Spin-Label Electron Spin Resonance Study of Bacteriophage M13 Coat Protein Incorporation into Mixed Lipid Bilayers†

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ABSTRACT: The major coat protein of bacteriophage M13 was incorporated in mixed dimyristoylphosphatidylcholine/dimyristoylphosphatidylglycerol (80/20 w/w) vesicles probed with different spin-labeled phospholipids, labeled on the C-14 atom of the *sn*-2 chain. The specificity for a series of phospholipids was determined from a motionally restricted component seen in the electron spin resonance (ESR) spectra of vesicles with the coat protein incorporated. At 30 °C and pH 8, the fraction of motionally restricted phosphatidic acid spin-label is 0.36, 0.52, and 0.72 for lipid/protein ratios of 18, 14, and 9 mol/mol, respectively. The ESR spectra, analyzed by digital subtraction, resulted in a phospholipid preference following the pattern cardiolipin = phosphatidic acid > stearic acid = phosphatidylserine = phosphatidylglycerol > phosphatidylcholine = phosphatidylethanolamine. The specificities found are related to the composition of the target *Escherichia coli* cytoplasmic membrane.

In vivo bacteriophage M13 enters the *Escherichia coli* cell leaving its coat protein in the cytoplasmic membrane (Marvin & Wachtel, 1975). After DNA duplication and coat protein synthesis, both progeny as well as parental coat protein are stored as integral membrane proteins (Wickner, 1976). During the membrane-bound assembly of new virions, the viral DNA is complexed with many copies of the major coat protein, without lysis of the host cell.

The major (gene 8 product) M13 coat protein (M_r 5240) consists of a basic carboxyl terminus, a central core of 19 hydrophobic amino acids, and an acidic amino terminus (Nakashima & Konigsberg, 1974; Hagen et al., 1978). It has been shown that this hydrophobic, integral membrane protein can only be incorporated at high levels in model membranes containing at least 20 wt % negatively charged phospholipids, which results in the formation of stable vesicles (Hagen et al., 1978; Datema et al., 1988a). Also, in vivo molecular association of the M13 coat protein with negatively charged cardiolipin in the *E. coli* membrane was suggested from altered host lipid metabolism after insertion of the M13 coat protein (Chamberlain & Webster, 1976).

Previously, the assembly and disassembly of plant viruses, the coat protein–nucleic acid interaction within the virus [for review, see Hemminga et al. (1985)], and the interaction of coat protein with model membranes (Datema et al., 1987a) have been studied by magnetic resonance. An important and related problem concerns the mechanism of phage infection of the host cell. Since, as noted above, M13 coat protein can be incorporated at high levels into phospholipid bilayers only if they contain negatively charged phospholipids (Hagen et al., 1978; Datema et al., 1988a), the interaction of the coat protein with negatively charged phospholipids may be of importance for the mechanism of the phage entry into the cell. Electron spin resonance (ESR)[1] measurements with spin-labeled phospholipids have been found to provide a particularly direct means of investigating the specificity of lipid interactions with integral proteins (Jost et al., 1973; Watts et al., 1979; Knowles et al., 1981; Marsh, 1981, 1985; Marsh & Watts, 1982; Griffith et al., 1982; Brophy et al., 1984; Esmann et al., 1985; Pates et al., 1985; Devaux & Seigneuret, 1985). However, the method has not yet been applied to study the mechanism of virus infection, apart from our preliminary results with M13 coat protein reported recently (Hemminga, 1987).

In the present work the bacteriophage M13 coat protein has been incorporated in mixed phospholipid membranes composed of 80 wt % dimyristoylphosphatidylcholine (DMPC) and 20 wt % dimyristoylphosphatidylglycerol (DMPG), probed with a variety of different spin-labeled phospholipids. The presence of the M13 coat protein was found to induce a second ESR spectral component, characteristic of a motionally restricted lipid population such as has been observed previously with other integral proteins. The two-component spectra were used to investigate the preference of the different spin-labeled phospholipids for the coat protein. A high specificity was found for cardiolipin, a negatively charged lipid of the target *E. coli* membrane.

Materials and Methods

Chemicals. DMPC and DMPG were purchased from Sigma Chemical Co. and used without further purification.

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[1] Abbreviations: ESR, electron spin resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; 14-SASL, 14-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; 14-PASL, 14-PESL, 14-PCSL, 14-PSSL, and 14-PGSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoric acid, -phosphoethanolamine, -phosphocholine, -phosphoserine, and -phosphoglycerol; 14-CLSL, 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]glycero-3-phospho]-*sn*-glycerol; L/P ratio, lipid to protein molar ratio; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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A series of spin-labeled phospholipids labeled on the C-14 atom of the *sn*-2 chain was used: 14-PASL, 14-PGSL, 14-PCSL, 14-CLSL, 14-PSSL, 14-PESL, and 14-SASL. All phospholipid spin-labels have been synthesized from lysophosphatidylcholine prepared from phosphatidylcholine from egg yolks, which was then acylated on the *sn*-2 position with the stearic acid spin-label, 14-SASL. The phospholipid labels thus contain predominantly palmitic acid chains in the *sn*-1 position. A description of the synthesis of the spin-labels can be found in Marsh and Watts (1982).

Sample Preparation and Characterization. Bacteriophage M13 was grown and isolated as described (Garssen et al., 1977). The major (gene 8 product) M13 coat protein was isolated according to Knippers and Hoffmann-Berling (1966). M13 coat protein was incorporated in mixed (80/20 w/w) DMPC/DMPG vesicles by cholate dialysis as described (Hagen et al., 1978) with some modifications. Typically, 32 mg of DMPC, 8 mg of DMPG, and 0.72 mg of spin-labeled phospholipid were suspended in 5 mL of 5 mM Tris-HCl buffer, pH 8.0, containing 8.0 M urea, 20 mM ammonium sulfate, 0.2 mM EDTA, and 2 wt % sodium cholate. Thus, the spin-label amount was 1.8 mol % of the total lipid. Dependent on the required lipid/protein ratio, M13 coat protein was added and dissolved by vortexing and incubation at 55 °C until a clear solution was obtained. After incubation, the suspension was dialyzed for 48 h at 4 °C against 3 mM phosphate buffer, pH 8.0, and 0.02 mM EDTA, containing 10% (v/v) methanol, with buffer changes at 12, 24, and 36 h. In the last dialysis, no methanol was added to the buffer. Samples were concentrated by freeze-drying and resuspended in 0.15 mL of H_2O. To obtain homogeneous samples of multilamellar vesicles, the samples were vortexed and heated through the phase transition several times. At this point, aliquots of the samples were taken to determine the protein and the phospholipid concentration (Bartlett, 1959; Peterson, 1977), from which the lipid/protein ratio was calculated. Error in these ratios was less than 10%. The sample homogeneity was checked by sucrose density gradient centrifugation. The material was loaded onto a continuous (5–40%) sucrose gradient and centrifuged (4 h, 182000*g*, 4 °C). In all cases, one single, sharp band was observed, indicating that each sample had a uniform L/P ratio. ^{31}P NMR experiments on similar samples have indicated that all lipids are arranged in a bilayer and that the samples do not contain small vesicles, which would give rise to isotropic averaging of the chemical shift anisotropy (Datema et al., 1988a). For ESR measurements samples were transferred into 0.05-mL glass capillaries.

Electron Spin Resonance Spectroscopy. ESR spectra were recorded on a Varian E-12 Century Line spectrometer with nitrogen gas flow temperature regulation. Spectra were digitized with a Digital Equipment Corp. LPS system and a dedicated PDP 11/10 computer with a VT 11 display. ESR spectrometer settings were 5-mW microwave power, 0.1-mT modulation amplitude, 128-ms time constant, 240-s scan time, 10-mT scan width, and 324-mT center field. Up to 13 spectra were accumulated to improve the signal/noise ratio.

Results

The ESR spectra of the 14-PASL spin-label in M13 coat protein–DMPC/DMPG multilayers are shown in Figure 1. The spectra were taken at 30 °C, which is well above the gel to liquid-crystalline phase transition region from 19 to 23 °C of the mixed DMPC/DMPG alone, as verified by the temperature dependence of the ESR spectra (data not shown). Apart from the motionally averaged sharp three-line spectrum, typical for liquid-crystalline phase lipid, the M13 coat protein

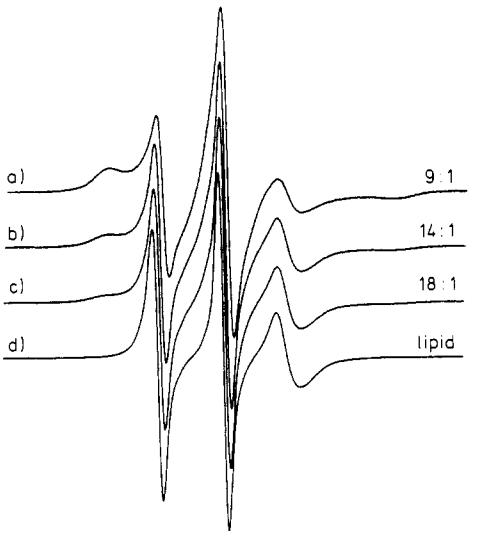

FIGURE 1: ESR spectra at 30 °C of the 14-PASL phosphatidic acid spin-label in M13 coat protein–DMPC/DMPG (80/20 w/w) complexes of different lipid/protein ratios: (a) lipid/protein = 9:1 mol/mol; (b) lipid/protein = 14:1 mol/mol; (c) lipid/protein = 18:1 mol/mol; (d) lipid alone. Total scan width = 10 mT.

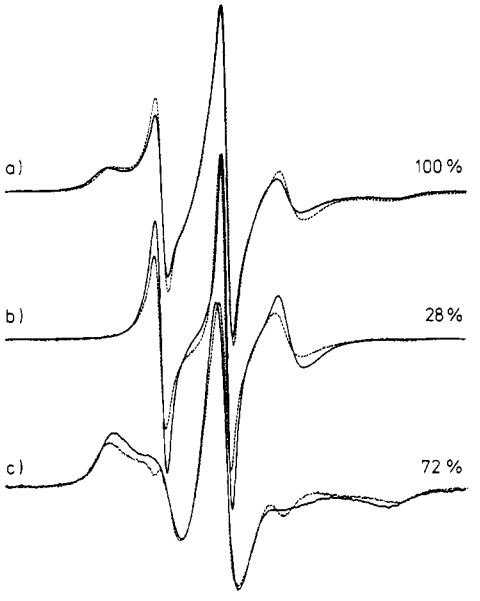

FIGURE 2: Spectral subtraction and addition with the 14-PASL spin-label. Full lines are original spectra. (a) M13 coat protein in DMPC/DMPG (80/20 w/w) at a lipid/protein ratio of 9:1 mol/mol, recorded at 30 °C; (b) DMPC/DMPG (80/20 w/w) alone recorded at 26 °C; (c) motionally restricted component comparison spectrum (14-PCSL in sonicated DMPC vesicles at 4 °C). Dashed lines are summed and difference spectra: (a) 28% lipid alone spectrum plus 72% motionally restricted spectrum; (b) protein–lipid spectrum minus 72% motionally restricted spectrum; (c) protein–lipid spectrum minus 28% lipid alone spectrum. Total scan width = 10 mT.

containing samples display a second, broader component with strongly restricted motion on the ESR time scale.

The two-component spectra were analyzed by spectral subtraction/addition as illustrated in Figure 2 [cf. Esmann et al. (1985)]. Digital subtraction of the lipid spectrum (Figure 2b, full line) from the spectrum of M13 coat protein–DMPC/DMPG multilayers (Figure 2a, full line) yields the spectrum of the motionally restricted component (Figure 2c, dashed line). Double integration gives the percentage of this component to be 72% of the total intensity. Complementary subtraction, i.e., of a broader gel phase lipid spectrum (Figure 2c, full line), yielded the spectrum of the fluid bilayer component (Figure 2b, dashed line), which resembles the lipid

Table I: Fraction of Motionally Restricted 14-PASL Spin-Label, f, in M13 Coat Protein–DMPC/DMPG (80/20 w/w) Complexes for Various Lipid/Protein Ratios (L/P)[a]

lipid/protein (mol/mol)	f	(L/P)f (mol/mol)
18	0.36	6.5
14	0.52	7.3
9	0.72	6.6

[a] Fractions were determined from the ESR difference spectra of the 14-PASL at 30 °C. Typical error in L/P mole/mole ratio is 10%. Typical error in f is 5%.

Table II: Fraction of Motionally Restricted Spin-Label, f, in M13 Coat Protein–DMPC/DMPG (80/20 w/w) Complexes for Various Spin-Labels[a]

spin-label	f	$[f/(1-f)]/[f/(1-f)]_{PCSL}$
14-CLSL	0.72	4.2
14-PASL	0.72	4.2
14-SASL	0.59	2.3
14-PSSL	0.56	2.1
14-PGSL	0.49	1.6
14-PCSL	0.38	1.0
14-PESL	0.36	0.9

[a] Fractions were determined from the ESR difference spectra of the spin-label at 30 °C for lipid/protein ratio of 9:1 mol/mol. Typical error in f is 5%. $f/(1-f)$ is the ratio of motionally restricted to fluid lipid spin-label component. Values are normalized to those for the 14-PCSL spin-label.

spectrum in the absence of M13 coat protein. By double integration of this spectrum, the relative intensity of the fluid component was determined to be 28% of the total spin-label intensity. These complementary quantitations agree to within an experimental error of 5%. The agreement of the addition spectrum (Figure 2a, dashed line) with the original experimental spectrum provides a further check on the consistency of the method. The fraction of motionally restricted lipid f, obtained from the mean value of the subtractions, is given for the different lipid/protein ratios in Table I. These values were found to vary relatively little with temperature over the range for which satisfactory subtractions could be obtained (data not shown). The values bear a direct relation to the lipid/protein ratio, L/P, as seen by the approximate constancy of the product (L/P)f in Table I.

The specificity of lipid interaction with the coat protein was investigated with different lipid species, all with the spin-label on the C-14 atom of the *sn*-2 chain. Figure 3 shows the ESR spectra at 30 °C of the various spin-labeled lipids in M13 coat protein–DMPC/DMPG multilayers of a lipid/protein molar ratio of 9:1. In all spectra a well-resolved motionally restricted component is superimposed on the fluid component. The motionally restricted components are very similar in maximum outer hyperfine splitting value, $2A_{max}$ = 6.2 mT, but have different relative intensities. The fraction of motionally restricted component, obtained by spectral subtraction, for each of the spin-labeled lipids is given in Table II. For comparison of the relative selectivities, the normalized ratio of motionally restricted to fluid components is also given in Table II. For a single lipid host, these values would give the ratios of the relative association constants for the different spin-labeled lipids [see, e.g., Marsh (1985)]. Because of the presence of the negatively charged lipid in the mixtures, the values in Table II, therefore, represent lower limits for the selectivity relative to the pure phosphatidylcholine.

Discussion

Incorporation of the M13 coat protein at high levels in stable vesicles requires the presence of negatively charged lipids (Hagen et al., 1978; Datema et al., 1988a). This is especially

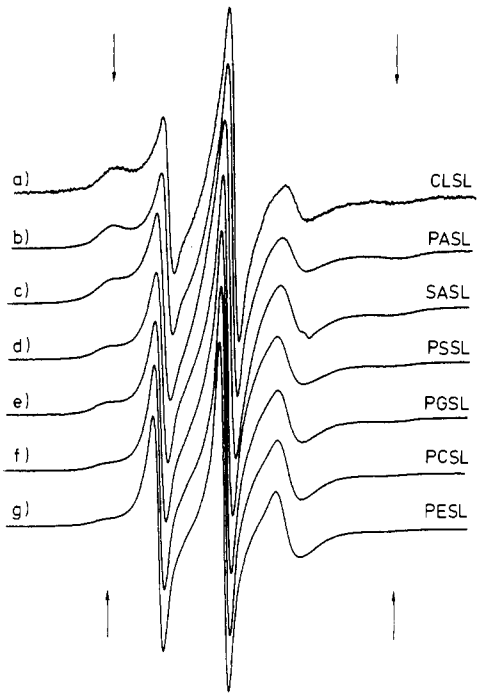

FIGURE 3: ESR spectra at 30 °C of DMPC/DMPG (80/20 w/w) dispersions with incorporated M13 coat protein at lipid/protein ratio = 9:1 mol/mol and containing different C-14-position spin-labeled lipids: (a) cardiolipin spin-label, 14-CLSL; (b) phosphatidic acid spin-label, 14-PASL; (c) stearic acid spin-label, 14-SASL; (d) phosphatidylserine spin-label, 14-PSSL; (e) phosphatidylglycerol spin-label, 14-PGSL; (f) phosphatidylcholine spin-label, 14-PCSL; (g) phosphatidylethanolamine spin-label, 14-PESL. The arrows in the outer wings of the spectra indicate the motionally restricted spin-label component, which is not present in the spectra of the lipids alone. Total scan width = 10 mT.

interesting in view of the lipid composition of the target *E. coli* membrane, which is as follows: phosphatidylethanolamine, 74%; phosphatidylglycerol, 19%; cardiolipin, 3% (Burnell et al., 1980). The spin-label ESR results presented here show that the coat protein exhibits different interactions with the different negatively charged spin-labeled lipids. A clear second component is seen in the ESR spectra of vesicles containing M13 coat protein. The fraction of motionally restricted component depends directly on the amount of protein present in the bilayer (see Figure 1). The product of the fraction of motionally restricted lipid and the lipid/protein ratio remains approximately constant (Table I), although for a mixed lipid system this can only indirectly be related to the total number of lipids interacting with the protein. Furthermore, the apparent temperature dependence of the fraction of motionally restricted lipid is small (data not shown). Also, the ratio of the fraction of restricted 14-PCSL and 14-PGSL is constant over the temperature range of 30–50 °C. The influence of M13 coat protein on the phase transition at 19–23 °C is small. The temperature range is only slightly broader, 18–25 °C in the presence of the protein (data not shown). These results clearly indicate that the motionally restricted component represents lipid molecules directly influenced by the coat protein [cf. Marsh (1985)].

The total percentage of motionally restricted lipid at L/P = 9 mol/mol, as determined from the fractions of motionally restricted 14-PCSL (f = 0.38) and 14-PGSL (f = 0.49) in the mixed DMPC/DMPG (80/20 w/w), is approximately 40%. This means that at L/P = 9 mol/mol approximately 4 mol of lipid molecules is directly associated with the protein per mole of M13 coat protein, while approximately 5 mol of lipid is in a fluid lipid environment. The ratio of motionally re-

stricted to fluid lipids of 0.40 is lower than one would expect on the basis of a model in which a single M13 coat protein molecule spans the membrane as a monomer. Therefore, the spin-label ESR results indicate protein aggregation, in agreement with time-resolved fluorescence anisotropy (Datema et al., 1987b) and ^2H NMR measurements (Datema et al., 1988b).

The protein-associated spectrum in Figure 2c has a hyperfine splitting of 6.2 mT, indicating a considerably reduced motion relative to that of the bilayer lipids. Since the fluid and the restricted components are well resolved (see Figure 2), the rate of exchange between the lipid molecules interacting with the protein and the free bilayer lipids is slow on the ESR time scale (Marsh, 1981; Devaux & Seigneuret, 1985). Comparing our spectra with simulations based on two-site exchange (L. I. Horvath, P. J. Brophy, & D. Marsh, personal communication) suggests that the exchange rate of the lipid molecules between the two components is of the order of 10^7 s^{-1}.

The specificity of the M13 coat protein for several phospholipids, determined from the fractions of restricted component for the different phospholipids, can be divided into three groups as seen in Table II. 14-PCSL and 14-PESL, both zwitterionic phospholipids, show little specificity ($f = 0.37$) and consequently little preferential interaction with the coat protein. Phosphatidylethanolamine, which accounts for 74% of the lipids in the *E. coli* target membrane (Burnell et al., 1980), has the smallest interaction with the coat protein and, therefore, provides a good reference for comparison with the other phospholipids. The negatively charged phospholipids, all bearing one negative charge per two fatty acid chains at pH 8, consist of two groups, one comprising 14-PSSL, 14-PGSL, and the fatty acid 14-SASL, displaying an intermediate specificity ($f \simeq 0.55$), and the other comprising 14-PASL and 14-CLSL, which show a larger specificity for the protein ($f = 0.72$). Consequently, the specificity cannot simply be explained by electrostatic interaction, and as the hydrocarbon chains are all the same for the phospholipid labels used, the configuration of the phospholipid head group must play a role in the interaction with the protein. A preference for negatively charged phospholipids, as found here, is seen also for most integral membrane proteins so far investigated, with the exception of rhodopsin (Watts et al., 1979), although the detailed specificity pattern differs between the different proteins (Marsh, 1985). The specificity of M13 coat protein for 14-CLSL is in good agreement with molecular association of cardiolipin with the coat protein as suggested from altered host lipid metabolism after infection of *E. coli* with M13 bacteriophage, indicating increased cardiolipin synthesis (Chamberlain & Webster, 1976). The limited selectivity for phosphatidylglycerol may also be significant, since there is a relatively high proportion of phosphatidylglycerol (19% of the total lipids) in the *E. coli* cytoplasmic membrane (Burnell et al., 1980), which does not increase after infection (Chamberlain & Webster, 1976).

The lipid specificity pattern for the M13 coat protein may therefore be essential for understanding the molecular mechanisms underlying (1) infection of the host cell by the bacteriophage, (2) storage of the coat protein in the host cytoplasmic membrane during reproduction of the viral DNA, and (3) membrane-bound assembly of the bacteriophage.

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Registry No. DMPC, 18194-24-6; DMPG, 61361-72-6; stearic acid, 57-11-4.

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