Interaction of dibutyltin-3-hydroxyflavone bromide with the 16 kDa proteolipid indicates the disposition of proton translocation sites of the vacuolar ATPase

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The organotin complex dibutyltin-3-hydroxyflavone bromide [$\text{Bu}_2\text{Sn}(\text{of})\text{Br}$] has been shown to bind to the 16 kDa proteolipid of *Nephrops norvegicus*, either in the form of the native protein or after heterologous expression in *Saccharomyces* and assembly into a hybrid vacuolar H^+ -ATPase. Titration of $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ against the 16 kDa proteolipid results in a marked fluorescence enhancement, consistent with binding to a single affinity site on the protein. Vacuolar ATPase-dependent ATP hydrolysis was also inhibited by $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$, with the inhibition constant correlating well with dissociation constants determined for binding of $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ complex to the proteolipid. The fluorescence enhancement produced by interaction of probe with proteolipid can be back-titrated by dicyclohexylcarbodiimide (DCCD), which covalently modifies Glu¹⁴⁰ on helix-4 of the

polypeptide. Expression of a mutant proteolipid in which Glu¹⁴⁰ was changed to a glycine resulted in assembly of a vacuolar ATPase which was inactive in proton pumping and which had reduced ATPase activity. Co-expression studies with this mutant and wild-type proteolipids suggest that proton pumping can only occur in a vacuolar ATPase containing exclusively wild-type proteolipid. The fluorescent enhancement or affinity of $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ for the mutant proteolipid was not significantly altered, with the organotin complex having no effect on residual ATPase activity. Interaction of the probe with mutant proteolipid was unaffected by DCCD. These data suggest an overlap in the binding sites for organotin and DCCD, and have implications for the organization and structure of proton-translocating pathways in the vacuolar H^+ -ATPase.

INTRODUCTION

The vacuolar H^+ -ATPases (V-ATPases) are a group of endomembrane pumps that are responsible for the acidification of plant and fungal vacuoles as well as intracellular compartments such as lysosomes, synaptic vesicles and the Golgi apparatus [1]. The V-ATPase is a multimeric enzyme, with a subunit composition analogous to that of the mitochondrial F_0F_1 -ATPase, containing a catalytic hydrophilic V_1 domain involved in the hydrolysis of ATP and a membrane-bound hydrophobic V_0 domain [2]. The principal component of the hydrophobic domain is the 16 kDa proteolipid (subunit *c*), with six copies being present per V_0 [3].

The 16 kDa proteolipid is a member of a group of highly conserved hydrophobic proteins. These proteins appear to have originated by a duplication event from a gene encoding the 8 kDa subunit *c* proteolipid of the F_0F_1 -ATPase [4]. Structural studies of the 8 kDa subunit *c* in organic solvent indicate that the protein is composed of a hairpin-like structure of two α -helices. The 16 kDa proteolipid has been modelled as a four-helical bundle and the polypeptide subunits as a hexamer surrounding a central pore [5,6]. Both the 8 kDa and 16 kDa proteolipids are involved in proton conductance across the membrane, and both contain a conserved carboxyl residue that is reactive with dicyclohexylcarbodiimide (DCCD) and essential for proton movement [7].

The 16 kDa proteolipid isolated from the hepatopancreas of the lobster *Nephrops norvegicus* exhibits sequence similarity with the vacuolar proteolipid. The *Nephrops* proteolipid has been

shown to be functionally interchangeable with the vacuolar proteolipid. In a yeast mutant in which the endogenous proteolipid gene (*VMA3*) has been disrupted, introduction of a cDNA encoding the *Nephrops* proteolipid results in complementation of the mutation and functional assembly of the lobster proteolipid into a hybrid V-ATPase [6,8]. The *vma3* mutant exhibits a pH-dependent conditional lethal phenotype, and complementation is manifested as restored capacity for growth at pH 7.5 [8,9].

The activity of the V-ATPase has been shown to be sensitive to a number of inhibitors, including DCCD, *N*-ethylmaleimide (NEM), bafilomycin [10–13] and the cyclic peptide destruxin B [14]. Incubation of the proteolipid with DCCD results in covalent modification of the 16 kDa subunit at Glu¹⁴⁰, which resides on helix-4 of the protein and is intimately involved in the proton-translocation mechanism of the V-ATPase. Glu¹⁴⁰ has also been shown to be the target for the virally encoded bovine papilloma-virus oncoprotein E5 [15]; interaction of E5 with the proteolipid leads to a loss of proton-pumping activity in the V-ATPase [16].

The F_0F_1 -ATPase isolated from mitochondrial membranes has been shown to be sensitive to organotin–flavone complexes, with inhibition of activity resulting from binding of the compound to the F_0 domain [17]. In particular, dibutyltin-3-hydroxyflavone bromide [$\text{Bu}_2\text{Sn}(\text{of})\text{Br}$] (Figure 1) is a fluorescent probe inhibitor of the F-type ATPase, which exhibits fluorescence enhancement on binding. Preliminary studies have also shown that $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ inhibits the activity of the vacuolar H^+ -ATPase by binding to a component of the V_0 domain [18]. Our investigations show that $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ directly titrates against the 16 kDa proteolipid component of the V_0 domain of the vacuolar ATPase, resulting

Abbreviations used: V-ATPase, vacuolar H^+ -ATPase; NEM, *N*-ethylmaleimide; $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$, dibutyltin-3-hydroxyflavone bromide; DCCD, *N,N'*-dicyclohexylcarbodiimide; NTA, nitrilotriacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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Figure 1 Structure of $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$

in both inhibition of ATPase activity and an increase in fluorescence intensity. Interaction of the $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ with the proteolipid is competitive with DCCD-modification, suggesting that the binding site for the probe is in the vicinity of Glu¹⁴⁰. These observations have implications concerning the location and mechanism of the proton-translocating pathway in V_o .

MATERIALS AND METHODS

Plasmid construction and expression

Expression of the *N. norvegicus* 16 kDa proteolipid was performed in the haploid *Saccharomyces cerevisiae* strain W303-1B (*MAT α* , *ade2*, *leu2*, *his1*, *trp1*, *ura3*) in which the *VMA3* gene for the endogenous 16 kDa proteolipid is disrupted by insertion of a *LEU2* gene (*LEU2::vma3*), kindly provided by N. Nelson [9]. Site-directed mutagenesis of the proteolipid was performed using a PCR methodology previously described [19]. For expression in yeast, cDNAs encoding forms of the *Nephrops* proteolipid were subcloned into the *Hind*III/*Xba*I sites of the galactose-inducible yeast-*E. coli* shuttle vector pYES2 (Invitrogen). For the purposes of simultaneous co-expression of two forms of proteolipid, a variant of pYES2 was constructed in which the *URA3* selectable marker of the original plasmid was replaced by the *TRP1* gene. Standard yeast transformation and manipulation techniques were carried out as described previously [8]. Yeast transformants were grown in selective yeast nitrogen base medium (pH 5.5) supplemented with 2% (v/v) glycerol/1% (w/v) galactose and the appropriate amino acids and bases. Complementation of the *vma3* mutation was monitored by testing for growth on yeast extract-peptone media buffered to pH 7.5 and supplemented with 1% (w/v) galactose or 1% (w/v) glucose [6,8].

Membrane purification and protein isolation

Yeast cells were grown to mid-log phase in the appropriate selective minimal medium supplemented with glycerol/galactose. Vacuolar membrane vesicles were prepared from spheroplasts as described previously [20]. Vesicles were stored in 10 mM Tris/HCl (pH 7.6)/0.1 mM EDTA with 10% (v/v) glycerol at -80°C . DCCD labelling of the 16 kDa proteolipid was performed as outlined previously [8]. Inhibition studies with NEM (50 μM) were performed at room temperature with membranes resuspended in 10 mM Tris/HCl/0.1 mM EDTA, pH 7.6, to a protein concentration of 0.5 mg/ml. After 40 min, NEM was quenched with 200 μM dithiothreitol, and the membranes were assayed for activity.

A V-ATPase-enriched fraction was prepared by glycerol-gradient fractionation, as previously described [8]. Briefly, vacuolar membrane vesicles (2 mg/ml) were solubilized with n-octyl glucopyranoside (25 mg/ml) for 30 min at 4°C . The solubilized fraction was recovered after centrifugation (100 000 g, 60 min, 4°C) and layered on to 10 ml 20–50% (v/v) glycerol gradients, prepared in 10 mM Tris/HCl/0.1 mM EDTA

(pH 7.9)/0.05% (w/v) n-octyl glucopyranoside/100 μM PMSF. Gradients were centrifuged at 150 000 g for 16 h at 4°C in a fixed-angle rotor (Ti 50) and then fractionated into 1 ml aliquots. For SDS/PAGE and immunoblotting, 150 μl of each fraction was sampled.

Affinity purification of n-octyl glucopyranoside-solubilized hexahistidine-tagged proteolipids on Ni^{2+} -nitriloacetic acid (NTA)-agarose was performed as in [8]. Ni^{2+} -NTA-bound proteins were eluted with increasing concentrations of imidazole hydrochloride.

The native 16 kDa proteolipid was prepared from the hepatopancreas of *Nephrops* as described previously [21]. Solubilization of the native material was achieved by treatment with lauroyl sarcosine (0.7%, w/v) at 50°C for 30 min, at a protein concentration of 1 mg/ml. The solubilized fraction was collected after centrifugation at 100 000 g for 60 min (20°C).

Proton pumping and ATP hydrolysis assay

Proton pumping was measured as ATP-dependent quenching of fluorescence yield from quinacrine (1 μM). Vacuolar membrane vesicles (20 μg of protein) were suspended in 50 mM Mes/Tris/5 mM MgSO_4 /0.01% (w/v) sodium azide, pH 6.9, and proton pumping was initiated by addition of 2.5 mM MgATP. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (0.2 μM) was used as uncoupler. Excitation and emission wavelengths were 425 nm and 495 nm respectively. Specific activity for ATP hydrolysis was measured in the same buffer system, as the rate of release of inorganic phosphate, as described previously [8].

Electrophoresis and immunoblotting

SDS/PAGE and immunoblotting was carried out on 12.5% acrylamide gels using the buffer system of Schagger and von Jagow [22]. Protein samples were prepared by precipitation with 9 vol. of acetone/ethanol (1:1, v/v) at 4°C . For immunoblotting, proteins were transferred to poly(vinylidene difluoride) membrane and probed using standard procedures with polyclonal antibodies raised against the native *Nephrops* 16 kDa proteolipid [23]. The alkaline phosphatase colour-development system was used to assay antibody binding.

Fluorescence titration of alkyltin binding

$\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ was synthesized according to [24] and stored in the dark as a 2 mM ethanolic solution. Fluorescence titrations were performed at 20°C using a Perkin-Elmer LS-50B fluorimeter with excitation and emission wavelengths of 400 and 450 nm respectively.

RESULTS

Interaction of dibutyltin flavone with the *Nephrops* 16 kDa proteolipid

The binding of the fluorescent probe, $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$, to the 16 kDa proteolipid was demonstrated using the native proteolipid purified directly from the hepatopancreas of the lobster *N. norvegicus*. As shown in Figure 2 (line A), there was a significant increase in the fluorescence emission intensity of the probe upon addition to the *Nephrops* proteolipid. The fluorescence enhancement obtained by interaction of the fluorophore with the proteolipid was found to be 40% lower when the protein was preincubated with 100 μM DCCD (Figure 2, line B).

Addition of dibutyltin flavone to the *Nephrops* proteolipid solubilized in lauroyl sarcosine resulted in fluorescence enhance-

Figure 2 Effect of DCCD on the interaction of $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ with the native 16 kDa proteolipid isolated from *N. norvegicus*

Proteolipid was labelled with DCCD according to the protocol outlined in the Materials and methods section. Proteolipid suspensions ($21 \mu\text{g}$ of protein/ml) were then incubated at pH 7.4 in 50 mM Hepes containing 200 mM sucrose in the absence (A) or presence (B) of $100 \mu\text{M}$ DCCD. Shown are the time-dependent fluorescence changes observed upon addition of $1.0 \mu\text{M}$ $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$, as indicated by the arrows. arb., arbitrary units.

Figure 3 Effect of detergent solubilization on the interaction of $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ with the native *Nephrops* 16 kDa proteolipid

Lauroyl sarcosine-solubilized proteolipid was labelled with DCCD as described in the legend to Figure 2. The fluorescence intensity was recorded upon the addition of $1.0 \mu\text{M}$ $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ (indicated by arrows) to the proteolipid solution ($8.0 \mu\text{g}$ of protein/ml) previously incubated in the absence (A) or presence (B) of $100 \mu\text{M}$ DCCD. arb., arbitrary units.

ment with an increased rate for the kinetics of binding (Figure 3, line A). The fluorescence signal for the sarcosyl-solubilized protein was also reduced by preincubation with $100 \mu\text{M}$ DCCD (Figure 3, line B).

Expression of *Nephrops* proteolipids in *Saccharomyces*

The apparent competition between DCCD and $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ implies possible involvement of the DCCD-reactive Glu^{140} residue of the 16 kDa proteolipid in the site of alkyltin interaction. This observation was further examined by monitoring $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ binding to *Nephrops* proteolipid after expression in the yeast complementation system [6,8]. Two further forms of proteolipid were constructed by site-directed mutagenesis and expressed in yeast; the first contained a single replacement of Glu^{140} by glycine ($\text{Glu}^{140} \rightarrow \text{Gly}$), whereas the second consisted of wild-type protein with a C-terminus hexahistidine affinity tag (16K-Hisx6) [8]. To facilitate simultaneous co-expression of two forms of proteolipid, the 16K-Hisx6 protein was expressed from a plasmid containing the *TRP1* marker, whereas the $\text{Glu}^{140} \rightarrow \text{Gly}$ and wild-type 16 kDa proteolipids were expressed from plasmids containing the *URA3* marker. The growth properties and V-ATPase activities *in vitro* of yeast strains transformed with cDNAs encoding mutant forms of the 16 kDa protein are given in Table 1. Replacement of Glu^{140} by a glycine residue resulted in failure to complement the *vma3* mutation, as shown by lack of growth at pH 7.5 [7]. Co-expression of the $\text{Glu}^{140} \rightarrow \text{Gly}$ with the 16K-Hisx6 protein also resulted in complete inhibition of the ability of the 16K-Hisx6 protein to complement the *vma3* mutation (Table 1).

In all strains, the vacuolar membranes retained azide-insensitive ATP-hydrolysing activity (Table 1). The ATPase activity of the $\text{Glu}^{140} \rightarrow \text{Gly}$ V-ATPase mutant was lower than that of the co-expression control 16K/16K-Hisx6 (0.23 compared with $1.25 \mu\text{mol}$ of phosphate/min per mg of protein respectively). ATPase activities of control strains (16K, 16K-Hisx6 and 16K/16K-Hisx6) showed a sensitivity to DCCD that was not observed with strains expressing the $\text{Glu}^{140} \rightarrow \text{Gly}$ proteolipid. Activity in all strains was, however, equally sensitive to low concentrations of NEM (70–78 % relative inhibition).

Growth at pH 5.5 on selective minimal media with glycerol/galactose allowed continued plasmid selection and induction of expression, as confirmed by immunoblot analysis (Figure 4A). Co-expression strains show two distinct bands, with the upper band corresponding to the hexahistidine-tagged wild-type pro-

Table 1 Functional characteristics of *S. cerevisiae* strains expressing mutant forms of the *Nephrops* 16 kDa proteolipid

The *S. cerevisiae* strain W303-1B (*LEU2::vma3*) was transformed with pYES2 plasmid containing the cDNA for the *Nephrops* 16 kDa proteolipid. Growth on yeast extract-peptone, pH 7.5, with 1 % galactose is shown relative to the growth of wild-type cells under the same conditions: + +, growth equivalent to the wild type; —, no growth.

Strain	Growth at pH 7.5	Specific activity*	Relative activity with inhibitor (%)		
			DCCD (100 μM)	$\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ (25 μM)	NEM (50 μM)
<i>Nephrops</i> 16K	+ +	0.35	29	42	23
16K-Hisx6	+ +	0.42	31	45	25
$\text{Glu}^{140} \rightarrow \text{Gly}$	—	0.23	103	98	30
16K/16K-Hisx6	+ +	1.25	28	45	24
16K-Hisx6/ $\text{Glu}^{140} \rightarrow \text{Gly}$	—	0.26	94	94	22

* ATP-hydrolysing specific activity is expressed as μmol of inorganic phosphate/min per mg of protein.

Figure 4 Immunoblot analysis of *Nephrops* proteolipid expression and assembly

(A) Co-expression of *Nephrops* proteolipid forms. Vacuolar membrane vesicles were prepared from *Saccharomyces* strains expressing forms of the *Nephrops* proteolipid, separated by SDS/PAGE (10 μ g of protein per lane) and analysed by immunoblotting. Lane 1, native proteolipid (0.5 μ g) isolated from the hepatopancreas of *Nephrops*; lane 2, wild-type *Nephrops* 16 kDa proteolipid; lane 3, wild-type *Nephrops* proteolipid with C-terminus hexahistidine tag; lane 4, Glu¹⁴⁰→Gly mutant proteolipid; lane 5, co-expression of hexahistidine-tagged and non-mutant wild-type *Nephrops* proteolipids; lane 6, co-expression of hexahistidine-tagged wild-type proteolipid with Glu¹⁴⁰→Gly mutant proteolipid. (B) Co-expressed forms of *Nephrops* proteolipid are both assembled into the V-ATPase. Vacuolar membranes from *Saccharomyces* cells expressing both the wild-type and hexahistidine-tagged wild-type *Nephrops* proteolipids were solubilized and fractionated by glycerol-gradient centrifugation, as described in the Materials and methods section. Fractions were analysed by SDS/PAGE and immunoblotting (shown). Fractions 1 and 10 correspond to the lowest and highest glycerol concentrations respectively. Identical results were obtained for co-expression of the Glu¹⁴⁰→Gly mutant and hexahistidine-tagged wild-type proteolipids (not shown). (C) Association between co-expressed forms of the *Nephrops* proteolipid. Pooled glycerol-gradient fractions [fractions 6–9 in (B)] were applied to a Ni²⁺-NTA-agarose column. The column was washed extensively (20 vol.) with 10 mM Tris/HCl (pH 7.9)/0.1 mM EDTA/10% (w/v) glycerol, with 0.05% (w/v) n-octyl glucopyranoside to remove unbound proteins. The column was then washed as above, but supplemented with 20 mM or 100 mM imidazole/HCl. Eluted proteins were sampled for SDS/PAGE and immunoblotting (shown). Lane 1, glycerol-gradient fraction pool; lane 2, material remaining unbound to Ni²⁺-NTA-agarose; lane 3, material eluting during initial wash; lanes 4 and 5, material eluting with 20 mM and 100 mM imidazole respectively.

tein, which migrates on SDS/PAGE with a larger molecular mass [8]. The co-expression of 16K-Hisx6 with wild-type 16K or the Glu¹⁴⁰→Gly mutant proteolipid resulted in equivalent levels of the proteins in the vacuolar membrane (Figure 4A, lanes 4 and 5).

Both forms of co-expressed proteolipid migrate together on glycerol gradients (Figure 4B) and correlate with azide-insensitive ATP-hydrolysing activity (results not shown), suggesting that both forms of co-expressed *Nephrops* proteolipid are incorporated into a population of hybrid V-ATPases. To confirm that both forms of co-expressed proteolipid can associate together within a V_o complex, the hexahistidine-tagged proteolipid was isolated by Ni²⁺ affinity chromatography (Figure 4C). Proteolipid lacking the hexahistidine tag was retained on the affinity resin even after extensive washing, indicating that it was strongly associated with the hexahistidine-tagged protein. The ratio of

Figure 5 Effect of Bu₂Sn(of)Br on the ATPase activity of vacuolar membranes

Vacuolar membranes vesicles (1.0 mg of protein/ml) isolated from yeast strains expressing the Glu¹⁴⁰→Gly mutant proteolipid (Δ) or expressing wild-type 16K/16K-Hisx6 proteolipids (□) were preincubated with the required concentration of Bu₂Sn(of)Br for 2 min. Samples were then removed for the ATPase assay as described in the Materials and methods section. Activities in the absence of Bu₂Sn(of)Br were 0.23 μ mol of phosphate/min per mg of protein for Glu¹⁴⁰→Gly and 1.25 μ mol of phosphate/min per mg of protein for 16K/16K-Hisx6, which are normalized to a value of 1.0 for data comparison. rel., relative units.

Figure 6 Proton uptake into vacuolar membrane vesicles

Vacuolar membranes containing the Glu¹⁴⁰→Gly mutant proteolipid (A), and co-expression mutants Glu¹⁴⁰→Gly/16K-Hisx6 (B) and 16K/16K-Hisx6 (C) were incubated in assay medium for 2 min. Proton uptake was initiated by addition of 2.5 mM MgATP (1), and reversed upon addition of 20 μ M FCCP (2). arb., arbitrary units.

affinity-tagged protein to untagged protein eluting from the column also showed some dependence on the concentration of the eluting imidazole (compare lanes 4 and 5, Figure 4C).

The concentration-dependent effect of Bu₂Sn(of)Br on ATP-hydrolysing activity is shown in Figure 5. Bu₂Sn(of)Br was found to have no significant effect on the activity of the Glu¹⁴⁰→Gly mutant at concentrations up to 25 μ M. The co-expression control (16K/16K-Hisx6) showed a maximal inhibition of 58% with a K_i^{app} of 0.76 ± 0.12 μ M. Similar inhibition effects were observed for strains expressing either the wild-type 16K or 16K-Hisx6 proteolipids (Table 1).

The ability of mutants to pump protons into the lumen of vacuolar vesicles was determined using a fluorimetric assay with the acridine dye Quinacrine. The Glu¹⁴⁰→Gly mutant showed no detectable proton-translocating activity (Figure 6, line A). The H⁺ uptake by membrane vesicles containing the

Figure 7 Effect of DCCD on $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ interaction with the 16 kDa proteolipid

At the time indicated by the lower arrows, $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ was added to a final concentration of $0.4 \mu\text{M}$ to a vacuolar membrane suspension ($1.0 \mu\text{g}$ of protein/ml) containing wild-type *Nephrops* proteolipid (A) or $\text{Glu}^{140} \rightarrow \text{Gly}$ mutant proteolipid (B). Fluorescence reversal was monitored following the addition of DCCD to a final concentration of $100 \mu\text{M}$. arb., arbitrary units.

$\text{Glu}^{140} \rightarrow \text{Gly}/16\text{K-Hisx6}$ co-expression mutant (Figure 6, line B) retained only 20 % of the uptake observed for the co-expression control $16\text{K}/16\text{K-Hisx6}$ (Figure 6, line C).

Dibutyltin flavone interaction at the vacuolar membrane

There was a rapid fluorescence response when $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ was added to vacuolar membranes isolated from yeast expressing wild-type *Nephrops* 16 kDa proteolipid (Figure 7, line A). A comparable fluorescence enhancement was also observed with vacuolar membranes containing the $\text{Glu}^{140} \rightarrow \text{Gly}$ mutant proteolipid (Figure 7, line B). The steady-state fluorescence showed a slow steady reversal upon the addition of $100 \mu\text{M}$ DCCD to the vacuolar membranes containing the wild-type proteolipid, which was not observed for the $\text{Glu}^{140} \rightarrow \text{Gly}$ mutant.

Figure 8 Titration of 16 kDa proteolipid by $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$

Aliquots of ethanolic solutions of $200 \mu\text{M}$ and 2 mM $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ were added to membrane or proteolipid suspensions ($1.0 \mu\text{g}/\text{ml}$), and the change in fluorescence intensity was recorded. The solid lines show fits to a single binding site with K_d^{app} values of: solubilized native *Nephrops* proteolipid, $0.18 \pm 0.03 \mu\text{M}$ (□); wild-type hexahistidine-tagged proteolipid, $0.82 \pm 0.13 \mu\text{M}$ (○); vacuolar membranes containing $\text{Glu}^{140} \rightarrow \text{Gly}$ mutant proteolipid, $0.10 \pm 0.02 \mu\text{M}$ (△). arb., arbitrary units.

Figure 9 Effect of DCCD on the fluorescence response due to $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ interaction with the 16 kDa proteolipid

Membranes or proteolipid suspensions (0.2 mg of protein/ml) were incubated with the required concentration of DCCD. Suspensions were then diluted to $2.0 \mu\text{g}/\text{ml}$, and the fluorescence response was recorded upon addition of $0.4 \mu\text{M}$ $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$. Plotted are the maximum fluorescence values obtained, relative to the fluorescence yield in the absence of DCCD, which is normalized to a value of 1.0 for comparison. Measurements were recorded for vacuolar membranes containing: $\text{Glu}^{140} \rightarrow \text{Gly}$ mutant proteolipid (△); $\text{Glu}^{140} \rightarrow \text{Gly}/16\text{K-Hisx6}$ (■); wild-type $16\text{K}/16\text{K-Hisx6}$ co-expressed proteolipids (□); purified 16K-Hisx6 proteolipid (○). rel., relative units.

Titration of $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ against detergent-solubilized native *Nephrops* proteolipid, affinity-purified hexahistidine-tagged proteolipid and vacuolar membranes containing the $\text{Glu}^{140} \rightarrow \text{Gly}$ mutant proteolipid resulted in fluorescence increases that could be fitted to a single site binding equation, with a K_d^{app} of 0.18 ± 0.03 , 0.82 ± 0.13 and $0.10 \pm 0.03 \mu\text{M}$ respectively (Figure 8).

Preincubation of vacuolar membranes containing wild-type *Nephrops* proteolipid with DCCD led to a reduction in the fluorescence response recorded upon the addition of $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$, with $200 \mu\text{M}$ DCCD leading to a 65 % reduction in signal (Figure 9). A similar effect was also observed with purified 16K-Hisx6 . The sensitivity of the fluorescence response to DCCD was found to be reduced with vacuolar membranes from the yeast strain co-expressing the $\text{Glu}^{140} \rightarrow \text{Gly}$ and 16K-Hisx6 proteolipids, with $200 \mu\text{M}$ DCCD causing a 30 % reduction in fluorescence. Vacuolar membranes containing the $\text{Glu}^{140} \rightarrow \text{Gly}$ mutant proteolipid were found to be totally insensitive to the effects of DCCD, with the increase in fluorescence upon addition of $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ being the same at all DCCD concentrations (Figure 9).

DISCUSSION

The 16 kDa proteolipid has been isolated in the form of paracrystalline protein arrays from the hepatopancreas of the crustacean *N. norvegicus* [18,20]. Analysis of this *Nephrops* preparation by SDS/PAGE reveals that it contains principally one major protein species, with buoyant density estimates indicating a high protein-to-lipid ratio [21]. Electron microscopy images show the functional unit of the *Nephrops* proteolipid to be a star-shaped hexameric complex [6].

In this study we have demonstrated that the fluorescent probe $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ can interact specifically with the 16 kDa proteolipid in these paracrystalline arrays (Figure 2). The rate of the fluorescence response upon binding of $\text{Bu}_2\text{Sn}(\text{of})\text{Br}$ to the proteolipid was markedly faster when the preparation was

solubilized using lauroyl sarcosine (Figure 2). Although it appears that the hexameric formation of proteolipid is resistant to detergent solubilization [21], increased accessibility to the dibutyltin flavone sites on the protein might occur as a consequence of increasing the inter-hexameric distances during the solubilization process. Preincubation of the proteolipids with the carboxyl-modifying reagent DCCD reduced the interaction of dibutyltin flavone with the proteolipid, indicating that the binding sites for these two hydrophobic molecules structurally overlap to some extent (Figures 2 and 3).

It is known that the site of action of DCCD at the 16 kDa proteolipid is a highly conserved carboxyl residue located on transmembrane helix-4 of the protein. Radiolabelling experiments have previously shown that DCCD covalently modifies the corresponding Glu¹⁴⁰ residue of the *Nephrops* 16 kDa proteolipid [5]. Covalent modification of this residue in 16 kDa proteolipid species that are subunits of the V-ATPase results in complete loss of proton-translocating activity [25,26]. In addition to these inhibition studies, site-directed mutagenesis studies [7] have reaffirmed the importance of this residue to the proton-translocation function of the V-ATPase. The competition between DCCD and Bu₂Sn(of)Br for binding to proteolipid would suggest a lipophilic environment for Glu¹⁴⁰ and therefore possibly for at least part of the pathway of proton conductance in the V_o sector of the enzyme. The involvement of Glu¹⁴⁰ in the formation of the Bu₂Sn(of)Br binding site in the V-ATPase was further studied using mutants generated from a *Nephrops* proteolipid template.

The *Nephrops* 16 kDa proteolipid has previously been shown to substitute structurally for the endogenous yeast proteolipid, giving rise to a functional V-ATPase and to viable cell growth at pH 7.5 [6,8]. However, expression of proteolipid in which Glu¹⁴⁰ was replaced by glycine, either as a single mutant or co-expressed with wild-type hexahistidine-tagged protein, resulted in failure of the yeast strains to grow at pH 7.5. Although the Glu¹⁴⁰→Gly mutant proteolipid was unable to support any proton translocation, it was stably expressed in the vacuolar membrane and appeared to be assembled into a non-functional hybrid V-ATPase. Folding and packing of the proteolipid does not therefore appear to be compromised by the loss of the glutamate side chain. This enzyme retained some ATP-hydrolysing activity in V₁ that, unlike the activities of hybrid V-ATPases containing wild-type *Nephrops* proteolipids, was insensitive to both DCCD and dibutyltin flavone. This residual activity therefore seems to be uncoupled completely from V_o. The ATPase activities in all mutant strains were, however, equally sensitive to low concentrations of NEM, an inhibitor that modifies a key catalytic residue in V₁ [10,11].

Equal levels of co-expression of the 16K-Hisx6 proteolipid with the Glu¹⁴⁰→Gly mutant were observed when the yeast strains were grown at low pH under conditions that maintain plasmid selection (Figure 4A). The ability of the co-expressed forms of the proteolipid to co-migrate on glycerol gradients, and of the polyhistidine-tagged form to retain the untagged proteolipid on an affinity column (Figures 4B and 4C), indicates that both forms of proteolipid are integrated together into the V_o population of the vacuolar membrane. Such a randomized association between equal amounts of functional and non-functional forms of proteolipid appears to result in a disproportionately large inhibition of V-ATPase function.

It seems clear that in order for a V-ATPase to retain any proton-pumping capacity, all copies of 16 kDa proteolipid in V_o must be functional. If co-expression yields equal amounts of both 16K-Hisx6 and Glu¹⁴⁰→Gly proteolipids, and assuming six copies of the proteolipid per V_o [3,6], then ≈ 14 % of the hexamers

formed would be expected to contain exclusively fully functional 16K-Hisx6 proteolipids. The proton-pumping activity of the 16K-Hisx6/Glu¹⁴⁰→Gly mutant is 20 % of the value determined for 16K/16K-Hisx6 control (Figure 6), which is in reasonable agreement with the theoretical value. The severely compromised proton-pumping capability of this co-expression mutant seems to be inadequate for supporting growth at pH 7.5.

A similar observation has recently been made for F₀F₁-ATPase, where introduction of a single subunit *c* proteolipid containing the mutation Asp⁶¹→Gly resulted in inhibition of reconstituted proton-translocating activity [27]. It appears that, in both F-type and V-type ATPases, inhibitory proteolipids exert a disproportionate effect on ATPase function. This observation is consistent with DCCD-binding effects, whereby modification of a single proteolipid in V_o [26] or in F_o [28] results in complete inhibition of proton pumping. This apparent interdependency or co-operative interaction between proteolipid subunits may support a cyclical mechanism for the V-ATPase, in which proton translocation occurs sequentially via successive proteolipids. A similar rotational mechanism has been proposed for the F₀F₁-ATPase [29].

Dibutyltin flavone binds specifically to the 16 kDa proteolipid in the vacuolar membranes, with binding also reversible upon the addition of DCCD (Figure 7). In the mutant in which Glu¹⁴⁰ was substituted by glycine, binding of Bu₂Sn(of)Br to the proteolipid remained largely unaffected, but was not reversed by the addition of DCCD. Interaction of the probe appears to be specific, with the *K_d* values determined from binding titrations for several forms of the proteolipid being similar (Figure 8). The *K_d* values for the fluorescence titrations are also comparable with the inhibition constant obtained for the direct effect of dibutyltin flavone on ATP hydrolysis. These data suggest that the binding site for dibutyltin flavone contains Glu¹⁴⁰, but that the side chain of this residue is not an essential structural component of this binding site. Furthermore, it appears that addition of a dicyclohexyl-*N*-acylurea adduct to the carboxyl side chain of Glu¹⁴⁰ by reaction with DCCD structurally alters this region of the proteolipid to the extent that dibutyltin is displaced.

Dibutyltin flavone inhibits ATP hydrolysis only in strains expressing a functional proteolipid, in which ATP hydrolysis and proton pumping are coupled. The manner of its inhibitory effect seems therefore to be analogous to that of DCCD. The binding of Bu₂Sn(of)Br at or close to a lipid-exposed structure involved in proton translocation might result in obstruction of the putative cyclical proton-translocation mechanism. The lipid solubility of dibutyltin flavone and DCCD certainly suggest that Glu¹⁴⁰ is lipid exposed, and therefore six distinct, but co-operative, proton pathways located close to the lipid interface of V_o might be responsible for conducting protons across the membrane.

The conserved acidic residue of 16 kDa proteolipids is also implicated in specific binding of the E5 oncoprotein [15]. The interaction between Bu₂Sn(of)Br and the proteolipid could prove useful in monitoring such protein interactions in the environment of the proton-translocation site.

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