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Stoichiometry of lipid-protein interaction and integral membrane protein structure

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Abstract The stoichiometries of lipid-protein interaction obtained from spin label electron spin resonance experiments with integral membrane proteins are compared with simple geometric models for the intramembranous perimeter that are based on the predicted numbers of transmembrane helices. Deviations from the predicted values provide evidence for oligomerization of the protein in the membrane and/or more complex arrangements of the transmembrane segments.

Electron spin resonance (ESR) spectroscopy of spin-labelled lipids has been used widely to determine the stoichiometry of the motionally restricted population that is attributed to lipids interacting directly with the intramembranous surface of integral membrane proteins (see e.g. Marsh 1985). The fraction, *f*, of motionally restricted lipid is quantified from the two-component ESR spectra that are observed with phospholipid probes (≤ 1 mol%) that bear the spin label group close to the terminal methyl of the *sn*-2 chain (Marsh 1983). Analysis is performed by spectral subtraction using libraries of single-component experimental spectra, combined with spectral integration (Marsh 1982). The requirement for reliable spectral subtraction is that the exchange rate between the two lipid populations should be much slower than the nanosecond timescale of conventional spin-label ESR (Marsh and Horváth 1989; Marsh 1989). By direct measurement it is observed that these lipid exchange rates are typically in the region of $10^7\ s^{-1}$, and hence satisfy this criterion (Horváth et al. 1988a, b, 1990a, b, 1993, 1994; Ryba et al. 1987; Wolfs et al. 1989; Peelen et al. 1992).

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The stoichiometry of the protein-interacting lipid population, N_b lipids per protein monomer, is determined from the equation for equilibrium lipid exchange association with the protein (Brotherus et al. 1981; Marsh 1985):

$$N_b = n_t / [1 + K_r (1–f) / f] \quad (1)$$

where n_t is the total lipid/protein ratio in the membrane and K_r is the association constant of the spin-labelled lipid probe relative to that of the background, unlabelled host lipid. This equation applies at the probe amounts of the spin-labelled lipid that are used for the ESR experiment. The lipid stoichiometry is determined from titration of the lipid/protein ratio, n_t, in which case both N_b and K_r can be determined simultaneously. In such titration experiments, it is found that the stoichiometry of motionally restricted lipid remains constant, independent of the total lipid/protein ratio, demonstrating that this population corresponds operationally to the first shell of lipid surrounding the protein (e.g. Marsh and Watts 1982; Marsh 1985; Brotherus et al. 1981). Alternatively, a spin-labelled lipid that shows no selectivity relative to the host lipid (i.e., $K_r = 1$) is used to determine N_b for membranes with a fixed lipid/protein ratio. In many cases, it has been demonstrated directly by lipid/protein titration that spin-labelled phosphatidylcholine in a host membrane of unlabelled phosphatidylcholine fulfills this requirement that $K_r = 1$ (Knowles et al. 1979; Brotherus et al. 1981; Brophy et al. 1984; Wolfs et al. 1989; Horváth et al. 1990a, 1995), and similarly for spin-labelled phosphatidylglycerol in unlabelled phosphatidylglycerol (Sankaram et al. 1991; Kleinschmidt et al. 1997). In general, it is also found that the fraction of motionally restricted lipids does not vary appreciably with temperature, for non-selective lipids. This holds as long as the conditions for slow exchange are met and the resolution of the two-component ESR spectra is adequate for spectral subtractions to be performed reliably (Watts et al. 1979; Brotherus et al. 1980; Esmann et al. 1985; Pates et al. 1985; Pates and Marsh 1987; Ryba et al. 1987; Horváth et al. 1988a).

Previously, it has been found that the stoichiometry of first-shell lipids determined by ESR correlates reasonably well with the square root of the molecular weight of the

protein and with estimates of the number of lipids that may be accommodated at the protein perimeter, based on the limited amount of low-resolution structural information then available (e.g. Knowles and Marsh 1991). Currently a large body of amino acid sequence information is available for integral membrane proteins from which the numbers of putative transmembrane segments may be predicted (Kyte and Doolittle 1982; Engelman et al. 1986). Additionally, there is a growing number of high-resolution structures of integral proteins appearing, against which these predictions may be tested (see e.g. Montal 1996). It is therefore now possible to reassess the available spin label data on lipid-protein interaction stoichiometries in light of these predictions regarding the intramembranous organisation of the proteins. The purpose of this, in principle, is several fold: to verify or otherwise the predictions of transmembrane topology from amino acid sequence; to test molecular models for the assembly of the intramembranous segments; and to provide evidence for possible oligomerization or other association of the proteins within the membrane.

The principle applied here is a straightforward geometric one based on the likely packing motifs of transmembrane α-helices. Association of helices reduces the intramembranous surface exposed to lipid and correspondingly reduces the lipid-protein stoichiometry. For a linear array of n_α transmembrane helices of diameter D_α, the number of diacyl lipids that can be accommodated around the intramembranous perimeter in a bilayer configuration is (Marsh 1993):

$$N_b = \pi\,(D_\alpha / d_{ch} + 1) + 2\,(n_\alpha - 1) D_\alpha / d_{ch} \qquad (2)$$

where d_{ch} is the diameter of a lipid chain. For a helical sandwich arrangement, the number is smaller and is given by (Marsh 1993):

$$N_b = \pi\,(D_\alpha / d_{ch} + 1) + n_\alpha\, D_\alpha / d_{ch} \qquad (3)$$

This latter expression is generally valid for two-layer sandwiches ($n_\alpha > 1$). It may readily be shown that it applies also to regular polygonal arrangements of helices which contain a central pore. For multilayer sandwiches, Eq. (3) is valid for $1 < n_\alpha < 7$, but for $n_\alpha \geq 7$ centred hexagonal arrangements in which one helix does not come into contact with lipid are also possible. For such centred arrangements, Eq. (3) would apply but with n_α replaced by $(n_\alpha - 1)$, at least over the range of validity $7 \leq n_\alpha \leq 13$.

The predictions of Eqs. (2) and (3) are illustrated in Fig. 1, for linear arrays and helical sandwich arrangements, respectively. A value of $D_\alpha = 1.0$ nm, which corresponds to the mean separation of adjacent transmembrane helices in bacteriorhodopsin (1.00 ± 0.09 nm for bacteriorhodopsin, and 0.97 ± 0.11 nm for the photosynthetic reaction centre; Sansom and Kerr 1995), and of $d_{ch} = 0.48$ nm corresponding to the diameter of a lipid chain segment, were used for this figure. This implies that approximately 10 diacyl lipids can be accommodated around an isolated transmembrane helix in a bilayer membrane. The latter value is in agreement with the number of lipid chains found to contact a transmembrane polyalanine α-helix by molecular

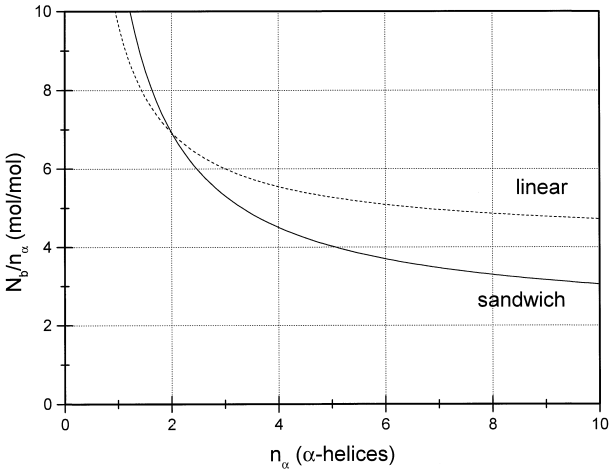

Fig. 1 Predicted dependence of the number of first-shell perimeter lipids, N_b, per transmembrane helix on the number of transmembrane helices, n_α, for monomeric polytopic integral proteins. The *dashed line* gives the prediction for a linear array of transmembrane helices, from Eq. (2), and the *full line* for a helical sandwich arrangement (or a regular polygon), from Eq. (3)

mechanics simulations (Wang and Pullman 1991). Intramembranous association of the transmembrane helices reduces the number of perimeter lipids per helix below the value of 10 expected for a monotopic integral protein, to an extent that depends on the number of helices per protein monomer and on their packing arrangement (Fig. 1). Similar considerations apply also to the oligomeric assembly of whole integral proteins. The number of lipids at the perimeter of the oligomer would be given by Eq. (2) or Eq. (3), depending on the oligomeric packing, where D_α is the diameter of the protein monomer and n_α then represents the aggregation number. Relations such as that given in Fig. 1 correspondingly would represent the decrease in number of perimeter lipids per protein monomer with degree of oligomerization (on the abscissa). In fact, because $N_b = 10$ for a single transmembrane helix (i.e. for $n_\alpha = 1$), the ordinate in Fig. 1 is just ten times the fractional number of perimeter lipids per monomer, relative to an isolated monomer, for this particular case.

The number of motionally restricted first-shell lipids per protein monomer, N_b, obtained from measurements with lipid spin labels, is given as a function of the predicted number of transmembrane helices per monomer, n_α, in Fig. 2. It is seen that the experimentally determined number of first-shell lipids increases with the intramembranous size of the protein (represented by n_α), as would be expected. Of interest, however, is the agreement with or deviation from the predicted dependence on the putative number of transmembrane helices. The continuous line in Fig. 2 represents the predictions from Eq. (3) for isolated protein monomers with a helical sandwich structure. In Eq. (3), the fractional error in the estimation of n_α is $(1 + \pi/n_\alpha)$ times the fractional uncertainty in D_α, for fixed N_b. Conversely, the range of predicted values of N_b arising from the standard deviation of the interhelical distances found for bac-

teriorhodopsin is indicated in Fig. 2, for $n_\alpha = 15$. The dotted and dashed lines in Fig. 2 represent predictions for protein dimers and hexamers, respectively. For these latter, it is assumed that oligomerization takes place with preservation of the helical sandwich structure. The number of perimeter lipids per protein monomer is then given from Eq. (3) by:

$$N_b = (\pi / n_{agg}) (D_\alpha / d_{ch} + 1) + n_\alpha D_\alpha / d_{ch} \quad (4)$$

where n_{agg}, the aggregation number, is the number of monomers per oligomer. The uncertainty in N_b arising from that in D_α is directly correlated between oligomers and monomers (i.e., the relative differences are preserved). If the helix packing at the oligomer interfaces is not so close as in a helical sandwich, the value of N_b will be greater than predicted by Eq. (4). Then the effective value of n_α must be increased by an amount corresponding to the voids at the oligomer interfaces.

It is seen from Fig. 2 that rhodopsin (Rho), which is a classical 7-helix sandwich (Schertler and Hargrave 1995) and is known to be monomeric in rod outer segment and certain reconstituted membranes (see e.g. Ryba and Marsh 1992), has a value of $N_b = 22–25$ which corresponds very well to the predictions of Eq. (3). (For the range in D_α given above for the analogous 7-helical bacteriorhodopsin, the range of values predicted from Eq. (3) is $N_b = 24 \pm 2$.). A mutant L37A of phospholamban (PLB) contains a single putative transmembrane stretch and has a value of $N_b = 12$ which is consistent with the prediction for a single helical monomer. Wild-type phospholamban and the phosphorylated forms exhibit lower lipid stoichiometries that are consistent with degrees of oligomerization varying from a dimer to a pentamer (Cornea et al. 1996). The M13 bacteriophage coat protein in its α-helical form also has a single putative transmembrane stretch, but its lipid stoichiometry is low, indicating the presence of an aggregated species as suggested originally for this reconstituted system (Peelen et al. 1992).

The myelin proteolipid protein (PLP) monomer is thought to consist of a 4-helix bundle (Weimbs and Stoffel 1992), but the low lipid stoichiometry is consistent with the predictions for a hexameric oligomer, for which there is independent experimental evidence (Smith et al. 1984). (For the lipid stoichiometry of the myelin proteolipid to correspond with a monomer in the membrane would require an effective helix diameter $D_\alpha = 0.46$ nm, which is much too small.) The 16-kD channel polypeptide from *Nephrops* is a proteolipid that is also thought to form a 4-helix bundle and has strong sequence similarities with the proteolipid subunits of the vacuolar ATPases (Holzenburg et al. 1993). Electron microscopy reveals hexameric arrays for the 16-kD polypeptide, with a central channel which accounts for the low stoichiometry relative to the prediction for a hexamer based on a helical sandwich structure (Páli et al. 1995).

Recent spin label studies on cytochrome *c* reductase (CR) have yielded a value of $N_b = 35–40$ lipids per cytochrome c_1 (Powell et al., submitted). The preliminary x-ray structure for the beef heart enzyme demonstrates that $n_\alpha = 13$ (Yu et al. 1996). This is reasonably consistent with the predictions of Eq. (3), although the crystal structure is dimeric and it is not yet known how much lipid is present at the dimer interface. The ADP/ATP translocator contains an apparent threefold internal repeat, each of which is thought to possess a hydrophobic and an amphipathic transmembrane helix (Aquila et al. 1985). The lipid stoichiometry of the ADP/ATP translocator is consistent with six, or more, transmembrane helices, but the interpretation is complicated by the fact that the active species is considered to be a dimer (Klingenberg 1985; Horváth et al. 1989).

Although the overall molecular masses of the Ca-ATPase and Na,K-ATPase differ considerably, the catalytic subunits of both these P-type ATPases are thought to have equal numbers of transmembrane segments, normally assumed to be 10 (Møller et al. 1996). The Na,K-ATPase has an additional transmembrane segment contributed by the β-subunit (Lingrel and Kuntzweiler 1994). Surprisingly, therefore, the lipid stoichiometries of the Ca-ATPase and the Na,K-ATPase (designated by Ca and NaK in Fig. 2) differ to a much greater extent than would be predicted merely by the additional presence of the β-subunit in the latter. This suggests that the intramembranous structures of these two ion-transport ATPases may differ more than has been previously assumed.

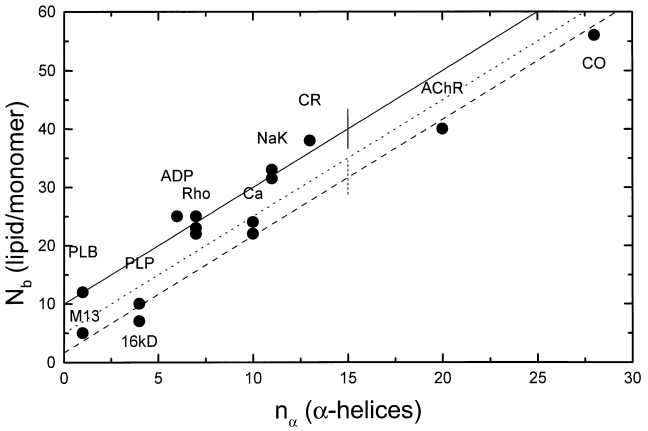

Fig. 2 Dependence of the number of first-shell motionally restricted lipids, N_b, per protein monomer on the predicted number of transmembrane helices per monomer for various integral membrane proteins. *M13*, M13 bacteriophage coat protein (Peelen et al. 1992); *PLB*, L37A mutant of phospholamban (Cornea et al. 1996); *PLP*, myelin proteolipid protein (Brophy et al. 1984; Sankaram et al. 1991); *16kD*, 16-kD channel polypeptide from *Nephrops norvegicus* (Páli et al. 1995); *ADP*, ADP/ATP translocator (Horváth et al. 1990a); *Rho*, rhodopsin (Watts et al. 1979; Pates et al. 1985; Ryba et al. 1987); *Ca*, Ca-ATPase (Thomas et al. 1982; Silvius et al. 1984); *NaK*, Na,K-ATPase (Brotherus et al. 1981; Esmann et al. 1985, 1988); *CR*, cytochrome *c* reductase (Powell et al. 1997); *AChR*, nicotinic acetylcholine receptor (Ellena et al. 1983); *CO*, cytochrome *c* oxidase (Knowles et al. 1979). The *solid line* is the predicted dependence for monomeric helical sandwiches [Eq. (3)], and the *dotted* and *dashed lines* are the corresponding predictions for protein dimers and hexamers, respectively [Eq. (4)]. The *vertical lines* at $n_\alpha = 15$ represent the (correlated) uncertainty in N_b corresponding to the standard deviation of the mean interhelical distance in bacteriorhodopsin ($D_\alpha = 1.00 \pm 0.09$ nm)

The nicotinic acetylcholine receptor (AChR) is an $\alpha_2\beta\gamma\delta$ heteropentamer, each subunit of which has been thought to contain four transmembrane segments. The lipid stoichiometry measured is, however, considerably smaller than that predicted for a pentamer of 4-helix bundles. The receptor channel structure determined by electron crystallography suggests, however, that only one transmembrane helix is contributed by each subunit and that the remainder of the transmembrane sections of the protein may be in a β-sheet structure (Unwin 1993). The recently determined x-ray structure for cytochrome *c* oxidase (CO) containing all 13 subunits shows that there are in total 28 transmembrane α-helices in the protein monomer (Tsukihara et al. 1996). In the crystal, the protein is present as a dimer. The lipid stoichiometry determined experimentally in reconstituted membranes (N_b = 56 per 204 kDa protein monomer) is, nonetheless, smaller than that predicted for a dimer of this size. This indicates that some of the helices are internal and do not contact lipid at all, in agreement with the x-ray structure. The effective number of internal helices estimated from Eq. (4) and the spin label data is approximately three. This is a lower estimate, however, because the dimer interface in the crystal structure does not correspond entirely to a close-packed helical sandwich that is assumed in Eq. (4).

The transmembrane segments for the preponderance of integral proteins whose structure is known consist of α-helices. Nevertheless, it is of considerable interest to compare the above analysis with the lipid stoichiometries expected for transmembrane β-sheet structures, such as the β-barrels of the porin family (e.g. Marsh 1996). These represent the other class of secondary structure that is energetically allowable for transmembrane protein segments exposed to lipid. The number of diacyl lipids that can be accommodated at the perimeter of a transbilayer β-barrel is given approximately by:

$$N_b = n_\beta D_\beta \; / \; (d_{ch} \cos \vartheta_\beta) \qquad (5)$$

where D_β (= 0.47 nm; Fraser and MacRea, 1973) is the interstrand separation, ϑ_β is the tilt of the β-strands relative to the membrane normal and n_β is the number of β-strands per protein monomer. Because β-strands are much more extended peptide structures than are α-helices, a tilt of $\vartheta_\beta \approx 60°$ is required for the same number of nonpolar residues to be accommodated within the membrane as for an α-helix (Marsh 1993, 1996). A tilt of this magnitude is achieved by staggering the hydrogen bonding between adjacent strands by two residues relative to an untilted structure (Mannella et al. 1992; Marsh 1997). Alternatively, doubling the number of transmembrane strands, by creating a β-hairpin structure with a reverse turn at the centre of the putative transmembrane segment, would achieve a similar effect. Noting that $D_\beta \approx d_{ch}$, it is predicted from Eq. (5) that $N_b \approx n_\beta$ for n_β untilted β-strands, and $N_b \approx 2n_\beta$ for n_β 60°-tilted strands or β-hairpins, in a β-barrel structure. The lipids are assumed to be accommodated only at the outer face of the β-sheet, in all these cases.

The stoichiometry of lipid interaction with transmembrane β-sheets has been determined by spin label ESR for a peptide corresponding to the single putative transmembrane sequence of a small protein (IsK) which, on expression, induces slowly activating voltage-gated K^+ channels (Horváth et al. 1995; Aggeli et al. 1996). This peptide has an apolar stretch of 23 amino acid residues, which is sufficient to form a membrane-spanning α-helix. However, the peptide reconstituted in phosphatidylcholine bilayer membranes is present wholly in a β-sheet conformation. The stoichiometry of motionally restricted lipids is N_b = 2.5 per peptide monomer. This low lipid stoichiometry is consistent with Eq. (5) if the peptide strands are transmembrane and are strongly tilted at an angle of $\vartheta_\beta \approx 60°$, for which there is independent evidence from infrared spectroscopy (Aggeli et al. 1996).

ESR determinations of lipid stoichiometry have also been made for the polymeric β-sheet form of the M13 bacteriophage coat protein (Wolfs et al. 1989; Peelen et al. 1992). This is a small bipolar protein with a single 19-residue hydrophobic stretch. The lipid stoichiometry was found to be $N_b \approx 4$ per protein monomer, which is considerably smaller than predicted for an isolated transmembrane helix, but greater than that obtained for the IsK peptide. This stoichiometry can be explained for a β-sheet structure if the strands are tilted at 60° or form β-hairpins, and lipids are trapped at *both* faces of the β-sheet within the polymeric aggregates. The hydrophobic stretch of the β-sheet form of the protein would then also match that of the lipid bilayer.

The survey of the available spin label results on lipid-protein stoichiometries given here demonstrates that such data can be of considerable help in investigating the intramembranous organization and assembly of integral proteins. In particular, these methods may now be used with some confidence to test molecular modelling (Páli et al. 1995), investigate the assembly of putative transmembrane peptides (Horváth et al. 1995; Marsh 1996), and for verification of the structural integrity of the intramembranous sections of large polytopic proteins that have been simplified by extensive proteolysis (Esmann et al. 1994). Furthermore, this approach can readily be applied to study quantitatively changes in the oligomeric association of integral proteins in membranes (Ryba and Marsh 1992; Cornea et al. 1996).

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