Biochimica et Biophysica Acta, 1147 (1993) 277–280

BBAMEM 70721

Rapid Report

Spin label saturation transfer EPR determinations of the stoichiometry and selectivity of lipid–protein interactions in the gel phase

László I. Horváth [a,1], Peter J. Brophy [b] and Derek Marsh [a]

[a] *Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, Göttingen (Germany) and* [b] *Department of Biological Science, Stirling University, Stirling (UK)*

(Received 5 February 1993)

Key words: Lipid–protein interaction; Spin label; Saturation transfer; Myelin; Proteolipid protein; Lipid gel phase; EPR

Lipid–protein interactions with the myelin proteolipid protein incorporated in the gel phase of dimyristoylphosphatidylcholine bilayers have been studied by saturation transfer EPR spectroscopy of spin-labelled phospholipids. The integrated intensities of the saturation transfer EPR spectra from spin-labelled phosphatidylcholine are linearly dependent on the protein/lipid ratio, and correspond to a fixed stoichiometry of approximately 11 lipids per monomer associated with the protein in the gel phase. The normalized saturation transfer intensities of spin-labelled phosphatidic acid, on the other hand, display a non-linear dependence on the protein/lipid ratio that can be described well by a selectivity for interaction with the protein in the gel phase with an average association constant relative to phosphatidylcholine of approx. 5.2. These values for the stoichiometry and selectivity of lipid–protein interaction in the lipid gel phase obtained from saturation transfer EPR spectroscopy are comparable to those found previously in fluid phase lipids by conventional EPR spectroscopy.

Lipid–protein interactions are essential determinants of the functional integrity of cell membranes. Conventional electron spin resonance (EPR) spectroscopy of spin-labelled lipids has proved to be extremely useful for characterization of both the stoichiometry and specificity of lipid interactions with integral proteins in membranes in the fluid phase (see, e.g., Ref. 1). This information is obtained from spectral subtractions with different spin-labelled lipids, generally as a function of the lipid/protein ratio of the sample. Such an approach, however, is not easily possible for membranes in the gel phase because of the lack of spectral resolution of the spin labels interacting directly with the protein from those in the bulk lipid gel phase. ^{2}H nuclear magnetic resonance (NMR), on the other hand, has been used to investigate the stoichiometry of phosphatidylcholine association with rhodopsin [2] and with cytochrome oxidase [3] reconstituted in the gel phase. However, such methods are not applicable also to the fluid phase because of the fast exchange of the lipids at the protein interface on the ^{2}H-NMR time scale (see, e.g., Ref. 4).

Lipid–protein interactions in the gel phase are of intrinsic interest because of the information that they may yield on the association state of the protein, and on the lipid mobility, in more tightly packed lipid membrane environments. From the point of view of spin label EPR measurements, methods for investigating lipid–protein interactions in the fluid phase where there is poor resolution between the protein- and bilayer-associated components, e.g., for lipids labelled close to the glycerol backbone (cf., Ref. 5), are also of considerable interest. This requires methods different from conventional EPR spectroscopy, and it seems that those based on the saturation properties of the spectrum which are sensitive to the spin-lattice relaxation of the spin label, and hence can distinguish differences in mobility on a timescale slower than that of conventional spin label EPR spectroscopy, might be appropriate.

Correspondence to: D. Marsh, Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, DW-3400 Göttingen, Germany.

[1] Permanent address: Institute of Biophysics, Biological Research Centre, Szeged, Hungary.

Abbreviations: STEPR, saturation transfer EPR; V_1, first harmonic EPR absorption signal detected in phase with respect to the field modulation; V_2', second harmonic absorption EPR signal detected 90° out-of-phase with respect to the field modulation; NMR, nuclear magnetic resonance; PLP, myelin proteolipid apoprotein; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; 14-PCSL, and -PASL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine, and -phosphoric acid.

In the present work, saturation transfer (ST) EPR experiments with spin-labelled lipids are used to study lipid–protein interactions in the gel phase of recombinants of the myelin proteolipid apoprotein (PLP) with dimyristoyl phosphatidylcholine (DMPC). It is known that the STEPR spectra of the protein-associated and lipid-associated spin labels differ in the gel phase of such systems [6]. Furthermore, the linear additivity of the normalized integral intensity of the STEPR spectra from multi-component systems [6] means that this method of analysis can be applied directly to titrations of the lipid/protein ratio of the gel-phase complexes, in a manner similar to that used for the conventional EPR spectra from the fluid phase. In this way, it is found possible to determine the stoichiometry of association of spin-labelled phosphatidylcholine and the selectivity of association of spin-labelled phosphatidic acid with the myelin proteolipid protein in gel phase lipid.

Representative STEPR spectra (V_2'-display) of spin-labelled phosphatidylcholine (14-PCSL) in gel phase DMPC bilayers, in a PLP/DMPC complex of lipid/protein ratio of 24:1 (mol/mol), and in association with delipidated PLP at 0°C are given in Fig. 1. Samples were prepared as described in Ref. 7 and spectra were recorded as described in Ref. 6, according to the standardized protocol given in Ref. 8. The line shapes of the STEPR spectra differ considerably between the different samples, indicating that the mobility of the spin-labelled lipid chains associated with the protein is considerably less than that in the gel phase lipid environment at the same temperature (cf., e.g., Ref. 9). However, the STEPR spectrum of the lipid/protein complex (Fig. 1b) cannot be resolved clearly into two components, as is to be expected because the STEPR spectra of the protein-alone (Fig. 1a) and of the pure lipid bilayers (Fig. 1c) both extend over the full spectral range. This is true, even more so, for the conventional EPR spectra recorded in the gel phase (data not shown). Verification and testing of the two-component nature of the spectra from the lipid/protein complex must therefore be made by evaluating the normalized integrals, I_{ST}, of the STEPR spectra (as defined in Ref. 6):

$$I_{ST} = \int V_2'(H) \cdot dH / \iint V_1(H) \cdot d^2H \qquad (1)$$

where H is the magnetic field strength.

The first integrals of the V_2' STEPR spectra normalized to the second integrals of the V_1 conventional EPR spectra for the 14-PCSL spin label in PLP/DMPC complexes at 0°C are given in Fig. 2, as a function of the total protein/lipid ratio of the complexes. It is seen that the STEPR integral of 14-PCSL depends linearly on the protein/lipid ratio, implying that there is a fixed stoichiometry of interaction with PLP in the gel phase, as is shown by the following analysis. Since the normalized integral, I_{ST}, of a composite STEPR spectrum is additive [6], the measured value for a spectrum composed of two components, b (protein-associated) and f (lipid bilayer-associated), is given by:

$$I_{ST} = f \cdot I^{o}_{ST,b} + (1 - f) I^{o}_{ST,f} \qquad (2)$$

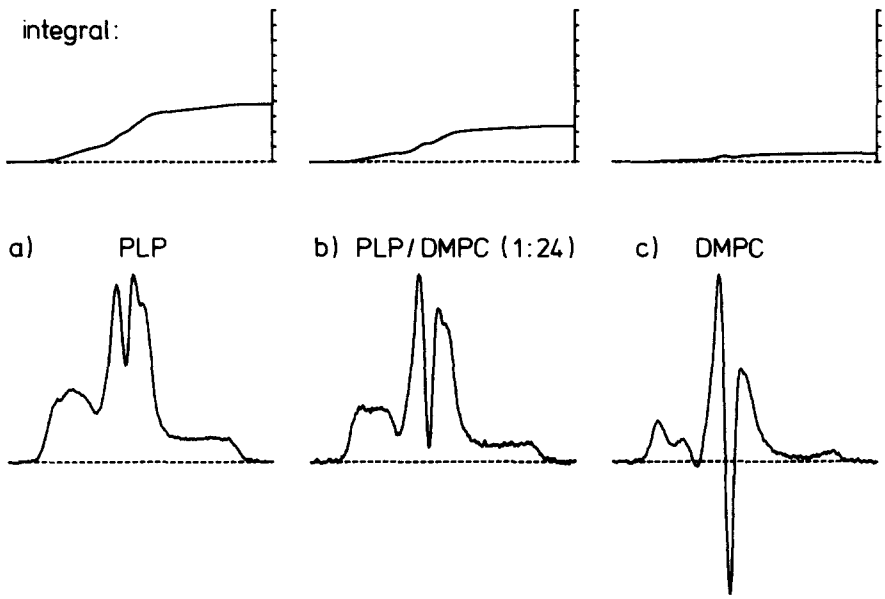

Fig. 1. Second harmonic, 90°-out-of-phase, absorption STEPR spectra (V_2'-display) and their normalized first integrals, for the 14-PCSL phosphatidylcholine spin label at 0°C. (a) Associated with the myelin proteolipid protein alone, (b) in a PLP/DMPC complex of lipid/protein ratio 24:1 mol/mol, and (c) in bilayers of DMPC alone. The spectra are given in the lower row and the integrals in the upper row. The ordinate is normalized to an integrated intensity of $2 \cdot 10^{-2}$ as full scale. Total scan width = 100 G.

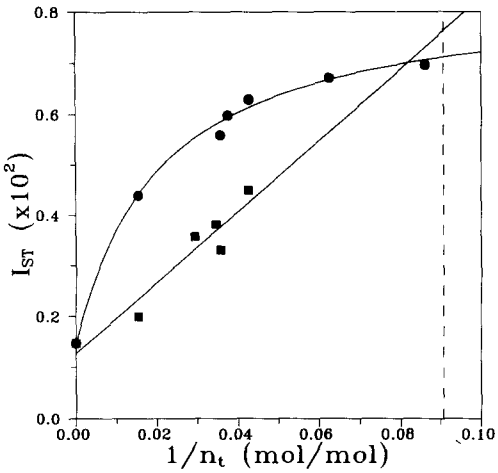

Fig. 2. Dependence on the protein/lipid ratio, $1/n_t$, of the normalized integrals, I_{ST}, from the saturation transfer EPR spectra (V_2'-display) of the 14-PCSL phosphatidylcholine spin label (■) and the 14-PASL phosphatidic acid spin label (●) in recombinants of the myelin proteolipid protein with dimyristoylphosphatidylcholine at 0°C. The full straight line represents a linear regression of the data for 14-PCSL according to Eqn. 4, with the parameters: $N_b(I^o_{ST,b} - I^o_{ST,f}) = 7.01$ and $I^o_{ST,f} = 0.127$. The curved line represents a non-linear least-squares fit of the data for 14-PASL to Eqn. 6, with the fitting parameters: $N_b = 11.4$, $K_r = 5.2$, $I^o_{ST,b} = 0.708$ and $I^o_{ST,f} = 0.146$. The vertical dashed line represents the lipid/protein stoichiometry $N_b = 11.0$.

where f is the fractional population of component b and $I^o_{ST,b}$ $I^o_{ST,f}$ are the normalized values of the saturation transfer integral for components b and f, respectively. If there is no selectivity between the spin-labelled lipid and the host lipid for association with the protein, the value of f is related to the total lipid/protein ratio, n_t, in the lipid/protein complex simply by:

$$f = N_b/n_t \tag{3}$$

where N_b is the number of lipid association sites with the protein. In this case of no selectivity, the saturation transfer integral is given from Eqns. 2 and 3 by:

$$I_{ST} = (I^o_{ST,b} - I^o_{ST,f})N_b/n_t + I^o_{ST,f} \tag{4}$$

and a linear dependence of I_{ST} on $1/n_t$ is expected. As noted from Fig. 2, this is the case for the 14-PCSL phosphatidylcholine spin label in complexes of the myelin proteolipid protein with dimyristoylphosphatidylcholine, indicating that there is no selectivity between the spin-labelled and unlabelled parent phosphatidylcholine for interaction with the PLP protein in the gel phase. A similar result has been found previously for the interaction of spin-labelled phosphatidylcholine with the PLP protein in DMPC complexes in the fluid phase [7]. The value of $I^o_{ST,f}$ deduced from the linear regression is close to that measured for the spin label in the gel-phase lipid alone at the same temperature. From the value of the gradient of the linear regression, together with the value of $I^o_{ST,b} = 0.763$ measured for a sample of the delipidated protein at 0°C (Fig. 1a), it can be estimated that the number of lipids associated with PLP in the gel phase is: $N_b = 11.0$. This value is very similar to the stoichiometry of lipid sites at the interface of the PLP in fluid phase DMPC, which was determined to be: $N_b = 9$–10 from lipid/protein titrations obtained by digital subtraction of the conventional EPR spectra [7].

Spin-labelled phosphatidic acid has a pronounced selectivity for interaction with the PLP protein in the fluid phase of DMPC [7,10]. The values of the STEPR integral as a function of protein/lipid ratio for 14-PASL in PLP/DMPC complexes in the gel phase are given in Fig. 2. It can be seen that the STEPR data for this spin-labelled lipid do not conform to the linear dependence on $1/n_t$ expected for a non-selective interaction with the protein in the lipid gel phase; also the values of I_{ST} are much larger, closer to those for the protein alone, than for 14-PCSL in complexes of similar protein/lipid ratios at the same temperature. This indicates a clear selectivity of the phosphatidic acid spin label over that of phosphatidylcholine for association with the PLP protein in gel phase lipid.

For spin-labelled lipids that show a selectivity for association with the protein, the value of f in Eqn. 2 is given by the equation for equilibrium association with the protein [1,11]:

$$f = K_r/(n_t/N_b + K_r - 1) \tag{5}$$

where K_r is the average association constant of the spin-labelled lipid with the protein, relative to that for the unlabelled host lipid, and $n_t \geq N_b$ Substituting Eqn. 5 in Eqn. 2 yields the following expression for the saturation transfer integral of spin-labelled lipids displaying a selectivity for the protein:

$$I_{ST} = [K_r I^o_{ST,b} + (n_t/N_b - 1)I^o_{ST,f}]/(n_t/N_b + K_r - 1) \tag{6}$$

The lipid/protein titration of the STEPR data for 14-PASL in gel phase PLP/DMPC complexes given in Fig. 2 can be described well by Eqn. 6, which allows for the selectivity of the spin-labelled lipid, with reasonable values of $I^o_{ST,f}$ and $I^o_{ST,b}$. The value of the former is identical to that measured for the spin label in the lipid alone, and the value for the latter lies reasonably close to that found with the delipidated protein: $I^o_{ST,b} = 0.763$. From calibrations with isotropically rotating spin-labelled haemoglobin [12], the value for 14-PASL corresponds to an effective rotational correlation time of 67 μs for the spin-labelled lipid associated with the PLP protein in the gel phase. The value deduced for the stoichiometry ($N_b = 11.4$) with 14-PASL is very similar to that obtained with the spin-labelled phos-

phatidylcholine in the gel phase. The relative association constant $K_r = 5.2$ deduced for 14-PASL evidences a considerable selectivity, relative to phosphatidylcholine, of the lipid–protein interaction in the gel phase and corresponds reasonably closely to that obtained recently ($K_r = 5.7$) in the fluid phase under otherwise comparable conditions [10].

It seems, therefore, that both the stoichiometry and specificity of the lipid–protein interactions with the PLP protein are similar in the gel phase to those in the fluid phase of the host lipid. Hence, it is tempting to speculate that the solvation of the integral protein by lipid is similar in the two phases. However, this must not absolutely be the case, although the lipid selectivity observed here does argue strongly in its favour. In particular, it cannot be excluded that the protein is more aggregated in the gel phase than in the fluid phase, even if it is fully solvated by lipid in both cases. The lipid stoichiometries of rhodopsin and cytochrome oxidase, for instance, that are obtained in the gel phase differ somewhat from those found in the fluid phase [4].

In conclusion, it has been demonstrated that lipid/protein titrations in reconstituted complexes with an integral protein can be used to investigate the stoichiometry and selectivity of lipid–protein interaction by saturation transfer EPR of spin-labelled lipids in the gel phase. These experiments are analogous to the usual lipid/protein titrations of the conventional spin label EPR spectra in the fluid phase. They rely for their success on the additivity of the saturation transfer EPR integral in multi-component systems [6], and on the fact that the dynamic properties of the protein-associated spin labels differ from those in the gel phase, resulting in a distinguishable spin-lattice relaxation and hence distinguishable saturation behaviour. This simple formulation is possible because, in the gel phase, any exchange with the bulk lipid of the lipids associated with the protein is too slow to affect the STEPR spectra [10].

We thank Mrs. S. Chatterjee and Frau B. Angerstein for their skillful technical assistance in the preparation of the protein and spin labels, respectively.

References

1 Marsh, D. (1985) in Progress in Protein–Lipid Interactions (Watts, A. and De Pont, J.J.H.H.M., eds.), Vol. 1, pp. 143–172, Elsevier, Amsterdam.
2 Bienvenue, A., Bloom, M., Davis, J.H. and Devaux, P.F. (1982) J. Biol. Chem. 257, 3032–3038.
3 Paddy, M.R., Dahlquist, F.W., Davis, J.H. and Bloom, M. (1981) Biochemistry 20, 3152–3162.
4 Bloom, M. and Smith, I.C.P. (1985) in Progress in Protein–Lipid Interactions (Watts, A. and De Pont, J.J.H.H.M., eds.), Vol. 1, pp. 61–88, Elsevier, Amsterdam.
5 Pates, R.D. and Marsh, D. (1987) Biochemistry 26, 29–39.
6 Horváth, L.I. and Marsh, D. (1983) J. Magn. Reson. 54, 363–373.
7 Brophy, P.J., Horváth, L.I. and Marsh, D. (1984) Biochemistry 23, 860–865.
8 Hemminga, M.A., De Jager, P.A., Marsh, D. and Fajer, P. (1984) J. Magn. Reson. 59, 160–163.
9 Thomas, D.D., Dalton, L.R. and Hyde, J.S. (1976) J. Chem. Phys. 65, 3006–3024.
10 Horváth, L.I., Brophy, P.J. and Marsh, D. (1993) Biophys. J. 64, 622–631.
11 Brotherus, J.R., Griffith, O.H., Brotherus, M.O., Jost, P.C., Silvius, J.R. and Hokin, L.E. (1981) Biochemistry 20, 5261–5267.
12 Marsh, D. and Horváth, L.I. (1992) J. Magn. Reson. 99, 323–331.