Lipid−Protein Interactions and Assembly of the 16-kDa Channel Polypeptide from *Nephrops norvegicus*. Studies with Spin-Label Electron Spin Resonance Spectroscopy and Electron Microscopy†

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ABSTRACT: The assembly of 16-kDa polypeptide channel units in membranes from the hepatopancreas of *Nephrops norvegicus* has been studied both by electron microscopy and by the lipid−protein interactions reported with spin-labeled lipids. Membranes prepared by extraction with *N*-lauroylsarcosine and Triton X-100 have a low lipid/protein ratio (ca. 4−6.5 phospholipids and 1 cholesterol per 16-kDa monomer), and those prepared by alkaline extraction have a higher lipid/protein ratio (ca. 12−16 phospholipids and 3.5−4 cholesterols per 16-kDa monomer). In the membranes extracted with detergents, the protein is assembled in membrane sheets as hexagonally packed hexameric complexes, whereas the alkali-extracted preparations consist of closed vesicles in which the channel complexes are near randomly distributed. The electron spin resonance (ESR) spectra from lipids spin-labeled at the C-14 position of the (*sn*-2) chain show lower mobility for the membranes extracted with *N*-lauroylsarcosine than for the alkaline-extracted membranes. At higher temperatures, the ESR spectra reveal a population of lipids whose mobility is restricted by direct interaction with the intramembranous sections of the channel assemblies. The population of protein-associated spin-labeled phosphatidylcholine in the alkali-extracted membranes corresponds to 4−5 phospholipid molecules plus 1 cholesterol molecule per 16-kDa polypeptide monomer. These numbers are only slightly smaller than the number of lipid molecules that can be accommodated around the perimeter of the model proposed for the channel complex [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] that consists of a hexameric arrangement of transmembrane four-helix bundles. The 16-kDa polypeptide displays a selectivity relative to phosphatidylcholine for the negatively charged spin-labeled lipids, stearic acid, phosphatidylserine, and phosphatidylglycerol, suggesting that basic amino acid residues (possibly Lys-53, Lys-78, Lys-156, Arg-120, and Arg-127) are located close to the lipid headgroups in the channel assembly.

The 16-kDa protein that is isolated from the hepatopancreas of *Nephrops norvegicus* as ordered membrane arrays (Finbow et al., 1992) bears a strong sequence homology with the 16-kDa proteolipid of vacuolar H^+-ATPases (Mandel et al., 1988; Nelson & Nelson, 1989), as well as with other related proteolipids such as that of the acetylcholine-transporting mediatophore complex from synaptosomes (Birman et al., 1990). Not only does the 16-kDa protein from *Nephrops* contain a DCCD-reactive site but it is also capable of substituting for the V-ATPase proteolipid in yeast cells (Holzenburg et al., 1993; Harrison et al., 1994). Thus, this class of proteolipid constitutes the principal subunit of the vacuolar proton channel and is also directly related to the 8-kDa proteolipid of the proton pore of the F-type ATPases, as well as to a wider range of ion transport systems (cf. Forgac, 1992). The *Nephrops* preparations therefore offer a system suitable for structural studies that are relevant to the membrane arrangement of all of these proteins.

The ordered arrays of the *Nephrops* protein bear a close resemblance to gap junctions and have allowed the determination of a low-resolution projection corresponding to the hexameric complexes of the 16-kDa monomer (Holzenburg et al., 1993). Together with the primary sequence information and molecular modeling, this has also led to the proposal of a structure that consists of six transmembrane bundles, each comprising a 16-kDa monomer that is composed of four α-helices (Finbow et al., 1992). An alternative approach to investigating the assembly of the proteolipid channels is provided by studying the lipid−protein interactions. It is known that spin-labeled phospholipids are specifically restricted in their chain motion by direct interaction with the intramembranous hydrophobic surface of integral membrane proteins [see, for example, Marsh (1985)]. This therefore constitutes a means for determining the intramembranous perimeter and, hence, the state of supramolecular organization of the channel monomers by using electron spin resonance

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(ESR)[1] spectroscopy (Marsh, 1993; Findlay & Marsh, 1995). In addition, the possibility of a selectivity of interaction with charged lipids may form the basis for identifying the location of those basic residues on the protein that are in the vicinity of the lipid headgroups.

In the present work, we have studied the lipid interactions with the 16-kDa protein in membranous preparations from *N. norvegicus* by using spin-labeled lipids in combination with ESR spectroscopy. Not only have the ordered arrays been studied but the alkali-extracted membrane preparations of higher lipid/protein ratios have also been used in order to examine systems in which the interaction of the protein with lipid is maximized. The two membrane preparations have been characterized by electron microscopy and image analysis. From the ESR results, we have concluded that 4−5 phospholipids and a smaller number of cholesterol molecules per monomer are accommodated at the intramembranous perimeter of the 16-kDa channel assemblies and that these assemblies display a selectivity of interaction with certain negatively charged lipids. These results are compared with the results of image reconstruction from electron microscopy and with the molecular model proposed for the hexameric assemblies of the 16-kDa polypeptide.

MATERIALS AND METHODS

Materials. Spin-labeled stearic acids (*n*-SASL) were synthesized according to Hubbell and McConnell (1971). The spin-labeled phospholipids 14-PCSL, 14-PGSL, 14-PSSL, and 14-PASL were synthesized from 14-SASL as described in Marsh and Watts (1982).

Membrane Preparation. Detergent-extracted membranes containing the 16-kDa protein were isolated from the hepatopancreas of *N. norvegicus* as described previously (Finbow et al., 1984, 1992; Buultjens et al., 1988). Membranes were also isolated by an alkali-based procedure (Hertzberg, 1984; Leitch & Finbow, 1990). For this latter procedure, the hepatopancreases from 35 animals were homogenized and the pellet from low-speed centrifugation was collected (Finbow et al., 1984). A crude plasma membrane fraction was prepared on a sucrose step gradient (0/42% (w/v) sucrose, Beckman SW28 rotor, 25 000 rpm, 100 min; Leitch & Finbow, 1990) by first suspending the pelleted material in a final volume of 240 mL of 42% (w/v) sucrose. Material at the 0/42% interface was collected, centrifuged, and resuspended in water to a final volume of 40 mL. To 5-mL aliquots was added 5 mL of 40 mM NaOH, followed by sonication and centrifugation (30 min at 20 000 rpm in a Sorvall SS34 rotor; Hertzberg, 1984; Finbow & Meagher, 1992). The pellets were combined and dispersed in 12 mL of 6 M urea, and 15 mg of trypsin (Sigma, type XI) was added. The suspension was incubated at 37 °C for 30 min before centrifugation. The pellet was washed free of urea before suspension in 12 mL of water, and the final membrane fraction was then isolated by centrifugation on sucrose step gradients (32%/60%, w/v) as described previously (Finbow et al., 1984). The final pellet was suspended to a volume of 1 mL in water by brief sonication, and particulate matter was removed by very brief centrifugation in a microfuge. Yields of protein from 35 animals were approximately 2 mg by alkaline extraction and 4 mg by detergent extraction.

Membrane lipids were extracted according to Bligh and Dyer (1959). Analysis by thin-layer chromatography on silica gel with various solvent systems revealed phosphatidylcholine to be the major phospholipid with relatively little detectable aminophospholipid. Lipid phosphate was determined according to Eibl and Lands (1969), protein was determined according to either Lowry et al. (1951) or the modified version of Peterson (1977), and cholesterol was determined by the cholesterol oxidase assay from Boehringer (Mannheim, Germany). Mole ratios are calculated on the basis of the sequence molecular weight of the protein, which is 17 500 (Finbow et al., 1992).

Isopycnic Centrifugation. The buoyant density of the membrane preparations was measured by equilibrium sedimentation on potassium iodide gradients (Finbow et al., 1984). Samples (200 μL) of membranes were pelleted in a microfuge and resuspended in 400 μL of 40% (w/v) potassium iodide. The samples were layered on the bottom of a continuous gradient (20−40% KI) in tubes for the Beckman SW 40 rotor, and the gradients were centrifuged for 18 h at 35 000 rpm and 15 °C. The gradients were harvested by upward displacement with fluorochemical oil, and 0.6-mL fractions were collected. The refractive index of the fractions was measured on an Abbé refractometer. Peak fractions were analyzed by SDS−PAGE, after pelleting of the membrane material.

Electron Microscopy. Specimens for electron microscopy were prepared as described previously (Holzenburg et al., 1993). Grids were examined on a Phillips CM10 electron microscope operated at an accelerating voltage of 100 keV. Electron micrographs were recorded on Agfa Scientia 23 D 56 electron image sheet film at calibrated magnifications. Selected electron micrographs were digitized on a Joyce-Loebl rotating drum microdensitometer at 25-μm increments. Digital image analysis and processing (lattice refinement, calculation of Fourier projection maps) were carried out using the MRC Cambridge Image Processing System and the CCP4 program suite run on a VAX workstation 4000/60 under VMS [for details, see Holzenburg et al. (1994)] or using the PC-based image processing package CRISP (Hovmöller, 1992). Images and power spectra were displayed either on the workstation using the DSLOAD software package (Dr. E. P. Morris, Imperial College, U.K.) or within CRISP.

Spin Labeling. Membranes were first washed in 10 mM Hepes, 10 mM NaCl, and 10 mM EDTA (pH 7.8). Spin label was added with vortex mixing from a stock solution in ethanol (ca. 1 mg/mL) at a level of approximately 0.5 mol % relative to membrane lipid, such that the resulting ethanol concentration was no greater than 1%. The mixture was incubated at room temperature overnight, and then the membranes were pelleted by centrifugation at 6000 rpm in a Biofuge. The pellets (ca. 2 mg of protein) were resuspended in 60 μL of buffer and loaded into 1-mm-diameter glass capillaries that were then centrifuged, and the excess supernatant was removed to give samples with a height of 5 mm. The capillaries were then flame-sealed and transferred to the ESR spectrometer.

[1] Abbreviations: *n*-SASL, *n*-(4,4-dimethyloxazolidinyl-*N*-oxy)stearic acid; 14-PCSL, 14-PGSL, and 14-PSSL, 1-acyl-2-[14-(4,4-dimethyloxazolidinyl-*N*-oxy)stearoyl]-*sn*-glycero-3-phosphocholine, -phosphoglycerol, and -phosphoserine; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; ESR, electron spin resonance.

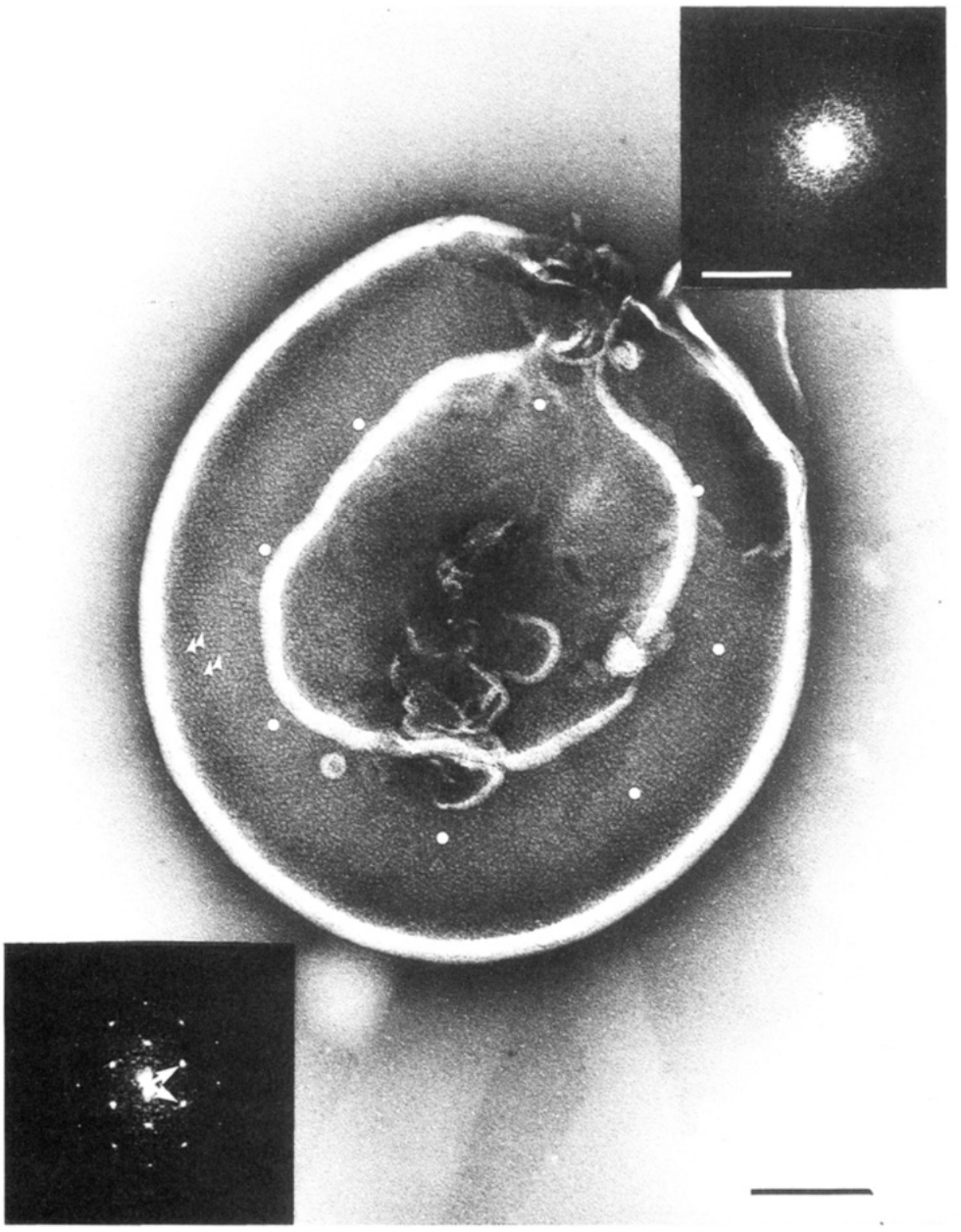

FIGURE 1: Figure 1. Electron micrograph of negatively stained (protein appears white) 16-kDa protein-containing membranes prepared by alkaline extraction. Stain-excluding complexes are clearly discernible (arrowheads). The scale bar corresponds to 200 nm. Upper insert: Power spectrum (scale bar = 0.2 nm^{-1}) of the area enclosed by the circles. Lower insert: Power spectrum from a corresponding micrograph of 16-kDa protein-containing membranes prepared by extraction with *N*-lauroylsarcosine. Unit cell dimensions derived from the lattice vectors (arrows) are $a = b = 8.8$ nm.

ESR Spectroscopy. ESR spectra were recorded on a Varian Century Line Series 9-GHz spectrometer equipped with a nitrogen gas-flow temperature regulation system. The sample capillaries were accommodated within standard 4-mm quartz ESR tubes containing light silicone oil for thermal stability. Temperature was measured by a fine-wire thermocouple located at the top of the microwave cavity within the silicone oil. Conventional, in-phase ESR spectra were recorded at a modulation amplitude of 0.5-G p-p and a modulation frequency of 100 kHz. Progressive saturation measurements were performed, and effective T_1T_2 relaxation time products were determined as described by Páli et al. (1993). Second-harmonic, out-of-phase saturation transfer ESR spectra were recorded and normalized integrals evaluated as described by Horváth and Marsh (1983). Spectral subtractions were performed as described in Marsh (1982) by using a spectral library for 14-PCSL in sonicated vesicles of dimyristoylphosphatidylcholine, and spectral simulations were performed according to Horváth et al. (1988).

RESULTS AND DISCUSSION

Membranous preparations of the 16-kDa protein from the hepatopancreas of *N. norvegicus* were prepared either by extraction with *N*-lauroylsarcosine or by alkaline extraction (see Materials and Methods). The membranes prepared by extraction with *N*-lauroylsarcosine were found to have a low phospholipid/protein ratio (4−6.5 mol/mol), whereas those prepared by alkaline extraction were found to have a higher phospholipid/protein ratio (12−16 mol/mol). As expected for a plasma membrane, the preparations were also found to contain cholesterol in addition to phospholipid. The cholesterol/protein ratio was found to be approximately 4 mol/mol for alkaline-extracted membranes and 1 mol/mol for those extracted with *N*-lauroylsarcosine. These values are

consistent with the buoyant densities of the two membrane preparations, which were measured to be 1.190 and 1.246 g/mL for the alkaline-extracted and sarcosyl-extracted preparations, respectively.

The characterization of the two membrane preparations by electron microscopy is presented first, followed by the analysis of the lipid−protein interactions resulting from spin-label ESR spectroscopy.

Electron Microscopy. A representative electron micrograph of negatively stained vesicular membranes prepared by alkaline extraction is shown in Figure 1. Electron micrographs from sarcosine-extracted membrane sheets have been found previously to display the ordered hexagonal lattice structure that is typical for these gap junction-like systems (Holzenburg et al., 1993). Electron microscope images of sarcosine-extracted membranes are also given in Finbow et al. (1984), Buultjens et al. (1988), and Leitch and Finbow (1990). In contrast, the images from the alkaline-extracted membranes in Figure 1 indicate a less ordered structure in which the stain-excluding regions are less densely packed. [This is particularly evident at higher magnification (data not shown) and from analysis of the power spectra, which is described in the following.] These differences of the alkaline-extracted preparation from the sarcosine-extracted preparation correlate with the higher lipid/protein ratio of the alkaline-extracted membranes.

Direct information on the lattice ordering and protein packing density is obtained from the power spectra of the micrographs that are given as inserts in Figure 1. The sarcosine-extracted membranes clearly show a hexagonal lattice in reciprocal space (lower insert, Figure 1). The corresponding center-to-center lattice spacing in real space is 8.8 nm, and the plane group is *P*6. The image from the alkaline-extracted preparation gives rise to a broad, halo ring in reciprocal space with no well-defined lattice points. The position of the center of this continuous ring corresponds to an average center-to-center distance in real space of 10 nm. Certain selected areas within the image give rise to power spectra with broad maxima at points arising from strongly distorted hexagonal domains arranged in a pseudo-polycrystalline fashion (data not shown). The average center-to-center distance within these areas also corresponds to approximately 10 nm. This increased center-to-center distance again correlates with the increased lipid/protein ratio of the alkaline-extracted membranes.

ESR Spectroscopy. The ESR spectra of the 14-PCSL phosphatidylcholine spin label in the 16-kDa protein-containing membranes prepared by extraction with *N*-lauroylsarcosine are given in Figure 2. The spectra are indicative of a high degree of restriction of lipid chain motion. This is consistent with the low lipid/protein ratio (ca. 4−6.5 mol/mol) of these samples that results in most of the lipids being in close contact with the 16-kDa protein assemblies. The 16-kDa protein has a high temperature stability (Holzenburg et al., 1993), allowing ESR measurements to be made up to elevated temperatures. The spectra in Figure 2 from the membranes extracted with *N*-lauroylsarcosine that are recorded at higher temperatures consist of two components, corresponding to the motionally restricted lipid chains in contact with the protein and to more fluid lipids (with intermediate spectral splittings) that are trapped within the protein aggregates [see, for example, Marsh (1989)]. Because of the extensive direct contacts between

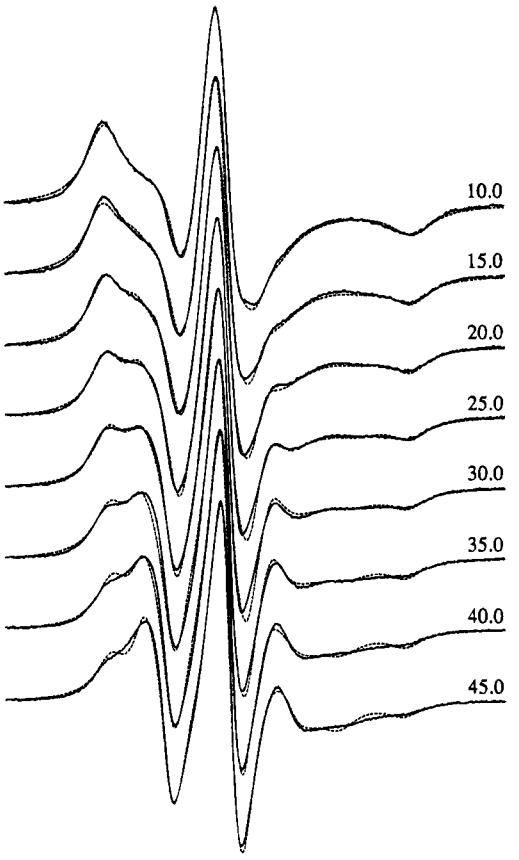

FIGURE 2: Temperature dependence of the ESR spectra of the 14-PCSL phosphatidylcholine spin label in 16-kDa protein-containing membranes from *N. norvegicus* prepared by extraction with *N*-lauroylsarcosine. Dashed lines are simulations of the spectra, assuming two components of different spectral anisotropy. Temperatures (°C) are indicated on the figure. Total scan width = 100 G.

the channel assemblies in the ordered arrays of the membranes extracted with *N*-lauroylsarcosine [see Holzenburg et al. (1993)], these spectra of the lipid spin label are unsuitable for determination of the intramembranous perimeter of a single channel unit. From simulations of the spectra obtained at higher temperatures (see Figure 2), approximately 30−40% of the lipids are found not to be motionally restricted.

The ESR spectra from the different spin-labeled lipids in 16-kDa protein-containing membranes of higher lipid/protein ratios (ca. 15 mol/16-kDa monomer) that are prepared by alkaline extraction are given in Figure 3. The spectra are recorded at higher temperature (45 °C) and clearly consist of two components: one corresponding to motionally restricted lipids and the other to fluid bilayer lipids (the sharper, three-line spectral component). The latter is rather similar to the spectrum of the same label in dispersions of the extracted membrane lipids, which consists of a single component but with considerably narrower line widths (spectra not shown). The relative population of the protein-interacting (i.e., motionally restricted) component for the phosphatidylcholine spin label clearly is smaller than that in the membranes prepared by *N*-lauroyl sarcosine extraction (cf. Figure 2), corresponding to the higher lipid/protein ratios of the alkali-extracted membranes. In addition, there is a clear selectivity of interaction with the protein between the different spin-labeled lipids given in Figure 3. The propor-

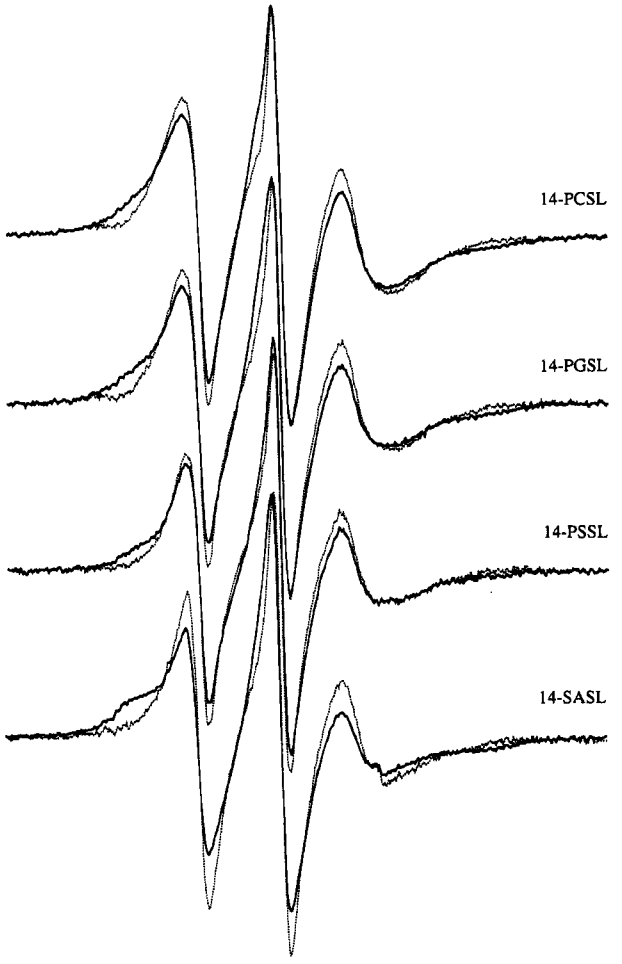

FIGURE 3: ESR spectra of spin-labeled lipids in alkali-extracted 16-kDa protein-containing membranes from *N. norvegicus*. Dashed lines are difference spectra obtained after subtracting the motionally restricted spectral component. Total scan width = 100 G; T = 45 °C.

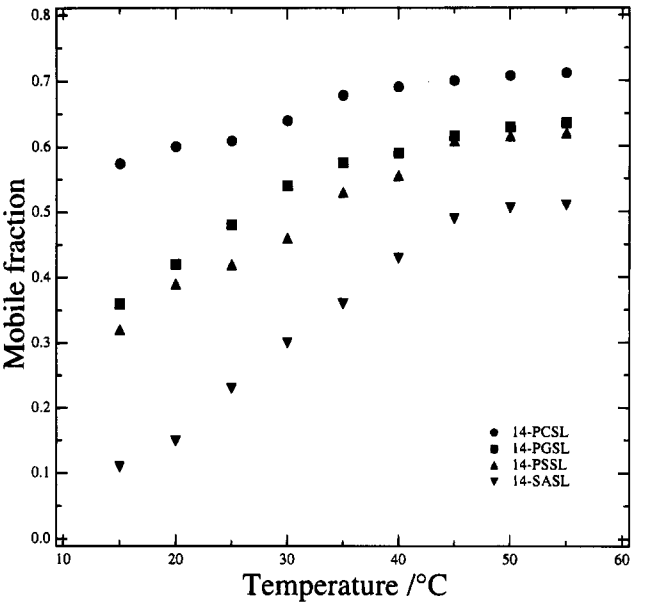

FIGURE 4: Temperature dependence of the fraction of fluid spin-labeled lipids, 1−f, in 16-kDa protein membranes from *N. norvegicus* prepared by alkaline extraction. Symbols for the different spin-labeled lipids are defined.

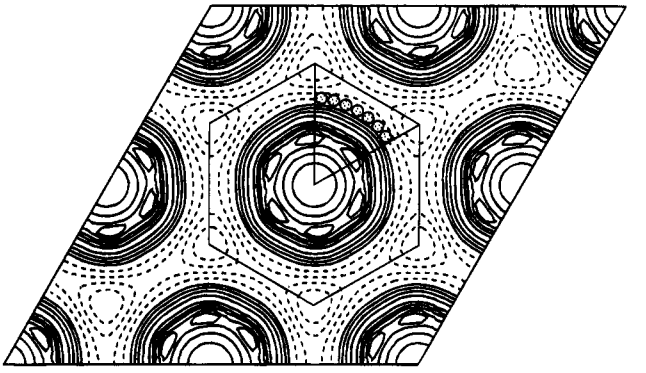

FIGURE 5: Fourier projection map (calculated in *P6*) of negatively stained ordered 16-kDa protein-containing membranes prepared by extraction with *N*-lauroyl sarcosine. The maximum level of stain exclusion corresponds to the six hexagonally arranged contours in the map. Contours shown for the exclusion of stain then decrease progressively upon moving radially outward and inward from this region. The outermost solid contour corresponds to the mean density of the map and to the 50% density level in the region of steepest contrast. The circles surrounding this contour have a diameter equal to that of a lipid chain (0.48 nm). The lengths of the sides of the four unit cells shown are 2 × 8.8 nm.

tion of the motionally restricted component is considerably greater for stearic acid, for instance, than it is for phosphatidylcholine.

The temperature dependence of the fraction, 1-f, of fluid lipids that is obtained by spectral subtraction (see Figure 3) for the four different spin labels in alkali-extracted membranes is given in Figure 4. The fraction of fluid lipids is lower at lower temperatures and increases with increasing temperature, reaching an approximately constant value at ca. 40−45 °C and above. This relatively steep temperature dependence of what ultimately becomes the full fluid lipid component presumably reflects the still rather high protein packing density, which has a very pronounced effect on the mobility of the lipids that are not in immediate contact with the protein. The limiting values at higher temperatures can be used to estimate both the stoichiometry and specificity of the lipid−protein interaction with the channel assemblies.

Stoichiometry of Lipid−Protein Interaction. Spin-labeled phosphatidylcholine has the lowest fraction, f, of motionally restricted lipid and therefore can be assumed to express little or no selectivity with respect to the background membrane lipids for interaction with the protein. In agreement with this, phosphatidylcholine is also found to be the major phospholipid of the 16-kDa membranes. Therefore, for spin-labeled phosphatidylcholine, the number of lipids surrounding the intramembranous perimeter of a channel assembly is given simply by $f \times n_t$, where n_t is the total number of membrane lipids per protein. This corresponds, in total, to approximately 4−5 phospholipid molecules plus 1 cholesterol molecule per 16-kDa protein monomer that are motionally restricted by the channel assemblies.

A Fourier projection map of the hexameric 16-kDa protein complex obtained from sarcosine-extracted membranes is given in Figure 5. This average image bears similarities to that of Sikewar et al. (1991) from *Homarus americanus* membranes, as might be anticipated (Holzenburg et al., 1993). The hexameric structure of the channel complex is seen from the density contours of the stain-excluding regions surrounding a central pit. Schematically, the lipid chains

directly surrounding the protein are indicated to scale by the circles in Figure 5. It is seen that approximately 14 lipid *chains* per monomer can be accommodated around the perimeter of the protein hexamer, allowing for both sides of the bilayer. A similar value of approximately 14 chains per monomer is also obtained from the perimeter of the molecular model for the protein that was proposed by Finbow et al. (1992). The latter consists of a hexamer of four-helix bundles, each of which corresponds to a 16-kDa monomer, surrounding a central channel in a star-shaped configuration. The value deduced from the molecular model directly relates to the intramembranous hydrophobic surface of the protein, but also correlates with the perimeter of the stain-excluding regions in Figure 5.

Because the cross-sectional area of a cholesterol molecule is approximately equal to that of a diacylphospholipid, the estimate from the molecular model is equivalent to approximately 5 diacylphospholipids and 1−2 cholesterols per 16-kDa monomer, at the phospholipid/cholesterol ratio determined for these membranes. This agrees quite well with the number of motionally restricted lipids deduced from the ESR measurements, although the value deduced from the latter is slightly smaller. The spin-label studies on the alkali-extracted membranes therefore are reasonably consistent with the mode of assembly of the monomer units, in terms of the extent of the intramembranous surface exposed to lipid, that was suggested on the basis of molecular modeling by Finbow et al. (1992).

It is interesting to note in comparison that another proteolipid, the myelin proteolipid protein, has also been proposed to be composed of a transmembrane four-helix bundle (Weimbs & Stoffel, 1992), for which approximately 18 phospholipids would be required to cover the intramembranous surface of the isolated monomer (Marsh, 1993). Lipid spin-label ESR measurements on this system have indicated, however, that only 10 ± 2 lipids per monomer are motionally restricted directly by this protein in reconstituted membranes (Brophy et al., 1984). This again is consistent with a hexameric arrangement of this proteolipid protein in the membrane, for which there is independent evidence from analytical ultracentrifugation studies in non-ionic detergents (Smith et al., 1984). It is possible that this is a common motif for such proteolipids with four putative transmembrane sections, including, for instance, the 16-kDa proteolipid of the vacuolar ATPase and possibly the dimer of the c-subunit of the F_1F_o-ATPase (cf. Holzenburg et al., 1993).

Selectivity of the Lipid−Protein Interaction. The relative selectivities of interaction of the various spin-labeled lipids with the channel assemblies can be deduced independent of assumptions regarding the effective lipid/protein ratios. The average relative association constant, K_r^{av}, of a lipid whose motionally restricted fraction is f, normalized to the corresponding values for phosphatidylcholine (PC), is given by (Marsh, 1989)

$$K_r^{av}/K_r^{PC} = [(1 - f)/f]^{PC}/[(1 - f)/f] \quad (1)$$

These average relative association constants for the different lipids are listed in Table 1, using the values of f deduced from the high-temperature measurements given in Figure 4 for the alkaline-extracted preparations. Qualitatively, a similar pattern of selectivity is seen in the spectra from these

Table 1: Fraction, f, of Motionally Restricted Spin-Labeled Lipids and Average Relative Association Constants, K_r^{av}, Normalized to the Values, K_r^{PC}, for Spin-Labeled Phosphatidylcholine[a]

spin label	f	K_r^{av}/K_r^{PC}
14-PCSL	0.29	1.0
14-PGSL	0.36	1.4
14-PSSL	0.38	1.5
14-SASL	0.49	2.4

[a] Deduced from the ESR spectra at 45−55 °C of 16-kDa protein-containing membranes from *N. norvegicus* prepared by alkali extraction (phospholipid/protein ratio ≈ 15 mol/16-kDa monomer).

Table 2: Saturation Transfer ESR Spectral Intensity (I_{ST}) and Effective T_1T_2 Relaxation Time Product, $(T_1T_2)^{eff}$, Determined from Progressive Saturation of the Conventional ESR Spectra for Spin-Labeled Stearic Acid (*n*-SASL) and Phosphatidylcholine (14-PCSL) in Sarcosyl-Extracted 16-kDa Membranes at 5 °C[a]

spin label	$I_{ST} \times 10^2$	$(T_1T_2)^{eff}$ (10^{-14} s^2)
14-SASL	0.33	5.6
16-SASL	0.43	5.2
14-PCSL	0.20	3.3

[a] I_{ST} is determined as described in Horváth and Marsh (1983), and $(T_1T_2)^{eff}$ is determined as in Páli et al. (1993).

labels in sarcosyl-extracted preparations (data not shown). In Table 1, selectivity is seen for the negatively charged lipids relative to zwitterionic phosphatidylcholine. It will be noted that the latter serves as a control for any nonspecific ionic interactions because it bears both a positively charged group and a negatively charged group and hence no net electrostatic charge. The selectivity found for negatively charged lipids even extends to phosphatidylglycerol, an anionic lipid that does not exhibit selectivity for many integral membrane proteins [see, for example, Marsh (1995)]. The higher selectivity observed for stearic acid may be due to the fact that this lipid possesses only a single chain, and this could allow the polar group to associate more closely with the protein side chains. The size of the average relative association constants does not, however, necessarily imply the existence of highly specific lipid binding sites. Rather it may correspond to a generalized increase in selectivity arising from the overall electrostatic environment in the region of the phospholipid headgroups adjacent to the protein (cf. Marsh, 1989).

Additional evidence for a preferential association of negatively charged lipids comes from the differential rates of reduction of the lipid spin labels at high pH and high temperature, presumably by reaction with the cysteine residue on the protein. The pseudo-first-order rate constants for reduction at 55 °C and pH 9.0 were 0.19, 0.12, 0.09, and 0.06 h^{-1} for 14-SASL, 14-PGSL, 14-PSSL, and 14-PCSL, respectively, which with the exception of phosphatidylserine are in the same order as the relative association constants given in Table 1. Also at low temperature, selectivity for stearic acid relative to phosphatidylcholine is evidenced by spin-label saturation experiments (see Table 2). Both the intensity of the saturation transfer ESR spectra and the effective T_1T_2 relaxation time product deduced from progressive saturation experiments are greater for stearic acid than for phosphatidylcholine, as a result of the preferential interaction of stearic acid with the protein (cf. Horváth et al., 1993). These results, therefore, all suggest that certain basic amino acid residues should be located close to the lipid headgroups in the three-dimensional structure of the channel

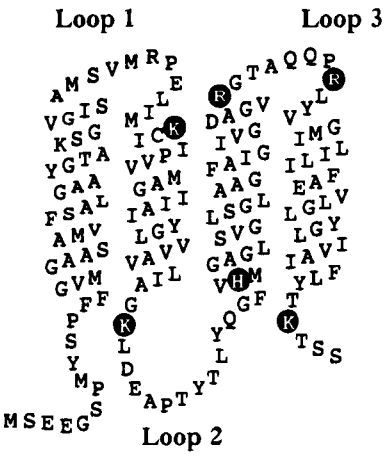

FIGURE 6: Transmembrane disposition of the amino acid residues of the 16-kDa polypeptide monomer according to the molecular model for the hexameric assembly proposed by Finbow et al. (1992). The extramembranous loops 1−3 linking the transmembrane segments 1 and 2, 2 and 3, and 3 and 4, respectively, are indicated. In the model, the transmembrane segments are arranged as four-helix bundles, with helix 1 directed toward the center of the assembly and helices 2−4 potentially accessible to lipid. Positively charged residues that may be located close to the lipid headgroup regions are highlighted. His-93 may be charged, depending on its pK_a.

assemblies. Again, this can be related to the molecular model proposed for the hexameric channel complexes (see Figure 6).

The *Nephrops* protein contains seven basic residues, six of which are conserved in the bovine 16-kDa protein and also in the 16-kDa proteins from *Drosophila* and yeast (Finbow et al., 1992). Of these, Lys-35 is on transmembrane segment 1, which is thought to line the interior aqueous pore, and therefore this residue would not be expected to come into contact with the lipid headgroups. Transmembrane segment 3 is that situated in the model at the outer perimeter of the channel and would have maximum contact with lipid. With the possible exception of His-93, this transmembrane segment has only one positively charged residue, Arg-120, that is part of loop 3 connecting with transmembrane segment 4. It is possible that this residue could be situated close to the lipid headgroups, as could Lys-156 located at the C-terminus beyond the end of transmembrane segment 4. Located near the beginning of transmembrane segment 4, possibly at the level of the lipid headgroups, is Arg-127, which is also in an orientation facing the lipid. The remaining basic residues are located in or near transmembrane segment 2. Arg-47 is in loop 1 connecting with transmembrane segment 1 and therefore is unlikely to come into close proximity with lipid. Lys-53 is contained in transmembrane segment 2 at a level within the phospholipid headgroup region and is oriented toward the lipid. Lys-78 (not conserved) is at the beginning of loop 2 joining transmembrane segments 2 and 3. Therefore, this residue is also in a region that is potentially exposed to the lipid headgroups. Thus, five of the seven basic residues (one not conserved) are located in the model of Finbow et al. (1992) in regions that potentially would give rise to selectivity for interaction with anionic lipids (cf. Figure 6).

In general, the observation of specificity for negatively charged lipids is consistent with the prediction that the transmembrane sections of the protein are connected by only short extramembranous loops, which brings several of the basic residues in the neighborhood of the phospholipid headgroups. Alternatively, the transmembrane segments may be shorter than those depicted in the model, which would place more basic residues in the connecting loops where they nonetheless might have the freedom to approach the phospholipid headgroup regions.

Conclusions. The lipid−protein interactions with the 16-kDa polypeptide in alkali-extracted membranes from *N. norvegicus* provide information on the way in which the monomer units are arranged in the supramolecular channel assemblies that are visualized by electron microscopy. The relatively small number of lipids that are in direct contact with the protein surface indicates that the protein monomers are assembled such that more than 50% of the protein surface is involved in intermolecular contacts and in lining the aqueous channel pore. This number is in reasonable agreement with the molecular model proposed on the basis of the primary sequence. The selectivity of interaction with negatively charged lipids locates some of the seven basic residues of the protein in the vicinity of the lipid headgroups. Among these, only Lys-35 and Arg-47 are excluded in the proposed model.

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