Volume 261, number 2, 464–467 FEBS 08054 February 1990

Interactions between *Salmonella typhimurium* lipopolysaccharide and the antimicrobial peptide, magainin 2 amide

Fazale R. Rana, Catherine M. Sultany+ and Jack Blazyk

Department of Chemistry and College of Osteopathic Medicine and +*University Research and Instrumentation Center,*
Ohio University, Athens, OH 45701, USA

Received 26 October 1989

Effects of magainin 2 amide on the phase behavior of *Salmonella typhimurium* lipopolysaccharide were characterized by FT-IR spectroscopy. This antimicrobial cationic peptide disorders the lipopolysaccharide at molecular ratios of lipopolysaccharide to magainin greater than 4, and can induce a temperature-dependent structural reorientation. The nature of the five phosphate groups of lipopolysaccharide was determined by ^{31}P NMR spectroscopy. At pH 7.4, the net charge on the phosphates is −7. Lipopolysaccharide undoubtedly plays an important role in modulating the interactions of magainin with the gram-negative cell envelope and may act as a molecular sponge to protect the plasma membrane.

Magainin; Lipopolysaccharide; Phase change; FT-IR spectroscopy; NMR spectroscopy, ^{31}P; (*Salmonella typhimurium*)

1. INTRODUCTION

Two low molecular weight cationic peptides, magainin 1 and magainin 2, have been isolated from the skin and glandular secretions of *Xenopus laevis* [1,2]. Both natural [1] and synthetic [3] magainins possess antimicrobial activity against a wide range of target organisms, including protozoa, fungi and gram-positive and gram-negative bacteria. The magainins are nonhemolytic and are assumed to be noncytotoxic [1,4]. Because of these unique properties, there is considerable interest in developing magainins or close analogs as therapeutic agents [4,5]. The antimicrobial mechanism of these peptides has not been fully characterized, but current evidence suggests that the magainins are membrane-active [1,3–8] and appear to interact strongly with acidic phospholipids, with only a weak affinity for zwitterionic phospholipids [8].

The outer membrane of the gram-negative bacteria, *Enterobacteriaceae*, which includes *Escherichia coli* and *Salmonella typhimurium*, is composed of proteins, the zwitterionic phospholipid, phosphatidylethanolamine, and the negatively charged glycolipid, lipopolysaccharide, or LPS [9,10]. LPS is located exclusively on the outer leaflet of the outer membrane and is important in determining the surface characteristics of gram-negative cells [10,11]. Because of its location and high negative charge, LPS undoubtedly interacts with magainin molecules as they come into contact with the cell surface and may play a role in modulating magainin-induced inhibition of the growth of gram-negative bacteria.

In this study, we have examined the effect of synthetic magainin 2 amide on the fluidity of smooth chemotype (Sm) LPS isolated from *S. typhimurium* using FT-IR spectroscopy. Magainin induces disordering of the hydrophobic region of Sm LPS in a concentration- and temperature-dependent manner. Furthermore, in order to determine the net charge on the LPS molecules, the nature of the monophosphate and diphosphate substitutions on the sugar residues of Sm LPS was examined by ^{31}P NMR spectroscopy. The stoichiometry of the binding between and magainin may prove to be an important factor in the potency of this antimicrobial peptide toward gram-negative organisms.

2. MATERIALS AND METHODS

Magainin 2 amide was synthesized by the Alberta Peptide Institute. A stock solution of magainin in D_2O (~2 mg/ml) was used for FT-IR experiments. Protein content was quantitated using the Pierce BCA protein assay with bovine serum albumin as the standard. *S. typhimurium* SL 3770 was maintained on Trypticase soy agar (BBL Microbiology), grown overnight at 37°C in Trypticase soy broth (BBL Microbiology) and diluted 150-fold into fresh media. The cells were cultured at 37°C to the late-log phase, harvested and washed with distilled H_2O, followed by acetone. The resulting cell paste was dried in vacuo and stored below 0°C until used. LPS was extracted as the natural salt form from dried cells using hot aqueous phenol and purified by differential centrifugation [12]. Lyophilized LPS was dispersed in D_2O for FT-IR experiments. The LPS concentration was determined using phosphorus as a marker by the method of Chen et al. [13].

Correspondence address: J. Blazyk, Department of Chemistry, Clippinger Research Laboratories, Ohio University, Athens, OH 45701, USA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

Samples for FT-IR spectroscopy were prepared by adding aliquots of LPS and magainin stock solutions to yield the desired ratio of magainin to LPS. To ensure equilibrium conditions, the LPS and magainin mixtures were vortexed vigorously and incubated at 37°C for 12 h. The LPS · magainin complex was sedimented in a Beckman Airfuge ultracentrifuge at 96 000 × *g* for 30 min at ambient temperature. The resulting pellet was transferred to a thermoelectrically controlled demountable liquid FT-IR cell with CaF_2 windows and a 50 μm Teflon spacer [14]. Infrared spectra were collected by coadding 250 interferograms at a nominal resolution of 4 cm^{-1} with triangular apodization using a Mattson Sirius 100 FT-IR spectrometer equipped with a narrow-band MCT detector. Data processing and the analysis of spectral parameters is described elsewhere [14,15].

Samples for ^{31}P NMR experiments were prepared by dispersing 50–70 mg of Sm LPS in 2 ml of 50% D_2O/50% H_2O containing 20 mg/ml sodium dodecyl sulfate and 10 mM EDTA, followed by sonication for 30 min using a Sonicor sonicating water bath. The pH of the sample was adjusted by the addition of either NaOH or HCl. ^{31}P NMR spectra were collected using a Varian VXR-400S spectrometer at 161 093 MHz using a 10 mm probe, at 19–20°C, with the field locked to D_2O in the sample. For quantitative measurements, 1500 transients were collected using a flip angle of 66°, a sweep width of 5624 Hz, an acquisition time of 1.5 s and a pulse delay of 30 s (a T_1 value of 2 s was measured for the slowest relaxing signal). Broadband proton decoupling (WALTZ sequence) was gated on only during acquisition for NOE suppression. Chemical shifts are relative to a phosphoric acid external standard. For pH titrations, 500–700 transients were accumulated with an interpulse delay of 3 s. All titration curves were reversible, indicating that little or no hydrolysis had taken place during the course of the experiment. The total number of phosphorus atoms and the average charge per phosphorus atom in Sm LPS at pH 7.4 were estimated by comparing the areas of monophosphate and diphosphate peaks in the ^{31}P NMR spectrum and using the pK_a values determined from the titration curves.

3. RESULTS AND DISCUSSION

Negatively charged substituents of Sm LPS consist of a free carboxyl group on each of the three KDO residues located in the core sugar region and five phosphorylation sites on the KDO and heptose residues of the core and the glucosamine disaccharide of the lipid A moiety. These sites contain either monophosphate or diphosphate, each of which can be free or esterified to either ethanolamine or arabinosamine in the case of lipid A phosphate [10,11]. Because of the variably substituted phosphorylation sites on the LPS molecules, the net negative charge of the molecules is modulated by the amounts of monophosphate and diphosphate monoesters and diesters [16]. An increase in the amount of diphosphate diester substituents has been implicated in the enhanced resistance toward cationic antibiotics such as polymyxin B shown by certain strains of *S. typhimurium* and *E. coli* [16–18]. Since the net charge on magainin at physiological pH is +3 to +4, electrostatic interactions between the peptide and LPS molecules undoubtedly play an important role in the initial binding process to the outer membrane.

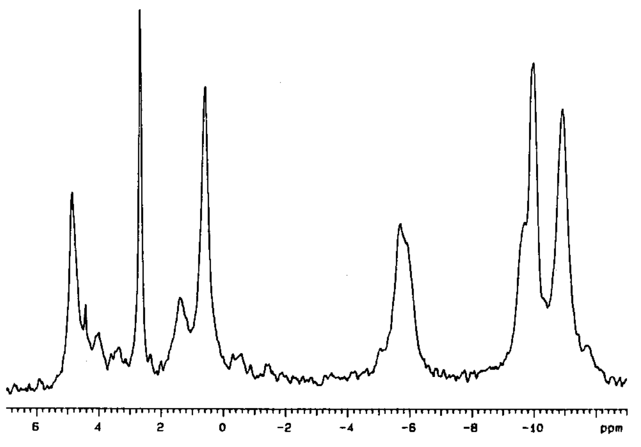

Fig.1. ^{31}P NMR spectrum of *S. typhimurium* SL 3770 LPS at pH 7.4.

The number of monophosphate and diphosphate substitutions and the net charge of Sm LPS at pH 7.4 was determined by ^{31}P NMR spectroscopy. In the spectrum shown in fig. 1, the signals between 0 and 5 ppm are attributed to both monophosphate monoesters and diesters, while those in the −5 ppm region arise from the β phosphorus of diphosphate monoesters and those between −10 and −13 ppm correspond to both the β phosphorus of diphosphate diesters and the α phosphorus of both diphosphate monoesters and diesters [19–21]. From the small pH dependence of the chemical shifts (fig.2), the peaks at 2.6 and 4.4 ppm were identified as monophosphate diesters [19,21]. By measuring peak areas, the phosphorylated substituents on Sm LPS are 45% monophosphate monoesters, 14% monophos-

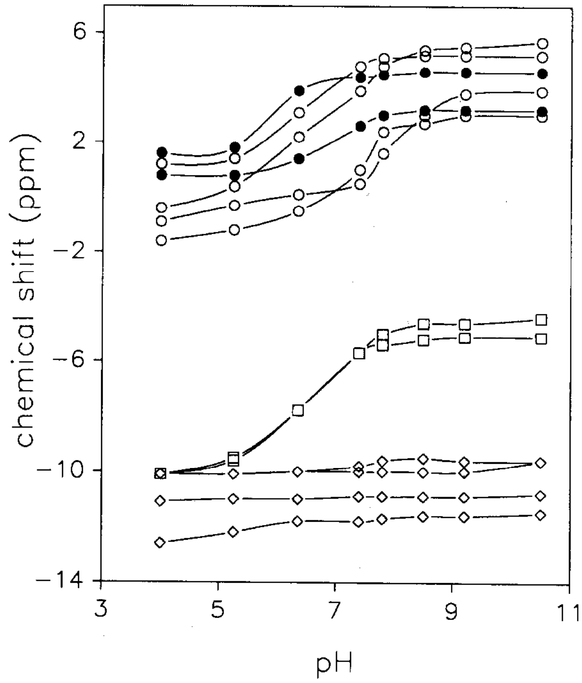

Fig.2. Effect of pH on ^{31}P chemical shifts of LPS monophosphate and diphosphate monoester and diester peaks, as follows: monophosphate monoesters (○); monophosphate diesters (●); β phosphorus of diphosphate monoesters (□); and α phosphorus of diphosphate monoesters and β phosphorus of diphosphate diesters (△).

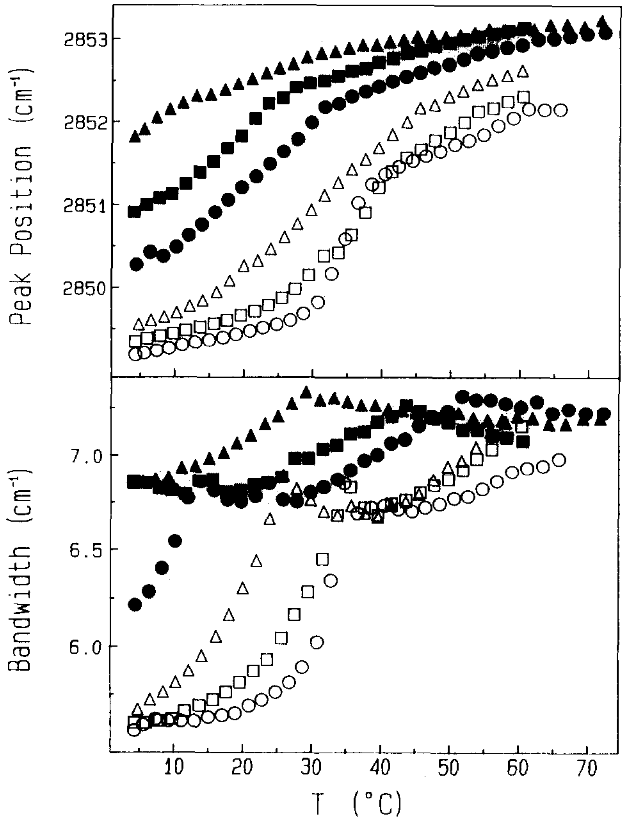

Fig.3. Temperature-dependent changes in the peak position and bandwidth of the symmetric methylene C-H stretching band of *S. typhimurium* Sl 3770 LPS (○) and from LPS · magainin complexes with molar ratios of: 5.0 (□); 3.6 (△); 2.1 (●); 1.4 (■); and 0.7 (▲).

phate diesters, 21% diphosphate monoesters and 20% diphosphate diesters. Since 59% of the five phosphorylated positions are substituted by monophosphates and 41% by diphosphates, the average number of phosphorus atoms per Sm LPS is 7.0. As determined from the distribution of the four types of phosphate groups and the pK_a values estimated from the titration curves in fig.2, the phosphate residues carry a net charge of about -7 at pH 7.4. Including the three carboxyl groups of the KDO residues, the total charge on the Sm LPS molecule is about -10.

Interactions between Sm LPS and magainin were monitored by FT-IR spectroscopy. The frequency and width of the symmetric methylene C-H stretching infrared band measure chain geometry and degree of motion of the hydrocarbon chains in lipid molecules [22]. Shifts towards higher frequency and increases in bandwidth are associated with the introduction of gauche conformers and greater motional freedom, respectively. These parameters can be used effectively to detect phase changes in model and native membranes [14,15,22,23]. Temperature-dependent changes in the frequency and width of this band in aqueous dispersions of *S. typhimurium* SL 3770 Sm chemotype LPS and complexes of LPS and magainin are shown in fig.3. In pure Sm LPS, a remarkably cooperative phase change, considering the heterogeneous nature of the LPS molecules, occurs near 35°C. This transition is very similar to that of the intact outer membrane-peptidoglycan complex of the *S. typhimurium* SL 3770 organism (data not shown). The addition of magainin to Sm LPS causes a concentration-dependent disordering of the lipid. At lower levels of peptide (i.e., LPS/Mag <4), magainin has little fluidizing effect. At higher peptide levels, the LPS hydrocarbon chains become progressively more disordered over the entire temperature interval and the cooperativity of the observed phase change is reduced considerably.

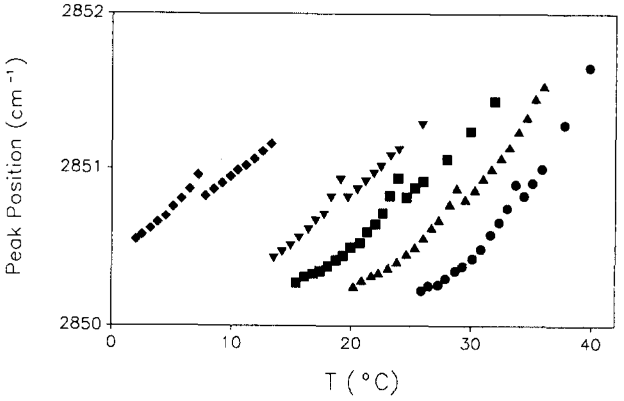

Fig.4. Temperature dependence of the frequency of the symmetric methylene C-H stretching band near the discontinuity temperature for LPS · magainin complexes with molar ratios of: 6.4 (●); 5.0 (▲); 3.6 (■); 2.8 (▼); and 2.1 (◆).

The temperature dependence of the bandwidth goes through a maximum during the phase transition for LPS/Mag ≥ 2. The temperature at which this maximum occurs decreases with increasing levels of peptide, from 34°C in the absence of magainin to 14°C at LPS/Mag of 2. In addition of this general disordering effect, magainin also induces a small but reproducible displacement (or 'spike') in thermal profiles of peak position at LPS/Mag ≥ 2, even at peptide levels where the overall phase change is affected very little. The temperature at which this spike occurs is dependent on the LPS/Mag, ranging from 33°C to 7°C at LPS/Mag of 6.4 to 2.1, respectively (fig.4). As the distance between adjacent LPS molecules increases during the transition from the gel to the liquid crystal state, a reorientation or rearrangement of magainin coordinated to negatively charged groups on LPS may account for the sudden displacement in peak position. Peptide-induced structural changes are known to occur with acidic phosphoglycerides. Polymyxin B has been shown to induce interdigitation in phosphatidylglycerol bilayers [24]. At higher levels of magainin, the phase change is abolished and the LPS hydrocarbon chains are extremely fluid even at the lowest temperature monitored (~ 5°C). The disruptive effects of magainin are significant at LPS/Mag <5 and are most pronounced at lower temperatures when LPS/Mag <2.

These results demonstrate that magainin binds effectively to Sm LPS. Wild-type *S. typhimurium* SL 3770 is relatively resistant to the bactericidal effects of magainin compared to 'rough' mutants of this organism which are deficient in LPS biosynthesis. Cationic peptides such as magainin and polymyxin B bind less effectively to the outer membranes from these rough mutants, where the concentration of LPS is lower and a significant portion of the carbohydrate moiety of the LPS molecules is missing (unpublished results). The outer membrane may serve as a sponge for cationic peptides, preventing them from reaching the plasma membrane where structural perturbations or permeability changes can result in cell death.

Acknowledgements: We wish to thank Dr M. Modrzakowski for use of microbiological facilities. This work was supported by the Ohio University College of Osteopathic Medicine and a Research Challenge grant from Ohio University.

REFERENCES

[1] Zasloff, M. (1987) Proc. Natl. Acad. Sci. USA 84, 5449-5453.

[2] Giovannini, M.G., Poulter, L., Gibson, B.W. and Williams, D.H. (1987) Biochem. J. 243, 113-120.

[3] Zasloff, M., Martin, B. and Chen, H.-C. (1988) Proc. Natl. Acad. Sci. USA 85, 910-913.

[4] Chen, H.-C., Brown, J.H., Morell, J.L. and Huang, C.M. (1988) FEBS Lett. 236, 462-466.

[5] Juretic, D., Chen, H.-C., Brown, J.H., Morell, J.L., Hendler, R.W. and Westerhoff, H. (1988) FEBS Lett. 236, 462-466.

[6] Terry, A.S., Poulter, L., Williams, D.H., Nutkins, J.C., Giovannini, M.G., Moore, C.H. and Gibson, B.W. (1988) J. Biol. Chem. 263, 5745-5751.

[7] Marion, D., Zasloff, M. and Bax, A. (1988) FEBS Lett. 227, 21-26.

[8] Matsuzaki, K., Harada, M., Handa, T., Susunu, F., Fujii, N., Yujima, H. and Miyajima, K. (1989) Biochim. Biophys. Acta 981, 130-134.

[9] Lugtenberg, B. and Van Alphen, L. (1983) Biochim. Biophys. Acta 737, 51-115.

[10] Hammond, S.M., Lambert, P.A. and Rycroft, A.N. (1984) The Bacterial Cell Surface, pp. 119-146, Kapitan Szabo, Washington, DC.

[11] Lüderitz, O., Freudenberg, M.A., Galanos, C., Volker, L., Rietschel, E.Th. and Shaw, D.H. in: Current Topics in Membranes and Transport (Razin, S. and Rottem, S. eds) vol. 17, Academic Press, New York.

[12] Westphal, O. and Jann, K. (1965) in: Methods in Carbohydrate Chemistry (Whistler, R.L. ed.) vol. 5, pp. 83-91, Academic Press, New York.

[13] Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756-1758.

[14] Blazyk, J. and Rana, F. (1987) Appl. Spectrosc. 41, 40-44.

[15] Loeffelholz, M.J., Rana, F., Modrzakowski, M.C. and Blazyk, J. (1987) Biochemistry 26, 6644-6648.

[16] Peterson, A.A., Fesik, S.W. and McGroarty, E.M. (1987) Antimicrob. Agents Chemother. 31, 230-237.

[17] Vaara, M., Vaara, T. and Sarvas, M. (1979) J. Bacteriol. 139, 664-667.

[18] Vaara, M., Vaara, T., Jensen, M., Helander, I., Nurminen, M., Rietschel, E.Th. and Makela, P.H. (1981) FEBS Lett. 129, 145-149.

[19] Crutchfield, M.M., Dugan, C.H. and Van Wazer, J.R. (1967) in: Topics in Phosphorus Chemistry (Grayson, M. and Griffith E.J. eds) vol. 5, pp. 2-74, Wiley, New York.

[20] Rosner, M.R., Khorana, H.G. and Satterthwait, A.C. (1979) J. Biol. Chem. 254, 5918-5925.

[21] Vogel, H.J. (1984) in: Phosphorus-31 NMR. Principles and Applications (Gorenstein, G.G. ed.) pp. 105-154, Academic Press, Orlando, FL.

[22] Cameron, D.G. and Dluhy, R.A. (1986) in: Spectroscopy in the Biomedical Sciences (Gendreau, R.M. ed.) pp. 53-86, CRC Press, Boca Raton, FL.

[23] Rana, F. and Blazyk, J. (1989) in: Biological and Synthetic Membranes (Butterfield, D.A. ed.) Prog. Clin. Biol. Res., vol. 292, pp. 77-85, Liss, New York.

[24] Ranck, J.L. and Tocanne, J.F. (1982) FEBS Lett. 143, 175-178.