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Evidence for a common structure for a class of membrane channels

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Electron microscopic analysis of gap-junction-like structures isolated from an arthropod (*Nephrops norvegicus*) and composed of a 16-kDa polypeptide, show the functional unit to be a star-shaped hexamer of protein arranged around a central channel which runs perpendicular to the plane of the membrane. Estimations of the molecular volume carried out on an averaged projection are consistent with a subunit mass of 16–18 kDa. Fourier transform infrared spectroscopy indicates a high α-helical content for the protein, supporting secondary-structure predictions of four transmembrane α helices/monomer. The averaged projection shows a close resemblance to a hexamer of the 16-kDa protein built on the basis of a four α-helical bundle [Finbow, M. E., Eliopoulos, E. E., Jackson, P. J., Keen, J. N., Meagher, L., Thompson, P., Jones, P. C. & Findlay, J. B. C. (1992) *Protein Eng. 5*, 7–15]. The reconstructed image is also similar to that obtained for gap-junction-like channels isolated from a related arthropod [*Homarus americanus*; Sikerwar, S. S., Downing, K. H. & Glaeser, R. M. (1991) *J. Struct. Biol. 106*, 255–263] whose protein content was unknown but which we demonstrate may be composed of a related 16-kDa protein.

Previous studies have shown a high sequence identity of the *Nephrops* 16-kDa protein with the 16-kDa proteolipid subunit c of the vacuolar H^+-ATPase, both of which in turn bear similarity to the 8-kDa proteolipid subunit of the F_1F_0-ATP synthase. Expression of cDNA coding for the *Nephrops* 16-kDa protein in *Saccharomyces cerevisiae*, in which the endogenous gene coding for the V-ATPase proteolipid has been inactivated, restores V-ATPase activity and cell growth.

A family of related proteolipids are known which appear to have diverse transport functions in prokaryotic and eukaryotic cells. The simplest member is the 8-kDa proteolipid (subunit c or subunit III) of ATP synthase. This proteolipid consists of two hydrophobic segments and is a major core component of the F_0 membrane sector, which forms an ion channel for proton translocation (for review see Futai et al., 1989).

A second member is the 16-kDa proteolipid of the vacuolar H^+-ATPase (V-ATPase). This ATPase is a proton pump in the endomembranes of eukaryotic cells responsible for the acidification of various intracellular compartments (Nelson and Taiz, 1989). The 16-kDa proteolipid consists of four hydrophobic segments and appears to be a tandem repeat of the 8-kDa proteolipid (Mandel et al., 1988). Like its F_0 counterpart, the 16-kDa proteolipid is a major component of the membrane sector (V_0) of the V-ATPase (Moriyama and Nelson, 1989; Parry et al., 1989).

Another 16-kDa proteolipid, which has a high degree of identity with the 16-kDa proteolipid of the V-ATPase, is a component of the mediatophore complex present in synaptosomal preparations isolated from the *Torpedo* electric organ (Birman et al., 1990). The mediatophore transports acetylcholine in response to elevated Ca^{2+} levels and therefore could be considered as a ligand-gated channel (Israel et al., 1986). In addition to this, another closely related 16-kDa polypeptide is the major component of a gap-junction-like structure isolated from various animal tissues (Finbow et al., 1984; Leitch and Finbow 1990; Finbow et al., 1992). These structures are protein/lipid arrays comprised of a pair of membranes containing a hexagonal lattice of particles. This suggests another transport function for this class of proteins, namely in the formation of channels for communication between contiguous membrane-bound compartments (Finbow et al., 1991).

A truncated form of the 16-kDa proteolipid has also been found in *Saccharomyces cerevisiae* (Shih et al., 1988). Expression of this protein gives resistance to toxic levels of the drug trifluoperazine. Finally, a gene in *S. cerevisiae* has been identified (Apperson et al., 1990) which codes for a 20-kDa protein homologous to the 16-kDa proteins, particularly over putative transmembrane regions. The function of this protein is unknown but inactivation of the gene is lethal (Apperson et al., 1990).

This family of related proteins are therefore of considerable interest in the study of membrane transport and, in particular, of bioenergetics, signal transduction and drug resistance. One of these proteins has been found to bind a viral

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Abbreviations. FTIR, Fourier-transform infrared; YEP, yeast extract peptone.

oncoprotein and this binding appears to be important for transforming activity (Goldstein et al., 1991).

The gap-junction-like structures composed of the 16-kDa protein provide a source of structural information which is relevant to all these proteins. They can easily be isolated from the hepatopancreas of *Nephrops norvegicus* in quantities suitable for biochemical and biophysical studies and their paracrystalline nature as ordered protein/lipid arrays, allows the use of imaging techniques (Finbow et al., 1984; 1992).

Protease studies have shown the *Nephrops* 16-kDa protein in these structures is, for the most part, buried in the bilayer and only the N-terminus is available for cleavage (Finbow et al., 1992). Chemical-labelling studies with the lipophilic reagent *N,N′*-dicyclohexylcarbodiimide has identified a conserved glutamic acid residue located in the fourth hydrophobic segment of the *Nephrops* protein. This residue appears to be functionally important for proton translocation in the vacuolar 16-kDa proteolipid (Arai et al., 1987; Noumi et al., 1991). This information, together with electron microscopic data, secondary-structure-prediction algorithms and crystallographic homologues, has made it possible to build a model of the *N. norvegicus* 16-kDa protein and the complex it forms in the bilayer (Finbow et al., 1992). We now present electron microscopic and biophysical evidence for the model.

Our investigations reveal a hexameric arrangement of the *Nephrops* 16-kDa protein around a central channel forming a star-shaped complex which constitutes the functional unit. Biophysical studies show a high degree of α-helical content and the image projection reveals a close similarity between the *Nephrops* gap-junction-like structures and the gap junctions isolated by others (Sikerwar et al., 1991) from a closely related species, *Homarus americanus*. We have also established a direct functional relationship between the *Nephrops* 16-kDa protein in these structures and the 16-kDa proteolipid of the V-ATPase, by showing that the former will substitute for the latter in a yeast model system. The image reconstruction data therefore have implications for understanding the organisation of the 16-kDa proteolipid in the V_0 sector of the V-ATPase and hence of subunit c in the F_0 sector of the H^+-ATP synthase.

MATERIALS AND METHODS

Isolation of 16-kDa protein gap-junction-like structures

The isolation of membranes containing the 16-kDa protein from the hepatopancreas of *N. norvegicus* has been previously described (Finbow et al., 1984; Finbow et al., 1992).

Electron microscopy

Negatively stained specimens for conventional electron microscopy were prepared according to Valentine et al. (1968) using a solution of uranyl acetate (4% mass/vol., pH 4.5). Samples containing about 200 µg protein/ml were allowed to adsorb to the carbon films for 20 s and washed on drops of double-distilled water before staining. Grids were examined in a Philips CM 10 transmission electron microscope, operated at an accelerating voltage of 100 kV. Electron micrographs were recorded at calibrated magnifications on Agfa Scientia 23 D 56 electron-image sheet film.

Electron micrographs were assessed by optical diffraction using a He/Ne laser-powered optical diffractometer LFO (Spindler and Hoyer) equipped with an image-reconstruction lens system. Image processing was carried out using the spatial-frequency-filtering technique in conjunction with image reconstruction according to Klug and DeRosier (1966). In order to avoid phase artefacts during optical filtering, opaque masks (amplitude filters) were used exclusively. Masks were made from Photoresist™-coated aluminium foil by photoetching; contact negatives of diffraction patterns recorded in the Fourier plane served as templates (after Misell, 1978). A range of zero-order beam attenuations were used in order to optimise the reconstructed image by reducing the zero-order background without introducing a frequency doubling. Filtered images were further processed using rotational averaging as described by Markham et al. (1963).

FTIR spectroscopy

Infrared spectra were recorded on a Bruker IFS 25 FTIR spectrometer with DTGS detector, at a resolution of 4 cm^{-1}, apodized with a triangular function and Fourier transformed after one level of zero-filling. Approximately 8 mg freeze-dried membranes were suspended in 100 µl 10 mM Hepes, 10 mM NaCl, pD 7.8, prepared in D_2O and the suspension was vortexed for 1 min. The sample was sealed and incubated for 4 h at 55 °C, followed by 20 h at 24 °C, to promote deuterium exchange. The sample was then introduced into a thermostatted cuvette with CaF_2 windows and a 50-µm teflon spacer for recording spectra. Peak positions in the amide I band were resolved by Fourier self-deconvolution (Kauppinen et al., 1981) and quantified by band fitting of the deconvoluted spectra using programmes kindly provided by Dr. H. H. Mantsch.

Expression of *Nephrops* 16-kDa protein in yeast

Nephrops cDNA containing the coding sequence (Finbow et al., 1992) was inserted into *Bam*H1 site of the yeast shuttle vectors YCp50 and pLGSD5-ATG (kindly provided by Dr. L Guarente, Department of Biology, Massachusetts Institute of Technology). Expression of inserted cDNA in the latter plasmid is under control of the *gal* promoter. The plasmids were transfected into a haploid strain of *S. cerevisiae* W303-1B (*MATa*, *leu2*, *his3*, *ade2*, *trp1*, *ura3*), in which the *VMA3* gene had been inactivated by insertion of the *LEU2* gene (*LEU2::vatc*; Nelson and Nelson, 1990; kindly provided by Dr. N. Nelson, Roche Institute for Molecular Biology, Nutley, New Jersey). Growth at pH 5.5 and pH 7.5 in yeast extract peptone (YEP) plates plus 1% galactose was tested as described by Nelson and Nelson (1990). The presence of the *Nephrops* 16-kDa protein was examined by harvesting an exponential 1-l culture growing in YEP medium containing galactose and preparing spheroplasts with zymolase. Vacuolar membranes were prepared as described by Uchida et al. (1985) and extracted on ice for 1 h with chloroform/methanol (1:2). The antiserum used (N2) has been described previously (Leitch and Finbow, 1990). Experimental protocols for yeast were those described in Guthrie and Fink (1991).

RESULTS

Electron microscopic analysis and image reconstruction

Preparations from the hepatopancreas of *N. norvegicus* contain large numbers of gap-junction-like structures as previously shown (Finbow et al., 1984; Buultjens et al., 1988; Leitch and Finbow, 1990). These structures are protein/lipid

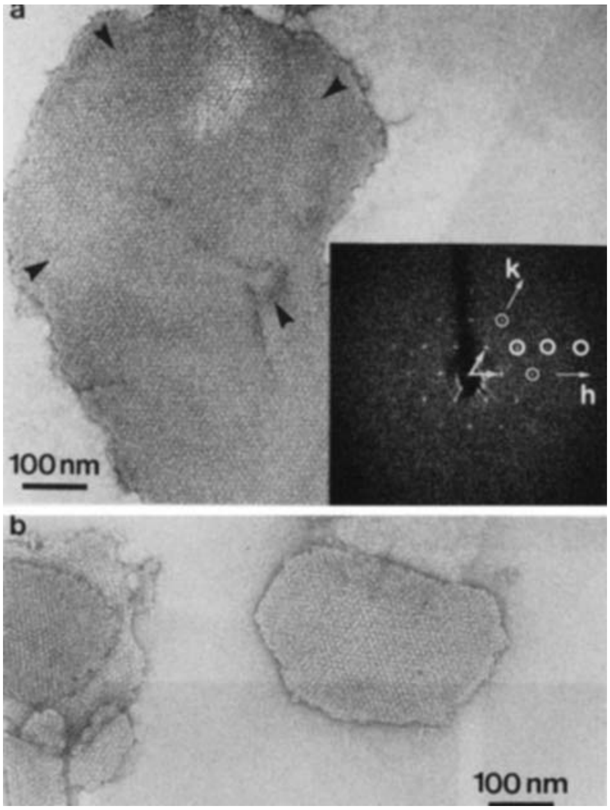


Fig. 1. Electron micrographs of negatively stained (protein appears white) gap-junction-like structures from (a) *N. norvegicus* and (b) *H. americanus*. In (a), the optical diffraction pattern obtained from the marked area (arrowheads) is shown in the inset. The reflections (*h,k*) = (1,1) (2,1) and (3,1) are circled in bold. The (3,1) reflection corresponds to 2.1 nm^{-1}. The *Homarus* preparation (b) exhibits essentially the same features and the unit-cell dimensions for both are $a = b = 8.8 \pm 0.5$ nm.

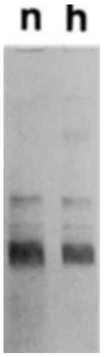


Fig. 2. SDS/PAGE of gap-junction-like structures isolated from the hepatopancreas of *N. norvegicus* (lane 'n') and *H. americanus* (lane 'h'). Arrowheads mark the position of the monomeric (18 kDa) and dimeric (28 kDa) forms of the *Nephrops* 16-kDa protein.

arrays formed from a hexagonal lattice of particles which are 6–8 nm in diameter and have a central stain-penetrable pore (Fig. 1). The principal protein component of these structures has been shown to be a protein with a molecular mass of 16-kDa (Buultjens et al., 1988, Leitch and Finbow, 1990; see Fig. 2).

Optical diffraction studies revealed a lattice constant of $a = b = 8.8 \pm 0.5$ nm with $\gamma = 120°$ for both the *Nephrops* (Fig. 1a) and *Homarus* (Fig. 1b) preparations. Highest-order reflections observed (*h,k*) = (3,1) correspond to 2.1 nm^{-1} (see Fig. 1a, inset). The reconstructed projection in Fig. 3a, after optical filtering (for details see Material and Methods) and rotational averaging over the two centres of threefold symmetry to emphasise the unit-cell symmetry, supports the idea that the 16-kDa protein forms hexameric complexes and suggests a p6 plane group. Although it seems that the hexamers interact with each other via the peripheral stain-excluding areas, the actual joins between the triangular protrusions should be treated with care since they are not resolved and can be caused by the slightest misalignment between the mask holes and reflections. To emphasise details within a single hexameric complex, a translationally averaged projection was subjected to an additional sixfold rotational averaging. The projection in Fig. 3b confirms that, normal to the membrane plane, the complexes can be described as hexagonal stars (overall diameter ≈7.5 nm). In the centre of the complexes there is a mass deficit (stain trap) with a diameter of ≈1.5 nm. The radius of the surrounding stain-excluding ring-like domain (Fig. 3b) measures approximately 1.9 nm from the centre of the complex. The ring-like domain is surrounded by six equally spaced stain-excluding areas, each assuming the shape of an equilateral triangle and covering an area of roughly 1.6 nm^2 (Fig. 3b). In contrast, imposing a fivefold rotational symmetry results invariably in a complete loss of structural detail and lattice integrity (Fig. 3c). Assuming (a) a partial specific volume (υ) of the 16-kDa protein of 0.74 ml/g (c.f. Harris and Holzenburg, 1989), (b) an extension of the protein along the *c* axis of 7 nm, as previously deduced from electron microscopy of sectioned material (Finbow and Leitch, 1990), and (c) that the central mass deficit constitutes a channel extending through the entire structure, the molecular mass (*m*) of one complex (Fig. 3b) can be estimated to be <110 kDa, according to the formula m = volume of the protein (ml) × Avogadro's number × $1/\upsilon$ (Harris and Holzenburg, 1989). Hence, the electron microscopy data are consistent with assembly of the 16-kDa protein into hexamers (the molecular mass from the deduced sequence is 17.5 kDa; Finbow et al., 1992) and are furthermore in agreement with the data obtained from secondary-structure-prediction algorithms (Fig. 3d and e).

Fourier-transform infra-red (FTIR) spectroscopy

FTIR spectroscopy provides a measure of the relative amounts of different secondary-structural elements and can be carried out directly on protein/lipid structures without recourse to extraction of the protein and its possible denaturation. Such an analysis carried out on *Nephrops* gap-junction-like structures is shown in Fig. 4 for the amide I region of the Fourier self-deconvoluted FTIR spectrum. The spectrum in this region changes little over the temperature range 15–95 °C, indicating that the protein is extremely stable with respect to heat denaturation. Control spectra from junction-like membranes which had been freshly dispersed in D_2O buffer (i.e. without prolonged incubation at higher temperatures to enhance deuterium exchange of the amide groups) are similar to those in Fig. 4 except for slight band shifts consistent with a lower degree of deuterium exchange.

Band-fitting of the Fourier deconvoluted spectrum (see Fig. 4) reveals the presence of two major bands at wavenumbers 1669 cm^{-1} and 1675 cm^{-1}, with smaller bands at 1647, 1639 and 1629 cm^{-1}, plus three other minor bands.

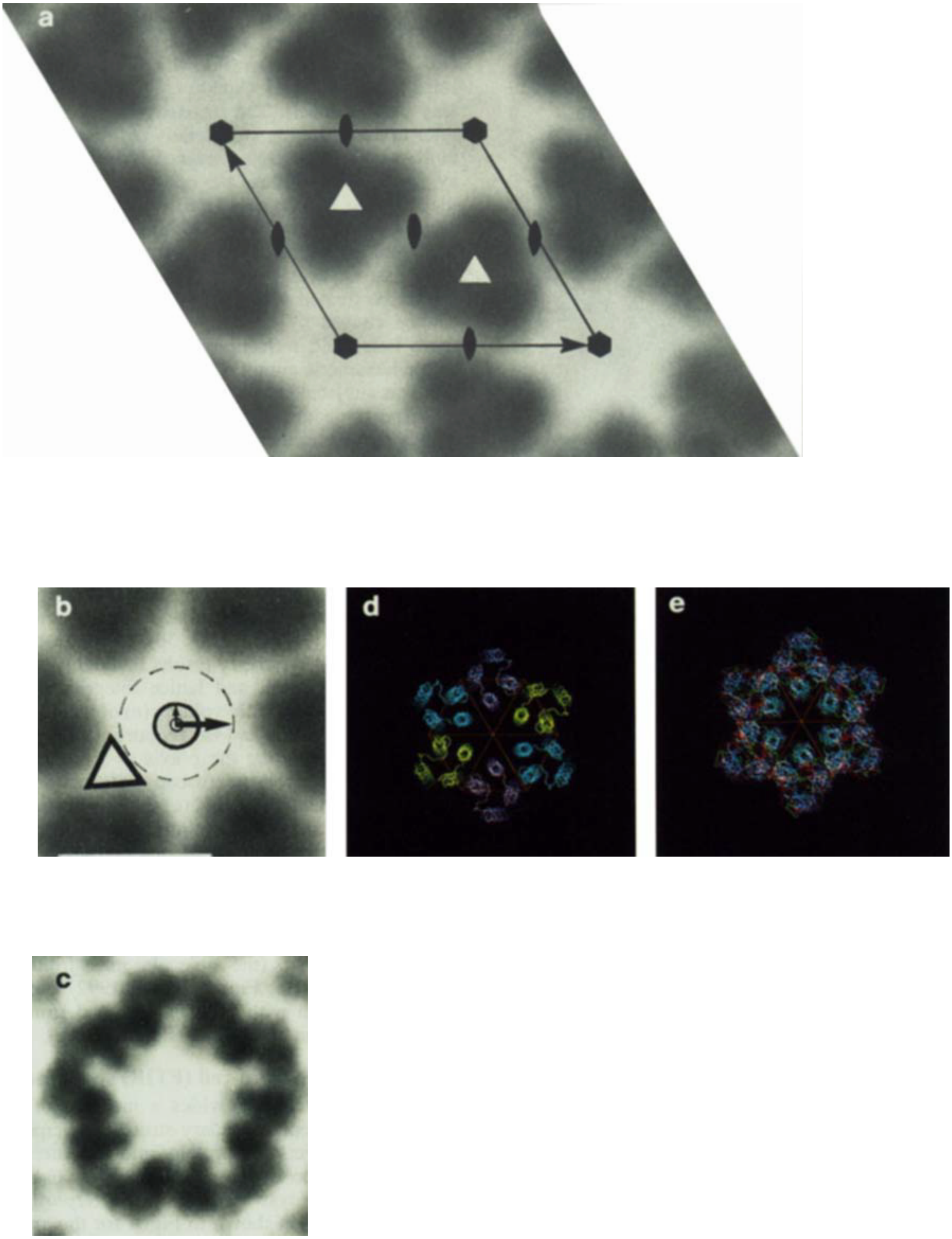


Fig. 3. Averaged projections of the unit cell (a, b and c) and predicted model of a hexamer of the 16-kDa protein (d and e) from Finbow et al. (1992). (a) Reconstructed projection (protein appears white) of the unit cell after optical filtering and rotational averaging over the two centres of threefold symmetry. The centres of two-, three- and sixfold symmetry are indicated. The architecture of the unit cell supports the idea that the quaternary structure of the 16-kDa protein is a hexamer. (b) Reconstructed projection of a single hexameric complex after optical filtering and sixfold rotational averaging reveals a central mass deficit (small arrow) with a radius of 0.75 nm. The radius of the surrounding ring measures 1.9 nm from the centre of the complex (large arrow). Each of the marked triangular protrusions covers an area of $\approx 1.6\,\mathrm{nm}^2$. The scale bar corresponds to 5 nm. In (c), a reconstructed projection after optical filtering and fivefold rotational averaging is depicted. (d and e) Computer-built models of a hexamer of the *Nephrops* 16-kDa protein taken from Finbow et al., 1992. In (d), the main-chain atoms are shown and in (e), main-chain and side-chain atoms are shown. The model is reproduced at the same magnification as the image in (b) and the corner−centre distance of the orange hexagon is 2 nm. In the earlier published models, the helices are arranged in an anti-clockwise rotation when viewed from the end opposite to the N-terminus and C-terminus. In the two examples shown here, the helices are arranged in a clockwise manner. The similarity of helices 2 and 4 resulting from gene duplication (see Figs 7 and 8), make it difficult to reliably predict whether the helices will be arranged in a clockwise or an anti-clockwise rotation.

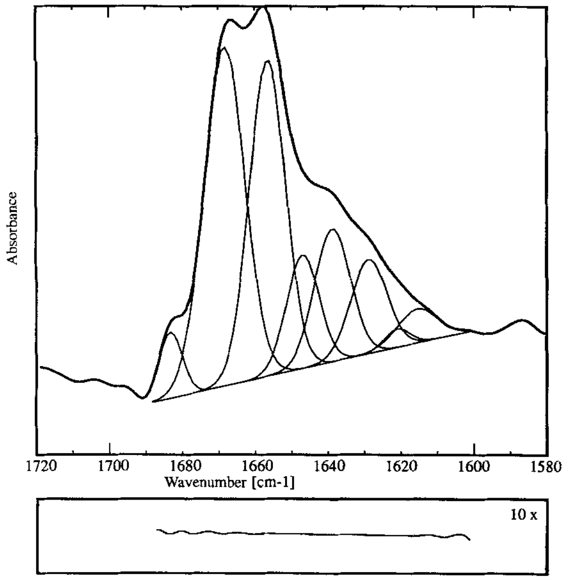


Fig. 4. FTIR spectrum (amide I region) of *Nephrops* gap-junction-like structures in D_2O buffer after Fourier self-deconvolution with a Lorentzian linewidth of 17 cm^{-1} and a band-narrowing factor of 2. The maximum of the original (non-deconvoluted) amide I band corresponded to 0.44 absorbance units. The spectrum was recorded at a temperature of 15 °C. The deconvoluted spectra are fitted by Gaussian bands as indicated, with residuals (lower box, with the same wavenumber axis as for the deconvoluted spectrum, but a 10 × vertical expansion) in the regions of the individual band positions of <0.02–0.1%.

The two major bands lie at positions of higher wavenumber than those normally assigned to defined secondary-structural elements such as the deuterated α helix (1654 cm^{-1}) or turns (1665 cm^{-1}; see Byler and Susi, 1986). The spectrum, however, bears a strong resemblance to that of bacteriorhodopsin (data not shown) which likewise contains two major bands shifted to unusually high wavenumber. These bands, which occur at wavenumbers of approximately 1665 cm^{-1} and 1658 cm^{-1} for the incident beam polarized parallel and perpendicular to the plane of incidence, respectively, have been attributed to the transmembrane α helices of bacteriorhodopsin (Earnest et al., 1990) which is the predominant secondary-structural element in this protein (Henderson et al., 1990). Therefore, it is likely that the two major bands observed for the *Nephrops* gap-junction-like structures correspond to α-helical structures similar to those found in bacteriorhodopsin. These two bands compose >60% of the total intensity of the amide I band (Fig. 4).

Of the remaining bands, those at 1639 cm^{-1} and 1629 cm^{-1} comprise approximately 20% of the total intensity and may correspond to extended β-like structures not necessarily in sheets (Byler and Susi, 1986). The poorly resolved band at 1647 cm^{-1} (9% of the total intensity) could correspond to disordered structures and the weak band at 1684 cm^{-1} (4% of total intensity) may arise from turns (Earnest et al., 1990).

Composition of gap-junction-like structures isolated from *H. americanus*

Gap-junction-like structures have also been isolated from the hepatopancreas of a related crustacean, *H. americanus* (Sikerwar et al., 1991). Electron microscopic studies in conjunction with digital image processing, carried out by Sikerwar et al. (1991) revealed unit cells similar in shape and dimension to the *Nephrops* gap-junction-like structures as analysed in the present study using electron microscopy in connection with optical methods. However, the protein composition of the *Homarus* structures was unknown. Therefore gap-junction-like structures were isolated from the hepatopancreas of *Homarus*, using the same isolation procedure as for *Nephrops* to investigate whether the 16-kDa protein is present in gap-junction-like structures of *Homarus*.

The preparations contain large numbers of gap-junction-like structures of similar morphology to those found in the *Nephrops* preparations (Fig. 1) and to gap-junction-like structures previously isolated from *Homarus* hepatopancreas.

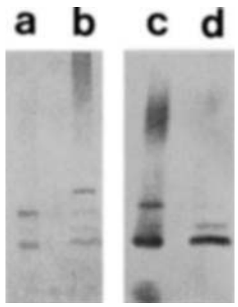


Fig. 5. SDS/PAGE (lanes a and b) and immunoblot (lanes c and d) of *Nephrops* gap-junction-like structures (lanes a and c) and chloroform/methanol extract of vacuolar membranes from *S. cerevisiae* transfected with *Nephrops* cDNA coding for the 16-kDa protein (lanes b and d). The immunoblots were probed with a rabbit antiserum (N2) raised against a total gap-junction-like preparation from *Nephrops* hepatopancreas. In addition to reacting to the *Nephrops* 16-kDa protein, the antiserum also reacts to a diffuse glycoprotein (54 kDa) present as a minor component in these preparations (Leitch and Finbow, 1990; Buultjens et al., 1988).

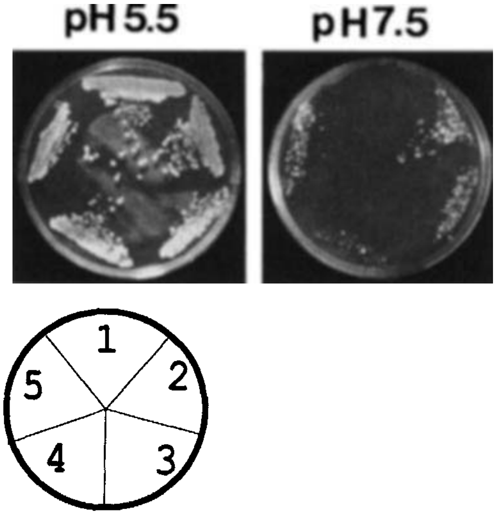
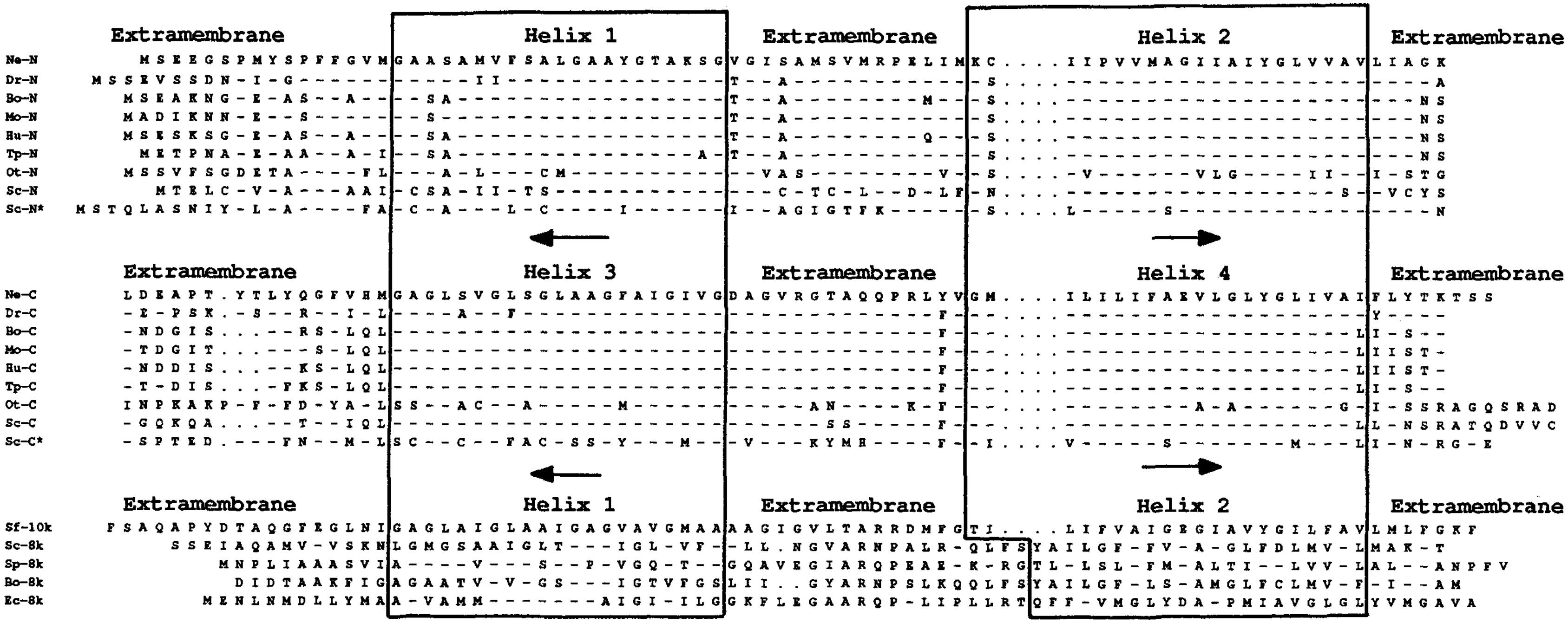


Fig. 6. Growth at pH 5.5 and 7.5 of *LEU2* : :*vatc* mutant *S. cerevisiae* before (sector 1) and after transformation with *Nephrops* cDNA coding for the 16-kDa protein in the pLGSD5-ATG (sectors 2 and 3) and YCp50 (sectors 4 and 5) yeast shuttle vectors. Cells grow irrespective of the carbon source at pH 5.5, since a functional V-ATPase is not required. Growth at pH 7.5 does, however, require a functional V-ATPase and occurs only in cells expressing the *Nephrops* 16-kDa protein under control of a galactose-inducible promoter. The YEP agar plates shown at pH 7.5 were supplemented with 1% galactose.



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           Extramembrane           Helix 1         Extramembrane           Helix 2          Extramembrane
Ne-N       MSEEGSPMYSPFFGVM GAASAMVFSALGAAYGTAKSG VGISAMSVMRPELIM KC....IIPVVMAGIIAIYGLVVAV LIAGK
Dr-N    MSSEVSSDN-I-G------ -----II-------------- T--A----------- -S....------------------- ----A
Bo-N      MSEAKNG-E-AS--A-- --SA----------------- T--A--------M-- -S....------------------- ---NS
Mo-N      MADIKNN-E--S----- --S------------------ T--A----------- -S....------------------- ---NS
Hu-N      MSESKSG-E-AS--A-- --SA----------------- T--A--------Q-- -S....------------------- ---NS
Tp-N       METPNA-E-AA--A-I --SA---------------A- T--A----------- -S....------------------- ---NS
Ot-N      MSSVFSGDETA----FL ---A-L---CM---------- --VAS--------V- -S....-V------VLG----II-- I-STG
Sc-N        MTELC-V-A---AAI -CSA-II-TS----------- ---C-TC-L--D-LF -N....-----------------S- -VCYS
Sc-N*  MSTQLASNIY-L-A----FA -C-A---L-C----I------ I--AGIGTFK----- -S....L-----S------------ ----N
                                     ←                                       →
           Extramembrane           Helix 3         Extramembrane           Helix 4          Extramembrane
Ne-C      LDEAPT.YTLYQGFVHM GAGLSVGLSGLAAGFAIGIVG DAGVRGTAQQPRLYV GM....ILILIFAEVLGLYGLIVAI FLYTKTSS
Dr-C      -E-PSK.-S--R--I-L ----A--F------------- -------------F- --....------------------- Y----
Bo-C      -NDGIS...--RS-LQL --------------------- -------------F- --....------------------L I-S--
Mo-C      -TDGIT...---S-LQL --------------------- -------------F- --....------------------L IIST-
Hu-C      -NDDIS...--KS-LQL --------------------- -------------F- --....------------------L IIST-
Tp-C      -T-DIS...-FKS-LQL --------------------- -------------F- --....------------------L I-S--
Ot-C      INPKAKP-F-FD-YA-L SS--AC--A-----M------ -----AN----K-F- --....--------A-A------G- I-SSRAGQSRAD
Sc-C      -GQKQA...--T--IQL --------------------- ------SS-----F- --....------------------L L-NSRATQDVVC
Sc-C*     -SPTED.---FN--M-L SC--C--FAC-SS-Y---M-- -V---KYMH----F- -I....V-----S-------M---L I-N-RG-E
                                     ←                                       →
           Extramembrane           Helix 1         Extramembrane           Helix 2          Extramembrane
Sf-10k   FSAQAPYDTAQGFEGLNI GAGLAIGLAAIGAGVAVGMAA AAGIGVLTARRDMFG TI....LIFVAIGEGIAVYGILFAV LMLFGKF
Sc-8k          SSEIAQAMV-VSKN LGMGSAAIGLT---IGL-VF- -LL.NGVARNPALR-QLFS YAILGF-FV-A-GLFDLMV-L MAK-T
Sp-8k             MNPLIAAASVI A----V---S--P-VGQ-T-- GQAVEGIARQPEAE-K-RG TL-LSL-FM-ALTI--LVV-L AL--ANPFV
Bo-8k              DIDTAAKFIG AGAATV-V-GS---IGTVFGS LII..GYARNPSLKQQLFS YAILGF-LS-AMGLFCLMV-F -I--AM
Ec-8k            MENLNMDLLYMA A-VAMM-------AIGI-ILG GKFLEGAARQP-LIPLLRT QFF-VMGLYDA-PMIAVGLGL YVMGAVA
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Fig. 7. Sequence alignment of 16-kDa proteins and F_0 8-kDa subunit-c proteins. The sequences for both types of protein are aligned with Pro residues of the extramembranous loops between helices 1 and 2 and helices 3 and 4 (Figs 7 and 11 in Finbow et al., 1992). The 16-kDa protein sequences shown are the N-terminal (-N) and C-terminal halves (-C) of *Nephrops* (Ne), *Drosophila melanogaster* (Dr), bovine (Bo), murine (Mo), human (Hu), *Torpedo californica* (Tp), Oat (Ot) and *S. cerevisiae VMA3* (Sc) and *VMA11* (Sc-*). Sequences are taken from Finbow et al. (1992), Meagher et al. (1990), Mandel et al. (1988), Hanada et al. (1991), Gillespie et al. (1991), Birmen et al. (1990), Lai et al. (1981), Nelson and Nelson (1990), and Umemoto et al. (1991), respectively. Sequences of representatives of the F_0 subunit-c protein [*Sulfolobus acidocaldarius* (Sf), *S. cerevisiae* (Sc), spinach (Sp), bovine (Bo) and *E. coli* (Ec)] were taken from Sebald and Hoppe (1981), Mandel et al. (1988) and Denda et al. (1989). Dashes correspond to identities to the top sequence (*Nephrops* for the 16-kDa proteins and *Sulfolobus* for the 8-kDa subunit-c proteins) and dots show insertions. The *Sulfolobus* sequence begins at Phe20. The boxed areas show the regions used for the helical wheels in Fig. 8 and the arrows indicate from which side the helical projections are being viewed.

Optical diffraction revealed a lattice constant of 8.8 nm for these hexagonal protein/lipid arrays. The slightly larger lattice constant (8.4 nm) than that found by Sikerwar et al. (1991) can be accounted for by a deoxycholate substitution step used in the earlier study.

SDS/PAGE shows that the *Homarus* preparations contain predominantly protein bands which co-migrate with the *Nephrops* 16-kDa protein and its dimeric form (Fig. 2). Sequencing of the N-terminus of the *Homarus* protein confirms it is a closely related 16-kDa protein (data not shown).

The *Nephrops* 16-kDa protein substitutes for the yeast vacuolar 16-kDa proteolipid

In relating the projected model of the hexamer of the *Nephrops* 16-kDa protein to the V_0 sector formed principally from the 16-kDa proteolipid subunit of the V-ATPase, it is important to know whether the two proteins are equivalent. This can, in part, be tested by examining the growth of mutants of *S. cerevisiae* where the endogenous gene (*VMA3*), coding for the 16-kDa proteolipid, has been inactivated (Nelson and Nelson, 1990; *LEU2: :vatc* mutant). These mutants grow at pH 5.5 but not at pH 7.5. However, their growth at pH 7.5 can be restored by transformation with a plasmid containing the yeast *VMA3* gene (Noumi et al., 1991).

cDNA encoding the *N. norvegicus* 16-kDa protein (Finbow et al., 1992) was incorporated into the yeast shuttle vector pLGSD5-ATG (high copy number in which expression of *Nephrops* cDNA is under control of the *Gal10* promoter) and YCp50 plasmid (low copy number). Organic-solvent extracts of vacuolar membranes from cells transformed with pLGSD5-ATG, containing the *Nephrops* cDNA and grown in the presence of galactose, show the presence of bands on SDS/PAGE of molecular mass 18, 22 and 28 kDa, which comigrate with bands in the gap-junction-like protein/lipid array preparations from *Nephrops* (Fig. 5). The 28-kDa band has previously been shown to be a dimeric species of the 16-kDa protein (Finbow et al., 1984). An endogenous 30-kDa polypeptide is also present. Immunoblotting analysis with antibodies to the *Nephrops* 16-kDa protein confirms the identity of the galactose-induced bands as the *Nephrops* 16-kDa protein.

The ability of the *Nephrops* 16-kDa protein to substitute functionally for the 16-kDa proteolipid is shown in Fig. 6. In the absence or presence of galactose, the mutant cells will grow at pH 5.5 but only the wild type cells will grow at pH 7.5. Transformation of the mutant cells with the *Nephrops* 16-kDa protein, however, restores growth at pH 7.5 in the presence of galactose, suggesting that the *Nephrops* protein can substitute for the inactivated yeast 16-kDa proteolipid.

DISCUSSION

Image reconstruction, secondary structure and similarity to proposed model

Electron microscopic studies of the *N. norvegicus* preparation show that the unit cell is composed of six 16-kDa protomers symmetrically disposed around a central channel. Stoichiometric studies on the vacuolar H^+-ATPase also suggest that there are six copies of the proteolipid (subunit c) in each complex (Arai et al., 1988; Nolta et al., 1991).

An earlier study on the mediatophore complex prepared from electric organ synaptosomes isolated from *Torpedo*, showed that it too is a multimeric assembly formed from a closely related 16-kDa protein (Israel et al., 1986; Birman et al., 1990). Visual analysis of negatively stained images indicated each complex to have similar dimensions and a similar appearance to the *Nephrops* 16-kDa preparations examined here, although only five subunits could be clearly distinguished. Imposing fivefold rotational symmetry on averaged projections of the *Nephrops* 16-kDa protein complexes showed no reinforcement of structural details (Fig. 3c). An additional polypeptide might be a component of the mediatophore, which could explain the apparent discrepancy (Morel et al., 1989).

The *Nephrops* structures are viewed *en face* to the plane of the paired membranes (Fig. 1) and therefore the projections contain contributions from two channel complexes joined end-end. Apposing subunits in the end-end association must align closely in register with each other because the hexamer array can be seen clearly. This suggests that there is a precise end-to-end interaction (see Finbow et al., 1991, for discussion on the functional significance of this channel pairing).

Band fitting of the Fourier-deconvoluted FTIR spectra suggests that >60% of the *Nephrops* 16-kDa protein is in an α-helical configuration, similar to that found for bacteriorhodopsin. Based on the comparison with the FTIR spectrum and the structure of bacteriorhodopsin (Henderson et al., 1990), this estimate for the *Nephrops* 16-kDa protein is likely to be a minimum value, i.e. no less than 95 residues of the 159 residues are in the α-helical array. This is in good agreement with secondary structure prediction algorithms which suggest four transmembrane α helices of average length 27 residues (Finbow et al., 1992). Such a disposition of the protein in the bilayer accounts for the protease insensitivity, the site of *N,N'*-dicyclohexyl carbodiimide modification and the position of epitopes.

Using a four α-helical bundle as the basis for tertiary-structural prediction, the helices would form a diamond arrangement in cross-sectional view to the plane of the bilayer (Fig. 3d and e). The resulting shape of a hexamer of subunits, taking into account symmetry considerations and the placement of hydrophobic regions in the lipid bilayer, is a star-like form with dimensions similar to the reconstructed image of the 16-kDa protein channel complex. This is consistent with the proposed structural model of the *Nephrops* 16-kDa protein and the quaternary structure of the complex it forms (Finbow et al., 1992).

Implications for the F_0 proton channel

The F_0 sector of the ATP synthase consists of a minimum of three subunits of which the most abundant is subunit c (Futai et al., 1989). There is, however, little information on the arrangement of the protein in this sector. The similarity between the *Nephrops* 16-kDa protein and subunit c raises the possibility that the reconstructed projection of the hexameric complex of the gap-junction-like form of the *Nephrops* protein is also a good representation of the F_0 sector structure.

The 16-kDa protein appears to be a tandem repeat of subunit c, which is thought to comprise two transmembrane α helices. A tandem repeat is consistent with the prediction of a four α-helical bundle for the 16-kDa protein. The level of sequence identity between subunit c and the *Nephrops* 16-kDa protein and 16-kDa proteolipids from different species is about 20% in each half, but comparison on a helical wheel

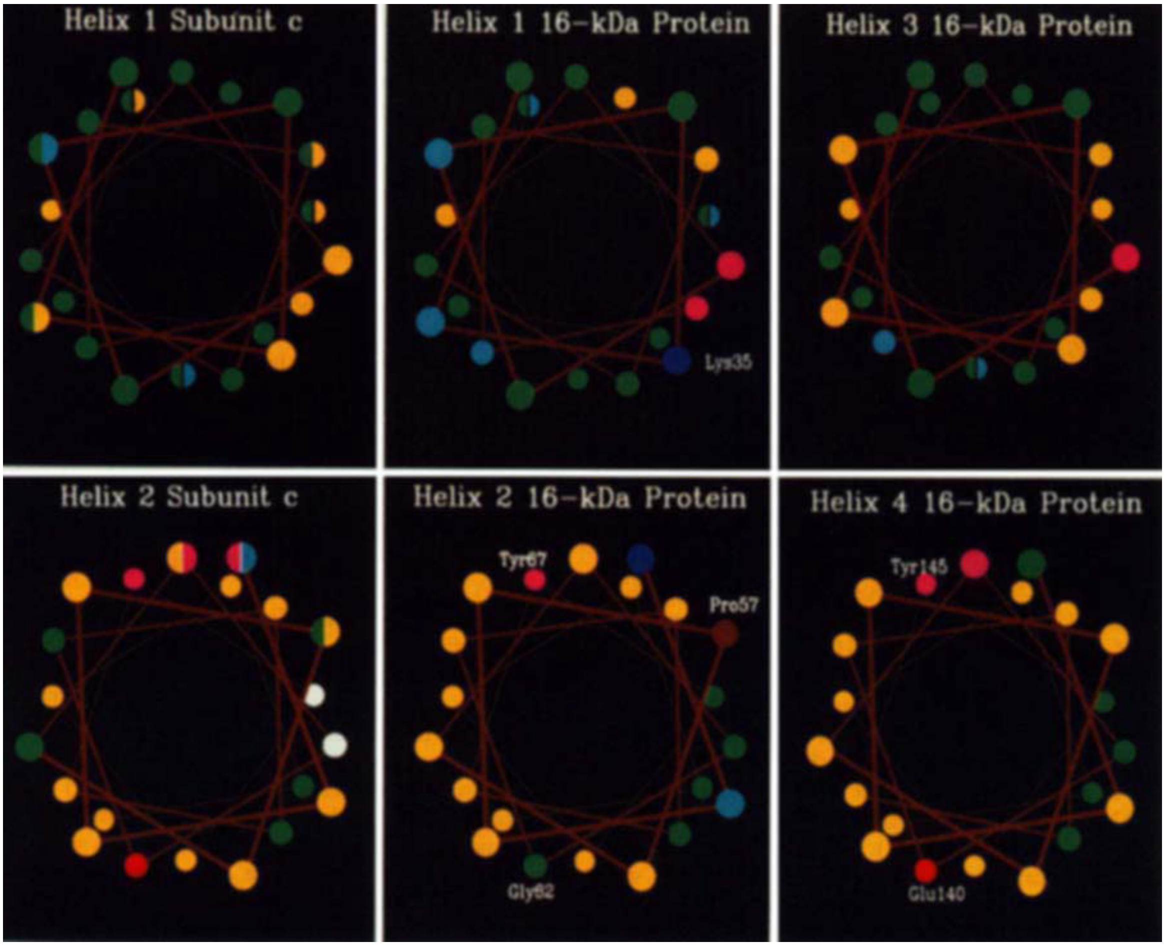

Fig. 8. Helical wheels of the four putative transmembrane helices of the 16-kDa protein and the two putative transmembrane helices of the F_0 8-kDa subunit-c protein. The predominant character of the amino acid at each position in the boxed regions of the sequences in Fig. 7 is shown. Helices 1 and 3 of the 16-kDa protein and helix 1 of subunit c are viewed with the C-terminus on top beginning Gly37 (helix 1, *Nephrops*) and Gly115 (helix 3, *Nephrops*). Helices 2 and 4 of the 16-kDa protein and helix 2 of subunit c are viewed from the N-terminus beginning with Lys53 (helix 2, *Nephrops*) and Gly131 (helix 4, *Nephrops*). The numbered residues shown are for the *Nephrops* 16-kDa protein. Green, Gly and Ala; yellow, Leu, Ileu, Val, Met; magenta, Tyr, Phe; brown, Pro; light blue, Ser, Thr; dark blue, Lys; red, Glu and Asp; white, non-conserved.

of the character of equivalent residues in the transmembrane domains shows a much higher degree of similarity (Figs 7 and 8). This similarity can be seen between helices 1 and 3 of the 16-kDa protein and helix 1 of subunit c, and between helices 2 and 4 of the 16-kDa protein and helix 2 of subunit c, as would be required for a tandem repeat. Therefore, it seems likely that the arrangement of the two polypeptides in the bilayer and the complexes they form will be similar.

Apart from the sequence similarity, there are two other lines of evidence which support the similarity in construction of the F_0 sector to the hexameric complex of the *Nephrops* 16-kDa protein. Firstly, the crystallisation of the F_1 sector (Bianchet et al., 1992) and earlier electron microscopic data (Boekema et al., 1986) show the two ATP-binding subunits, of which there are three copies each, are alternately arranged in a clover-leaf structure around a central sixfold symmetry axis that is perpendicular to the plane of the membrane. Such an axis is present in the hexameric complex of the 16-kDa protein. Secondly, an SDS-stable form of subunit c (CF_0 subunit III) has been isolated (Fromme et al., 1987) which appears to contain 12 copies (i.e. equivalent to six copies of the 16-kDa protein) and has similar dimensions as measured from negative-stained images to the hexameric complex of the 16-kDa protein. Like the *Nephrops* membrane complex, this multimeric form of the subunit c is remarkably stable to heat.

In summary, our findings are consistent with the core structure of the F_0 sector being formed from six dimers of subunit c, with each dimer organised as a four α-helical bundle and arranged around a central sixfold symmetry axis.

Relationship to the connexon channel of gap junctions

The projection map of the gap-junction-like channels isolated from *Homarus* by Sikerwar et al. (1991) shows a close similarity to the hexamer complex of the 16-kDa protein isolated from *Nephrops*. Our results, although using a different isolation procedure, show that the 16-kDa protein is present in *Homarus* preparations and therefore suggest that the structural basis of the *Homarus* gap-junction-like structures studied by Sikerwar et al. is the 16-kDa protein.

A slight difference can be seen in the averaged projections of the unit cells from *Homarus* (Sikerwar et al., 1991) and *Nephrops* gap-junction-like structures; the *Nephrops* hexameric complex resembles a star while the *Homarus* complex appears more like a rounded hexagon. This is due to the differences in image-processing procedures employed (A. H., unpublished results) and may not necessarily reflect any inherent structural differences. The reconstructed projections of *Homarus* gap-junction-like channels and the *Nephrops* hexameric complex of the 16-kDa protein are also similar to the connexon channels of gap junctions isolated from rodent liver (Makowski et al., 1977; Caspar et al., 1977; Unwin and Zampighi, 1979; Unwin and Ennis, 1984) and seen in freeze-fracture replicas from rat liver gap junctions (Rash and Yasamura, 1992). The liver connexon is formed from a hexamer of similar sized, rod-shaped subunits producing a cylinder, 7–7.5 nm in length with a central channel of 1–2 nm diameter and centre-centre spacing in the hexagonal array of 8.2–8.8 nm. The comparative figures for the *Nephrops* structures are length ≈7 nm, central channel 1.5 nm, centre-centre spacing 8.8 nm; figures which are similar to those derived from *Homarus* gap junctions. The connexons in gap junctions isolated from heart also have similar dimensions (Yeager and Gilula, 1992).

The connexons of isolated rat liver and heart gap junctions are thought to be composed of connexin 32 (Milks et al., 1988; Stauffer et al., 1991) and connexin 43 (Yeager and Gilula, 1992), respectively. There is no sequence similarity between these connexins and the 16-kDa proteins, although both connexins, as all other connexins so far identified, have four hydrophobic segments of sufficient length to span the bilayer as α helices. Connexin 32 is nearly twice the size of the 16-kDa protein and connexin 43 more than 2.5-times larger, yet the lengths of the respective hexagonal complexes they form are similar. This suggests there must be a larger amount of protein present in the region radial to the hexad axis of the connexon than that for the hexagonal complex of the 16-kDa protein. From the published data, it is unclear how this extra mass can be accommodated in the proposed model of connexin disposition in the bilayer as four transmembrane helices. For further discussion on this point see Yeager and Gilula (1992).

A family of related 16-kDa proteins; the *Ductins*

One of the results of our study shows that a protein which can form a gap-junction-like structure is the membrane component of a proton pump. The work of Israel and his collaborators (Israel et al., 1986; Birman et al., 1990) indicates that a highly similar 16-kDa protein is an essential component of the mediatophore thought to be involved in neurotransmitter release. Recent work (Umemoto et al., 1991) has shown that the yeast *VMA11* gene codes for a very similar (80% identity with the *VMA3* gene product) 16-kDa protein and that inactivation of this gene produces a similar phenotype as the *VMA3* mutants (growth at pH 5.5 but not at pH 7.5). A protein closely related to the *VMA11* product although smaller, has been identified in yeast. This product endows resistance to the drug trifluoperazine (Shih et al., 1990). Thus, there seems to be a family of closely related proteins which may have different transport functions. Perhaps a suitable name for this family of proteins is 'Ductins' (from the Latin *ducere*, *ductus*) to emphasise the role in membrane transport.

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REFERENCES

Apperson, M., Jensen, R. E., Suda, K., Witte, C. & Yaffe, M. P. (1990) *Biochem. Biophys. Res. Comm. 168*, 574–579.

Arai, H., Berne, M. & Forgac, M. (1987) *J. Biol. Chem. 262*, 11006–11011.

Arai, H., Terres, G., Pink, S. & Forgac, M. (1988) *J. Biol. Chem. 263*, 8796–8802.

Bianchet, M., Ysern, X., Hullihen, J., Pederson, P. L. & Amzel, L. M. (1991) *J. Biol. Chem. 266*, 21197–21201.

Birman, S., Meunier, F.-M., Lesbats, B., Le Caer, J-P., Rossier, J. & Israel M.(1990) *FEBS Lett. 261*, 303–306.

Boekema, E. J., Berden, J. A. & van Heel, M. G. (1986) *Biochim. Biophys. Acta 851*, 353–360.

Buultjens, T. E. J., Finbow, M. E., Lane, N. J. & Pitts, J. D. (1988) *Cell Tissue Res. 251*, 571–580.

Byler, D. M. & Susi, H. (1986) *Biopolymers 25*, 469–487.

Caspar D. L. D., Goodenough, D. A., Makowski, L. & Phillips, W. C. (1977) J. *Cell Biol. 74*, 605–628.

Denda, K., Konishi, J., Oshima, T., Date, T. & Yoshida, M. (1989) *J. Biol. Chem. 264*, 7119–7121.

Earnest, T. N., Herzfeld, J. & Rothschild, K. J. (1990) *Biophys. J. 58*, 1539–1546.

Finbow, M. E., Buultjens, T. E. J., Lane, N. J., Shuttleworth, J. & Pitts, J. D. (1984) *EMBO J. 3*, 2271–2278.

Finbow, M. E., Pitts, J. D., Goldstein, D. J., Schlegel, R. & Findlay, J. B. C. (1991) *Mol. Carcinog. 4*, 441–444.

Finbow, M. E., Eliopoulos, E. E., Jackson, P. J., Keen, J. N., Meagher, L., Thompson, P., Jones, P. C. & Findlay, J. B. C. (1992) *Protein Eng. 5*, 7–15.

Fromme, P., Boekema, E. J. & Graber, P. (1987) *Z. Naturforsch. 42c*, 1239–1245.

Futai, M. Noumi, T. & Maeda, M. (1989) *Annu. Rev. Biochem. 58*, 111–136.

Gillespie, G. A., Somlo, S., Germino, G. G., Weinstat-Saslow, D. & Reeders, S. T. (1991) *Proc. Natl Acad. Sci. USA 88*, 4289–4293.

Goldstein, D. J., Finbow, M. E., Andresson, T., McLean, P., Smith, K., Bubb, V. & Schlegel, R. (1991) *Nature 352*, 347–349.

Guarente, L., Yocum, R. R. & Gifford, P. (1982) *Proc. Natl Acad. Sci. USA 79*, 7410–7414.

Guthrie, C. & Fink, G. M. (1991) *Methods Enzymol. 194*, Academic Press.

Hanada, H., Hasebe, M., Moriyama, Y., Maeda, M. & Futai, M. (1991) *Biochem. Biophys. Res. Comm. 176*, 1062–1067.

Harris, J. R. & Holzenburg, A. (1989) *Micron Microsc. Acta 20*, 223–238.

Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E. & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899–929.

Israel, M., Morel, N., Lesbats, B., Birman, S. & Manaranche, R. (1986) *Proc. Natl Acad. Sci. USA 83*, 9226–9230.

Klug, A. & DeRosier, D. J. (1966) *Nature 212*, 29–32.

Lai, S., Watson, J. C., Hansen, J. N. & Sze, H. (1991) *J. Biol. Chem. 266*, 16078–16084.

Leitch, B. & Finbow, M. E. (1990) *Exp. Cell Res. 190*, 218–226.

Kauppinen, J. K., Moffat, D. J., Mantsch, H. H. & Cameron, D. G. (1981) *Appl. Spectrosc. 35*, 271–276.

Makowski L., Caspar, D. L. D., Phillips, W. C. & Goodenough, D. A. (1977) *J. Cell Biol. 74*, 629–645.

Mandel, M., Moriyama, Y., Hulmes, J. D., Pan, Y-C. E., Nelson, H. & Nelson, N. (1988) *Proc. Natl Acad. Sci. USA 85*, 5521–5524.

Markham, R., Frey, S. & Hills, G. J. (1963) *Virology 20*, 88–102.

Meagher, L., McLean, P. & Finbow, M. E. (1990) *Nucleic Acid Res. 18*, 6712.

Milks, L. C., Kumar, N., Houghten, R., Unwin, N. & Gilula, N. B. (1988) *EMBO J. 7*, 2967–2975.

Misell, D. L. (1978) *Practical methods in electron microscopy* (Glauert, A. M., ed.) Elsevier/North Holland, Amsterdam.

Morel, N., Israel, M., Synguelakis, M. & Le Gal la Salle, G. (1989) *Neurochem.* Int. 15, 169–177.

Moriyama, Y. & Nelson, N. (1989) *J. Biol. Chem. 264*, 3577–3582.

Nelson, H. & Nelson, N. (1990) *Proc. Natl Acad. Sci. USA 87*, 3503–3507.

Nelson N. & Taiz, L. (1989) *Trends Biol. Sci. 14*, 113–116.

Nolta, K. V., Padh, H. & Steck, T. L. (1991) *J. Biol. Chem. 266*, 18318–18323.

Noumi, T., Beltran, C., Nelson. H. & Nelson, N. (1991) *Proc. Natl Acad. Sci. USA 88*, 1938–1942.

Parry, R. V., Turner, J. C. & Rea, P. A. (1989) *J. Biol. Chem. 264*, 20025–20032.

Rash, J. E. & Yasamura, T. (1992) *Microsc. Res. Tech. 20*, 187–204.

Sebald, W. & Hoppe, J. (1981) *Curr. Top. Bioenerg. 12*, 1–64.

Shih, C-K., Wagner, R., Feinstein, S., Kanik-Ennulat, C. & Neff, N. (1988) *Mol. Cell. Biol. 8*, 3094–3103.

Sikerwar, S. S., Downing, K. H. & Glaeser, R. M. (1991) *J. Struct. Biol. 106*, 255–263.

Stauffer, K. A., Kumar, N. M., Gilula, N. B. & Unwin, N. (1991) *J. Cell. Biol. 115*, 141–150.

Uchida, E., Ohsumi, Y. & Anraku, Y. (1985) *J. Biol. Chem. 260*, 1090–1095.

Umemoto, N., Ohya, Y. & Anraku, Y. (1991) *J. Biol. Chem. 266*, 24526–24532.

Unwin, P. N. T. & Ennis, P. D. (1979) *Nature 307*, 609–613.

Unwin, P. N. T. & Zampighi, G. (1984) *Nature 283*, 545–549.

Valentine, R. C., Shapiro, B. M. & Stadtman, E. R. (1968) *Biochemistry 7*, 2143–2152.

Yeager, M. & Gilula, N. B. (1992) *J. Mol. Biol. 223*, 929–948.