding to the mid-helix 3 region. Helix 3-helix 4 and helix 2-helix 3 double cysteine mutants were constructed by superimposing the sequence mutation onto single cysteine mutant template cDNAs, using the PCR method described above. In the case of the helix 1 double cysteine replacement at positions 25 and 27, both mutations were incorporated into a single, naturally occurring cysteine in the proteolipid.

RESULTS

Complementation of the vma3 Phenotype—Growth of S. cerevisiae at pH 7.5 requires a functional V-ATPase (25), and the ability of cells to grow at this pH after transformation with cDNAs for mutant proteolipids provides a useful diagnostic test of function. Using this complementation assay, mutation of the single, naturally occurring cysteine in the Nephrops proteolipid (Cys) to serine was found not to affect V-ATPase function, and transformants were able to assemble an active V-ATPase and grow at non-acidic pH (20). A number of single cysteine replacements subsequently introduced into putative helix 2 of the proteolipid abolished the ability of the cDNA to complement the vma3 mutation (Table 1). Four cysteine replacements on helix 2 were found not to affect function. Assuming α-helical periodicity, the positions of two of these residues (positions 58 and 61) would approximate to the same face of helix 2. On helix 3, a number of glycine residues which occur with helical periodicity were all sensitive to mutation. On the same segment of the proteolipid, three mutationally insensitive positions (103, 107, and 111) would also be spaced approximately one α-helical turn apart. Replacement of two hydrophobic Leu and one Ala residue in helix 4 also gave rise to functional proteolipids, but all other changes on this segment were detrimental to function. The effects of cysteine mutations in helix 1 on V-ATPase function have previously been described (20). In general, cysteine replacement of Ser, Ala, or Val tended to be less compromising of V-ATPase function, whereas Tyr, Ile, and especially Gly residues tended to be more mutationally sensitive.

In a number of cases, addition of a second cysteine appeared to have a suppressing effect on the inhibitory effect of the initial mutation (Table 1). Combinations of cysteines in helix 1 and helix 2 at positions Ala , Leu , and, to a lesser extent, Gly, were well tolerated.

Fluorimetry—Fluorimetric measurement of PM modification was made with 200–300 ng of solvent-extracted proteolipid in a cuvette volume of 2 ml (50 mM HEPES-NaOH, pH 7.5, 0.2% SDS). Fluorescence emission (at 20 °C) was measured over the range 355–455 nm, with excitation at 342 nm, using a Perkin-Elmer LS50 scanning fluorometer. Excitation and emission monochromator slit widths were set at 7.5 nm.
Complementation of the vma3 disruption in Saccharomyces by cysteine replacement mutants of the Nephrops 16-kDa proteolipid

Saccharomyces cells (vma3::LEU2) were transformed with plasmids harboring Nephrops proteolipid cDNAs with double cysteine replacements. Transformants were screened for vma3 complementation by testing for growth on media buffered to pH 7.5, and growth rates were scored relative to the rate for the strain expressing the wild type Nephrops proteolipid: ††, rate equal to the control strain; †, up to approximately 50% of control; † † † †, up to approximately 20% of control; † † † † † †, no detectable growth. Filled cells indicate apparent suppressor pairs, and unfilled cells indicate values not determined. The positions of the initial single cysteine replacement and corresponding growth characteristics of each mutant are listed in the table.

### Table I

| Helix 2: | I55 +† | I56 +† | P57 +† | V58 +† | V59 +† | M60 +† | A61 +† | G62 +† | I63 +† | I66 +† | Y67 +† |
|---------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Helix 1: |        |        |        |        |        |        |        |        |        |        |        |
| F24 +†  | —      | —      | —      | —      | —      | —      | —      | —      | —      | —      | —      |
| S25 +†  | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
| A26 ++  | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
| L27 +†  | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
| G28 —   | —      | —      | —      | —      | —      | —      | —      | —      | —      | —      | —      |
| A29 +†  | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
| A30 + +†| +      | +      | +      | +      | +      | +      | +      | +      | +      | +      | +      |
| Y31 +†  | —      | —      | —      | —      | —      | —      | —      | —      | —      | —      | —      |

* Described in Ref. 20.

**Helix 1-Helix 1 Disulfide Cross-linking—** Work from our laboratory has previously shown that helix 1 of the 16-kDa proteolipid is in contact with the same helix of an adjacent proteolipid monomer, with Ala26 of helix 1 oriented toward a central, water-accessible pore (20). A testable prediction of these conclusions is that cysteines placed diametrically opposite each other within a single turn of helix 1 should result in close proximity between sulfhydryl side chains of residues on neighboring monomers. Helix 1-helix 1 contacts can then be verified by oxidative disulfide cross-linking. Accordingly, cysteines were introduced in place of Ser25 and Leu27 in helix 1 of the proteolipid, and their ability to form cross-links in *vivo* in the vacuolar membrane analyzed (Fig. 1). This double cysteine mutant proteolipid was fully functional, and isolated vacuolar membranes showed high rates of ATPase activity (6.8 μmol of phosphate/mg of protein/h). Treatment with Cu(II) phenanthroline resulted in formation of a cross-linked proteolipid dimer of apparent mass 29 kDa, with subsequent reduction by DTT resulting in reversion of this species to monomer (Fig. 1). Approximately 50% of the expressed Nephrops proteolipid was incorporated into dimer. This proportion could not be increased either by extended oxidation time, higher oxidant concentration, or incubation with bis-maleimide homobifunctional cross-linkers (data not shown). Oxidizing treatment also reversibly inhibited 70–80% of ATP hydrolyzing activity, presumably as a consequence of disulfide formation between native cysteines within the active site of V1 (31).

Cysteine replacements of residues Ser6 and Ser6 in the extramembranous N-terminal and helix 1-helix 2 loop regions of the proteolipid, respectively, also form disulfide cross-links (20). In this study, we combined these mutations into an active double mutant in an effort to produce higher order cross-linked structures that might reflect proteolipid stoichiometry in V0. However, even with both cysteines present on each polypeptide, only a dimeric species could be generated (Fig. 1). Unlike in the case of the Ser25/Ala27 cysteine replacement combination, oxidation resulted in a complete shift to the dimeric species.

**Site-specific Labeling of Cysteine Replacement Mutants—** Because of its ability to partition into the hydrophobic phase of the membrane, and to form a fluorescent species only after reaction with thiols, N-(1-pyrene)maleimide is particularly useful as a probe for cysteines at lipid/protein interfaces (21, 23). In this study, vacuolar membranes were isolated from *Saccharomyces* cells constitutively expressing single cysteine mutant proteolipids and incubated with PM in order to assess lipid accessibility of the individual replacement sites. Expression from the pPMA1 plasmids gave equivalent levels of all mutant proteolipids, as assessed by immunoblotting of vacuolar membranes (data not shown). PM-modified proteolipids were extracted into chloroform/methanol and the extent of modifica-
tion determined from the fluorescence yield of the pyrenyl adduct (Fig. 2). Fluorescence spectra for PM-modified proteolipids in SDS solution showed an emission maximum at 382 nm, with excitation at 342 nm. For accurate normalization of fluorescence yield, concentration of the extracted proteolipids was determined using the fluorescent CBQCA method, which we found to be sensitive in the 20–500 ng range. Typically, 0.5–1.0 μg of proteolipid was recovered from an initial 0.2 mg of vacuolar membrane protein. There was a variable degree of PM labeling of all helix 4 cysteine replacements, with significant labeling being consistently found only in the case of the cysteine replacement of Leu142 (Fig. 2A). Although cysteines introduced into helix 3 appeared overall less susceptible to modification (Fig. 2B), a cysteine replacement of Ile111 did show significant levels of labeling. None of the individual replacements on helix 2 showed significantly elevated labeling (data not shown). Quantitatively, the Leu142 → Cys mutant proteolipid gave 113 fluorescence units/μg of protein, equivalent to approximately 19 units/pmol of proteolipid, assuming a mass of 16,420 Da for the Nephrops proteolipid (17). Standard curves of fluorescence yield from DTT-reduced PM gave a yield of 2.1 units/pmol of PM (data not shown), suggesting approximately 90% modification of the cysteine replacing Leu142. Typical examples of chloroform/methanol-extracted 16-kDa proteolipids are shown in Fig 3A; only small, and variable, amounts of contaminating polypeptides were detected. The presence of dye-maleimide adducts has been reported to cause a shift in proteolipid migration on SDS-PAGE (20), but no such effect was observed with PM in this study. Although PM modification always resulted in a 70–80% loss of ATP hydrolyzing activity, we were not able to discriminate between inhibitory effects of proteolipid modification and effects due to modification of other V-ATPase subunits.

Introduction of single cysteine mutants did not appear to significantly compromise assembly of the enzyme, as judged by the ability of the hybrid V0 sector to facilitate binding of the Vma1p and Vma2p subunits to the vacuolar membrane (Fig. 3B). No apparent differences in Vma1p/Vma2p association with the membrane could be observed when comparing membranes containing functional or non-functional proteolipids (Fig. 3B). It is reasonable to surmise therefore that folding and assembly of any of the mutant proteolipids does not differ significantly from the native fold, and that the labeling pattern described in Fig. 2 authentically represents the disposition of individual sites in the native proteolipid. It follows that the inhibitory effects of some of these single-site changes must be exerted through relatively subtle changes in V0 organization.

**Fig. 1.** Helix 1-helix 1 intermolecular disulfide cross-linking. The oxidative catalyst Cu(II) orthophenanthroline was used to induce cross-linking in vacuolar membranes containing Nephrops proteolipids bearing double cysteine replacements at positions Ser6 and Ser44 (left panel), and at Ser25 and Leu27 (right panel). Vacuolar membrane proteins were separated by SDS-PAGE under non-reducing conditions and analyzed by immunoblotting with polyclonal antiserum to the Nephrops proteolipid. Incubation of the Ser6 and Ser44 double mutant (Cys6/44) with oxidant resulted in complete dimer formation, whereas the corresponding single cysteine mutants (Cys6, Cys44) showed only partial shift to dimer (see Ref. 20). The Ser6/Leu27 double cysteine mutant (Cys25/27) also showed incomplete dimer formation, although the corresponding single cysteine mutants (Cys25, Cys27) showed no dimeric species whatsoever. In all cases, subsequent incubation with DTT resulted in shift of dimeric species to monomer. 16-kDa indicates native proteolipid isolated from Nephrops.

**Fig. 2.** Site-specific labeling of 16-kDa proteolipid cysteine replacement mutants with N-(1-pyrene)maleimide. Vacuolar membranes from Saccharomyces cells expressing (A) helix 4 and (B) helix 3 single cysteine mutant proteolipids were modified with PM as described under "Experimental Procedures." Proteolipids were extracted with chloroform/methanol, and the extent of modification determined from pyrene fluorescence yield at 376 nm with excitation at 342 nm. Fluorescence yield was normalized for protein concentration, and corrected for background fluorescence quantified from the C54S cysteine-less mutant proteolipid (7 ± 2 fluorescence units/μg of protein). Wild-type proteolipid showed similar labeling to the negative control (11 ± 4 units/μg of protein). Values are the means of four separate experiments (± S.D.). Analysis of variance and f tests indicates that only labeling of cysteine replacements at positions Leu142 (p < 0.01) and Ile111 (p < 0.05) was significantly different from the mean labeling and from the labeling of any other individual mutant.

**Fig. 3.** Interaction—Helix 1-Helix 2 and Helix 3-Helix 4 Interactions—A survey of helix 1-helix 2 double cysteine mutants (listed in Table I) revealed four combinations giving rise...
to disulfide cross-links under oxidizing conditions (Fig. 4). Two of these cysteine combinations, at positions Ser25/Ile56 and Leu27/Met60, are also apparent suppressor pairs (see Table I). The second pairings, at positions Ala30/Met60 and Ser25/Met60, are non-functional mutants but were stably expressed and assembled at the vacuolar membrane. As in the case of helix 1-helix 3 cysteine combinations, only dimeric cross-linked species were observed, which reverted to monomer in the presence of reductant. The formation of this 27-kDa cross-linked species could be prevented by preincubation with N-ethylmaleimide.

A broad spectrum of helix 3-helix 4 cysteine combinations (Table I) were also analyzed, but none yielded dimeric cross-linked species. However, oxidative treatment of vacuolar membranes containing a proteolipid with a cysteine replacement at positions Ser103/Ile56 resulted in formation of a proteolipid species migrating on SDS-PAGE with an apparent mass of 9-kDa (Fig. 5). The appearance of this species was time-dependent, reaching steady-state after approximately 30 min of incubation, and was reversed by treatment with reductant. The generation of this species was also inhibited by pretreatment with N-ethylmaleimide (Fig. 5), confirming that its formation requires free sulfhydryls. Analysis by two-dimensional SDS-PAGE (first dimension non-reducing, second dimension reducing) demonstrated that this apparently low mass species undergoes a mobility shift upon reduction, subsequently comigrating with the bulk proteolipid monomer (Fig. 5, lower panel). The mutational combination Ser103 → Cys/Ile56 → Cys also appears to be a suppressor pair, implying proximity of the side chains of these residues. We conclude therefore that this species most likely arises as a consequence of low yield intramolecular disulfide cross-link formation.

**Disulfide Cross-linking: Helix 2-Helix 3 Interactions.**—Intermolecular contact between helix 2 and helix 3 of the proteolipid is predicted by an interim model constructed on the basis of the cross-linking and labeling data described above. Explicitly to test this prediction, a number of double cysteine combinations were introduced into these helices (see Table I) and the expressed proteolipids analyzed for disulfide cross-link formation (Fig. 6). Single cysteine replacements of glycine residues in helix 3 (Gly103, Gly104, and Gly108), and the functional Leu105 → Cys mutation, were combined with cysteine replacements of Ile55, Val58, Gly62, Ile66, and Tyr67 of helix 2. The glycine residues were all found to be sensitive to mutation (Table I), consistent with a role in mediating crucial protein-protein contacts. Cysteine replacements of Gly62, Ile66, and, to a lesser extent, Val58 all formed reductant-sensitive cross-links with a cysteine replacing the helix 3 residue Leu105 (Fig. 6A). No cross-linked species were observed with the Leu105 → Cys mutant in combination with cysteine replacements of Ile55 or Tyr67. Again, the only cross-linked species that could be generated was a dimer, migrating with an apparent molecular mass of 27 kDa.

Cysteine substitutions within the glycine-rich face of helix 3 produced sulfhydryls that readily formed cross-links with cysteines on helix 2 (Fig. 6B). In particular, combinations of the mutant Gly104 → Cys with cysteine substitutions of residues Tyr67, Ile66, or (to a lesser extent) Gly62 gave a high yield of cross-linked dimer of apparent mass 26 kDa that was extremely resistant to reduction (Fig. 6B). The same helix 3 residue did not form disulfides with cysteine replacements of Ile55 or Val56. Combinations of helix 2 cysteines with a cysteine replacement at position Gly104 resulted in significant cross-link formation only with the pairing Gly104 → Cys/Gly62 → Cys, giving a dimer with an apparent mass of only 24 kDa. Incubation of cross-linked proteolipids with reductant under nitrogen for up to 3 h at 60 °C resulted only in incomplete reversion to monomer, a phenomenon that has previously been described in cysteine mutagenesis studies (32, 33). In this study, resistance to reduction appears specifically to be a characteristic of double cysteine mutants incorporating Gly to Cys changes on helix 3 of the proteolipid. Uniquely among the wide range of cysteine replacements tested in this study, the mutation Gly108 → Cys appeared to destabilize expression and assembly, as proteolipids combining this mutation with helix 2 cysteine substitutions were indetectable by immunoblotting of vacuolar membrane proteins (data not shown). The reason for this unique property remains unclear. Disulfide cross-linking between helices 2 and 3 of the 16-kDa proteolipid is summarized in Fig. 7.

None of the helix 2-helix 3 double cysteine mutant proteolipids gave rise to functional ATPases (Table I). In order to validate the cross-linking data in Fig. 6, we sought to establish the authenticity of assembly of the corresponding hybrid enzyme complexes. Because of its large mass, the solubilized vacuolar ATPase shows characteristic migration during centrifugation
on glycerol gradients. The solubilized enzyme incorporating the 66/104 double cysteine proteolipid migrated through 20–50% glycerol gradients to become most concentrated in a fraction containing approximately 40% glycerol (Fig. 8A, fraction 6). Distribution of the V-ATPase in the gradient was verified by immunoblotting with antibodies recognizing both V1 (Vma 1p and Vma2p) and V0 (Vma6p) subunits. Solubilized complexes containing the cross-linkable double cysteine proteolipids 62/104, 67/104, 58/105, 62/105, and 66/105 showed distribution on glycerol gradients identical to that shown in Fig. 8A (data not shown). The corresponding peak fraction from each gradient was additionally screened for the presence of the V1 subunit Vma5p and V0 subunit Vph1p (Fig. 8B); all of the complexes incorporating the cross-linkable proteolipids contained levels of each subunit comparable to control (C54S mutant) vacuolar membranes. V1 complexes will only assemble at the vacuolar membrane in the presence of a fully assembled V0 complex, containing both Vph1p and the 16-kDa proteolipid in a stable association (34, 35). The presence of the V1 subunits in the peak glycerol gradient fraction must indicate stable formation of V0 in the case of each mutant proteolipid. Since each double cysteine proteolipid is able to make the associations with Vma6p
DISCUSSION

Stoichiometry studies on the V-ATPase (36) and electron microscopy of proteolipid-enriched membranes from Nephrops (17) indicate that the proteolipid most likely assembles as a hexameric complex in V₀, and that this complex has six-fold symmetry. It is reasonable to suppose therefore that each proteolipid four-helix bundle occupies a 60° sector of the V₀ complex. Disulfide cross-linking data from this and previous studies (20) indicate that helix 1 will line a pore at the center of this complex. The helices will be oriented with respect to each other such that Ser⁵⁰ of one monomer and Leu⁷⁰ of the neighboring polypeptide are sufficiently close that they could cross-link when changed to cysteine (Fig. 9). Using the experimentally determined position of helix 1 as a foundation, the organization of the remaining transmembrane helices can be approximated on the basis of the functional impact of cysteine mutations. This approximation can then be refined by considering the relative positioning of cross-linking and suppressor pairs to produce a structural description of the 16-kDa proteolipid which converges both functional and protein chemical data into a consensus model.

Although cysteine has a relatively small and amphipathic side chain, its insertion into the proteolipid was, in the majority of cases, disruptive to function. Helical packing in the proteolipid must therefore be acutely sensitive even to small perturbations, and it follows therefore that the proteolipid must adopt a very precise architecture in order to function. Generalized rules for the internal location of the most conserved residues in proteins (37) cannot be applied to the 16-kDa proteolipid because of the high degree of conservation throughout its sequence. However, sites within the proteolipid that were sensitive to cysteine mutation tended to be clustered onto specific helical faces, suggesting that these faces may be play a more predominant role in the formation of helix-helix contacts. In particular, opposing faces on helix 2 broadly centered around the positions of Met⁶⁰ and Gly⁶² (Fig. 9A), and the internal face of helix 1 (20) were all mutationally sensitive. The glycine-rich face of helix 3 (positions 101, 104, and 108), which was also acutely sensitive to mutation, is diametrically opposite another sensitive helix 3 region, centered around the position of Ala¹⁰⁶. It is reasonable to suppose that these faces, along with the sensitive regions of helix 2, will be involved in helical contacts. The likely orientation of helix 4 with respect to helix 3 is suggested by the suppressor/cross-linking cysteine pair at positions 148 and 103. Orientation of helix 4 such that mutationally insensitive sites are excluded from helical contacts, while maintaining proximity of Ile¹⁴⁸ to Ser¹⁰³, places sensitive sites adjacent to the core of the four-helical bundle (Fig. 9A).

Of course, it is not possible to make an unambiguous assignment of the positions of helices 2–4 on the basis of the functional effects alone, and several models of helix organization could accommodate the data. However, additional constraints introduced in line with the helix 1-helix 2 cross-linking (Fig. 4) and suppressor mutation data (Table I) leave only one model as the best fit to the data (Fig. 9A). In addition to making an intramolecular contact with helix 1, the cross-linking data dictate that helix 2 must also be reasonably close to helix 1 of a neighboring monomer. Specifically, a cysteine at position 25 on helix 1 must be able to make contact with cysteines at positions
56 and 60 on helix 2 of the adjacent polypeptide (Fig. 9B). Similarly, a cysteine at position 27 must be able to contact position 60 on helix 2. These constraints can be accommodated in the experimental model of the proteolipid, although with relatively large distances between the side chains of these residues. These distances account for the low yield of dimer in helix 1-helix 2 cross-linking experiments. Normal vibrational movement of the polypeptide chain would allow for a low frequency of contacts between side chains at these positions.

Suppressor mutations also imply proximity between the residues forming each suppressor pair. Changes in mass of the side chain of the suppressing residue apparently compensate for local disturbances in side chain packing caused by the initial, inhibitory mutation (38). Although these compensatory effects do not necessarily have to be short range, the model presented here, although we would suggest that the mutation at position 137 in the 104/137 pairing will exert its suppressing effect by compensating indirectly for disturbance of a helix 3-helix 2 intermolecular contact.

The exclusive ability of cysteines introduced at positions 111 and 142 to become modified by PM identifies these two residues as being accessible from the hydrophobic region of the membrane. The site-specific labeling approach therefore provides independent evidence that the faces of helix 3 and helix 4 that, respectively, carry these residues must interface with the lipid phase. Such an orientation for these helices would be consistent with the described experimental model. In a separate study, we have applied fluorescent and spin-labeled analogues of DCCD as probes for glutamate residues accessible from the lipid phase. Using this approach, we have demonstrated lipid accessibility not only of the wild type Glu140, but also of glutamate residues introduced mutagenically at positions 64 and 107 on helices 2 and 3, respectively. Proximity of all three residues to the lipid phase is predicted from the model in Fig. 9A.

The model of helix organization constructed on the basis of functional, cross-linking and site-specific labeling data predicts

\[2\text{ M. A. Harrison, B. Powell, T. Pali, D. Marsh, M. E. Finbow, and J. B. C. Findlay, submitted for publication.}\]
that the glycine-rich face of helix 3 and the Gly\textsuperscript{62} region of helix 2 will form the predominant intermolecular contacts within the proteolipid oligomer. Disulfide cross-linking experiments confirm that this is indeed the case (Fig. 6). It might be expected that cross-linking throughout the proteolipid hexamer should produce higher order species, reflecting the stoichiometry of the proteolipid in V\textsubscript{0}. However, it was not possible to produce species greater than dimer, irrespective of the position of the cysteine residues, and as a consequence the proteolipid can only be confidently modeled as a dimer. This phenomenon suggest that the proteolipid is organized not as a regular hexamer, but rather with a superimposed three-fold symmetry that might introduce greater spacing between dimeric units. Alternatively, cross-linking within the oligomer of Nephrops 16-kDa proteolipids may be interrupted by the presence of endogenous yeast proteolipids Vma11p and Vma16p. These polypeptides are certain present in the vacuolar membrane (40) and are able to interact with the Nephrops proteolipid both

in vivo and in vitro.\textsuperscript{3} We are currently investigating the possibility that these polypeptides are present in the hybrid yeast V\textsubscript{0} sector.

The organization described in Fig. 9 shows some notable parallels with a recently described model of the subunit c oligomer in the F\textsubscript{1}F\textsubscript{0}-ATPase (41, 42). This model shows subunit c forming a dodecameric ring, with helix 1 oriented toward the center of the complex. Helix 2, which contains the DCCD-reactive Asp\textsuperscript{61}, is at the periphery of the complex, forming the protein/lipid interface. Asp\textsuperscript{61} itself is in close proximity to Ala\textsuperscript{24} on helix 1 of the adjacent polypeptide, with both residues oriented toward the center of the pseudo-four helical bundle formed by the association of two subunit c proteolipids. This proximity explains the ability of an aspartate substitution at Ala\textsuperscript{24} to complement mutation of Asp\textsuperscript{61} in the E. coli F-ATPase (43). Alignment of proteolipid sequences indicates that, in accordance with the tandem repeat relationship between the two proteins, Gly\textsuperscript{26} and Glu\textsuperscript{140} on helix 2 and helix 4 of the 16-kDa proteolipid correspond to Asp\textsuperscript{61} of subunit c (7, 10). These two residues therefore represent equivalent positions in the first and second elements of the tandem repeat that constitutes the 16-kDa proteolipid. Similarly, Ala\textsuperscript{206} of helix 1 and Ala\textsuperscript{107} of helix 3 of the 16-kDa proteolipid align with the position of Ala\textsuperscript{64} of subunit c. In our experimentally derived model of the 16-kDa proteolipid, the relative positions of the Gly\textsuperscript{26}/Ala\textsuperscript{206} and, in particular, Glu\textsuperscript{140}/Ala\textsuperscript{107} residue pairs within each tandem repeat element are broadly similar to the positions of the corresponding Asp\textsuperscript{61}/Ala\textsuperscript{24} pair within each subunit c monomer (41, 42). The helical organization within the 16-kDa proteolipid complex therefore mirrors to some extent the organization within the subunit c dodecamer, with helices 2 and 4 external to the proteolipid complex. However, the complexes differ in that the helix 3/helix 4 element of the 16-kDa proteolipid has been radically displaced from center, a structural adjustment that presumably occurred concomitantly with the loss of a second acidic residue at the Gly\textsuperscript{62} position. Our best-fit model does place Glu\textsuperscript{140} somewhat further away from the interface with helix 3 than is the case for the corresponding Asp\textsuperscript{61} of subunit c, which appears to be relatively close to the intramolecular helix 1-helix 2 interface (41). However, the overt similarities between the 16-kDa and subunit c complexes should make it feasible to model a molecular replacement model of the 16-kDa proteolipid based on the NMR structure of the subunit c monomer (30). A complete description of this model will appear elsewhere.

The positions of helices in the model (Fig. 9) were fixed in accordance with the experimental constraints, such that lipid-exposed residues were external to the complex and distances between residues that form strong helix 2/helix 3 intermolecular cross-links were minimized. The helix 1-helix 1 contact that ensues from this helical packing produces an intermolecular distance of approximately 8 Å between Ala\textsuperscript{25} and Leu\textsuperscript{27} \textalpha-carbons, in reasonable agreement with an \textalpha-carbon separation between disulfide-linked cysteines of 4–7 Å (44). The relatively large distances between helix 1-helix 2 cross-linking sites suggest that the low yield disulfides formed by these residues must therefore result from conformational trapping. As a consequence, these cross-links may not be representative of the mean position of the respective residues, but should still provide a valid assay for the general position of helices within the proteolipid complex. Similarly, although suppressor effects are not necessarily short range, we estimate (from the subunit c-based molecular replacement model) that intramolecular distances between side chains of suppressor sites may be small (between 1–3 Å for 60/137 and 60/141, 9 Å for 59/141 and 103/148). The trapping of sulphydryls into cross-links across apparently large distances would imply that there is considerable vibrational movement of helices in directions both perpendicular and parallel to the plane of the membrane. We are unable to determine at this time whether these trapped conformations are active or inactive for proton translocation.

Most hypothetical models for the mechanism that couples ATP-driven rotation to proton movement in the F- and V-ATPases require that the transmembrane acidic residue must become exposed to the lipid at some stage during cycling of the pump (14, 45–47). Lipid exposure of Glu\textsuperscript{140} of 16-kDa proteolipid is clearly a feature of our structural model, with the residue sited toward the external surface of the V\textsubscript{0} sector. There are certainly significant functional differences between F- and V-ATPases, for example relating to the H\textsuperscript{-}/ATP stoichiometries in the two classes of ATPase, and the apparent unidirectionality of the vacuolar proton pump. This aside, we propose that structural parallels between proteolipid complexes in V\textsubscript{0} and F\textsubscript{0} sectors may reflect some fundamental similarities in their mechanism of action.

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