Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1611 (2003) 243–248
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Lipid–protein interactions in *Escherichia coli* membranes overexpressing the sugar–H^+ symporter, GalP EPR of spin-labelled lipids

Anne Hubert[a], Peter J.F. Henderson[a], Derek Marsh[b,*]

[a] *School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK*
[b] *Max-Planck-Institut für biophysikalische Chemie, Abt. Spektroskopie, 37070 Göttingen, Germany*

Received 6 January 2003; received in revised form 18 February 2003; accepted 21 February 2003

Abstract

The D-galactose–H^+ symport protein (GalP) of *Escherichia coli* is a homologue of the human glucose transport protein, GLUT1. After amplified expression of the GalP transporter in *E. coli*, lipid–protein interactions were studied in gradient-purified inner membranes by using spin-label electron paramagnetic resonance (EPR) spectroscopy. Phosphatidylethanolamine, -glycerol, -choline and -serine, in addition to phosphatidic and stearic acids, were spin-labelled at the 14 C-atom of the *sn*-2 chain. EPR spectra of these spin labels at probe amounts in GalP membranes consist of two components. One component corresponds to a lipid population whose motion is restricted by direct interaction with the transmembrane sections of the integral protein. The other component corresponds to a lipid population with greater chain mobility, and is similar to the single-component EPR spectrum of the spin-labelled lipids in membranes of *E. coli* lipid extract. Quantitation of the protein-interacting spin-label component allows determination of the stoichiometry and selectivity of lipid–protein interactions. On average, approximately 20 mol of lipid are motionally restricted per 52 kDa of protein in GalP membranes. At the pH of the transport assay, there is relatively little selectivity between the different phospholipids tested. Only stearic acid displays a stronger preferential interaction with this protein.

Keywords: Protein–lipid interaction; Membrane; Electron paramagnetic resonance; GLUT1; Sugar transport

1. Introduction

The D-galactose–H^+ membrane transport protein (GalP) is the hexose-specific member of a family of sugar–H^+ symporters that are responsible for proton-driven uptake of sugars by *Escherichia coli* [1,2]. These bacterial transporters are homologous to the GLUT family of mammalian passive facilitated glucose transporters [3]. In particular, GalP possesses a sugar specificity very similar to that of the GLUT1 transporter, and is similarly inhibited by the antibiotics forskolin and cytochalasin B.

In common with all transporters, GalP is a membrane-embedded integral protein. Therefore, its stability and function are likely to be governed, at least in part, by lipid–protein interactions. Electron paramagnetic resonance (EPR) spectroscopy with spin-labelled lipids has proved to be an especially fruitful method for studying lipid interactions with integral membrane proteins [4,5]. Here we use this technique to determine the stoichiometry and selectivity of lipid–protein interactions in the native environment of the GalP protein, viz., the *E. coli* inner membrane. To maximise lipid interactions with the sugar–H^+ transporter, expression of GalP is amplified to levels of 50%, or more, of total membrane protein (Fig. 1) by using a plasmid containing the *galP* gene under control of its own promoter [6,7].

There are significant advantages to studying transporters in their native membrane, when such high expression levels are achieved. The native state of assembly is assured,

Abbreviations: 14-SASL, 14-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; 14-PESL, 14-PGSL, 14-PCSL, 14-PSSL and 14-PASL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphoethanolamine, -phosphoglycerol, -phosphocholine, -phosphoserine and -phosphoric acid; EPR, electron paramagnetic resonance

* Corresponding author. Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, Am Fassberg 11, D-37070 Göttingen, Germany. Tel.: +49-551-201-1285; fax: +49-551-201-1501.
E-mail address: dmarsh@gwdg.de (D. Marsh).

0005-2736/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0005-2736(03)00081-6

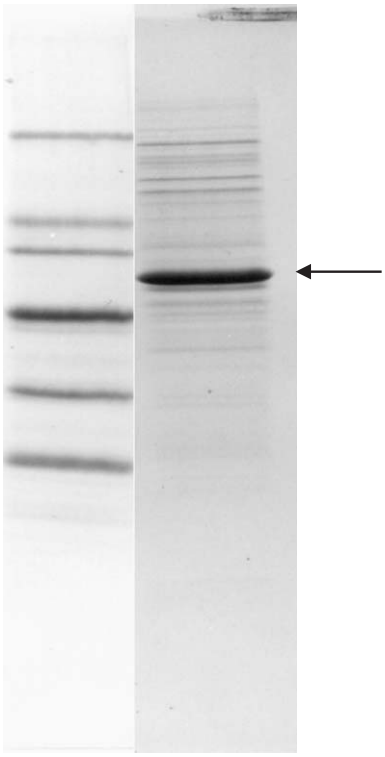

Fig. 1. Coomassie-stained SDS-PAGE gel of the inner membrane preparation used in this study. The GalP protein is indicated with an arrow. Lane 1 contains molecular weight standards 14–66 kDa; lane 2 is the inner membrane preparation.

without extensive reconstitution studies. Currently, a high-resolution three-dimensional structure is not available for a sugar transporter, either in a crystal or in detergent, and even less so in a native membrane environment. The stoichiometry and selectivity of lipid–protein interactions that are determined by EPR spectroscopy, however, provide valuable indicators for the mode of membrane integration and assembly [8]. The lipid stoichiometry is related directly to the intramembranous perimeter of the protein and hence to the assembly of the transmembrane helices [9]. Selectivity for negatively charged lipids can be related to the disposition of basic amino acid residues at the membrane–water interface [4]. Both of these are essential features of the protein structure.

2. Materials and methods

2.1. Materials

E. coli lipids were obtained from Avanti Polar Lipids (Birmingham, AL). Spin-labelled stearic acid was synthesised according to Ref. [10]. Spin-labelled phosphatidylcholine, 14-PCSL, was synthesised by acylating lysophosphatidylcholine with 14-SASL, as described in Ref. [11]. Other spin-labelled phospholipids, 14-PGSL, 14-PESL, 14-PSSL and 14-PASL, were synthesised from 14-PCSL by phospholipase D-catalysed headgroup exchange according to Ref. [12] (and see also Ref. [11]).

2.2. Preparation of GalP membranes

E. coli strain JM1100 (pPER3), which overexpresses GalP, was grown on minimal medium as described [6,7]. Inside-out vesicles of the inner cytoplasmic membrane were prepared by explosive decompression of intact cells in a French press [13], purified on a sucrose density-gradient [14], and resuspended in 50 mM potassium phosphate, 10 mM $MgSO_4$, pH 6.6 buffer at about 1–5 mg/ml. On Coomassie-stained SDS gels, GalP constitutes 52 mol% of the 14 bands, with the next most intense accounting for 8 mol% (see Fig. 1). Protein concentration was determined according to Ref. [15] and lipid phosphate according to Ref. [16].

2.3. Spin labelling

GalP membranes were doped with spin-labelled lipids, at a level of ca. 1 mol% relative to total lipid, by adding the spin label to the membrane dispersion from a concentrated solution (1 mg/ml) in ethanol. For spin-labelled phospholipids, unincorporated spin label was removed by resuspending and washing the membrane pellet repeatedly with buffer. Finally, the membrane pellet was resuspended in a minimal volume of buffer and transferred to a 1-mm ID glass capillary for EPR measurements. The sample was packed in the capillary by centrifugation on a bench-top centrifuge, excess supernatant removed, and the capillary sealed. For 14-SASL, the washing steps were omitted because spin-labelled fatty acids partition into the membrane, at equilibrium with the aqueous spin label. Unless otherwise noted, the buffer used was that of the transport assay: 50 mM potassium phosphate, 10 mM $MgSO_4$, pH 6.6.

2.4. EPR spectroscopy

EPR spectra were recorded on a Varian Century Line 9 GHz spectrometer equipped with gas-flow temperature regulation. The sample capillaries were accommodated in a standard 4-mm quartz EPR tube that contained light silicone oil for thermal stability. Temperature was measured with a fine-wire thermocouple located in the silicone oil at the top of the microwave cavity. Conventional EPR spectra were recorded in the standard in-phase, first-harmonic, absorption mode. Spectral subtractions were performed as described in Ref. [17], by using interactive software written by Dr. J. Kleinschmidt of this Institute. Single-component reference spectra were taken from libraries of 14-PCSL in sonicated dimyristoyl phosphatidylcholine vesicles, and in egg phosphatidylcholine liposomal dispersions. Subtraction endpoints were established by overlaying the difference spectrum with a single-component reference spectrum complementary to that used for the subtraction.

3. Results and discussion

3.1. EPR spectra of 14-position labels

Fig. 2 gives the EPR spectra of the different spin-labelled lipids in GalP membranes at 25 °C. All spectra from the membranes consist of two components: a motionally restricted component, corresponding to lipid chains directly interacting with transmembrane protein segments, and a more mobile component arising from the fluid bilayer region of the membrane (see, e.g., Refs. [4,18]). The identity of the latter component is confirmed by the similarity to the single-component spectrum that is obtained from membranes of extracted *E. coli* lipids (see bottom spectrum in Fig. 2).

3.2. Difference spectra

With the exception of 14-SASL, the membrane spectra of the phospholipid spin labels in Fig. 2 appear rather similar, suggesting a similar degree of interaction with the protein. Quantitation of the relative populations of the two spectral components is performed by difference spectroscopy, as illustrated in Fig. 3. For this, it is necessary to obtain spectra that match the individual components in the two-component membrane spectra. These were chosen from libraries of spectra from 14-PCSL in sonicated dimyristoyl phosphatidylcholine vesicles and egg phosphatidylcholine liposomes at different temperatures. Although the spectra from membranes of *E. coli* lipids are similar to those of the fluid component in GalP membranes, it was found that egg phosphatidylcholine membranes give a better match when the temperature was optimised. Evidently, these spectra can better represent the longer range perturbations of the fluid bilayer lipids in GalP membranes.

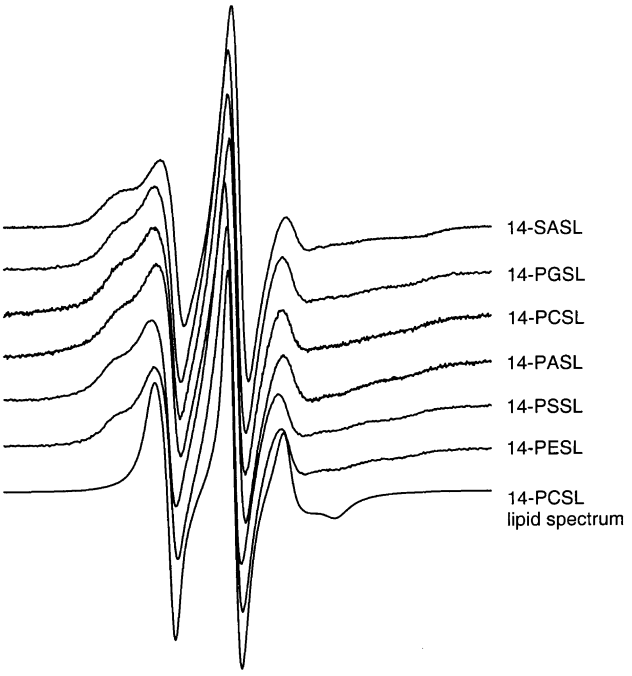

Fig. 2. EPR of different spin-labelled phospholipid species, 14-PXSL (as indicated on figure) and spin-labelled stearic acid, 14-SASL, in GalP membranes. The bottom spectrum is 14-PCSL in aqueous dispersion of *E. coli* lipids, given for comparison. $T = 25$ °C; total scan width = 10 mT.

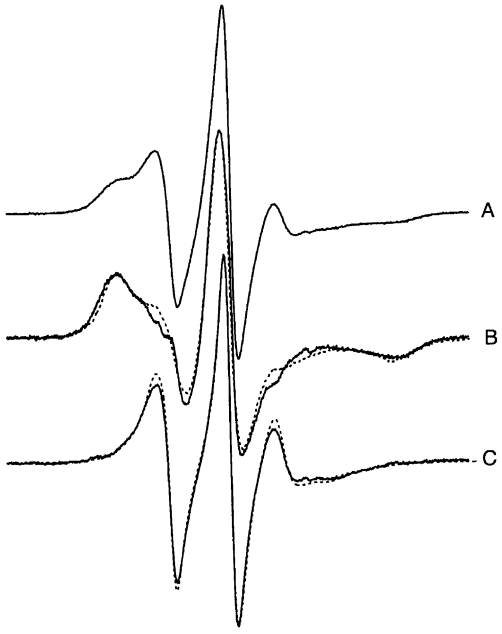

Fig. 3. EPR difference spectra. (A) Two-component GalP membrane spectrum of 14-SASL at 25 °C; (B) Solid line: difference spectrum obtained by subtracting dotted line, single-component spectrum in C from spectrum A.; dotted line: single-component comparison spectrum, 14-PCSL in sonicated DMPC vesicles at 2 °C. (C) Solid line: difference spectrum obtained by subtracting dotted line, single-component spectrum in B from spectrum A; dotted line: single-component comparison spectrum, 14-PCSL in egg phosphatidylcholine membranes at 7 °C. Spectra are normalised to the same maximum line height to aid comparison of the spectral line shapes. Total scan width = 10 mT.

In order to facilitate comparison of the line shapes with the reference spectra, the difference spectra in Fig. 3B and C are normalised to the same maximum line height. Therefore, they do not reflect the relative proportions of the two components in the composite spectrum (Fig. 3A). Because EPR spectra are recorded as the first derivative, it is necessary to determine the fractional double-integrated intensity that is subtracted from the two-component membrane spectrum to produce a single-component end point. Table 1 gives the fractional populations, f, of the motionally restricted (i.e., protein-interacting) component that are obtained by spectral subtraction for the different spin-labelled lipids. The overall size of these values reflects the number of lipids associated with the protein and their relative values reflect the selectivity of the lipid–protein interaction.

3.3. Lipid stoichiometry and selectivity

The equation for equilibrium lipid–protein association gives the following expression for the ratio of fluid to

Table 1
Fractions, f, of motionally restricted spin-labelled lipid in GalP membranes, and relative association constants, K_r, normalised to that for 14-PESL, K_r^{PE}

Spin label	pH	f	K_r/K_r^{PE}	$\Delta G-\Delta G_{PE}$ (kJ/mol)
14-PESL	6.6	0.43	1.0	0.0
	8.6	0.43	1.0	0.0
14-PCSL	6.6	0.43	1.0	0.0
14-PASL	6.6	0.43	1.0	0.0
	8.6	0.69	2.9	− 2.7
14-PGSL	6.6	0.40	0.9	0.3
14-PSSL	6.6	0.43	1.01	− 0.02
14-SASL	6.6	0.60	1.9	− 1.7
	8.6	0.76	4.3	− 3.6

T = 25 °C.
Selectivities, K_r/K_r^{PE} are calculated from Eq. (2). Difference in free energy of association is given by: $\Delta G - \Delta G_{PE} = -RT\ln(K_r/K_r^{PE})$.

motionally restricted spin-labelled lipid populations [18,19]:

$$(1/f) - 1 = \left(\frac{n_t}{N_b} - 1\right) \Big/ K_r \qquad (1)$$

where N_b is the number of lipid sites on the protein, K_r is the mean association constant of the spin-labelled lipid, relative to the background membrane lipid and n_t is the total lipid/protein ratio in the membrane.

From Table 1, it is seen for the lipids with lowest selectivity that $f \approx 0.43$. For these lipids, it can be assumed that $K_r \approx 1$, i.e., they display no selectivity relative to the background membrane lipid. In support of this assumption, we see that this value is appropriate for 14-PESL, and that phosphatidylethanolamine is the majority lipid in *E. coli* inner membranes. Further, lipid–protein titrations in reconstituted membranes show that there is little selectivity of 14-PCSL relative to the parent unlabelled phosphatidylcholine as host lipid (e.g., Refs. [20,21]). The stoichiometry, N_b, of the lipid–protein interaction can then be determined from the spin-label experiments and a knowledge of n_t. The total lipid/protein ratio in the GalP membranes was determined from protein and lipid–phosphate assays to be: $n_t = 44$ mol phospholipid/52 kDa protein. Correspondingly, the mean lipid–protein stoichiometry in the purified GalP membranes is $N_b = fn_t = 19$ mol phospholipid/52 kDa protein.

Irrespective of the lipid/protein ratio, it is possible to determine the relative values of the association constants for the spin-labelled lipids, i.e., the lipid selectivity. From Eq. (1) we have, relative to 14-PESL:

$$K_r/K_r^{PE} = (1/f_{PE} - 1)/(1/f - 1) \qquad (2)$$

where f_{PE} is the value of f determined with 14-PESL. The values, K_r/K_r^{PE}, of the association constant relative to 14-PESL are given in Table 1. The free energy of association, $\Delta G - \Delta G_{PE}$, of a particular spin-labelled lipid relative to 14-PESL can then be obtained immediately. These values are also included in Table 1.

3.4. Temperature dependence

Fig. 4 shows the EPR spectra of the 14-PESL spin label in GalP membranes, as a function of temperature. All spectra consist of two components, characteristic of lipid–protein interaction with the integral protein. The relative heights of the two components vary with temperature because the temperature dependence of the line shape and line widths of the fluid component, which is in the motional narrowing regime, is much greater than that of the motionally restricted component, which is in the slow-motional regime (see, e.g., Ref. [22]). Difference spectroscopy and double integration, as described in the previous subsection, allow for these changes in intrinsic line shape when calculating the relative proportions of the two components.

Fig. 5 shows the temperature dependence of the ratios (K_r/K_r^{PE}) of the association constants for the different spin-labelled lipid species, relative to PE. The spread in lipid selectivity is greater at 37 °C than that indicated in Table 1, at 25 °C. Differences in temperature dependence can, in principle, be attributed to a differential enthalpy of interaction, $\Delta H - \Delta H_{PE}$ (see, e.g., Ref. [23]). The steepest temperature dependence in Fig. 5 is for the negatively charged phospholipid 14-PSSL. Those for both 14-PGSL and 14-SASL are small, whereas that for the zwitterionic lipid 14-PCSL is of opposite sign. Note that, although we are dealing with a complex lipid mixture, and an inhomogeneous protein content, this reversal in sign of the temperature dependence cannot be attributed to any intrinsic tendency of the lipid to phase separation (modulated by protein) in GalP membranes. A temperature dependence of the lipid selectivity, and hence of the lipid environment of

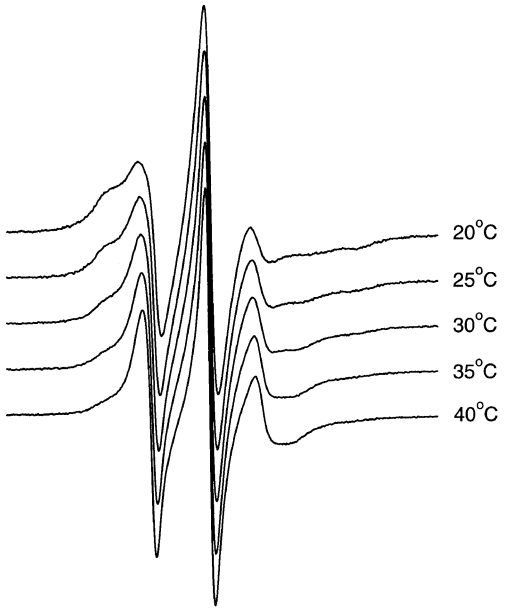

Fig. 4. EPR spectra of 14-PESL in GalP membranes, as a function of temperature. The sample temperature is indicated on the figure. Total scan width = 10 mT.

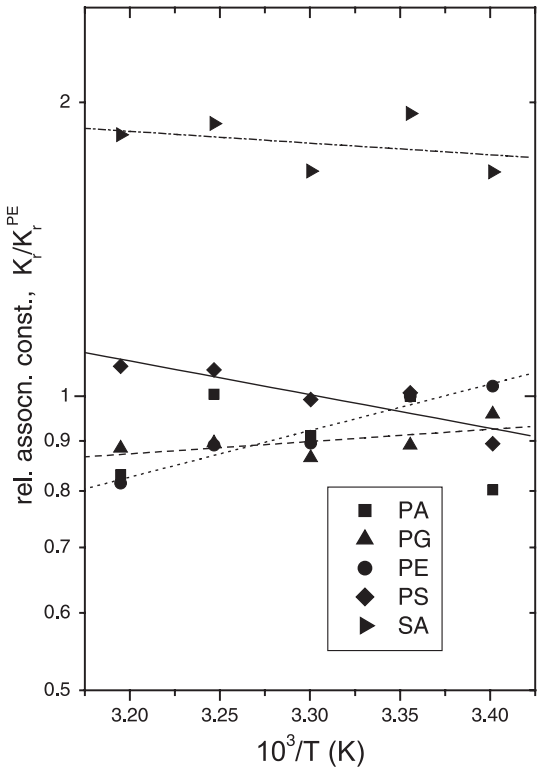

Fig. 5. Temperature dependence of the lipid selectivity, K_r/K_r^{PE}, relative to PE. Data are deduced from spectral subtractions of the different 14-position spin label in GalP membranes by using Eq. (2). The different spin-label species (14-PASL, 14-PGSL, 14-PCSL, 14-PSSL and 14-SASL) are indicated on the figure. Straight lines are linear regressions for 14-SASL (dashed-and-dotted), 14-PASL (solid) and 14-PGSL (dashed). For a maximum uncertainty of $\delta f = \pm 0.01$ in spectral subtraction factor, the fractional error in K_r/K_r^{PE} is $\pm 0.04-0.06$.

the protein, could contribute materially to the temperature dependence of the transport activity.

4. Conclusions

Amplified expression of GalP in *E. coli* achieves levels that constitute 50–55%, or more, of the total inner membrane protein (see Fig. 1 and Refs. [6,7]). Thus, it is likely that the greater part of the lipid–protein interactions studied here with gradient-purified membranes reflects that with the GalP protein itself. Hydropathy plots deduced from the membrane sequence predict that GalP possesses 12 α-helical membrane-spanning domains (see, e.g., Ref. [24]). Therefore, it is to be expected that GalP will present an appreciable hydrophobic surface to the inner membrane lipids. The detection here of a substantial motionally restricted lipid population in GalP membranes is consistent with this expectation for the intramembranous structure of the transporter. A trilayer sandwich (see Fig. 7C in Ref. [21]) composed of 12 helices would accommodate approximately 30 lipids around the perimeter (see, e.g., Ref. [9]). This estimate is considerably greater than the stoichiometry of approximately 20 mol of motionally restricted lipids per 52 kDa of protein that is determined here. The other proteins present in the inner membrane are therefore unlikely to have such a high proportion of their molecular mass within the membrane as does GalP.

At a pH of 6.6, which is normally used for the proton-linked sugar transport studies, there is little selectivity among the different lipids at 25 °C, with the exception of stearic acid (see Table 1). At pH 6.6, stearic acid in an interfacial location is expected to be protonated (see, e.g., Ref. [25]). The selectivity for 14-SASL therefore arises from a non-electrostatic specificity, or association, probably associated with the single-chain nature of this amphiphile. The lack of selectivity for the nominally anionic phosphatidylserine and phosphatidic acid, relative to the zwitterionic lipids, arises from the low pH, although this is probably not the case for phosphatidylglycerol. In the future, it will be of interest to investigate the pH dependence of the lipid selectivity in detail, but this will be better done with purified, reconstituted GalP. Such work is currently in progress.

Acknowledgements

We wish to thank J. O'Reilly for membrane preparations and Frau B. Angerstein for spin-label synthesis. This work was supported by a joint European Travel Grant from the Royal Society. A.H. is the recipient of a research studentship from the BBSRC. Research in PJFH's laboratory is funded by BBSRC, MRC and EU, and supported by the BBSRC North of England Centre for Structural Biology.

References

[1] P.J.F. Henderson, A.J.S. McPherson, Assay, genetics, proteins, and reconstitution of proton-linked galactose, arabinose, and xylose transport systems of *Escherichia coli*, Methods Enzymol. 125 (1986) 387–429.
[2] P.J.F. Henderson, The 12-transmembrane helix transporters, Curr. Opin. Cell Biol. 5 (1993) 708–721.
[3] S.A. Baldwin, P.J.F. Henderson, Homologies between sugar transporters from eukaryotes and prokaryotes, Annu. Rev. Physiol. 51 (1989) 459–471.
[4] D. Marsh, L.I. Horváth, Structure, dynamics and composition of the lipid–protein interface. Perspectives from spin-labelling, Biochim. Biophys. Acta 1376 (1998) 267–296.
[5] D. Marsh, Specificity of lipid–protein interactions, in: A.G. Lee (Ed.), Biomembranes, vol. 1, JAI Press, Greenwich, CT, 1995, pp. 137–186.
[6] P.E. Roberts, The galactose–H^+ symport protein of *Escherichia coli*, PhD Thesis, University of Cambridge, 1992.
[7] P.J.R. Spooner, N.G. Rutherford, A. Watts, P.J.F. Henderson, NMR observation of substrate in the binding site of an active sugar–H^+ symport protein in native membranes, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 3877–3881.
[8] D. Marsh, The nature of the lipid–protein interface and the influence of protein structure on protein–lipid interactions, in: A. Watts (Ed.), Protein–Lipid Interactions, New Comprehensive Biochemistry, vol. 25, Elsevier, Amsterdam, 1993, pp. 41–66.

[9] D. Marsh, Stoichiometry of lipid–protein interaction and integral membrane protein structure, Eur. Biophys. J. 26 (1997) 203–208.
[10] W.L. Hubbell, H.M. McConnell, Molecular motion in spin-labelled phospholipids and membranes, J. Am. Chem. Soc. 93 (1971) 314–326.
[11] D. Marsh, A. Watts, Spin-labeling and lipid–protein interactions in membranes, in: P.C. Jost, O.H. Griffith (Eds.), Lipid–Protein Interactions, vol. 2, Wiley-Interscience, New York, 1982, pp. 53–126.
[12] P. Comfurius, R.F.A. Zwaal, The enzymatic synthesis of phosphatidylserine and purification by CM-cellulose column chromatography, Biochim. Biophys. Acta 488 (1977) 36–42.
[13] M. Futai, Experimental systems for the study of active transport in bacteria, in: B.P. Rosen (Ed.), Bacterial Transport, Marcel Dekker, New York, 1978, pp. 7–41.
[14] A. Ward, N.M. Sanderson, J. O'Reilly, N.G. Rutherford, B. Poolman, P.J.F. Henderson, The amplified expression, identification, purification, assay and properties of histidine-tagged bacterial membrane transport proteins, in: S.A. Baldwin (Ed.), Membrane Transport—A Practical Approach, Blackwell, Oxford, 2000, pp. 131–166.
[15] W. Schaffner, C. Weissman, A rapid, sensitive, and specific method for determination of protein in dilute solution, Anal. Biochem. 56 (1973) 502–514.
[16] G. Rouser, S. Fleischer, A. Yamamoto, Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots, Lipids 5 (1970) 494–496.
[17] D. Marsh, Electron spin resonance: spin label probes, in: J.C. Metcalfe, T.R. Hesketh (Eds.), Techniques in Lipid and Membrane Biochemistry, vol. B4/II, Elsevier, Shannon, 1982, pp. B426/1–B426/44.
[18] D. Marsh, Experimental methods in spin-label spectral analysis, in: L.J. Berliner, J. Reuben (Eds.), Biological Magnetic Resonance, vol. 8, Plenum, New York, 1989, pp. 255–303.
[19] J.R. Brotherus, O.H. Griffith, M.O. Brotherus, P.C. Jost, J.R. Silvius, L.E. Hokin, Lipid–protein multiple binding equilibria in membranes, Biochemistry 20 (1981) 5261–5267.
[20] P.F. Knowles, A. Watts, D. Marsh, Spin label studies of lipid immobilization in dimyristoylphosphatidylcholine-substituted cytochrome oxidase, Biochemistry 18 (1979) 4480–4487.
[21] P.J. Brophy, L.I. Horváth, D. Marsh, Stoichiometry and specificity of lipid–protein interaction with myelin proteolipid protein studied by spin-label electron spin resonance, Biochemistry 23 (1984) 860–865.
[22] D. Marsh, L.I. Horváth, Spin-label studies of the structure and dynamics of lipids and proteins in membranes, in: A.J. Hoff (Ed.), Advanced EPR. Applications in Biology and Biochemistry, Elsevier, Amsterdam, 1989, pp. 707–752.
[23] M. Esmann, D. Marsh, Spin-label studies on the origin of the specificity of lipid–protein interactions in Na^+, K^+-ATPase membranes from *Squalus acanthias*, Biochemistry 24 (1985) 3572–3578.
[24] D. Marsh, P.J.F. Henderson, Specific spin labelling of the sugar–H^+ symporter, GalP, in cell membranes of *Escherichia coli*: site mobility and overall rotational diffusion of the protein, Biochim. Biophys. Acta 1510 (2001) 464–473.
[25] J. Miyazaki, K. Hideg, D. Marsh, Interfacial ionization and partitioning of membrane-bound local anaesthetics, Biochim. Biophys. Acta 1103 (1992) 62–68.