Interactions between Magainin 2 and *Salmonella typhimurium* Outer Membranes: Effect of Lipopolysaccharide Structure†

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ABSTRACT: The role of the outer membrane and lipopolysaccharide (LPS) in the interaction between the small cationic antimicrobial peptide magainin 2 and the Gram-negative cell envelope was studied by FT-IR spectroscopy. Magainin 2 alters the thermotropic properties of the outer membrane–peptidoglycan complexes from wild-type *Salmonella typhimurium* and a series of LPS mutants which display differential susceptibility to the bactericidal activity of cationic antibiotics. These results are correlated with the LPS phosphorylation pattern and charge (characterized by high-resolution ^{31}P NMR) and outer membrane lipid composition, and are compared to the bactericidal susceptibility. LPS mutants show a progressive loss of resistance to killing by magainin 2 as the length of the LPS polysaccharide moiety decreases. Disordering of the outer membrane lipid fatty acyl chains by magainin 2, however, depends primarily upon the magnitude of LPS charge rather than the length of the LPS polysaccharide, contradicting the proposal by Weiss et al. [Weiss, J., Beckerdite-Quagiata, S., & Elsbach, P. (1980) *J. Clin. Invest. 65*, 619–628] that the sugar side chain of LPS shields the negative charges of the outer membrane surface. While disruption of outer membrane structure most likely is not the primary factor leading to cell death, the susceptibility of Gram-negative cells to magainin 2 is associated with factors that facilitate the transport of the peptide across the outer membrane, such as the magnitude and location of LPS charge, the concentration of LPS in the outer membrane, outer membrane molecular architecture, and the presence or absence of the O-antigen side chain.

Recently, the cationic antimicrobial peptides magainin 1 and magainin 2 were isolated from the skin (Zasloff, 1987) and glandular secretions (Giovanni et al., 1987) of the African clawed frog *Xenopus laevis*. Both natural and synthetic magainins have been shown to inhibit the growth of a broad range of microorganisms (Zasloff, 1987; Zasloff et al., 1988), including Gram-positive and Gram-negative bacteria, fungi, and protozoa. Soravia et al. (1988) showed that synthetic PGLa (peptide between glycine and leucine amide) and XPF (xenopsin precursor fragment), which are also components of *X. laevis* skin secretions, are comparable to magainins 1 and 2 with respect to size, amphiphilicity, antimicrobial potential, and hemolytic activity, suggesting that these and other peptides present in the skin secretion, such as the caerulin precursor fragments and levitide precursor fragment, may form a large family of antimicrobial peptides (Giovannini et al., 1987; Hunt et al., 1988; Poulter et al., 1988).

Structural analysis of magainin 2 by two-dimensional nuclear magnetic resonance (NMR)[1] spectroscopy (Marion et al., 1988) and circular dichroism (Chen et al., 1988) indicates that the peptide adopts a primarily random-coil conformation in aqueous solution but shows increased α-helical structure upon addition of small amounts of trifluoroethanol. Edmundson wheel diagrams of magainins demonstrate that these peptides may be capable of forming amphipathic α-helices (Terry et al., 1988), a characteristic shared by many membrane-active peptides (Kaiser & Kézdy, 1987). Magainins 1 and 2 interact strongly with acidic phospholipids, such as PS and PG, but not with zwitterionic phospholipids, such as egg yolk PC (Matsuzaki et al., 1989; Williams et al., 1990). While the molecular mechanism of their antimicrobial activity remains uncertain, recent evidence indicates that these peptides may cause cell death by permeabilizing the energy-transducing membranes of the target organism (Zasloff, 1987; Westerhoff et al., 1989), perhaps by aggregating in the lipid bilayer to form anion-selective channels composed of four to six monomers (Zasloff, 1987; Zasloff et al., 1988; Westerhoff et al., 1989; Duclohier et al., 1989). Williams et al. (1990) have disputed this model, suggesting that magainins disrupt membrane structure by acting as detergents.

The cell envelope of Gram-negative bacteria is a complex structure composed of two membrane systems. The inner membrane, typically containing the electron-transport chain and the enzymatic apparatus necessary for oxidative phosphorylation (Lugtenberg & Van Alphen, 1983), is the site where magainins would be expected to exert their antimicrobial effect, according to the mechanism proposed by Westerhoff et al. (1989a,b). The outer membrane, which serves as a barrier imparting Gram-negative cells with increased resistance to most hydrophobic antibiotics (Nikaido, 1979; Lugtenberg & Van Alphen, 1983), is relatively ineffective at protecting the cell against cationic antibiotics, such as polymyxin B. These agents bind to negatively charged LPS molecules located on the exterior of the outer membrane, thus disrupting its structure (Strom et al., 1977; Vaara et al., 1979, 1981). We

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[1] Abbreviations: BPI, bactericidal/permeability increasing; CL, cardiolipin; EDTA, ethylenediaminetetraacetic acid sodium salt; FT-IR, Fourier transform infrared; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; KDO, 2-keto-3-deoxyoctonate; LPS, lipopolysaccharide; OM–P, outer membrane–peptidoglycan; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; Sm, smooth (wild type); Tris, tris(hydroxymethyl)aminomethane.

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have shown that magainin 2 disorders the fatty acyl chains of wild-type *Salmonella typhimurium* LPS (Rana et al., 1990), thereby implicating LPS as the initial site of interaction between the peptide and the Gram-negative cell envelope. *S. typhimurium* is an excellent model for systematically examining the role of both the outer membrane and LPS in mediating the interactions of antimicrobial agents with the Gram-negative cell envelope, since the structures of its outer membrane and LPS are well-known (Lugtenberg & Van Alphen, 1983). Moreover, mutant strains containing LPS with incomplete sugar side chains are available which are increasing sensitivity to antimicrobial cationic peptides, such as those found in polymorphonuclear granules, as the length of the sugar side chain of the LPS molecule is diminished (Rest et al., 1977, 1978; Modrzakowski et al., 1979; Hodinka & Modrzakowski, 1983).

In the present study, FT-IR spectroscopy is used to monitor the effects of synthetic magainin 2 on the thermotropic properties of the OM–P complexes of four strains of *S. typhimurium*: (1) the smooth wild-type strain SL 3770 with Sm chemotype LPS, containing a full complement of core sugars and O-antigen side chain; (2) the rough strain SL 3749 with Ra chemotype LPS, lacking only the O-antigen side chain; (3) the rough strain SL 3769 with Rd_1 chemotype LPS, lacking both the O-antigen side chain and the core sugars beyond the heptose residues; and (4) the rough strain SL 1102 with Re chemotype LPS, which lacks the entire core region and O-antigen side chain (see Figure 1). Since the use of lysozyme to remove peptidoglycan from the Gram-negative outer membrane can result in reorientation of LPS (Muhlradt & Golecki, 1975), examination of the OM–P complex offers a more physiologically relevant insight into the structure of the cell envelope.

Changes in the rate of motion and geometry of the hydrocarbon interior of lipid bilayers and membranes can be detected by measuring the temperature dependence of the methylene C–H stretching band by FT-IR spectroscopy (Cameron & Dluhy, 1986; Blazyk & Rana, 1987). This method has been used successfully to characterize the gel-to-liquid-crystal transition in the plasma membrane of *Acholeplasma laidlawii* (Casal et al., 1980), the inner and outer membranes of *Acinetobacter calcoaceticus* (Loeffelholz et al., 1987) and the OM–P complex from wild type (LT-2) and two LPS mutant strains (Ra and Re) of *S. typhimurium* (Rana & Blazyk, 1989), and to study lipid–protein interactions (Casal & Mantsch, 1984).

The interaction of a cationic peptide, such as magainin 2, with LPS in the Gram-negative outer membrane undoubtedly has an electrostatic component (Rana et al., 1990). The phosphate substituents of LPS, which are located on one KDO and both heptose residues of the core sugar as well as the 1- and 4′-positions of the glucosamine disaccharide (Figure 1), are major contributors to the total negative charge of LPS. These heterogeneous phosphorylation sites are either monophosphates or diphosphates existing as the free acid, or esterified with positively charged ethanolamine or arabinosamine (Muhlradt et al., 1977; Lüderitz et al., 1982). Since the nature of the phosphorylation sites in wild type and mutant LPS would profoundly affect the outer membrane surface charge, we have used high-resolution ^{31}P NMR spectroscopy to determine the phosphorylation pattern of LPS isolates from the four *S. typhimurium* strains. By use of these data, combined with the effects of LPS mutations on the ratio of LPS to PE in the outer membrane as determined by high-resolution ^{31}P NMR (Rana, 1990), the surface charge characteristics of the Gram-negative cell envelope are detailed in this work.

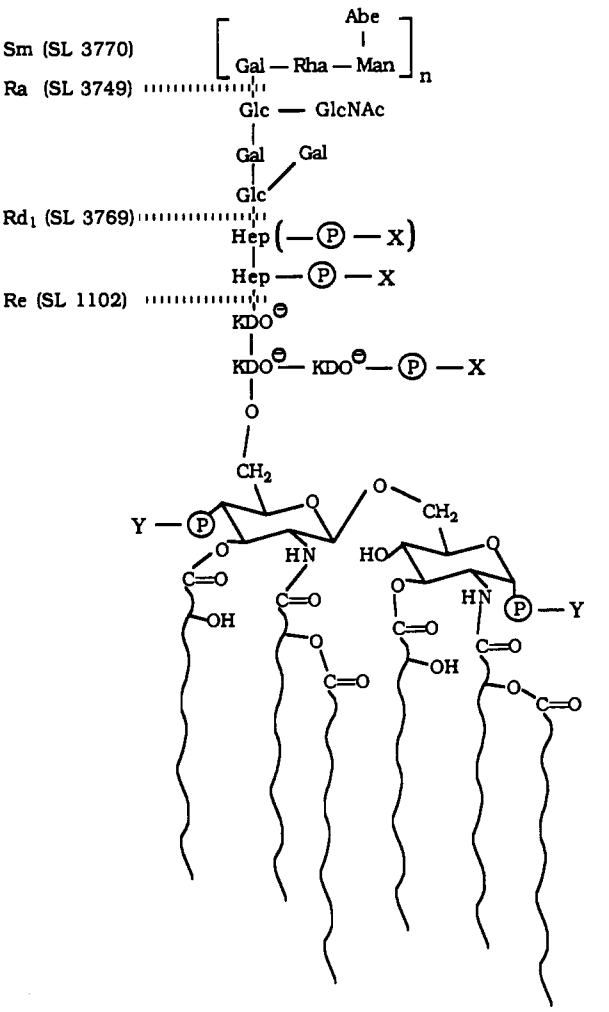

FIGURE 1: Structure of wild-type and LPS mutant *S. typhimurium* LPS molecules (KDO, 2-keto-3-deoxyoctonate; Hep, heptose; Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Rha, rhamnose; Man, mannose, Abe, abequose). The phosphorylation site on the outermost heptose residue is absent in Rd_1 LPS. Ethanolamine or arabinosamine may be substituted at the positions labeled X and Y, respectively.

Magainin 2 causes a concentration-dependent increase in the fluidity of the outer membrane lipids from both the wild-type and LPS mutant strains of *S. typhimurium*. The extent of disordering is nearly the same for all four membrane systems at 37 °C at relatively low levels of the peptide (i.e., magainin to LPS stoichiometries of 1:1 or less); however, at both lower temperatures and higher magainin concentrations, the degree of membrane disordering depends upon the magnitude of the charge of the LPS chemotype. These results also suggest that perturbing the fluidity of the outer membrane is not immediately responsible for cell death. On the basis of these data, we propose a model for the role of the outer membrane and LPS in the antimicrobial mechanism of magainins.

Materials and Methods

Bacterial Growth Conditions and Bactericidal Assays. S. typhimurium LT-2 strains SL 3770, SL 3749, and SL 1102 were maintained as streak cultures on trypticase soy agar (BBL Microbiological Systems). Cultures were grown to late-log phase in trypticase soy broth (BBL Microbiological Systems) at 37 °C before being harvested for membrane preparation and LPS extraction. For bactericidal assays, cells were grown

to mid-log phase and diluted serially by 10^6 in tryptone–saline solution [0.5% Bacto-Tryptone (Difco Laboratories) containing 0.5% NaCl, pH 7.0]. A 600-μL aliquot of this solution (containing about 500 cells) was added to each assay mixture. Magainin 2 was synthesized with solid-phase peptide synthesis by Dr. Milind Deshpande at the Laboratory for Rational Drug Design, Boston University Medical Center. Aliquots of a magainin 2 stock solution (0.14 mg/mL) were added, and the final volume was adjusted to 800 μL. Duplicate 100-μL aliquots of each assay mixture were plated on trypticase soy agar immediately after the addition of magainin (T_0). Following incubation of the assay mixture for 1 h at 37 °C, duplicate 100-μL aliquots were plated again (T_{60}). The number of colony-forming units were counted after incubation at 37 °C for 18–24 h. The percentage of viable cells at T_{60} compared to T_0 was determined. Comparative growth values were calculated by ratioing the percentage of viable cells in the magainin-treated samples to those in the control groups.

Isolation of OM–P Complexes and LPS. OM–P complexes were isolated according to the method of Schnaitman (1970) with the modifications of Koplow and Goldfine (1974) and Smit et al., (1975). Late-log-phase cells were harvested at 4 °C and washed in cold 50 mM Tris, pH 7.8, then resuspended in the presence of ribonuclease and deoxyribonuclease (Sigma Chemical Co.), and passed through a French pressure cell at 14000–16000 psi 3 times. The crude membranes were sedimented at 23500*g* and resuspended in cold 10 mM HEPES, pH 7.4. The OM–P complex was separated from the inner membrane and cell debris by a three-step discontinuous sucrose gradient (0.77, 1.44, and 2.02 M) in an SW25.2 rotor (Beckman Instruments) at 107000*g* for 12 h. The OM–P complex, which concentrated as a grayish white band at the lower interface of the gradient, was washed extensively in cold 10 mM HEPES, pH 7.4, followed by three washings in D_2O. Phosphorus content was determined by the method of Chen et al. (1956). Protein was measured by using the BCA assay (Pierce) with bovine serum albumin as the standard. LPS was extracted as the natural salt form from dried late-log-phase cells with hot aqueous phenol (Westphal et al., 1965) for smooth chemotype LPS, or by the method of Galanos et al. (1969) for Ra, Rd_1, and Re chemotype LPS.

FT-IR Spectroscopy. Membrane samples for FT-IR spectroscopy were sedimented from a D_2O suspension by centrifugation at 149000*g* in a Beckman Airfuge for 30 min at room temperature. Samples containing magainin 2 were prepared by adding an aliquot from a stock solution of the peptide in D_2O to a D_2O suspension of OM–P to give the desired molar ratio of membrane phosphorus to peptide. The stoichiometries of membrane LPS to magainin 2 were determined from the number of P atoms per LPS (see below) and the mole fraction of LPS and phospholipid in the OM (Rana, 1990) as determined from ^{31}P NMR spectroscopy. The mixtures were vortexed vigorously and incubated for at least 12 h at 37 °C prior to centrifugation to ensure equilibrium conditions. The resulting pellets were analyzed in a thermoelectrically controlled cell with CaF_2 windows and a 50-μm Teflon spacer (Blazyk & Rana, 1987). FT-IR spectra were collected as described previously (Loeffelholz et al., 1987) using a Mattson Polaris FT-IR spectrometer equipped with a HgCdTe detector. A total of 250 interferograms were coadded and Fourier-transformed with triangular apodization to generate absorbance spectra with 4 cm^{-1} resolution and data points encoded every 2 cm^{-1}, with a signal-to-noise ratio of better than 500. The frequency and bandwidth (at 80% peak height) of the symmetric methylene C–H stretching band were calculated as described previously (Blazyk & Rana, 1987). Typical error for the frequency and bandwidth values is ±0.1 cm^{-1}.

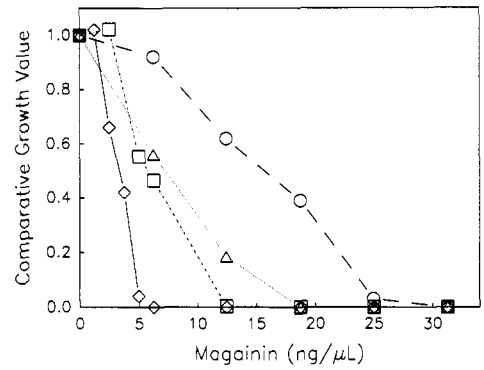

FIGURE 2: Inhibitory effect of magainin 2 on the growth of wild-type and LPS mutant strains of *S. typhimurium*: wild-type (SL 3770) Sm chemotype (○); Ra (SL 3749) chemotype (△); Rd_1 (SL 3769) chemotype (□); Re (SL 1102) chemotype (◇).

^{31}P NMR Spectroscopy. Samples for ^{31}P NMR experiments were prepared by dispersing 50–70 mg of LPS in 2 mL of 50% D_2O/50% H_2O containing 20 mg/mL sodium dodecyl sulfate and 10 mM EDTA, followed by a brief period in a Sonicor sonicating water bath until the solutions appeared clear. The pH of the samples was adjusted with either NaOH or HCl and was measured with a Markson 88 portable pH meter. ^{31}P NMR measurements were performed with a Varian VXR-400S spectrometer at 161.093 MHz with a 10-mm probe at ambient temperature (19–21 °C) and the field locked to D_2O in the sample. For quantitative measurements, 1500–2000 transients were collected by using a flip angle of 66°, a sweep width of 5625 Hz, an acquisition time of 1.5 s, and a pulse delay of 30 s (based on the T_1 value of 2 s for the slowest relaxing signal). Broad-band proton decoupling was performed by using the WALTZ pulse sequence. The decoupler was gated on only during the acquisition for nuclear Overhauser effect suppression. For pH titrations, 500–700 transients were collected with a pulse delay of 3 s, which was sufficient to identify chemical shifts accurately. ^{31}P NMR titration curves were reversible, indicating that little or no hydrolysis of phosphate residues took place during the course of the experiment. Chemical shifts were referenced to an 85% phosphoric acid external standard as described previously (Rana et al., 1991). The average number of phosphorus atoms for each LPS molecule was determined from the relative occurrence of phosphate and diphosphate substituents and the number of phosphorylation sites, which is five for Sm and Ra, four for Rd_1, and three for Re chemotype LPS (Lüderitz et a., 1982). The charge per LPS molecule was calculated as a weighted average using the relative amounts and the estimated charges of each phosphate substituent corresponding to the NMR signals, computed from the Henderson–Hasselbach expression using pK_a values determined by NMR pH titration curves from spectra between pH 4 and pH 10 (listed in Table I), with the β-phosphorus of diphosphate diesters and the α-phosphorus of both diphosphate monoesters and diphosphate diesters assigned charges of 0 and −1, respectively.

RESULTS

Antimicrobial Activity of Magainin 2. The growth response of the smooth parent strain and the Ra, Rd_1, and Re LPS mutant strains of *S. typhimurium* to increasing concentrations of magainin 2 is shown in Figure 2. The strain characterized by Re chemotype LPS (deep rough) showed the greatest sensitivity to the bactericidal activity of magainin 2, with

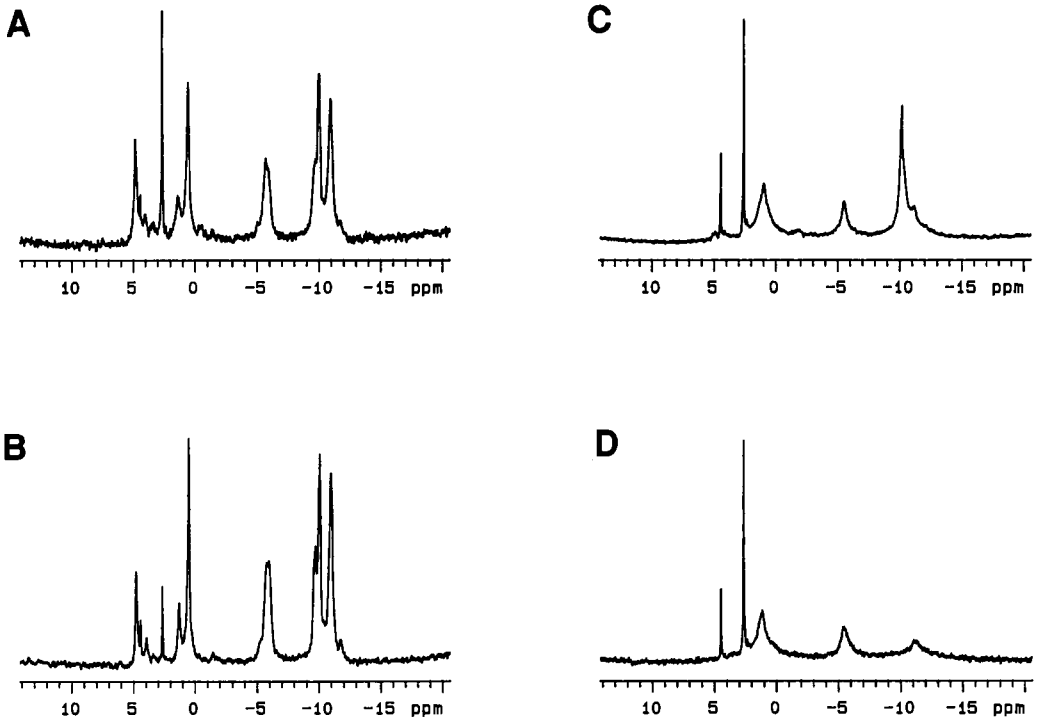

FIGURE 3: High-resolution ^{31}P NMR spectra of *S. typhimurium* LPS dispersed in 50% D_2O/50% H_2O containing 20 mg/mL sodium dodecyl sulfate and 10 mM EDTA at pH 7.4: (A) wild-type (SL 3770) Sm chemotype; (B) Ra (SL 3749) chemotype; (C) Rd_1 (SL 3769) chemotype; (D) Re (SL 1102) chemotype.

Table I: pK_a Values for Phosphorus Signals Detected by ^{31}P NMR

		pK_a value			
peak (ppm)	class[a]	Sm	Ra	Rd_1	Re
4.9	P_m	6.3	6.3		
4.4	P_d	5.7	5.9	5.9	6.0
3.9	P_m	6.7	6.8		
2.6	P_d	6.9	7.0	6.9	6.9
1.2	P_m	6.8	7.1	6.7	6.7
0.6	P_m	8.2	8.2		
−5.7	β-PP_m	6.7	6.8	6.2	6.3

[a] P_m, phosphate monoester, P_d, phosphate diester; β-PP_m, β-phosphorus in diphosphate monoester.

bacterial cell viability eliminated at 6 ng of peptide per microliter of assay mixture. The smooth parent strain, showing the greatest resistance to the bactericidal activity of magainin 2, was killed at a concentration of 25 ng/μL. The sensitivity of the intermediate chemotype strains was proportional to the degree of roughness in LPS chemotype.

High-Resolution ^{31}P NMR Spectroscopy of LPS. ^{31}P NMR spectra of Sm, Ra, Rd_1, and Re LPS at pH 7.4 are shown in Figure 3. The signals located between 0 and 5 ppm are due to both monophosphate monoesters and monophosphate diesters; the broad signal near −5 ppm arises from the β-phosphorus of diphosphate monoesters, and those observed between −10 and −13 ppm correspond to both the β-phosphorus of diphosphate diesters and the α-phosphorus of both diphosphate monoesters and diphosphate diesters (Vogel, 1984; Rana et al., 1990). Monophosphate diester signals were identified on the basis of the relatively limited pH dependence of their chemical shift values (Crutchfield et al., 1967; Strain et al., 1983; Vogel, 1984; Bately et al., 1985; Rana et al., 1990). Since the peak areas are proportional to the number of phosphorus atoms and the diphosphate monoester β-phosphorus atoms correspond to the signal at −5 ppm, the relative number of diphosphate diester α- and β-phosphorus atoms was calculated by subtracting the area of signal at −5 ppm from the total area of the signal located between −10 and −13 ppm. It is therefore possible to determine the proportion of each type of phosphorus atom in the LPS molecules even though the identity of the individual peaks in the −10 to −13 ppm region remains ambiguous.

The pK_a values for the phosphate monoesters and diesters and the β-phosphorus atom of the diphosphate monoesters are listed in Table I. It is evident from the consistency of these values among the wild-type and mutant strains that altering the polysaccharide moiety of LPS does not markedly influence the ionization properties of the phosphorylation sites. The composition of the phosphorylation sites is shown in Table II. Monophosphate monoesters are the most predominant substituent, while diphosphate monoesters and diesters occur at roughly the same levels, except for Re chemotype LPS which contains no detectable diphosphate diesters. Similar results were obtained by Rosner et al. (1979) for a heptose-less LPS

Table II: Phosphate Structure of LPS from Wild-Type and Mutant Strains

		% occurrence						
strain	chemotype	P_m[a]	P_d[b]	PP_m[c]	PP_d[d]	av P/LPS[e]	av charge/P[f]	av charge/LPS[g]
SL 3770	Sm	46	14	21	20	7.0	−1.04	−7.3
SL 3749	Ra	42	6	29	24	7.7	−1.05	−8.2
SL 3769	Rd_1	38	21	21	21	5.7	−1.04	−5.9
SL 1102	Re	48	26	26	0	2.8	−1.26	−4.8

[a] Phosphate monoesters. [b] Phosphate diesters. [c] Diphosphate monoesters. [d] Diphosphate diesters. [e] Average number of phosphorus atoms per LPS molecule. [f] Average charge per phosphorus atom on the LPS molecule. [g] Average total charge of the phosphate residues on the LPS molecule (these values do not include the contribution from the KDO residues, which adds an extra −3 to the total charge on the LPS molecule).

Table III: Composition of the Outer Membrane

strain	LPS chemotype	LPS[a]	PE[a]	PG + CL[a]
SL 3770	Sm	35	53	12
SL 3749	Ra	27	63	10
SL 3769	Rd_1	38	48	13
SL 1102	Re	41	46	12

[a] Percentage of total polar lipid in the outer membrane. Typical error is ±3%.

isolated from *Escherichia coli*. This difference largely accounts for the increased average charge per Re LPS phosphorus atom of −1.26 as compared to the other LPS chemotypes, in which the average charge per phosphorus atom is about −1. The ^{31}P NMR spectra and composition of the phosphorylation sites of Sm and Ra LPS, which share the same five phosphorylation sites, are strikingly similar, suggesting that the loss of the O-antigen side chain from the LPS polysaccharide does not dramatically influence the phosphorylation pattern of LPS; however, Ra LPS does show an increase in the number of phosphorus atoms resulting in a higher negative charge per molecule. Similar results were observed for Rb, Rb_2, and Re LPS, all of which lack the O-antigen side chain and outermost core sugars, but have the same phosphorylation sites as Sm and Ra LPS (Rana, 1990). The reduced overall charge on Rd_1 and Re LPS is due mainly to the loss of one and two phosphorylation sites, respectively, rather than neutralization of negative charge by ethanolamine or arabinosamine substitution, as observed in LPS from polymyxin-resistant strains of *E. coli* and *S. typhimurium* (Peterson et al., 1987; Rana, 1990).

The mole fractions of LPS and phospholipid in the outer membrane of each strain, determined by ^{31}P NMR of OM–P complexes at pH 10 (Rana, 1990), are listed in Table III. LPS constitutes 30–40 mol % of the outer membrane lipids; the phospholipids include zwitterionic PE as the major component at 50–60 mol %, along with anionic PG and CL which together comprise about 10 mol % of the total lipids. On the basis of these data, it is clear that greater than 90% of the outer membrane surface charge arises from LPS for all four strains. The effect of LPS mutations on the outer membrane lipid composition, as deduced from high-resolution ^{31}P NMR spectroscopy, is much less dramatic than reported in earlier studies (Smit et al., 1975; Gmeiner & Schlecht, 1979).

Phase Behavior of the OM–P Complexes. Temperature-dependent changes in the frequency and bandwidth of the symmetric methylene C–H stretching band are shown for the OM–P complexes isolated from the Sm, Ra, Rd_1, and Re strains in Figure 4. This band is sensitive to the conformation and motion of the hydrocarbon chains of the membrane lipids (Cameron & Dluhy, 1986), thus serving as an indicator of the physical state of the hydrophobic core of the membrane. Shifts in the position of this band toward higher frequency correlate with the introduction of gauche conformers which accompany the disordering of the fatty acyl chains, while the bandwidth is proportional to the relative motion of the chains. The OM–P complexes from all of the strains show similar phase behavior. All undergo a broad thermotropic phase change centered near 40 °C. The temperature dependence of the peak position is quite large over the entire temperature interval studied, in contrast to dipalmitoylphosphatidylcholine, where the frequency of the band remains nearly constant below the phase transition temperature (Blazyk & Rana, 1987). All of the bandwidth curves exhibit a maximum between 25 and 30 °C, significantly below the T_m indicated by the frequency curves, which is probably indicative of complex phase behavior in these heterogeneous membranes. At the growth temperature of the organisms (37 °C), all of the membranes appear to be in a quasi-fluid state (intermediate between the gel and liquid-crystal phases).

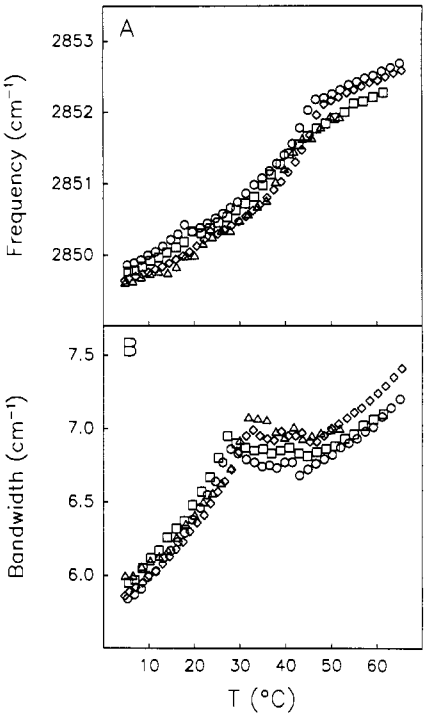

FIGURE 4: Temperature dependence of the frequency and bandwidth of the symmetric methylene C–H stretching band for *S. typhimurium* outer membrane–peptidoglycan complexes: wild-type (SL 3770) Sm chemotype (○); Ra (SL 3749) chemotype (△); Rd_1 (SL 3769) chemotype (□); Re (SL 1102) chemotype (◇).

Effects of Magainin on the Fluidity of the OM–P Complexes. The effects of magainin 2 on the temperature-dependent behavior of the OM–P complexes from the wild-type (Sm), Ra, Rd_1, and Re strains are shown in Figures 5 and 6. The peptide causes concentration-dependent increases in both the number of gauche conformers and the motional freedom of the lipid acyl chains, as determined from the frequency and bandwidth of the methylene symmetric C–H stretching band, respectively. Furthermore, the phase change of the OM–P complex for each strain shows a progressive decrease in cooperativity, with both T_m and the bandwidth maximum occurring at successively lower temperatures as the magainin 2 to LPS ratio is increased. The peptide induces the disordering of the lipid hydrocarbon chains, thus increasing the fluidity of the outer membrane lipids. This fluidizing effect is most pronounced at lower temperatures, where the lipids in the OM–P complex are normally relatively ordered.

The influence of LPS structure on the magainin 2 induced disordering of the OM–P complex is evaluated from the change in frequency of the symmetric methylene C–H stretching band, as compared to the OM–P control, as a function of the magainin 2 to LPS ratio at 15 and 37 °C (Figure 7). The disordering of outer membrane lipids by magainin 2 is not correlated with the length of the LPS polysaccharide chain (Sm ≫ Ra > Rd_1 > Re) according to the model describing the interaction between cationic BPI protein and wild-type and LPS mutant cell envelopes (Weiss et al., 1980). At 15 °C, when the lipids are relatively ordered (approaching the gel state), disordering by magainin 2 occurs as follows: Ra ≫ Sm ≈ Re > Rd_1, with differences most pronounced at a magainin 2 to LPS ratio of about 2. At 37 °C and ratios of magainin 2 to LPS less than 1, the fluidizing effects of magainin 2 are similar in all four membrane systems; however,

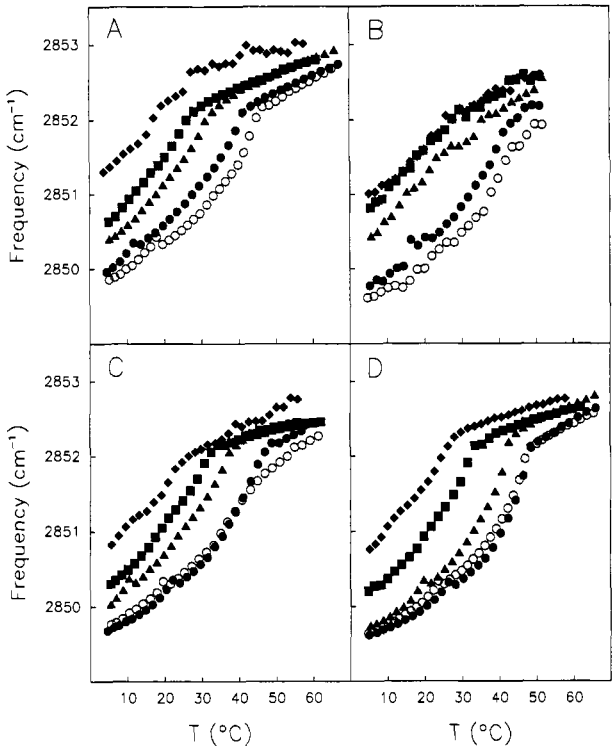

FIGURE 5: Temperature dependence of the frequency of the symmetric methylene C–H stretching band for outer membrane–peptidoglycan complexes in the absence and presence of magainin 2: (A) wild-type (SL 3770) Sm chemotype at molar ratios of magainin 2 to LPS of 0 (○), 0.34 (●), 0.83 (▲), 1.67 (■), and 3.33 (♦); (B) Ra (SL 3749) chemotype at molar ratios of magainin 2 to LPS of 0 (○), 0.34 (●), 0.91 (▲), 1.67 (■), and 3.33 (♦); (C) Rd_1 (SL 3769) chemotype at molar ratios of magainin 2 to LPS of 0 (○), 0.28 (●), 0.67 (▲), 1.43 (■), and 3.33 (♦); and (D) Re (SL 1102) chemotype at molar ratios of magainin 2 to LPS of 0 (○), 0.19 (●), 0.47 (▲), 1.00 (■), and 2.50 (♦).

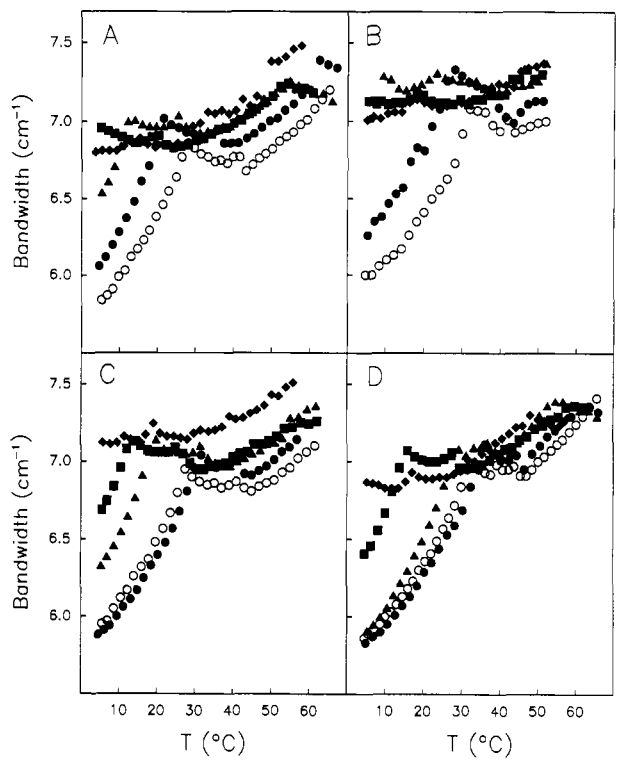

FIGURE 6: Temperature dependence of the bandwidth of the symmetric methylene C–H stretching band for outer membrane–peptidoglycan complexes in the absence and presence of magainin 2: (A) wild-type (SL 3770) Sm chemotype at molar ratios of magainin 2 to LPS of 0 (○), 0.34 (●), 0.83 (▲), 1.67 (■), and 3.33 (♦); (B) Ra (SL 3749) chemotype at molar ratios of magainin 2 to LPS of 0 (○), 0.34 (●), 0.91 (▲), 1.67 (■), and 3.33 (♦); (C) Rd_1 (SL 3769) chemotype at molar ratios of magainin 2 to LPS of 0 (○), 0.28 (●), 0.67 (▲), 1.43 (■), and 3.33 (♦); and (D) Re (SL 1102) chemotype at molar ratios of magainin 2 to LPS of 0 (○), 0.19 (●), 0.47 (▲), 1.00 (■), and 2.50 (♦).

at higher levels of peptide, differential disordering of the outer membrane lipids is observed ($Ra \approx Re > Sm \approx Rd_1$). Only the Ra OM–P complex shows greatest disordering at both 15 and 37 °C at a magainin 2 to LPS ratio below 2. The other OM–P complexes require a higher level of magainin 2 for maximal disordering at 15 °C than at 37 °C.

Discussion

The effects of the antibiotic peptide magainin 2 on the phase behavior of the lipid fatty acyl chains in the OM–P complexes from wild-type and rough mutants of *S. typhimurium* were monitored by FT-IR spectroscopy. The broad and relatively noncooperative phase change observed near growth temperature in the outer membrane lipids of the wild-type strain (Figure 4) is consistent with previous results obtained by a variety of physical and spectroscopic techniques (Lugtenberg & Van Alphen, 1983). In the rough strains, alterations in the length of the LPS sugar side chain cause only slight changes in the fluidity of the OM–P complex. In contrast, Rottem and Leive (1977) employed electron paramagnetic resonance spectroscopy to show that the outer membrane fluidity of LPS mutant *E. coli* strains was decreased significantly when LPS contained a shorter sugar side chain compared to its wild-type counterpart, as detected by measuring the temperature dependence of the signal from a nitroxide-labeled fatty acid. This discrepancy may arise from real differences between the two organisms or experimental considerations. The nitroxide probe used for electron paramagnetic resonance spectroscopy may selectively partition to a specific domain in the membrane and may not reflect the overall fluidity characteristics as detected

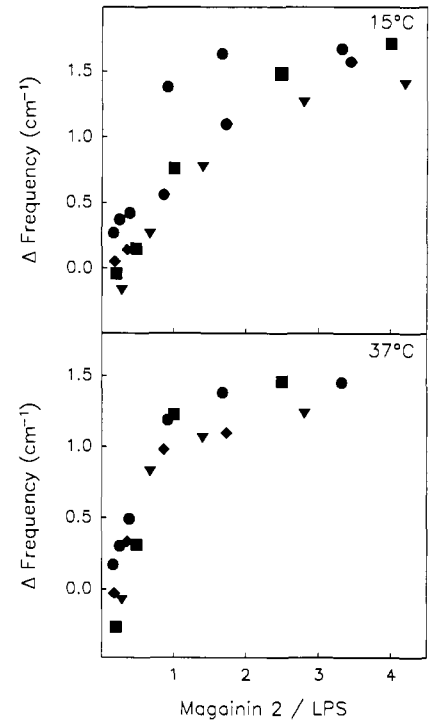

FIGURE 7: Change in frequency of the symmetric methylene C–H stretching band at 15 and 37 °C as a function of the ratio of magainin 2 to LPS for *S. typhimurium* outer membrane–peptidoglycan complexes: wild-type (SL 3770) Sm chemotype (♦); Ra (SL 3749) chemotype (●); Rd_1 (SL 3769) chemotype (▼); Re (SL 1102) chemotype (■).

by FT-IR spectroscopy. Nikaido et al. (1977) reported that nitroxide-labeled stearic acid molecules preferentially associate with phospholipid-rich domains in the outer membrane of *S. typhimurium* HN202. FT-IR spectroscopy is a nonperturbing method which, by analyzing changes in the symmetric methylene C–H stretching band in which the contribution of protein is quite small, provides a sensitive measure of the bulk fluidity of the hydrocarbon chains from all of the membrane lipids.

Zasloff et al. (1988) measured a minimum inhibitory concentration of magainin 2 against *S. typhimurium* (ATCC 14028) of 35–70 μg/mL. This value is comparable to the concentration of magainin 2 necessary to cause complete inhibition of growth for wild-type *S. typhimurium* (~25 μg/mL from Figure 2). Hodinka and Modrzakowski (1983) examined the bactericidal activity of fractionated granular contents from rat polymorphonuclear leukocytes using the same set of rough LPS mutant strains of *S. typhimurium* studied in this paper. The active components of these granules are a collection of small cysteine- and arginine-rich cationic peptides called defensins, which were purified and characterized recently (Eisenhauer et al.,1989). The relative susceptibility of these strains to killing by defensins in the granular extract was identical with the results obtained with magainin 2. The concentration of peptide necessary to induce cell death decreased as the length of the LPS sugar side chain decreased. These results suggest that the native structure of the OM of *S. typhimurium* may afford protection against the bactericidal effects of magainin 2 and defensins by similar mechanisms.

The binding of magainin 2 to the OM–P complexes from smooth and rough strains of *S. typhimurium* disorders the hydrocarbon chains of the outer membrane lipids. The outer membrane of *S. typhimurium* is composed of LPS, PE, small amounts of PG and CL, and proteins. In the wild-type strain, LPS is the sole lipid species in the outer monolayer of the outer membrane, in contrast to the rough mutants, where the outer monolayer contains both LPS and PE (Ames et al., 1974; Smit et al., 1975: Lugtenberg & Van Alphen, 1983). Since PE is zwitterionic, the cationic magainin 2 should bind primarily to the negatively charged sugar and phosphate residues of LPS in a manner similar to another antimicrobial cationic peptide, polymyxin B (Strom et al., 1977; Vaara et al., 1979, 1981). Moreover, magainin 2 induces changes in the phase behavior of purified Sm chemotype LPS which are strikingly similar to that of the Sm OM–P complex (Rana & Blazyk, 1990).

Weiss et al. (1980) have proposed that the enhanced resistance of wild-type *S. typhimurium* to the cationic BPI protein (purified from neutrophil granules) compared to LPS mutant strains results from the large Sm LPS sugar side chain which shields the negatively charged residues of the core and interfacial region of the wild-type LPS molecule from the positive charges of the BPI protein. Presumably, the negative charges are more exposed in LPS molecules with a deficient polysaccharide moiety, thus allowing cationic peptides easier access for enhanced interaction with LPS on the outer membrane surface. This widely accepted model has been used to explain the ordered loss of resistance of LPS mutant strains to the bactericidal activity of cationic materials in general. On the basis of this model and the order of susceptibility of the wild-type and LPS mutant strains to magainin 2, the length of the LPS sugar side chain should be inversely proportional to the magnitude of interaction of magainin 2 with the OM–P complexes.

The FT-IR data presented here demonstrate that the sugar side chain of wild-type LPS is no more effective at shielding magainin 2 from the negative charges of the LPS molecule (and presumably the membrane surface) than the LPS polysaccharide chains of mutant LPS molecules (Figure 7). The extent of disordering induced by magainin 2 levels at both 15 °C and 37 °C correlates much better with LPS charge than the size of the polysaccharide moiety, indicating that the primary interaction between magainin 2 and the outer membrane is most likely electrostatic in nature. The membrane lipids of the OM–P complexes from the Sm and Ra strains which produce LPS chemotypes bearing a larger negative charge than Rd_1 LPS, as determined by ^{31}P NMR spectroscopy, are more readily disrupted by magainin 2 than the Rd_1 OM–P complex. The OM–P complexes from a polymyxin-sensitive strain of *S. typhimurium* (SH 5014), which produces a Rb_2 chemotype LPS bearing a −10.4 charge, and a polymyxin-resistant strain (SH 5357), also possessing Rb_2 chemotype LPS but with a reduced negative charge of −7.5, are disordered by magainin 2 to the same extent as the wild-type and Rd_1 OM–P complexes, respectively, supporting the notion that net charge is a principal factor (Rana, 1990).

The anomalous nature of the interaction of magainin 2 with the Re OM–P complex may be due to the localization of virtually all of the negatively charged residues near the hydrophilic/hydrophobic boundary (interfacial region) of Re LPS (Labishinski et al., 1985) (Figure 1) and the high average charge per LPS phosphorus (Table II). The binding of magainin 2 to the interfacial region of the outer membrane might be expected to have a more dramatic effect on the membrane structure than binding to negatively charged sites located on heptose and KDO sugar residues of the core region, which is more likely to occur in the wild-type, Ra, and Rd_1 strains. At 37 °C, the Re OM–P complex experiences significantly greater magainin 2 induced disordering than at 15 °C. This is reasonable since interfacial charges are less likely to be accessible at lower temperatures when the membrane lipid molecules are more closely packed. A similar but less pronounced temperature dependence is observed for magainin 2 interactions with the Rd_1 OM–P complex, which also may result from a concentration of negative charges near the polar/nonpolar interface.

A proposed killing mechanism for magainins involves the disruption of energy metabolism in the target organism by increasing the permeability of energy-transducing membranes (Westerhoff et al., 1989a,b). The inner membrane of *Enterobactericae*, containing both the electron-transport chain and enzymes necessary for oxidative phosphorylation, is the most probable site of attack. The lack of correlation between the magainin 2 induced disordering of the OM–P complexes and the susceptibility of the *S. typhimurium* strains to the bactericidal effects of the peptide suggests that loss of outer membrane native structure is not the primary factor responsible for cell death. Instead, the differential susceptibility of the wild-type and LPS mutants to the killing activity of magainin 2 may be related to the ease by which the peptide traverses the outer membrane.

The data reported here and elsewhere (Rana et al., 1990) suggest that the mechanism by which magainin crosses the outer membrane may be similar to the self-promoted pathway proposed for polymyxin B (Hancock, 1984), where the peptide compromises the outer membrane barrier by binding to LPS and disrupting membrane structure. The enhanced resistance of the wild-type strain may be related to the presence of the O-antigen side chain and differences in the molecular architecture of wild-type and rough outer membranes. Using differential scanning calorimetry and FT-IR spectroscopy, we detected a phase change corresponding to a cooperative re-

arrangement of the LPS sugar side chain in aqueous dispersions of wild-type LPS, but not in LPS from rough mutants (Rana, 1990). The large volume of the polar region of Sm LPS may stabilize the bilayer structure of the outer membrane even though the hydrophobic region is disordered by the peptide, thus accounting for decreased susceptibility to magainin 2 in the wild-type organism. Furthermore, PE in the outer leaflet of the outer membrane of LPS mutants does not mix with LPS, but rather forms phospholipid-rich domains that may serve as a pathway across the outer membrane for hydrophobic materials (Nikaido, 1979). This pathway may not exist in the wild-type strain, where LPS is the sole lipid species in the outer monolayer. It is possible that magainin 2 may be able to exploit this hydrophobic pathway of LPS mutants in addition to the self-promoted pathway, thus enhancing its entry into the cell.

The differential susceptibility of the LPS mutants to magainin 2 is undoubtedly influenced by LPS charge. It is expected that magainin will be held more tightly at the cell surface by electrostatic forces in strains with more negatively charged LPS. Moreover, magainins may cross the outer membrane more readily when the electrostatic interaction with LPS molecules occurs at the interfacial region rather than at more remote sites in the core sugar region. In summary, increased ability of the outer membrane to bind magainin 2 away from the hydrophobic interior, most evident in the wild-type organism, enhances survival by preventing entry of the peptide to the plasma membrane. Any alteration in outer membrane structure which enables cationic peptides to reach the hydrophobic boundary of the outer membrane more easily, such as removal of core sugars from LPS or inclusion of phosphoglycerides on the outer surface, will increase the susceptibility of the organism to bactericidal agents such as magainin 2.

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Lipid-Protein and Protein-Protein Interactions in Double Recombinants of Myelin Proteolipid Apoprotein and Myelin Basic Protein with Dimyristoylphosphatidylglycerol

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ABSTRACT: The integral proteolipid apoprotein (PLP) from bovine spinal cord has been reconstituted in dimyristoylphosphatidylglycerol (DMPG) bilayers, and the mutual interactions on binding the peripheral myelin basic protein (MBP) have been studied. Quantitation of protein and lipid contents in the MBP-PLP-DMPG double recombinants at different PLP:DMPG ratios led to the conclusion that MBP binds only to the DMPG lipid headgroups and is hindered from interaction with the first shell of lipids surrounding the PLP. No specific PLP-MBP association could be detected. Electron spin resonance (ESR) spectra of phosphatidylglycerol spin-labeled at position $n = 5$ in the *sn*-2 chain showed that complexation of MBP with the PLP-DMPG recombinants leads to a decrease in lipid chain mobility to an extent which correlates with the degree of MBP binding. At low DMPG:PLP ratios, the perturbations of lipid mobility by both proteins are mutually enhanced. In single recombinants of PLP with DMPG, the ESR spectra of phosphatidylglycerol spin-labeled at position $n = 14$ in the *sn*-2 chain indicated that approximately 10 lipids/protein are motionally restricted by direct contact with the intramembranous surface of the protein. This number is in agreement with earlier results for reconstitutions of PLP in dimyristoylphosphatidylcholine (DMPC) [Brophy, P. J., Horváth, L. I., & Marsh, D. (1984) *Biochemistry 23*, 860-865] and is consistent with a hexameric arrangement of the PLP molecules in DMPG bilayers. The selectivity of interaction of different spin-labeled lipids with PLP in single recombinants with DMPG is in the order cardiolipin ≈ stearic acid > phosphatidic acid > phosphatidylglycerol > phosphatidylethanolamine > phosphatidylserine > phosphatidylcholine, which differs from that found previously in recombinants with DMPC, due to differences in lipid-lipid interactions with the background lipid and shifts in the lipid pK_a due to the electrostatic surface charge. Binding of MBP to the PLP-DMPG recombinants decreased the specificity of interaction of the different lipids for the PLP, and modified the selectivity pattern which was found to be in the order cardiolipin > stearic acid > phosphatidylglycerol ≈ phosphatidic acid > phosphatidylethanolamine ≥ phosphatidylserine ≈ phosphatidylcholine. These changes in specificity can be attributed to the interaction of MBP with the bulk lipid regions of the double recombinants, and the extent of competition correlates with the previously measured selectivity of interaction of the different lipids with MBP bound to DMPG [Sankaram, M. B., Brophy, P. J., & Marsh, D. (1989) *Biochemistry 28*, 9699-9707].

Integral membrane proteins span the hydrophobic core of the lipid bilayer and present an apolar face to the surrounding acyl chain milieu. Peripheral proteins are associated with membrane surfaces through interactions with phospholipid headgroups or with other membrane proteins. These two different modes of membrane associations of proteins are manifest in their operational behavior. Relatively mild conditions, mostly high ionic strength buffers, release membrane-bound peripheral proteins whereas purification of in-

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