Stoichiometry, Selectivity, and Exchange Dynamics of Lipid-Protein Interaction with Bacteriophage M13 Coat Protein Studied by Spin Label Electron Spin Resonance. Effects of Protein Secondary Structure†

Sjaak J. C. J. Peelen,‡,§ Johan C. Sanders,‡ Marcus A. Hemminga,‡ and Derek Marsh*,§

Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, D-3400 Göttingen, Federal Republic of Germany, and Department of Molecular Physics, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

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ABSTRACT: Bacteriophage M13 major coat protein has been isolated with cholate and reconstituted in dimyristoyl- and dioleoylphosphatidylcholine (DMPC and DOPC, respectively) bilayers by dialysis. Fourier transform infrared spectra of DMPC/coat protein recombinants confirmed that, whereas the protein isolated by phenol extraction was predominantly in a β-sheet conformation, the cholate-isolated coat protein contained a higher proportion of the α-helical conformation [cf. Spruijt, R. B., Wolfs, C. J. A. M., & Hemminga, M. A. (1989) *Biochemistry 28*, 9158-9165]. The cholate-isolated coat protein/lipid recombinants gave different electron spin resonance (ESR) spectral line shapes of incorporated lipid spin labels, as compared with those from recombinants with the phenol-extracted protein that were studied previously [Wolfs, C. J. A. M., Horváth, L. I., Marsh, D., Watts, A., & Hemminga, M. A. (1989) *Biochemistry 28*, 9995-10001]. Plots of the ratio of the fluid/motionally restricted components in the ESR spectra of spin-labeled phosphatidylglycerol were linear with respect to the lipid/protein ratio in the recombinants up to 20 mol/mol. The corresponding values of the relative association constants, K_r, and number of association sites, N_1, on the protein were $K_r \approx 1$ and $N_1 \approx 4$ for DMPC recombinants and $K_r \approx 1$ and $N_1 \approx 5$ for DOPC recombinants. Simulation of the two-component lipid spin label ESR spectra with the exchange-coupled Bloch equations gave values for the off-rate of the lipids leaving the protein surface of 2.0×10^7 s^{-1} at 27 °C in DMPC recombinants and 3.0×10^7 s^{-1} at 24 °C in DOPC recombinants. These rates are up to 4-5 times faster than the lipid exchange measured previously in DMPC recombinants with the phenol-extracted protein. A selectivity was found for phosphatidic acid whereas stearic acid, phosphatidylserine, and phosphatidylglycerol displayed only a slight preference, relative to phosphatidylcholine, for interaction with the cholate-isolated protein, in contrast to the considerably stronger lipid selectivity found previously with the phenol-extracted protein.

The filamentous bacteriophage M13 (Ray, 1977) enters the *Escherichia coli* cell, leaving its major coat protein (the gene 8 product) in the cytoplasmic membrane (Marvin & Wachtel, 1975). During replication and synthesis of phage proteins, the coat protein is stored in the cytoplasmic membrane as an integral protein (Wickner, 1976). Together with the newly synthesized proteins, this parental coat protein is used for the membrane-bound assembly of new phage particles (Armstrong et al., 1983) without lysis of the *E. coli* cell. The major coat protein (M_r 5240) with a total length of 50 amino acids consists of three domains: an acidic N-terminus (residues 1-20), a basic C-terminus (residues 40-50), and a hydrophobic core (residues 21-39) (Van Wezenbeek et al., 1980). The conformation of the major coat protein in the intact phage is known to be α-helical (Van Asbeck et al., 1969; Day, 1969).

The coat protein when extracted with phenol has essentially a β-sheet structure, whereas that isolated with cholate is predominantly α-helical in structure (Nozaki et al., 1976; Spruijt et al., 1989). The cholate-isolated protein is able to form small aggregates, whereas the phenol-extracted protein forms larger (polymeric) aggregates (Spruijt et al., 1989; De Jongh et al., 1990). Recent molecular dynamics simulations (Sanders et al., 1991) suggest that the α-helical and β-sheet conformations of the coat protein in membranes become more comparable energetically as the degree of polymerization of the β-sheet structure increases. Experimentally it is found that retention of the α-oligomeric structure of the coat protein in reconstituted systems depends on both the lipid headgroup and the degree of unsaturation of the lipid chains, as well as on ionic strength and the lipid/protein ratio (Spruijt et al., 1989; Spruijt & Hemminga, 1991). A predominantly β-sheet structure is characteristic of the outer membrane proteins of *E. coli*, e.g., the porins (Vogel & Jähnig, 1986), whereas the integral proteins of cytoplasmic and intracellular membranes are thought to have intramembranous portions consisting mostly of α-helices. It is therefore of considerable interest to compare the membrane properties, in particular the lipid-protein interactions, of these two different conformational forms of the M13 phage coat protein reconstituted in lipid bilayer systems.

Spin label electron spin resonance (ESR)[1] has previously been used to study lipid-protein interactions with the phe-

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* Author to whom correspondence should be addressed.
‡ Agricultural University, Wageningen.
§ Max-Planck-Institut, Göttingen.

[1] Abbreviations: ESR, electron spin resonance; FTIR, Fourier transform infrared spectroscopy; HPLC, high-performance liquid chromatography; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; 14-SASL, 13-(4,4-dimethyl-3-oxy-2-butyl-2-oxazolidinyl)tridecanoic acid; 14-PASL, -PSSL, -PGSL, -PESL, and -PCSL, 1-acyl-2-[13-(4,4-dimethyl-3-oxy-2-butyl-2-oxazolidinyl)tridecanoyl]-*sn*-glycero-3-phosphoric acid, -phosphoserine, -phosphoglycerol, -phosphoethanolamine, and -phosphocholine; L/P, lipid/protein molar ratio; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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nol-extracted M13 phage coat protein (Datema et al., 1987; Wolfs et al., 1989; Van Gorkom et al., 1990). In the present work, the interactions with the cholate-isolated form of the protein are examined in reconstitutions with dimyristoyl- and dioleoylphosphatidylcholine. Lipid/protein titrations have been performed to determine the number of lipid association sites and the relative association constants for lipids of different headgroups (PASL, SASL, PSSL, PGSL, PESL, and PCSL) with the cholate-isolated protein. The rates of lipid exchange at the association sites on the protein have been determined by spectral simulation, and the results have been compared with those obtained previously from reconstitutions with the phenol-extracted protein over the same range of lipid/protein ratios. It is found that the lipid exchange rates at the surface of the α-helical form of the protein are considerably faster and also that the lipid selectivity is less marked than with the polymeric β-sheet form of the protein.

Materials and Methods

Materials. Sodium cholate, DMPC, and DOPC were obtained from Sigma (St. Louis, MO). The phospholipids were shown to migrate as a single spot on thin-layer chromatography. EDTA and sodium chloride were from Merck (Darmstadt, FRG), and Tris-HCl was from Fluka (Buchs, Switzerland). Sepharose 6B-Cl was from Pharmacia (Uppsala, Sweden). Spin-labeled phospholipids with the nitroxide group on the 14-C atom of the *sn*-2 chain, 14-PCSL, -PASL, -PGSL, -PESL, and -PSSL, were synthesized by acylation of lysophosphatidylcholine with spin-labeled stearic acid, 14-SASL, and subsequent headgroup modification mediated by phospholipase D. Details of the spin label synthesis can be found in Marsh and Watts (1982).

Sample Preparation. Bacteriophage M13 was grown as described by Garssen et al. (1977) and isolated as described by Spruijt et al. (1989). The coat protein was isolated with sodium cholate by a modification of the procedure used by Makino et al. (1975). To dissociate the bacteriophage, 6 mL of a bacteriophage solution (concentration 10–15 mg/mL) was mixed with 12 mL of 120 mM cholate in 10 mM Tris-HCl, 0.2 mM EDTA, 140 mM NaCl, pH 8.0, and chloroform (4% v/v). The suspension was incubated at 37 °C with occasional vortexing until a clear, nonopalescent solution was obtained. This solution was loaded on a Sepharose 6B-Cl column (2.6 × 39 cm) and eluted with 10 mM cholate, 10 mM Tris-HCl, 0.2 mM EDTA, and 140 mM NaCl, pH 8.0, buffer to separate the major coat protein from the viral DNA. Fractions with an absorbance ratio A_{280}/A_{260} greater than 1.5 were collected, the cholate concentration was increased to 50 mM, and the resulting coat protein solution was then stored at 4 °C.

For reconstitution of the protein in lipid bilayers, typically 5 or 10 mg of freeze-dried phospholipid was suspended in 2 mL of 50 mM cholate, 10 mM Tris-HCl, 0.2 mM EDTA, and 140 mM NaCl, pH 8.0, buffer so that a clear solution was obtained. The required quantity of cholate-isolated M13 coat protein was added to achieve the desired lipid/protein ratio. The mixture was then dialyzed against a 100-fold excess of 10 mM Tris-HCl, 0.2 mM EDTA, and 140 mM NaCl, pH 8.0, buffer for 48 h at room temperature with buffer change every 12 h. It was found that adding NaCl to the buffer decreased the degree of aggregation of the M13 coat protein as assayed by HPLC [Spruijt & Hemminga, 1991; for HPLC conditions see Spruijt et al. (1989)]. After dialysis, the samples were concentrated by centrifugation (40 000 rpm, Beckmann 50Ti rotor, 4 °C). The supernatant was removed and the pellet resuspended in 1 mL of 10 mM Tris-HCl, 0.2 mM EDTA, and 140 mM NaCl, pH 8.0, buffer.

Spin label was incorporated from a stock solution in ethanol (1 mg/mL) at a level of approximately 1 mol % with respect to total lipid such that the total ethanol concentration was less than 0.1% (v/v). After incubation at room temperature for 2 h, unincorporated label and ethanol were removed by washing with excess buffer and concentration of the lipid/protein complex by centrifugation. The reconstituted sample was then transferred to a glass capillary (1 mm o.d.) and pelleted for 30 min in a bench centrifuge (10 000 rpm). Excess buffer was removed and the capillary sealed with the sample under argon.

Lipid phosphate and protein concentrations were determined according to Eibl and Lands (1969) and Peterson (1977), respectively. SDS–polyacrylamide (15%) gel electrophoresis was performed according to Laemmli (1970) and revealed no aggregates in SDS with a molecular weight greater than 40 000. Sucrose density gradient centrifugation demonstrated that reconstitution by the above method produced samples that were homogeneous in lipid/protein ratio.

ESR Spectroscopy. ESR spectra were recorded on a 9-GHz Varian E-12 Century Line spectrometer with nitrogen gas flow temperature regulation. The sample capillaries were contained within 4-mm quartz ESR tubes, containing silicone oil for thermal stability. Spectra were digitized using a Digital Equipment LPS system with a PDP 11/10 minicomputer. Instrumental settings were 10-mW microwave power, 1.25-G modulation amplitude, 100-kHz modulation frequency, 0.25-s time constant, 4-min scan time, 100-G scan range, and 3245-G center field. Up to 10 scans were accumulated to improve the signal/noise ratio. Spectral subtractions and additions were performed as described by Marsh (1982). The deviations between the values for the fraction of motionally restricted spin label obtained by the two complementary subtractions and by addition were less than 7.5%. Continuous temperature scans were performed at a rate of 2 °C/min by recording the height of the central ESR peak at increased vertical gain against the thermocouple output on an x–y recorder. The magnetic field was locked to the central peak during the temperature scan by feeding the output from the second modulation channel (1 kHz) to the field-frequency lock unit; cavity coupling was maintained by a servo mechanism [see Marsh and Watts (1981)].

Line shape simulations of the two-component spectra were performed using the exchange-coupled Bloch equations (Horváth et al., 1988a,b). The exchange model with uncorrelated orientations was used, corresponding to model II of Davoust and Devaux (1982). The motionally restricted component used as starting point for the exchange-coupled calculations was obtained by simulation of a matching spectrum of 14-PCSL in sonicated DMPC vesicles at 4 °C, and the mobile component used was obtained from the spectrum of 14-PGSL reconstituted in lipid without protein recorded at 25 °C for DMPC and at 20 °C for DOPC. Root mean square differences between the simulated and experimental two-component spectra evaluated over the entire scan range of 1000 spectral points were in the range of 1–2% of the total absolute spectral intensity. In addition to the rms deviation, a further criterion in fitting was that the value for the fraction of motionally restricted lipid obtained by simulation should be reasonably close to that obtained by spectral subtractions. Comparison of the data from subtractions and simulations and the consistency with variation of the lipid/protein ratio (see Tables I–III) gives an estimate of the likely errors involved.

Infrared Spectroscopy. Phenol-extracted M13 coat protein [prepared according to Knippers and Hoffman-Berling (1966)]

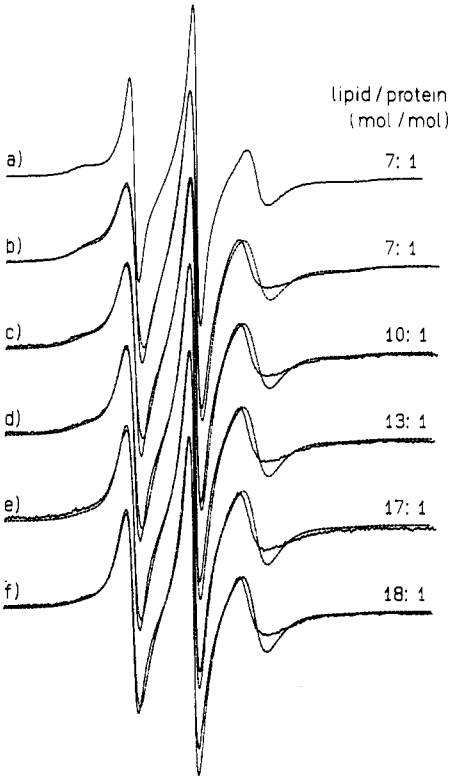

FIGURE 1: ESR spectra of the 14-PGSL spin label in M13 coat protein/DMPC recombinants as a function of lipid/protein ratio. (a) Phenol-extracted protein recorded at 30 °C; (b–f) cholate-isolated protein recorded at 27 °C. The lipid/protein ratios of the samples are (a) 6.7:1 mol/mol, (b) 7.0:1 mol/mol, (c) 10.0:1 mol/mol, (d) 13.0:1 mol/mol, (e) 17.5:1 mol/mol, and (f) 18.0:1 mol/mol. The full lines are experimental spectra and the dashed lines are spectra simulated using the exchange-coupled Bloch equations. Total scan range is 100 G.

and cholate-isolated protein were reconstituted in DMPC as described above. After reconstitution, the samples were pelleted by centrifugation at 4 °C (40 000 rpm, Beckmann 50Ti rotor). The supernatant was then removed, and the samples were freeze dried for 48 h. The samples were then rehydrated in D_2O and transferred to a thermostated cell holder with CaF_2 windows and 25-μm sample thickness. Infrared spectra were recorded on a Bruker IFS-25 Fourier transform spectrometer equipped with a DTGS detector. Approximately 1000 interferograms were collected at a spectral resolution of 4 cm^{-1}, apodized with a trapezoidal function, and Fourier transformed after one level of zero filling.

Results

Lipid/Protein Titrations. The ESR spectra of the 14-PGSL spin label in cholate-isolated M13 coat protein/DMPC recombinants of various lipid/protein ratios are given in Figure 1. The spectra are recorded at 27 °C, which is above the gel–fluid phase transition of the recombinants, as indicated by ESR continuous temperature scans (data not shown). The spectra clearly consist of two components: a narrow three-line component in the central part of the spectrum whose line shape corresponds to spin labels in a fluid lipid environment and a second broader component which is resolved in the outer wings of the spectrum and corresponds to spin labels undergoing restricted motion [cf. Marsh (1985)]. This second component arises from a direct interaction of the spin label chains with the coat protein, as indicated by the increase in its relative intensity with increasing protein content of the recombinants.

The ESR spectrum of the phenol-extracted protein (Wolfs et al., 1989) in a DMPC recombinant of lipid/protein ratio similar to that for the cholate-isolated protein recombinant of Figure 1b is given in Figure 1a for comparison. The two spectra are very clearly different in that the line widths of the fluid component of the spectrum for the phenol-extracted protein recombinant are considerably narrower than those for the cholate-isolated protein recombinant. This spectral difference can be attributed to differences in the exchange rate of the spin-labeled lipids off the surface of the protein and in the longer-range perturbations of the lipid mobility by the protein, as discussed later.

Table I: Lipid/Protein Ratio Dependence of the Fraction of Motionally Restricted 14-PGSL Spin Label, f, and Off-Rates for Exchange, τ_b^{-1}, in Cholate-Isolated M13 Coat Protein/DMPC Recombinants at 27 °C[a]

lipid/protein (mol/mol)	f_{sub}[b]	f_{sim}	τ_b^{-1} (s^{-1})
7.0	0.41	0.45	2.0×10^7
10.0	0.33	0.37	2.2×10^7
13.0	0.26	0.30	2.1×10^7
17.5	0.20	0.22	2.0×10^7
18.0	0.18	0.20	1.9×10^7

[a] Values of f are derived both from spectral subtractions (f_{sub}) and from simulations (f_{sim}). [b] Averaged value from the two complementary spectral subtractions and from spectral addition.

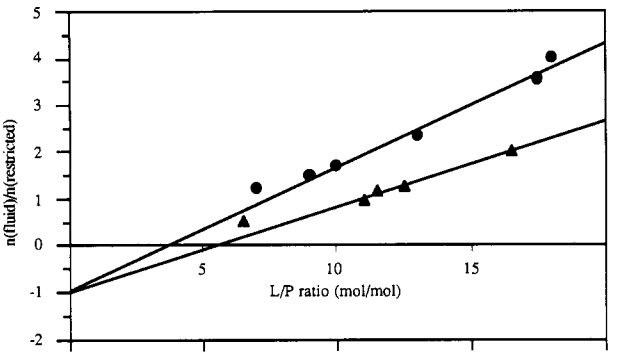

FIGURE 2: Lipid/protein titration of cholate-isolated M13 coat protein/phosphatidylcholine recombinants obtained from simulation of the ESR spectra of incorporated 14-PGSL spin label. (●) DMPC recombinants at 27 °C; (▲) DOPC recombinants at 24 °C. The ordinate is the ratio of the double-integrated intensity of the fluid and motionally restricted spectral components, n_f^*/n_b^*, and the abscissa is the total lipid/protein ratio, n_t, in the recombinant (cf. eq 1).

The relative proportions of the two components in the spectra of Figure 1 have been quantitated by digital subtraction and addition [see, e.g., Marsh (1982, 1985) and Datema et al. (1987)] and by two-component spectral simulation using the exchange-coupled Bloch equations [see, e.g., Ryba et al. (1987) and Horváth et al. (1988a,b)]. To allow for the broadening effects of exchange (and of longer-range lipid–protein interactions affecting the fluid component), fluid and gel-phase spectra from sonicated DMPC vesicles that best fit the fluid and motionally restricted components, respectively, in the recombinant spectra were chosen for the spectral subtractions/additions. For the fluid component, a sonicated DMPC vesicle spectrum recorded at a few degrees lower in temperature than that of the recombinant (but still above the phase transition) was taken. The two complementary subtractions, involving the two separate single spectral components, and spectral addition gave consistent values for the fraction, f, of motionally restricted spin labels. These values are given in Table I, along with those obtained from simulations (dashed lines in Figure 1) which were found to give essentially similar results.

The ratio of the fractions of fluid and motionally restricted spin labels, $(1-f)/f$, obtained by simulations is plotted as a function of lipid/protein ratio in Figure 2. The values ob-

tained by subtraction give a similar plot (cf. Table I). The results conform to the equation for equilibrium association of the spin-labeled lipids with the protein (Brotherus et al., 1981; Knowles et al., 1979):

$$n_f^*/n_b^* = (1/K_r)(n_t/N_1 - 1) \quad (1)$$

where $n_f^*/n_b^* = (1 - f)/f$ is the ratio of the fluid to the motionally restricted components in the ESR spectrum, n_t is the lipid/protein molar ratio in the recombinant, N_1 is the number of first-shell lipid association sites on the protein, and K_r is the average association constant of the spin-labeled lipids relative to the background DMPC host lipid. The values for K_r and N_1 found by linear regression on the data obtained from subtractions are 1.2 and 3.0, respectively, and those from the data obtained by simulations are 1.0 and 3.6. (In each case the point at lowest lipid/protein ratio has been omitted from the regressions because of deviations from linearity caused by direct protein–protein interactions). The number of first-shell sites, N_1, is approximately 4 per protein monomer, which is a similar number to that found for the phenol-extracted M13 coat protein in DMPC (Wolfs et al., 1989). The value of the relative association constant, K_r, is approximately 1, indicating that the 14-PGSL spin label has much the same affinity for the protein as have the host DMPC lipids.

Exchange Simulations. The simulations of the two-site exchange-coupled spectra from the lipid/protein titration series are given by the dashed lines in Figure 1. The simulated spectra show a consistent fit with the experimental spectra at 27 °C. Some discrepancies between the two are seen, particularly in the high-field region of the spectrum. These are most probably caused by perturbations of the lipids beyond the first motionally restricted shell. A fluid single-component spectrum corresponding to that of 14-PGSL in cholate-dialyzed DMPC, but recorded at a slightly lower temperature than that for the recombinant, was used for the exchange simulations in order to compensate partially for these effects (cf. Materials and Methods). However, this was not able to emulate entirely the obvious increase in anisotropy of the high-field lines, and, therefore, the fitting concentrated on the low-field and central regions of the spectrum which are most sensitive to exchange and least sensitive to these longer-range perturbations (Horváth et al. 1988a). The off-rates, τ_b^{-1}, for exchange from the surface of the protein found by these simulations are given in Table I. The intrinsic off-rates are independent of the total lipid/protein ratio in the recombinants, as expected (Marsh & Horváth, 1989; Horváth et al., 1988a). These off-rates for recombinants with the cholate-isolated protein are more than three times larger than the corresponding values reported for recombinants of the phenol-extracted protein in DMPC (Wolfs et al., 1989).

Lipid Selectivity. The ESR spectra of spin-labeled lipids with different polar groups in cholate-isolated M13 coat protein/DMPC recombinants of fixed lipid/protein ratio are given in Figure 3. The spectra were recorded at 30 °C; the full lines are the experimental spectra and the dashed lines are the simulations. The motionally restricted component is relatively larger in the spectrum of 14-PASL, indicating a preferential selectivity of the protein for phosphatidic acid over that for the other lipids. The fraction of motionally restricted component and the off-rates for exchange at the lipid–protein interface for the different spin labels were obtained by spectral subtraction and simulation and are given in Table II. Essentially similar results were obtained by both methods. The values of the fraction of motionally restricted spin label clearly demonstrate the selectivity of the protein for phosphatidic acid but reveal only a very slight selectivity of the other negatively

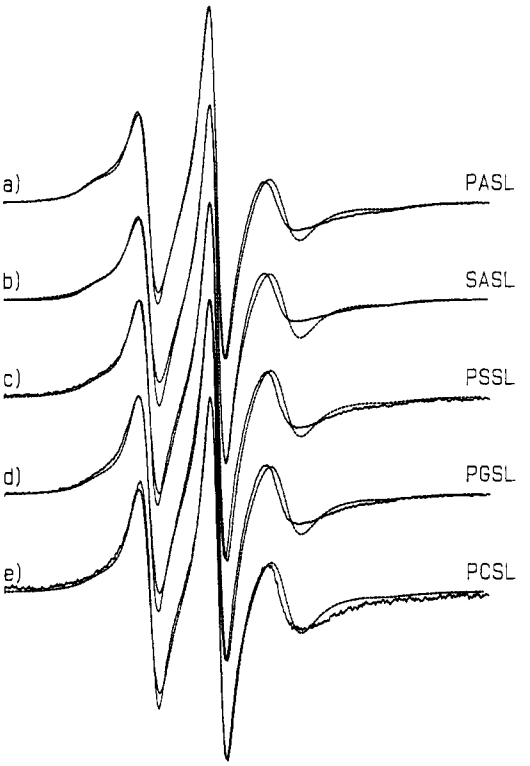

FIGURE 3: ESR spectra of different spin-labeled lipids in cholate-isolated M13 coat protein/DMPC recombinants of lipid/protein ratio 9:1 mol/mol, recorded at 30 °C. (a) Spin-labeled phosphatidic acid, 14-PASL; (b) spin-labeled stearic acid, 14-SASL; (c) spin-labeled phosphatidylserine, 14-PSSL; (d) spin-labeled phosphatidylglycerol, 14-PGSL, and (e) spin-labeled phosphatidylcholine, 14-PCSL. The full lines are experimental spectra, and the dashed lines are spectra simulated using the exchange-coupled Bloch equations. Total scan range is 100 G.

Table II: Dependence on Lipid Polar Headgroup of the Fraction of Motionally Restricted Spin Label, f, and Off-Rates for Exchange, τ_b^{-1}, in Cholate-Isolated M13 Coat Protein/DMPC Recombinants of Lipid/Protein Ratio 9:1 mol/mol at 30 °C[a]

spin label	f_{sub}[b]	f_{sim}	τ_b^{-1} (s^{-1})
14-PASL	0.47	0.49	1.4×10^7
14-SASL	0.39	0.42	1.8×10^7
14-PSSL	0.37	0.42	2.0×10^7
14-PGSL	0.36	0.40	2.2×10^7
14-PESL	0.33	0.38	2.2×10^7
14-PCSL	0.34	0.37	2.3×10^7

[a] Values of f are derived both from spectral subtractions (f_{sub}) and from simulations (f_{sim}). [b] Averaged value from the two complementary spectral subtractions and from spectral addition.

charged lipids over that for phosphatidylcholine. This pattern of lipid selectivity is rather different from that reported for recombinants of the phenol-extracted protein (Datema et al., 1987).

From eq 1, it is clear that it is possible to determine the ratio between the relative association constants of different spin-labeled lipids from measurements on recombinants of fixed lipid/protein ratio. Using the data of Table II obtained from simulation yields the following values for the different negatively charged lipids relative to phosphatidylcholine: K_r(PA)/K_r(PC) = 1.6, K_r(SA)/K_r(PC) = 1.2, K_r(PS)/K_r(PC) = 1.2, K_r(PG)/K_r(PC) = 1.1, and K_r(PE)/K_r(PC) = 1.0. The value obtained for 14-PGSL is in good agreement with that derived from the lipid/protein titration in Figure 2 and confirms that there is little selectivity of 14-PCSL over that for the parent unlabeled DMPC. The differential free energies of association relative to phosphatidylcholine [$\Delta G - \Delta G$(PC)

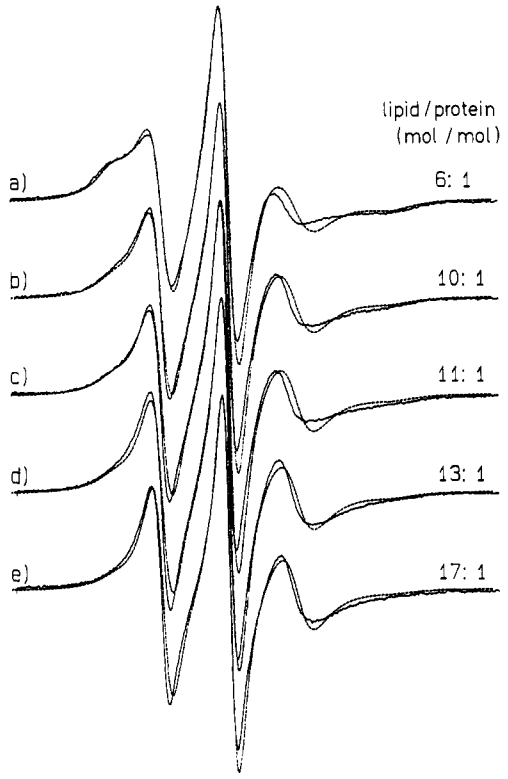

FIGURE 4: ESR spectra of the 14-PGSL spin label in cholate-isolated M13 coat protein/DOPC recombinants as a function of lipid/protein ratio, recorded at 24 °C. The lipid/protein ratios of the samples are (a) 6.5:1 mol/mol, (b) 11.0:1 mol/mol, (c) 11.5:1 mol/mol, (d) 12.5:1 mol/mol, and (e) 16.5:1 mol/mol. The full lines are experimental spectra, and the dashed lines are spectra simulated using the exchange-coupled Bloch equations. Total scan range is 100 G.

= $-RT \ln [K_r/K_r(PC)]$] can be obtained from the ratios of the relative association constants yielding values of −1.2 kJ·mol^{-1} for 14-PASL and −0.5 kJ·mol^{-1} for 14-SASL and very small values for 14-PSSL and 14-PGSL. The selectivity for phosphatidic acid given in Table II is also reflected in the off-rate for exchange, which is markedly slower than that for the other spin-labeled lipids. The data on the exchange dynamics in fact display the expected dependence on the thermodynamics of selectivity (Marsh, 1985; Horváth et al., 1988a,b):

$$\tau_b^{-1}(PC)/\tau_b^{-1}(L) = K_r(L)/K_r(PC) \qquad (2)$$

where L refers generally to a spin-labeled lipid and PC to spin-labeled phosphatidylcholine. From Table II, the ratios of the off-rates are $\tau_b^{-1}(PC)/\tau_b^{-1}(PA) = 1.6$, $\tau_b^{-1}(PC)/\tau_b^{-1}(SA) = 1.3$, $\tau_b^{-1}(PC)/\tau_b^{-1}(PS) = 1.2$, $\tau_b^{-1}(PC)/\tau_b^{-1}(PG) = 1.0$, and $\tau_b^{-1}(PC)/\tau_b^{-1}(PE) = 1.0$, which are in good agreement with the reciprocal ratios of the relative association constants given above.

Recombinants with Unsaturated Phosphatidylcholine. Because most of the phospholipids in the *E. coli* plasma membrane have at least one unsaturated acyl chain (Burnell et al., 1980), it is of interest to investigate recombinants with an unsaturated lipid. The ESR spectra of the 14-PGSL spin label in recombinants of cholate-isolated M13 coat protein with DOPC are given in Figure 4. The spectra are recorded at a temperature of 24 °C that is well above the gel–fluid transition temperature (−16 °C) of pure DOPC (Van Dijck et al., 1976). Compared with the spectra from recombinants of the cholate-isolated protein with DMPC, the fluid components in the spectra from recombinants with DOPC are yet even broader. It seems likely that the longer-range perturbations of the lipid shells by the protein and/or the lipid exchange rate is greater in the unsaturated lipid than in DMPC. The fraction of motionally restricted spin-labeled lipid has again been quantitated by spectral subtractions and additions and by exchange-coupled simulations (dashed lines in Figure 4). To correct for exchange and for the extra broadening of the fluid component a spectrum of cholate-dialysed DOPC recorded at 12 °C was used for the fluid single component in the spectral subtractions. Again a fluid single-component spectrum recorded at a few degrees lower in temperature (cholate-dialyzed DOPC at 20 °C) than that of the recombinant was used as the nonexchanging fluid component in the exchange-coupled simulations, in order to allow for the longer-range perturbations.

Table III: Lipid/Protein Ratio Dependence of the Fraction of Motionally Restricted 14-PGSL Spin Label, f, and Off-Rates for Exchange, τ_b^{-1}, in Cholate-Isolated M13 Coat Protein/DOPC Recombinants at 24 °C[a]

lipid/protein (mol/mol)	f_{sub}[b]	f_{sim}	τ_b^{-1} (s^{-1})
6.5	0.75	0.65	2.0×10^7
11.0	0.45	0.51	3.0×10^7
11.5	0.40	0.46	2.9×10^7
12.5	0.35	0.44	3.0×10^7
16.5	0.28	0.33	3.1×10^7

[a] Values of f are derived both from spectral subtractions (f_{sub}) and from simulations (f_{sim}). [b] Averaged value from the two complementary spectral subtractions and from spectral addition.

The quantitative results obtained from the spectral subtractions and simulations are given in Table III. The values for the fraction of motionally restricted spin label are somewhat larger than those obtained from the DMPC recombinants. The lipid/protein titration for the DOPC recombinants is also plotted in Figure 2. The values for the relative association constant, K_r, and the number of first-shell lipid association sites, N_1, obtained by linear regression are 0.9 and 5.1, respectively, from the spectral subtractions, and 1.0 and 5.6 from the spectral simulations. The point at the lowest lipid/protein ratio is again excluded from the linear regression because of the deviation from linearity caused by protein–protein interactions. The value of $K_r \approx 1$ is the same as that obtained for the DMPC recombinants and therefore indicates that the unsaturated chains do not confer a special selectivity of interaction with the protein relative to the saturated chains. This is in contrast to the effects of the lipid headgroups. The number of first-shell sites, $N_1 \approx 5$, is somewhat higher than found with DMPC but does not indicate that the hydrophobic surface of the protein available to the lipid is grossly different from that in the DMPC recombinants.

Infrared Spectra. The different conformations of the cholate-isolated and phenol-extracted forms of the M13 coat protein and of the closely related fd bacteriophage coat protein in phospholipids and ionic detergents have been characterized previously by circular dichroism measurements (Nozaki et al., 1976, 1978; Spruijt et al., 1989). These results have been confirmed here from the FTIR spectra in the amide region of the phenol-extracted and the cholate-isolated M13 coat protein, both reconstituted in DMPC and dispersed in D_2O buffer. The spectra recorded both above and below the gel-to-fluid phase transition of DMPC were essentially similar in this region. The cholate-isolated M13 coat protein showed a major absorption at 1650–1660 cm^{-1} (1654 cm^{-1} by deconvolution) in the amide I region of the spectrum. The residual intensity in the amide II region had its major absorption at 1540–1550 cm^{-1}. Both of these absorption maxima are typical for an α-helical conformation of the protein. On the other hand, the phenol-extracted M13 coat protein showed

principal absorptions at 1625 cm^{-1} (amide I) and 1520–1525 cm^{-1} (amide II), which are typically found for β-sheet structures.

Discussion

The conformational polymorphism of the M13 phage coat protein is well known (Nozaki et al., 1976, 1978; Spruijt et al., 1989; Spruijt & Hemminga, 1991). Previous spin label ESR studies of lipid–protein interactions with the M13 coat protein have been performed with phenol-extracted preparations (Datema et al., 1987; Wolfs et al., 1989) for which it has been shown that the protein has about 70% β-sheet structure (Spruijt et al., 1989). These studies are of extreme interest since all other spin label characterizations of lipid–protein interactions [see, e.g., Marsh (1985)] have been performed with integral proteins the intramembranous sections of which are thought to be composed of α-helices. Since the porins and other integral proteins of the *E. coli* outer membrane are known to contain a high proportion of β-sheet structure (Vogel & Jähnig, 1986; Jap et al., 1990), it is of great interest to compare the lipid–protein interactions of a protein with β-sheet structure, in this case the phenol-extracted M13 phage coat protein, with those of the same protein in an α-helical conformation. The comparison system is provided here by measurements on the cholate-isolated M13 coat protein reconstituted in either saturated or unsaturated phosphatidylcholines. The presence of α-helical structure in the lipid recombinants with the cholate-isolated protein is confirmed by infrared spectroscopy.

Stoichiometry. Over the range of lipid/protein ratio up to approximately 20:1 (mol/mol), the stoichiometry of the motionally restricted spin-labeled lipid population remains essentially constant for recombinants of the cholate-isolated protein with both DMPC and DOPC. Only at the lowest lipid/protein ratio studied (ca. 6–7 mol/mol) do the lipid/protein titrations given in Figure 2 deviate significantly from linearity due to an appreciable amount of nonspecific protein–protein contacts. The values deduced for the stoichiometry of the motionally restricted lipid component (ca. 4–5 mol/mol) are remarkably similar to those obtained previously for the phenol-extracted protein in DMPC (Wolfs et al., 1989). They correspond neither to the number of lipids (approximately 10, assuming a lipid chain diameter of 4.8 Å and a helix diameter of 10 Å) that can be accommodated around the intramembranous perimeter of a single transmembrane α-helix nor to the number of lipids per monomer (approximately 2)[2] that would directly contact the hydrophobic surface of an antiparallel β-sheet polymer structure. The stoichiometry of lipid–protein interaction with the phenol-extracted protein has been explained previously by assuming that the protein is incorporated in the lipid in an aggregated state which is accompanied by lipid trapping (Datema et al., 1987; Wolfs et al., 1989). In this case, the β-polymeric structure is so large that the stoichiometry of the motionally restricted lipid is insensitive to any heterogeneity in size of the aggregates. For the cholate-isolated protein studied here, it must be assumed that, over the range of lipid/protein ratios studied, the α-helical part of the preparation is also aggregated, to a limited extent, as suggested additionally by the results from SDS–polyacrylamide gel electrophoresis. This would have the effect of reducing the hydrophobic surface of the protein accessible to the lipid. For example, a tetrameric degree of oligomerization would be required, on average, to reduce the number of lipids associated with the intramembranous section of the α-helical form of the protein to four per monomer. It is also likely that an assembly of several intramembranous α-helices is more able to provide a hydrophobic surface capable of causing a detectable motional restriction of the lipid chains, such as is observed with larger integral proteins (Marsh, 1985), than is an isolated intramembranous helix. Although a heterogeneity may exist in the size of the aggregates of the α-oligomeric protein, the constancy of the stoichiometry of motionally restricted lipid with lipid/protein ratio implies the existence of a well-defined mean degree of aggregation. It should also be noted that the results of density gradient centrifugation indicate that there is no heterogeneity with respect to the lipid/protein ratio of the samples.

Lipid Selectivity. The extent of lipid selectivity found here for recombinants with the cholate-isolated protein is not so marked as that observed previously for recombinants with the phenol-extracted protein (Wolfs et al., 1989; Datema et al., 1987). The values of the relative association constants for the different negatively charged lipids are all considerably smaller in the recombinants with the cholate-isolated protein than in those with the phenol-extracted protein, for which relative association constants between 2 and 4 times that of phosphatidylcholine have been obtained for different anionic lipids. Although the present experiments were conducted at a somewhat higher ionic strength, it is unlikely that this can account wholely for the observed difference in lipid selectivity [cf. Esmann and Marsh (1985), Powell et al. (1987), and Horváth et al. (1988b)]. Indeed, another series of experiments at lower ionic strength showed no significant change in the lipid selectivity (data not shown). At least part of the difference in lipid selectivity may be attributed directly to conformational differences in the protein for the two cases. The more extended nature of a β-sheet structure relative to the rather compact α-helix would tend to locate those amino acid residues that give rise to the selectivity in a different position with respect to the lipid headgroups, resulting in a less optimal configuration for the α-helical form of the protein.

Exchange Rates. The most striking difference between the lipid interactions with the cholate-isolated and phenol-extracted proteins in DMPC recombinants is found for the rate of exchange at the lipid–protein interface. This is very clear from the spectral lineshapes obtained in the two systems [compare Figure 1 here with Figure 1 of Wolfs et al. (1989)]. The intrinsic off-rates for exchange of 14-PCSL and 14-PASL are 4–6 times greater in recombinants with the cholate-isolated protein than in recombinants with the phenol-extracted protein [see Table II and Wolfs et al. (1989)]. The exchange rates found here for the cholate-isolated protein are comparable to those found previously for larger integral proteins that have predominantly α-helical intramembranous domains [see Knowles and Marsh (1991) for a review]. It is the phenol-extracted protein that is exceptional in having unusually slow exchange rates at the lipid–protein interface. The reason for these strongly retarded exchange rates, which has been discussed previously (Wolfs et al., 1989), is that part of the lipid may be trapped in association with the β-sheet structure and

[2] The separation of the peptide strands in a β-sheet structure is 4.9 Å, which is comparable to the diameter of one lipid chain. Therefore approximately two phospholipid molecules could contact the intramembranous surface of one peptide strand (allowing for molecules on both sides of the bilayer), if the strands are oriented parallel to the membrane normal. The β-strands would have to be tilted by 60° with respect to the membrane normal in order to accommodate ca. four lipids per monomer at the hydrophobic surface. For a 19-residue hydrophobic stretch, as in the M-13 coat protein, the thickness of the hydrophobic region would then be comparable to that of the lipid bilayer, but this orientation is likely to be inconsistent with the existence of large polymeric aggregates.

hence have a very much reduced exchange rate. This trapping has been suggested to arise from the polymeric nature of the β-sheet structure (Van Gorkom et al., 1990). Additionally, it is possible that the extended nature of the β-sheet conformation may require the lipid chains at the protein interface to adopt a more extended configuration, hence reducing the chain flexibility and lipid exchange rates. The fact that in spectral subtractions the motionally restricted component was matched by a spectrum from sonicated DMPC vesicles recorded at 10 °C for recombinants with the cholate-isolated protein and at 4 °C for recombinants with the phenol-extracted protein demonstrates that the rotational mobility of the lipid chains associated with the β-polymeric form of the protein is lower. The faster exchange rates observed with the cholate-isolated protein indicate the absence of any trapped lipid in this system or of any appreciable slowing of the lipid exchange by the β-structures in the preparation, as was found previously for the phenol-extracted protein in DMPG bilayers (Wolfs et al., 1989).

Longer-Range Perturbation. The spin-labeled lipid spectra from recombinants with the phenol-extracted protein were adequately simulated by calculating the effects of exchange on the fluid lipid component (Wolfs et al., 1989). To account fully for the greatly increased broadening of the fluid spectral component from recombinants with the cholate-isolated protein, however, it was necessary to include an additional perturbation due to longer-range interactions of the protein beyond the first lipid shell [cf. Knowles et al. (1979)]. This was done by using a fluid lipid spectral component in the exchange simulations which was characteristic of a lower temperature than that at which the spectrum of the recombinant was recorded (cf. Results). This can be explained by the fact that the effective mole fraction of the phenol-extracted protein is lower (because of its higher degree of aggregation), and this limits the effects of longer-range perturbations as compared with those from the more highly dispersed cholate-isolated protein.

Conclusions. Lipid–protein interactions with the cholate-isolated M13 phage coat protein differ considerably from those with the phenol-extracted protein. The most pronounced effects are in the lipid exchange rates at the hydrophobic surface of the protein, in the lipid selectivity, and in the perturbation of the chain mobility of lipids beyond the first solvation shell. These differences must be attributed to the different conformational populations and aggregation states of the two forms of the protein. The comparison of the two forms reveals some of the features that might be expected to be found in the interaction of lipids with integral proteins, such as those of the *E. coli* outer membrane, whose secondary structure is predominantly of the β-type.

Registry No. DMPC, 18194-24-6; DOPC, 4235-95-4; stearic acid, 57-11-4.

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^1H and ^{51}V NMR Studies of the Interaction of Vanadate and 2-Vanadio-3-phosphoglycerate with Phosphoglycerate Mutase†

Susana Liu,‡,§ Michael J. Gresser,*,‡ and Alan S. Tracey*
Department of Chemistry, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6
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ABSTRACT: The formation of complexes of vanadate with 2-phosphoglycerate and 3-phosphoglycerate have been studied using ^{51}V nuclear magnetic resonance spectroscopy. Signals attributed to two 2,3-diphosphoglycerate analogues, 2-vanadio-3-phosphoglycerate and 2-phospho-3-vanadioglycerate, were detected but were not fully resolved from signals of inorganic vanadate and the anhydride formed between vanadate and the phosphate ester moieties of the individual phosphoglycerates. Equilibrium constants for formation of the two 2,3-bisphosphate analogues were estimated as 2.5 M^{-1} for 2-vanadio-3-phosphoglycerate and 0.2 M^{-1} for 2-phospho-3-vanadioglycerate. The results of the binding study are fully consistent with noncooperativity in the binding of vanadiophosphoglycerate to the two active sites of phosphoglycerate mutase (PGM). 2-Vanadio-3-phosphoglycerate was found to bind to the dephospho form of phosphoglycerate mutase with a dissociation constant of about 1×10^{-11} M at pH 7 and 7×10^{-11} M at pH 8. Three signals attributed to histidine residues were observed in the ^1H NMR spectrum of phosphoglycerate mutase. Two of these signals and also an additional signal, tentatively attributed to a tryptophan, underwent a chemical shift change when the vanadiophosphoglycerate complex was bound to the enzyme. The results obtained here are in accord with these vanadate–phosphoglycerate complexes being much more potent inhibitors of phosphoglycerate mutase than either monomeric or dimeric vanadate. The dissociation constant of 10^{-11} M for 2-vanadio-3-phosphoglycerate is about 4 orders of magnitude smaller than the K_m for PGM, a result in accordance with the vanadiophosphoglycerates being transition state analogues for the phosphorylation of PGM by 2,3-diphosphoglycerate. These results strongly support the view that phosphoryl transfer in this enzyme involves a pentacoordinate phosphate intermediate and suggests that the two active sites operate independently of each other.

Phosphoglycerate mutase (PGM) (EC 5.4.2.1) catalyzes the interconversion of 3-phosphoglycerate and 2-phosphoglycerate. The mammalian muscle enzyme is a dimer of about 55 000 molecular weight that requires 2,3-diphosphoglycerate for full activity. It also catalyzes, at much lower rates than the above interconversion, the hydrolysis of 2,3-diphosphoglycerate to inorganic phosphate and phosphoglycerate as well as the conversion of 1,3-diphosphoglycerate to 2,3-diphosphoglycerate. Information concerning this and related enzymes, which are involved in phosphoglycerate and diphosphoglycerate metabolism, has been reviewed recently (Fothergill-Gilmore & Watson, 1989).

There has been a number of reports on the inhibition of PGM by inorganic vanadate (V_i) (Carreras et al., 1980, 1982; Climent et al., 1981; Ninfali et al., 1983). It has been proposed that V_i inhibits PGM by destablizing the phosphoenzyme, which is the catalytically active form (Carreras et al., 1982; Ninfali et al., 1983). Vanadate does activate the 2,3-diphosphoglycerate phosphatase activity of PGM. Kinetic studies of this activity have been rationalized in terms of divanadate (V_2) binding at the catalytic site of the phosphoenzyme as a substrate analogue and activating the hydrolysis of the phosphoenzyme to inorganic phosphate and dephosphoenzyme (Stankiewicz et al., 1987). The dephosphoenzyme is then rephosphorylated by 2,3-diphosphoglycerate to complete the phosphatase cycle. Additional support for this mechanism was provided by the observation that V_2 binds noncooperatively to the two subunits of PGM with an intrinsic dissociation constant of 4×10^{-6} M (Stankiewicz et al., 1987). No binding of the vanadate monomer (V_1) was detected. It seems probable that low-affinity binding of V_1 does occur and could have been detected if the much higher affinity binding of V_2 did not compete so effectively.

More recently, it was reported that 2-phosphoglycerate or 3-phosphoglycerate potentiates inhibition of PGM by V_i and that development of the inhibition was time-dependent and required the presence of both V_i and phosphoglycerate (Stankiewicz & Hass, 1986a,b). This behavior is consistent with the inhibitor being a complex of phosphoglycerate and vanadate binding at the catalytic site of the dephosphoenzyme as a transition-state analogue for the transfer of a phosphoryl group between the enzyme and phosphoglycerate. An anal-

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* Authors to whom correspondence should be addressed.
‡ Present address: Merck Frosst Centre for Therapeutic Research, Merck Frosst Canada, Inc., CP1005 Point Claire-Dorval, Quebec H9R 4P8, Canada.
§ This work is abstracted in part from the M.Sc. thesis of S.L.

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