Communication

Location of Fatty Acids in Lipid A Obtained from Lipopolysaccharide of Rhodopseudomonas sphaeroides ATCC 17023*

(Received for publication, December 28, 1987)

Nilofer Qureshi; Jeffrey P. Honovich, Hisako Hara, Robert J. Cotter, and Kuni Tukayama

From the Mycobacteriology Research Laboratory, William S. Middleton Memorial Veterans Hospital, Madison, Wisconsin 53705, and the Department of Bacteriology, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706, and the Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205.

Monophosphoryl lipid A (MLA) obtained from the lipopolysaccharide of Rhodopseudomonas sphaeroides ATCC 17023 was initially purified by silicic acid column chromatography to yield a single major pentaacyl MLA fraction. This fraction was methylated and further purified by reverse-phase high performance liquid chromatography to yield three prominent peak fractions. Laser desorption mass spectrometry of these three fractions allowed us to complete the important structural analysis of lipid A from this source.

Three structurally distinct forms of dimethyl MLA were identified where $M_\text{r} = 1447, 1449, and 1451$, respectively. These forms differed only by the presence or absence of unsaturation and keto group in the fatty acids. We established that the acyloxyacyl group (either $\Delta^2$-tetradecenoyloxytetradecanoate or tetradecanoyloxytetradecanoate) and the 3-ketotetradecanoyloxytetradecanoate occupied the 2'- and 3-positions of the glucosamine disaccharide, respectively. Analysis of several minor fractions suggests that there is considerable structural heterogeneity in the MLA. With this new knowledge, the study of the structure-to-function relationship of the reported lack of toxicity of lipopolysaccharide from R. sphaeroides can be completed.

Lipopolysaccharide (LPS) is an amphiphilic macromolecule found on the outer surface of the outer membrane of Gram-negative bacteria (1). It consists of the hydrophilic polysaccharide and lipophilic lipid A moieties (2). The lipid A component has been shown to have numerous beneficial biological properties, i.e. antitumor activity, protection against x-irradiation, and protection against bacterial infection (3), but because of its characteristic high toxicity, its beneficial properties have not been utilized clinically.

The toxicity of Salmonella LPS was attenuated considerably by an appropriate mild acid treatment to yield the MLA (4, 5). Such a preparation retained the beneficial biological properties (6, 7). Strittmatter et al. (8) reported the isolation of LPS from Rhodopseudomonas sphaeroides ATCC 17023, which was naturally nontoxic and yet had structural similarities in the lipid A region to the toxic LPS from the Salmonella strain. This group has continued their study of the LPS from this source in an effort to determine the structural basis for this surprising lack of toxicity (9, 10). Although much is now known, the structural analysis of the lipid A from this source is still incomplete.

We examined the HPLC-purified dimethyl MLA fractions obtained from the LPS of R. sphaeroides ATCC 17023 by LDMS and report that the main structural ambiguities have now been resolved. We have established the precise locations of the fatty acids in the major lipid A fractions from this source.

**EXPERIMENTAL PROCEDURES**

**Growth of Bacteria and Preparation of LPS**—The microaerophilic R. sphaeroides ATCC 17023 (American Type Culture Collection, Washington, D. C.) was grown phototrophically in medium 550 as recommended by the American Type Culture Collection at 34 °C, harvested at late-log growth, and washed with 0.9% saline. The cell paste (569 g) was extracted by the method described by Strittmatter et al. (8) to yield 2.86 g of crude LPS. We further extracted this crude LPS preparation with chloroform/methanol (2:1, v/v) to yield 545 mg of the rough-type LPS.

**Preparation of Purified MLA**—MLA was prepared from 200 mg of LPS as previously described (11) and purified on a 2 x 14-cm silicic acid column as described by Qureshi et al. (12). A linear gradient of 0-25% methanol in chloroform (500 ml) was used, and 5.0-ml fractions were collected. The following pooled fractions were obtained (pooled according to spot charring and analytical thin layer chromatography): A, 25-36 (5.2 mg); B, 37-39 (3.2 mg); C, 40-42 (5.8 mg); D, 43-53 (13.0 mg); E, 54-70 (6.4 mg); F, 71-90 (3.8 mg); G, 91-100 (3.7 mg). Fraction D, which represented the major fraction and contained the desired pentaacyl MLA, was converted to the dimethyl derivative as previously described (12).

**HPLC Fractionation**—HPLC was performed on the dimethyl MLA as described by Qureshi et al. (13). An 8 mm x 10 cm C$_{18}$-bonded silica cartridge (4 µm Nova Pak, Millipore Corp., Waters Chromatography Division, Milford, MA) was used with a linear gradient of 20-80% isopropl alcohol/water (93:7, v/v) over a period of 60 min at a flow rate of 2.0 ml/min. The wavelength of the detector was set at 210 nm.

**Mass Spectrometry**—The HPLC-purified dimethyl MLA fractions were analyzed by LDMS, fast atom bombardment mass spectrometry, and other methods.
RESULTS AND DISCUSSION

LPS obtained from R. sphaeroides was hydrolyzed under mild acid conditions to yield crude MLA. This preparation was then fractionated on a silicic acid column to yield the dimethyl pentaacyl MLA and subjected to reverse-phase HPLC (Fig. 1). Each of the three major fractions thus obtained was analyzed by LDMS, and the resulting spectra are shown in Fig. 2.

LDMS of peak 1 gave a molecular ion $M+K^+$ at $m/z$ 1486. Cleavage of OHC$_{10}$ at the sugar-oxygen linkage yielded $M+K^+-OHC_{10}$ ion at $m/z$ 1298. Previously, we showed that the reducing-end sugar of dimethyl MLA from LPS of Neisseria gonorrhoeae can undergo three major types of fragmentations on LDMS (14,16). These were simultaneous cleavages of (A) $C_1-O$ and $C_2-C_3$, (B) $C_4-C_5$ and $C_1-O$, which resulted in the appearance of (distal unit + reducing unit) fragment + $K^+$ ions. Such fragmentations allowed us to determine the fatty acid distribution within the distal and reducing-end sugars. Cleavages A, B, and C of the reducing-end sugar of peak 1 occurred to give fragment + $K^+$ ions at $m/z$ 1204, 1032, and 972 (calculated mass, 972.5), respectively. These results showed that the $M_r = 1447$ atomic mass units, $R_1 = OHC_{10}$, $R_2 = OHC_{14}$, $R_3 = OHC_{10}$, and $R_4 = 3KC_{14}$ (Table I). This was supported by the results of plasma desorption mass spectrometry (data not shown). We observed an oxonium ion of C$_{46}H_{55}NO_12$ at $m/z$ 874, which is diagnostic of the distal sugar fragment (11) and consistent with the fatty acid assignments.

LDMS of peak 2 gave a molecular ion $M+K^+$ at $m/z$ 1488. The loss of OHC$_{10}$ yielded $M+K^+-OHC_{10}$ ion at $m/z$ 1301. An additional loss of dimethylphosphate group gave $M+K^+-OHC_{10}PO_3H(CH_3)_2$ ion at $m/z$ 1174. Cleavages A and C gave fragment + $K^+$ ions at $m/z$ 1205 and 974 (calculated mass, 974.6), respectively. These results showed that the $M_r = 1449$ atomic mass units, $R_1 = OHC_{10}$, $R_2 = C_9OC_{14}$, $R_3 = OHC_{10}$, and $R_4 = 3KC_{14}$ (Table I). This was supported by the results of both plasma desorption and fast atom bombardment mass spectrometry, where we obtained M+Na$^+$ ion at $m/z$ 1472 and an oxonium ion at $m/z$ 876 (data not presented). Gas chromatography-mass spectrometry analysis of the fatty acids of this fraction showed the presence of small amounts of $\Delta^7$-tetradecenoate and OHC$_{14}$, which suggested the possible existence of a structural component containing $R_1 = OHC_{10}$, $R_2 = OHC_{10}$, $R_3 = OHC_{10}$, and $R_4 = OHC_{14}$ (data not presented).

LDMS of peak 3 gave a molecular ion $M+K^+$ at $m/z$ 1490. Cleavage of OHC$_{10}$ yielded $M+K^+OHC_{14}$ ion at $m/z$ 1303. Cleavages A and C gave fragment + $K^+$ ions at $m/z$ 1205 and 975 (calculated mass, 974.6), respectively. These results showed that the $M_r = 1451$ atomic mass units, $R_1 = OHC_{10}$, $R_2 = C_9OC_{14}$, $R_3 = OHC_{10}$, and $R_4 = OHC_{14}$ (data not presented). This was supported by the results of both plasma desorption and fast atom bombardment mass spectrometry.

FIG. 1. Reverse-phase HPLC of a silicic acid columns-purified dimethyl pentaacyl MLA. C$_{18}$-bonded silica cartridge was used, and the mobile phase was a linear gradient of isopropyl alcohol/water in acetonitrile/water. This graph was base line corrected.

FIG. 2. LDMS spectra of the HPLC peaks: A, peak 1; B, peak 2; C, peak 3. Refer to Fig. 1 for the source of the peaks.
atom bombardment mass spectrometry.

LDMS of the minor peak 4 (data not shown) gave molecular ion M+K+ at m/z 1577 (M, = 1588 atomic mass units). Cleavages of the reducing-end sugar allowed us to suggest a fatty acid distribution of R1 = OHC16, R2 = hydroxylated C14OC14, R3 = OHC16, R4 = OHC16. There appeared to be an additional methyl group at an undetermined site on the distal portion of the molecule.

A complex mixture of multiple structural forms of hexacycl dimethyl MLA was found in the minor silicic acid column fraction B. The size of the molecular ions and the fragmentation patterns by LDMS suggested the presence of two acyloxyacyl groups on the distal sugar (data not presented). This is similar to the MLA from the Salmonella strain (17).

However, these MLA appeared to have either hydroxylated, methoxylated, or keto acyloxyacyl groups. This type and extent of structural heterogeneity is reminiscent of the MLA from the LPS of N. gonorrhoeae (14). The extra methyl groups found in some fractions might be due to the diazomethane derivatization reaction.

Work done by Salimath et al. (9) on the structural analysis of the unfraccionated free lipid A obtained from LPS of R. sphaeroides had established the following for the major component: (a) backbone sugar is β-D-glucosaminyl-1,6-D-gluco-samine, (b) phosphate groups are at the 1- and 4'-positions of the disaccharide, (c) OHC16's are ester-linked at both C-3 and C-3', (d) Δ⁵-C14OC14 and 3KC14 are N-linked, (e) hydroxyl groups at C-4 and C-6' are free. Salimath et al. (10) have taken this study further by establishing the structures of the small polysaccharide region of this LPS.

We have now assigned the Δ⁵-C14OC14 or C14OC14 to the nitrogen of C-2' (R3) and 3KC14 or OHC14 to the nitrogen of C-2 (R4). We also showed considerable structure heterogeneity by the presence of three distinct and prominent structural forms of MLA as well as other minor components, which might be highly relevant to the question of why the LPS/lipid A from this source is nontoxic. Both Strittmatter et al. (8) and Salimath et al. (9) have inferred that this lack of toxicity is caused by the presence of unsaturation in Δ⁵-C14OC14 and keto group in 3KC14. However, other factors might also be involved. This study completes the structural analysis of the important features of free lipid A obtained from the LPS of R. sphaeroides. The structures as the dimethyl derivatives are given in Fig. 3. A study of the precise nature of the structure-function relationship of this lack of toxicity of LPS from this source is planned.

Acknowledgments—We thank Todd Sievert, Laura Phillips, and Sajeev Khan for their help in growing the cells and the extraction of LPS. Mass spectral determinations were carried out at the Middle Atlantic Mass Spectrometry Laboratory, a National Science Foundation Shared Instrumentation Facility, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

REFERENCES
1. Nikaido, H., and Vaara, M. (1985) Microbiol. Rev. 49, 1-32
2. Rietschel, E. T., Galanos, C., Loderitz, O., and Westphal, O. (1982) in Immunopharmacology and the Regulation of Leukocyte Function (Wells, D. R., ed.) pp. 183-225, Marcel Dekker, Inc., New York
3. Nowotny, A. (1980) Beneficial Effects of Endotoxins, Plenum Publishing Corp., New York
4. Qureshi, N., Takayama, K., and Ribi, E. (1983) J. Biol. Chem. 258, 11806-11813
5. Takayama, K., Qureshi, N., Ribi, E., and Cantrell, J. L. (1984) Rev. Infect. Dis. 6, 439-443
6. Takayama, K., Qureshi, N., Ribi, E., Cantrell, J., and Amano, K. (1983) in Bacterial Lipopolysaccharides: Structure, Synthesis, and Biological Activities (Anderson, L., and Unger, F. M., eds) ACS Symposium Series, No. 231, pp. 219-253, American Chemical Society, Wash., D. C.
7. Ribi, E., Cantrell, J., and Takayama, K. (1985) Clin. Immunol. Neurit. 6, 33-36
8. Strittmatter, R., Weckesser, J., Salimath, P. V., and Galanos, C. (1983) J. Bacteriol. 155, 153-168
9. Salimath, P. V., Weckesser, J., Strittmatter, W., and Mayer, H. (1983) Eur. J. Biochem. 136, 195-200
10. Salimath, P. V., Tharanathan, R. N., Weckesser, J., and Mayer, H. (1984) Eur. J. Biochem. 144, 227-232
11. Qureshi, N., Takayama, K., Heller, D., and Fenselau, C. (1983) J. Biol. Chem. 258, 12947-12951
12. Qureshi, N., Cotter, R. J., and Takayama, K. (1986) J. Microbiol. Methods 5, 45-77
13. Qureshi, N., Mascagni, P., Ribi, E., and Takayama, K. (1985) J. Biol. Chem. 260, 5271-5276
14. Takayama, K., Qureshi, N., Hyver, K., Honovich, J., Cotter, R. J., Mascagni, P., and Schneider, H. (1986) J. Biol. Chem. 261, 10624-10631
15. Alai, M., Demirev, P., Fenselau, C., and Cotter, R. J. (1986) Anal. Chem. 58, 1305-1307
16. Cotter, R. J., Honovich, J., Qureshi, N., and Takayama, K. (1987) Biomed. Environ. Mass. Spec. 14, 591-598
17. Takayama, K., Qureshi, N., and Mascagni, P. (1983) J. Biol. Chem. 258, 12601-12603

Fig. 3. Structure of dimethyl pentaacyl MLA. HPLC peak 1 obtained from the LPS of R. sphaeroides. The configuration of the double bond in R4 is not established. Refer to Table I and the text for the other fatty acid distributions.