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Minireview

Lipid-protein interactions in membranes

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The interactions of lipids with integral and peripheral proteins can be studied in reconstituted and natural membranes using spin label electron spin resonance (ESR) spectroscopy. The ESR spectra reveal a reduction in mobility of the spin-labelled lipid chains on binding of peripheral proteins to negatively-charged lipid bilayers. A selectivity of interaction has been established for different lipid species, and in certain cases evidence is obtained for a partial penetration of the peripheral proteins into the membrane. The latter may be relevant to the import mechanism of apocytochrome *c* into mitochondria. Integral proteins induce a more direct motional restriction of the spin-labelled lipid chains, allowing the stoichiometry and specificity of the interaction, and the lipid exchange rate at the protein interface to be determined from the ESR spectra. In this way, a population of very slowly exchanging cardiolipin associated with the mitochondrial ADP-ATP carrier has been identified. The residues involved in the specificity for charged lipids of the myelin proteolipid protein have been localized to the deletion in the DM-20 mutant, and the difference in lipid-protein interactions with the β-sheet and α-helical conformations of the M-13 coat protein, has been characterized.

Integral protein; Peripheral protein; Lipid-protein interaction; Spin label; Electron spin resonance

1. INTRODUCTION

The basic building block of biological membranes is the lipid bilayer. Peripheral proteins, which may have either a structural or a functional role, are associated, largely electrostatically, at the membrane surface. Integral proteins, which are responsible for transmembrane communication and many of the functional interactions within the plane of the membrane, are embedded in and traverse the lipid bilayer. Nascent peripheral proteins, and also many soluble proteins, must pass through one or more membranes during biosynthesis. Lipid-protein interactions are, therefore, an essential feature of membrane makeup and, indeed, of the life of the cell itself.

Various peripheral proteins are activated on binding to membranes, in a manner which in several cases is critically dependent on the lipid composition of the membrane. Certain integral proteins require specific lipids for optimal function and can be inhibited by other lipid species. In general, the activity of integral membrane enzymes is often sensitive to the lipid bilayer dynamics, and the aggregation state of integral proteins is frequently dependent on the lipid composition and state.

The structural and dynamic aspects of lipid-protein interactions can only be investigated directly by physical methods. Of these methods, magnetic resonance spectroscopy, particularly electron spin resonance of spin-labelled membrane components, has proved to be especially useful. The timescale of spin label ESR is such that, even if the lipid diffusion rate is comparable to that in fluid lipid bilayers, the population of lipids interacting directly with the membrane proteins can be resolved from that of the bulk lipids. In this way, it has been possible to identify the boundary layer, or first shell, of lipids surrounding integral proteins.

For peripheral proteins, the surface interactions have a less pronounced effect on the mobility of the lipid chains than does the intramembranous section of integral proteins, and the lipids interacting directly with the protein are not resolved from the bulk lipids. However, direct interactions with the lipid chains are detected for certain peripheral proteins. These can be taken as indicative of membrane penetrant sections of the protein, which may have particular significance for the transport of precursor proteins across membranes.

2. LIPID-PROTEIN ASSOCIATIONS

The association of lipids with membrane proteins can be analysed by the multi-site models for the association of ligands with soluble proteins. The difference is that, in the membrane case, the lipid sites on the protein are always fully occupied. The exchange equilibrium between two lipids, L and L*, competing for sites on the

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protein is depicted by:

$$PL + L^* \rightleftharpoons PL^* + L$$

and the association constant for lipid L* relative to that for lipid L is given by:

$$K_r = [PL^*]\,[L]/[PL]\,[L^*] \quad (1)$$

The corresponding equation for lipid-protein association is [1]:

$$N_b = f\{n_t^* + n_t/[f + K_r(1-f)]\} \quad (2)$$

where f is the fraction of the lipid L* which is associated with the protein, n_t^* and n_t are the total numbers of L* and L lipids, respectively, per protein, and N_b is the number of lipid association sites on the protein (cf. Fig. 1). This equation may therefore be used to analyse both the stoichiometry (N_b) and the specificity (K_r) of the lipid/protein interaction. In the case of spin-labelled lipids, L* is present only in probe amounts, i.e. $n_t^* \approx 0$.

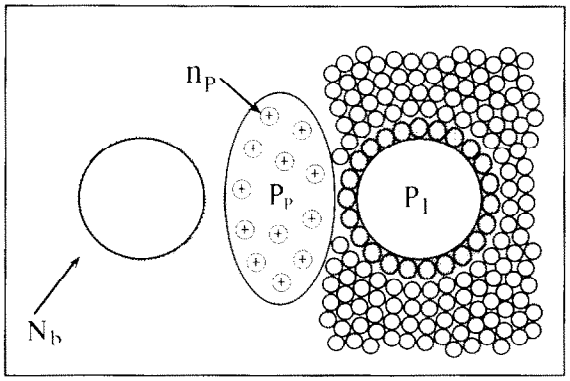

Fig. 1. Schematic indication of the association of lipids with integral and peripheral membrane proteins. The integral protein (P_I) is surrounded by a first shell of N_b lipids which interact with the hydrophobic surface of the protein. The peripheral protein (P_P) is bound at the polar surface of the lipid bilayer and bears n_P specific association sites ⊕ at which the lipid headgroups can associate. The exchange equilibrium association is described by Eqn 2, where $N_b \equiv n_P$ in the case of peripheral proteins (see [2,30,31]).

3. ASSOCIATION WITH INTEGRAL PROTEINS

3.1. *Lipid stoichiometry*

In experiments with spin-labelled lipids, the fraction, f, of lipids associated with the integral protein can be determined directly by quantitating the resolved lipid components in the ESR spectrum. In general, the stoichiometry of association (N_b) agrees well with the size of the intramembranous perimeter of the proteins involved and therefore the association sites may be identified with the first shell, or boundary layer, of lipids solvating the protein (cf. Fig. 1; reviewed in [2]). In reconstituted systems, there is found to be a fixed number of sites per protein independent of the total lipid/protein ratio. Only at low lipid/protein ratios does the stoichiometry decrease, corresponding to non-specific aggregation. In several systems, the stoichiometry scales with the square root of the protein molecular weight, as would be expected for proteins of roughly cylindrical intramembranous cross-section with approximately equivalent proportions of the protein protruding from the membrane. Values vary from approximately 60 lipids per Na,K-ATPase dimer to 22 lipids per rhodopsin monomer. In cases where the proteins form stable aggregates, the monomeric stoichiometry is correspondingly reduced, e.g. for the myelin proteolipid protein [3] and for the M13 phage coat protein [4].

3.2. *Lipid specificity*

A selectivity of interaction for different lipid species is found with many integral proteins, rhodopsin and the Ca-ATPase being exceptions (reviewed in [5]). This selectivity can be quantitated in terms of the relative association constants, K_r, which again are obtained from the values of the fractional degree of association, f, deduced directly from the ESR spectra of different spin-labelled lipids (cf. Eqn 2). In general, phosphatidylcholine exhibits one of the lowest selectivities, with that for phosphatidylethanolamine sometimes being somewhat lower. Preferential selectivities are normally found for negatively charged lipids, an exception being phosphatidylglycerol. Either cardiolipin (diphosphatidylglycerol), phosphatidic acid, or free fatty acid, are found to have one of the highest selectivities, depending on the particular protein.

Values of K_r range up to 5–6 (or even higher), relative to phosphatidylcholine, e.g. for cardiolipin associated with cytochrome oxidase [6]. The latter may be of functional significance in the inner mitochondrial membrane, since cardiolipin is found to be an efficient activator of cytochrome oxidase ([7,8] and references therein). Interestingly, gangliosides show relatively little selectivity relative to phosphatidylcholine in the membrane systems so far tested [9,10]. However, gangliosides are not deselected for, indicating that their unique headgroup structure may play a significant role at the lipid-protein interface. In plant membranes, the lipid composition is very different from mammalian and other systems. For thylakoid membranes, monogalactosyl diglyceride appears to take the part played by phosphatidylcholine in non-plant systems, and phosphatidylglycerol displays a marked selectivity in this system [11].

In general, lipid selectivity is dependent on ionic strength and on the protonation state of the lipid [12–14]. Such experiments indicate that the source of the selectivity is not in all cases solely electrostatic. Covalent modification has indicated that lysine residues are involved in the lipid selectivity of cytochrome oxidase [13], and the subunits bearing some of the lysine groups involved have been identified [15]. The polar region (residues 116–150) that makes a major contribu-

tion to the lipid selectivity of the myelin proteolipid protein has been located with the help of the DM-20 proteolipid mutant which lacks this portion of the sequence [16].

In addition, spin-labelled local anaesthetic analogues have been shown to substitute, with a greater specificity than for phospholipids, in sites at the lipid-protein interface [17,18]. These associations correlate directly with the function of the local anaesthetics, which have been shown to act as non-competitive blockers at multiple sites on the acetylcholine receptor [19].

3.3. *Lipid exchange rates*

Analysis of the line broadening in the two-component ESR spectra from spin-labelled lipids, can yield information on the dynamics of lipid exchange at the protein interface [20,21]. The exchange rate is determined by τ_b^{-1}, which is the first-order rate constant for exchange of the protein-interacting lipid component. These off-rates are generally in the region of 10^7 s^{-1} [22], which is of the same order but significantly slower than the free diffusion rates in lipid bilayers [23,24]. An interesting exception is the M13 phage coat protein, for which the interfacial lipid exchange rate is 4× slower for the β-sheet conformation than for the α-helical conformation of the protein [25]. This difference in the lipid-protein interaction with the β-sheet conformation may be directly relevant to the outer membrane proteins of *E. coli*.

The selectivity of the lipid-protein interaction is reflected in the exchange dynamics. Combination of Eqn 2 with the condition for mass balance, yields the following relationship between the lipid exchange rate and relative association constant [2]:

$$\tau_b^{-1}/\tau_f^{-1} = (n_t/N_b - 1)/K_r \quad (3)$$

If it is assumed that the on-rate, τ_f^{-1}, is the same for all lipids (i.e. is diffusion-controlled), the ratios of the off-rate constants for different lipids would be expected to be in the inverse ratio of the relative association constants. This is found to be the case both for the myelin proteolipid protein [14,20,21] and for the M-13 coat protein [4]. A very significant exception is the interaction of cardiolipin with the ADP-ATP carrier, for which a specific population of sites was found, at which the exchange of cardiolipin was extremely slow [26].

4. ASSOCIATION WITH PERIPHERAL PROTEINS

4.1. *Surface effects*

The binding of most peripheral proteins to negatively charged lipids is predominantly of an electrostatic nature, being reduced by increased ionic strength, and also being susceptible to pH titration. The overall result of this surface interaction is generally to increase the lipid packing density, which has the effect of reducing the extent of lipid chain motion as recorded by spin label ESR spectroscopy [27–29]. The extent of this perturbation, at saturation binding, is found to be in the order: myelin basic protein ≈ apocytochrome *c* » cytochrome *c*, corresponding to the better ability of the random coil proteins to adapt to the surface distribution of lipid charges, than for the compact globular cytochrome *c* molecule. The lipid/protein stoichiometries determined by saturation binding are, for example, approximately 8–9 mol/mol for cytochrome *c* and apocytochrome *c* [27], and 36 mol/mol for myelin basic protein [28]. This corresponds with the available number of positively charged residues (31) in the case of myelin basic protein and with the *net* positive charge (+9 at neutral pH) in the case of (apo)cytochrome *c*, and also approximately with the maximum amount of protein that can be accommodated at the lipid surface.

4.2. *Lipid selectivity*

A selectivity of interaction is evidenced by the varying extents to which the chain mobility of different spin-labelled lipids is perturbed by the binding of peripheral proteins to negatively charged lipid bilayers [30,31]. The selectivity patterns so established are listed in Table I. These sequences correspond only to low concentrations of the test lipid in the negatively charged lipid host which is obligatory for the overall binding of the protein. Although, for myelin basic protein, the selectivity of interaction corresponds approximately with the strength of binding to the individual lipids, especially with respect to the strong binding to phosphatidylserine [32], this need not necessarily be the case. With myelin basic protein and the simple homo-polypeptide polylysine, the specificity patterns conform to the expectation that negatively charged lipids display a selective interaction over zwitterionic lipids, although there is some selectivity between lipids with the same formal charge. For the other proteins, a more complex specificity pattern is evidenced, indicating the importance of factors other than simple electrostatics, e.g. steric interactions and hydration.

In addition to the proteins already mentioned, an interaction of the mitochondrial creatine kinase with negatively charged cardiolipin, most likely the receptor site for this protein, has been detected by spin label ESR spectroscopy [33]. The lipid selectivity between the different protonation states of fatty acids have also been determined for a range of different peripheral proteins [34]. The shifts in the pK_a of the fatty acid have provided strong evidence for a dehydration of the lipid surface on binding of the peripheral proteins.

Experiments with myelin basic protein [31] and with apocytochrome *c* [35] have been performed with mixtures of negatively charged lipids with zwitterionic phosphatidylcholine. At relatively low concentrations, the effects of phosphatidylcholine admixture are those that would be predicted from Eqn 2, on the basis of the

Table 1

Selectivity of interaction of spin-labelled phospholipids with peripheral proteins bound to dimyristoyl phosphatidylglycerol bilayers [30,31]

myelin basic protein	$PS^- > CL^- > PA^{2-} > PG^- > PI^- > PA^- > PE^\pm > PC^\pm$
apocytochrome *c*	$PI^- > CL^- > PS^- > PC^\pm > PG^- > PE^\pm$
cytochrome *c*	$PI^- > PG^- > CL^- > PS^- \approx PC^\pm > PE^\pm$
lysozyme	$CL^- > PG^- >> PE^\pm > PC^\pm > PS^- > PI^-$
polylysine	$CL^- > PS^- \geq PG^- > PI^- > PC^\pm > PE^\pm$

PS, phosphatidylserine; CL, cardiolipin; PA, phosphatidic acid; PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

relative selectivities (K_r) (cf. Fig. 1; [31]). At higher phosphatidylcholine concentrations the behaviour is more complex, indicating additional mixing effects which are almost certainly present in the heterogeneous lipid composition of biological membranes. Particularly interesting in this respect is the finding that sulphatide, the unique anionic lipid of myelin, displays a selectivity relative to phosphatidylglycerol for the basic protein bound to sulphatide, but in a phosphatidylglycerol host the reverse is true [36]. This gives some indication of the way in which the lipid interactions with peripheral proteins may be modulated by the complex lipid composition of biological membranes.

4.3. *Protein penetration*

For the mitochondrial precursor protein, apocytochrome *c*, a more direct interaction with the lipid chains is observed, additionally to the generalized surface effects on the lipid packing density that is present for all peripheral proteins [27]. In analogy with the results for integral proteins, this provides evidence for a partial penetration of apocytochrome *c* into the lipid membrane, indicating a mechanism whereby negatively charged lipids may be involved in the import of the precursor protein into mitochondria. Experiments with various fragments of apocytochrome *c* have localized the major site of penetration to the amino-terminal region [37]. Such results are consistent with the precursor first inserting into the lipid regions of the outer mitochondrial membrane, prior to interaction of the N-terminal portion of the protein with the internally-facing haem lyase.

Similar evidence has been obtained for a partial membrane penetration of the myelin basic protein [28]. The penetrant portions were found not solely to be localized in the larger (12.6 kDa) N-terminal fragment of the protein [38]. Negatively charged lipids were found to be preferentially associated with the penetrant segments of the protein, and the proportion of lipid affected decreased with decreasing proportion of negatively charged components in lipid mixtures, according to the predictions of Eqn 2 (cf. Fig. 1; [31]). In comparison with apocytochrome *c* (11.5 kDa), the penetrant sections of the myelin basic protein (18.4 kDa) interact with a considerably larger number of lipid chains. This, together with the less localized nature of the penetrant sequences, points to a structural role in myelin compaction for the membrane penetration of the basic protein, as opposed to the transient functional role in apocytochrome *c* import.

5. CONCLUSLION

The binding of peripheral proteins is controlled mostly by electrostatics. However, partial membrane penetration occurs for certain peripheral proteins and can be of either structural or functional significance. Additionally, specificity in the lipid-peripheral protein interaction can lead to inhomogeneous lipid distributions within the membrane. Hydrophobic associations dominate the lipid interactions with integral proteins, and exchange at the lipid-protein interface provides the necessary couple for the response of activity to bulk lipid mobility. In addition, the selectivity of interaction with specific lipids provides a further means of modulating activity by preferential enrichment in the immediate environment of the protein.

Finally, the studies on lipid-protein interactions in mixed lipid systems, and recent experiments on double reconstitutions of the myelin proteolipid protein and the myelin basic protein with negatively charged lipids [39], represent further steps in the direction of systems with ever increasing molecular complexity. In the latter case, the results of ESR and binding experiments provide evidence for mutual lipid-protein interactions and demonstrate that the myelin basic protein is excluded from association with the first shell of lipids surrounding the proteolipid protein (cf. Fig. 1). The finding that the whole can, in a variety of ways, be different from the sum of the component parts gives a rationale for the heterogeneous composition of biological membranes.

REFERENCES

[1] Brotherus, J.K., Griffith, O.H., Brotherus, M.O., Jost, P.C., Silvius, J.R. and Hokin, L.E., (1981) Biochemistry 20, 5261–5267.

[2] Marsh, D. (1985) in: Progress in Protein-Lipid Interactions, vol. 1 (Watts, A. and De Pont, J.J.H.H.M. eds) pp. 143–172, Elsevier, Amsterdam.
[3] Brophy, P.J., Horváth, L.I. and Marsh, D. (1984) Biochemistry 23, 860–865.
[4] Wolfs, C.J.A.M., Horváth, L.I., Marsh, D., Watts, A. and Hemminga, M.A. (1989) Biochemistry 28, 9995–10001.
[5] Marsh, D. (1987) J. Bioenerg. Biomembr. 19, 677–689.
[6] Knowles, P.F., Watts, A. and Marsh, D. (1981) Biochemistry 20, 5888–5894.
[7] Abramovitch, D.A., Marsh, D. and Powell, G.L. (1990) Biochim. Biophys. Acta (in press).
[8] Marsh, D. and Powell, G.L. (1988) Bioelectrochem. Bioenerg. 20, 73–82.
[9] Esmann, M., Marsh, D., Schwarzmann, G. and Sandhoff, K. (1988) Biochemistry 27, 2398–2403.
[10] Horváth, L.I., Marcheselli, V., Daniotti, J.L., Vidal, A.C., Maccioni, H.J.F., Arias, H.R., Schwarzmann, G., Sandhoff, K., Marsh, D. and Barrantes, F.J. (1990) to be published.
[11] Li, G., Knowles, P.F., Murphy, D.J., Nishida, I. and Marsh, D. (1989) Biochemistry 28, 7446–7452.
[12] Esmann, M. and Marsh, D. (1985) Biochemistry 24, 3572–3578.
[13] Powell, G.L., Knowles, P.F. and Marsh, D. (1987) Biochemistry 26, 8138–8145.
[14] Horváth, L.I., Brophy, P.J. and Marsh, D. (1988) Biochemistry 27, 5296–5304.
[15] McMillen, D.A., Volwerk, J.J., Ohishi, J., Erion, M., Keana, J.F.W., Jost, P.C. and Griffith, O.H. (1986) Biochemistry 25, 182–193.
[16] Horváth, L.I., Brophy, P.J. and Marsh, D. (1990) Biochemistry 29, 2635–2638.
[17] Earnest, J.P., Limbacher, H.P. Jr, McNamee, M.G. and Wang, H.H. (1986) Biochemistry 25, 5809–5818.
[18] Horváth, L.I., Arias, H.R., Hankovszky, H.O., Hideg, K., Barrantes, F.J. and Marsh, D. (1990) Biochemistry (in press).
[19] Heidmann, T., Oswald, R.E. and Changeux, J.-P. (1983) Biochemistry 22, 3112–3127.
[20] Horváth, L.I., Brophy, P.J. and Marsh, D. (1988) Biochemistry 27, 46–52.
[21] Marsh, D. and Horváth, L.I. (1989) in: Advanced EPR in Biology and Biochemistry (Hoff, A.J. ed) pp. 707–752, Elsevier, Amsterdam.
[22] Ryba, N.J.P., Horváth, L.I., Watts, A. and Marsh, D. (1987) Biochemistry 26, 3234–3240.
[23] Sachse, J.-H., King, M.D. and Marsh, D. (1987) J. Magn. Reson. 71, 385–404.
[24] King, M.D., Sachse, J.-H. and Marsh, D. (1987) J. Magn. Reson, 72, 257–267.
[25] Peelen, S., Sanders, J.C., Hemminga, M.A. and Marsh, D. (1990) to be published.
[26] Horváth, L.I., Drees, M., Beyer, K., Klingenberg, M. and Marsh, D. (1990) (submitted).
[27] Görrissen, H., Marsh, D., Rietveld, A. and De Kruijff, B., (1986) Biochemistry 25, 2904–2910.
[28] Sankaram, M.B., Brophy, P.J. and Marsh, D. (1989) Biochemistry 28, 9685–9691.
[29] Marsh, D. and Watts, A. (1988) in: Advances in Membrane Fluidity, vol. 2 (Aloia, R.C., Curtain, C.C. and Gordon, L.M. eds) pp. 163–200, Alan R. Liss, New York.
[30] Sankaram, M.B., De Kruijff, B. and Marsh, D. (1989) Biochim. Biophys. Acta 986, 315–320.
[31] Sankaram, M.B., Brophy, P.J. and Marsh, D. (1989) Biochemistry 28, 9699–9707.
[32] Boggs, J.M. and Moscarello, M.A. (1978) J. Membr. Biol. 39, 75–96.
[33] Cheneval, D., Carafoli, E., Powell, G.L. and Marsh, D. (1989) Eur. J. Biochem. 186, 415–419.
[34] Sankaram, M.B., Brophy, P.J., Jordi, W. and Marsh, D. (1990) Biochim. Biophys. Acta 1021, 63–69.
[35] Rietveld, A., Berkhout, T.A., Roenhorst, A., Marsh, D. and De Kruijff, B. (1986) Biochim. Biophys. Acta 858, 38–46.
[36] Sankaram, M.B., Brophy, P.J., Schwarzmann, G., Rotscheidt, D., Sandhoff, K. and Marsh, D. (1990) (submitted).
[37] Jordi, W., De Kruijff, B. and Marsh, D. (1989) Biochemistry 28, 8998–9005.
[38] Sankaram, M.B., Brophy, P.J. and Marsh, D. (1989) Biochemistry 28, 9692–9698.
[39] Sankaram, M.B., Brophy, P.J. and Marsh, D. (1990) (submitted).