Effect of Lipoteichoic Acid on Thermotropic Membrane Properties

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Lipoteichoic acid, diglucosyldiacylglycerol, and phosphatidylglycerol isolated from Staphylococcus aureus were embedded in dipalmitoylphosphatidylglycerophosphoglycol vesicles, and their thermotropic influence on this matrix was studied by differential scanning calorimetry. The natural fatty acids of phosphatidylglycerol effected peak broadening and a decrease in molar heat capacity. These effects were more pronounced with the glycolipid, which also increased the main transition temperature. With the lipoteichoic acid mixtures, two broad main transition peaks were observed, possibly due to different levels of lipoteichoic acid in vesicles. Both peaks showed a further upshift in transition temperatures and a pronounced decrease in molar heat capacity. Since the diacylglycerol moieties of all three amphiphiles were practically identical, the differences in the thermotropic effects have to be ascribed to the different structures of the head groups. Diglucosyldiacylglycerol is proposed to exert an additional effect by hydrogen bonding the hydroxyls of the sugar rings to their phospholipid neighbors. The stronger effect of lipoteichoic acid points to dynamic interactions of the long hydrophilic chain with the vesicle surface, which stabilize the membrane structure.

Characteristic lipid components of the cytoplasmic membrane of many gram-positive bacteria are glycolipids and lipoteichoic acids (LTAs) (7). For example, the cytoplasmic membrane of Staphylococcus aureus contains LTA, diglucosyl-diacylglycerol (DGDG), phosphatidylglycerol (PG), diacylglycerol, and lysylphosphatidylglycerol, which are present during exponential growth at 6, 8, 50, 24, and 10 mol%, respectively. Trace amounts of monoglucosyldiacylglycerol are also present, and toward the end of exponential growth, variable amounts of bisphosphatidylglycerol (cardiolipin) are formed (16). LTA is biosynthetically derived from DGDG, on which a linear chain of an average of 25 1,3-linked glycerophosphate residues is polymerized by the transfer of glycerophosphate from PG. Owing to the long hydrophilic chain, LTA contains more than 50% of the total membrane lipid glycerol. The concentration of LTA in the outer membrane layer is 12 mol%, which means that each LTA molecule is surrounded by eight lipid molecules.

Although LTA was discovered 25 years ago and has been considered an essential cellular component, little is known about its physiological functions (2, 7). Several biological activities associated with the hydrophilic chain were described, but the structural diversity of the hydrophilic chain with regard to d-alanine ester content, substitution with glycosyl residues, and in particular net electric charge rendered a general function based on the chain structure unlikely. We therefore focused on LTA’s role as a membrane constituent and searched for its influence on the physicochemical properties of the membrane.

Recent X-ray scattering analysis revealed that LTAs are unique because they form micellar supramolecular structures in aqueous dispersion (18), in contrast to membrane lipids and to the lipopolysaccharides of gram-negative bacteria (17, 20, 21). Accordingly, LTAs are not able to form stable monolayers (12) and need to be inserted in vivo into layers of membrane lipids. Here we report studies on the influence of LTA on lipid phase transition with a well-defined matrix of synthetic PG containing palmitoyl residues.

We tried to differentiate the effects of the natural fatty acid pattern, the DGDG moiety, and the long hydrophilic chain by separately inserting PG, DGDG, and LTA, all isolated from S. aureus, into the synthetic matrix.

MATERIALS AND METHODS

Lipids and LTA. 1,2-Dipalmitoyl-sn-glycerol-3-phospho(1-acyl-glycerol) sodium salt (DPPG) was purchased from Sigma GmbH (Deisenhofen, Federal Republic of Germany). To isolate membrane lipids and LTA, S. aureus (DSM 20233) was grown to the late exponential growth phase (11). After harvesting, cells were suspended in 0.1 M sodium citrate, pH 3.5, and were disintegrated with glass beads in a homogenizer (Braun, Melsungen, Federal Republic of Germany). Lipids were extracted by a modified Bligh Dyer procedure, separated by column chromatography on DEAE cellulose, and analyzed as described previously (6). The lipids were pure, as shown by two-dimensional thin-layer chromatography. LTA was extracted from the disintegrated bacteria with the hot phenol-and-water procedure and was purified from the aqueous layer by hydrophobic interaction chromatography (8). The preparation was free of proteins, nucleic acids, and polysaccharides. It contained β-gentibiosi-diacylglycerol as a lipid anchor and a linear chain of 21 to 25 glycerophosphate residues, on average (5, 10, 11). d-Alanine ester was removed by mild alkaline treatment, pH 8, for 24 h, followed by ultratrituration (10). Phospholipids and LTA were used in the sodium salt form. Fatty acids were released from LTA (15) and lipids (13, 14) as described previously and were identified and quantified by gas-liquid chromatography as methyl esters (9). The positional distribution of fatty acids in phospholipids was analyzed with phospholipase A2 (hog pancreas), as detailed earlier (6). Salts and enzymes were removed from the incubation mixture by Folch partition at pH 4.

For positional analysis of glycolipids and LTA, the 1-position-specific lipase from Rhizopus arrhizus was used in 0.04 M Tris buffer, pH 7.2, containing Triton X-100 (2 mg/ml). Cleavage products of LTA were separated by hydrophobic interaction chromatography, and the lysyglycolipid was released from the chain with 96% (vol/vol) acetic acid (11). Fatty acids and lysy compounds were separated by thin-layer chromatography, the lysy compounds were deacylated, and the fatty acid methyl esters were analyzed as described above.

DSC measurements. Lipids and LTAs were dispersed in 10 mM NaCl dissolved in water, which was triple distilled in a quartz apparatus. Sonication was performed with a bath sonifier. Vesicles were prepared from a 10−3 molar dispersion of DPPG in 10 mM NaCl by sonication and slight warming. Appropriate volumes of the dispersions of LTA and PG from S. aureus were added to the dispersion of DPPG vesicles. The mixtures were sonicated and warmed until the dispersions were clear or slightly opaque. To obtain DPPG vesicles containing S. aureus glycolipids, appropriate amounts of both lipids dissolved in CHCl3 were mixed, dried, dispersed in 10 mM NaCl, and sonicated.

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About 1.5 ml of lipid dispersions was used for differential scanning calorimetry (DSC). Each sample was run three times between 20 and 60°C at a rate of 20°C/h in an MC-2 microcalorimeter (Microcal, Northampton, Mass.) with 10 mM NaCl as a reference. Heat capacity, $C_p$ (in joules per degree Celsius), was measured at atmospheric pressure as a function of temperature (in degrees Celsius). $C_p$ was converted to molar heat capacity, $C_p^m$ (in kilojoules per mole per degree Celsius), by introducing the cell volume ($V = 1.2249 \text{ ml}$) and the sample concentration. The DSC upscans of a given sample were reproducible within the experimental error. The second and third upscans were used for calculations and comparison. Phase transition temperatures, $T$ (in degrees Celsius), were obtained from the DSC curves after baseline subtraction. $T_{\text{main}}$ is the temperature in the DSC curve where $C_p^m$ was at its highest. The molar enthalpy change, $\Delta H^m$ (in kilojoules per mole), was calculated from the area under the DSC curve in the temperature range of the phase transition.

### RESULTS AND DISCUSSION

**Fatty acid composition and distribution.** The fatty acid compositions of PG, DGDG, and LTA from *S. aureus* are shown in Table 1. The positional distributions of the fatty acids on the glycerol moieties of LTA, MGDG, DGDG, PG, and bisphosphatidylglycerol from *S. aureus* are shown in Fig. 1.

The fatty acid compositions of PG and DGDG are almost identical. In the LTA, 13-methyltetradecanoic acid (15i), 12-methyltetradecanoic acid (15ai), and 18:0 fatty acids are accumulated, mainly at the cost of the 19:0 and 20:0 fatty acids. In spite of this, the mean chain lengths, calculated from the data in Table 1 were 17.0, 16.9, and 16.6 for PG, DGDG, and LTA, respectively. Consistent with the biosynthetic origin of lipids and LTA from the same diacylglycerol pool (16), the distribution of the fatty acids between the two positions of their glycerol moieties follows the same principle; longer-chain, unbranched fatty acids (16:0, 18:0, and 20:0) are at position 1, and shorter-chain (14:0), mostly branched iso- and anteiso-fatty acids (15i and 15ai), are at position 2. Minor modifications are seen in the phospholipid and glycolipid-LTA series.

**Thermotropic properties.** As depicted in Fig. 2, synthetic DPPG exhibited a pretransition at $33 \pm 0.3^\circ$C (mean $\pm$ standard deviation) and a sharp main transition into the fluid phase at $39.0 \pm 0.1^\circ$C, with a $T_{\text{main}}^{1/2}$ of 0.47 $\pm$ 0.05°C and a $C_p^m$ of 51.5 kJ/mol (Table 2). This well-known behavior (23, 24) changed when bacterial PG was added. At a concentration in the vesicle of 10 mol% of the bacterial lipid, the pretransition was no longer visible, and the main transition broadened ($T_{\text{main}}^{1/2} = 2.0$ $\pm$ 0.1°C) and shifted slightly to 38.5 $\pm$ 0.1°C. The DSC peak at the main transition was not symmetrical, showing a small shoulder and broadening in the descent. $C_p^m$ was reduced drastically to a value of 14.2 kJ/mol (Fig. 2; Table 2).

The broadening of the main transition peak and the decrease in $C_p^m$ were apparently caused by the pairing of shorter-

### TABLE 1. Fatty acid composition of lipids and LTA from *S. aureus*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>PG</th>
<th>DGDG</th>
<th>LTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>14i</td>
<td>3.8</td>
<td>5.3</td>
<td>3.0</td>
</tr>
<tr>
<td>14:0</td>
<td>11.9</td>
<td>8.9</td>
<td>5.8</td>
</tr>
<tr>
<td>15i</td>
<td>4.7</td>
<td>5.7</td>
<td>9.1</td>
</tr>
<tr>
<td>15ai</td>
<td>13.9</td>
<td>19.1</td>
<td>24.9</td>
</tr>
<tr>
<td>15:0</td>
<td>2.3</td>
<td>1.8</td>
<td>0.6</td>
</tr>
<tr>
<td>16i</td>
<td>1.1</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>16:0</td>
<td>12.1</td>
<td>10.4</td>
<td>11.3</td>
</tr>
<tr>
<td>17i</td>
<td>0.6</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>17ai</td>
<td>1.5</td>
<td>1.3</td>
<td>0.8</td>
</tr>
<tr>
<td>18:0</td>
<td>11.1</td>
<td>12.9</td>
<td>24.5</td>
</tr>
<tr>
<td>18:1</td>
<td>4.8</td>
<td>1.8</td>
<td>2.4</td>
</tr>
<tr>
<td>19:0</td>
<td>4.2</td>
<td>6.1</td>
<td>2.4</td>
</tr>
<tr>
<td>20:0</td>
<td>25.6</td>
<td>22.1</td>
<td>12.9</td>
</tr>
<tr>
<td>20:1</td>
<td>1.0</td>
<td>1.7</td>
<td>-----</td>
</tr>
</tbody>
</table>

*Abbreviations: 14:0, tetradecanoic acid; 18:1, octadecenoic acid; 15i, 13-methyltetradecanoic acid; 15ai, 12-methyltetradecanoic acid.*
chain, branched and longer-chain, unbranched fatty acids in natural PG. Around the natural diacylglycerol moieties, the packing of the alkyl chains of DPPG is disturbed, resulting in an increase of gauche rotamers. As a consequence, the number of van der Waals interactions is reduced, and $C_p^{m}$ decreased accordingly.

A more drastic effect was seen when the same molar amount of natural DGDG was incorporated into the DPPG matrix.

![DSC curves (second upscan) of vesicles containing DPPG alone and DPPG in 9:1 molar ratio mixtures with PG, DGDG, and LTA. The $T_{\text{main}}$ of PG- and DGDG-containing vesicles are shown in the insets. Vesicles were prepared and analyzed in 10 mM NaCl.](image)

**TABLE 2. Thermotropic properties of DPPG–bacterial-lipid mixtures**

<table>
<thead>
<tr>
<th>DPPG–bacterial-lipid mixture $^b$</th>
<th>$T_{\text{pre}}$ (°C)</th>
<th>$C_p^{m}_{\text{pre}}$ (kJ/mol/°C)</th>
<th>$T_{\text{main}}$ (°C)</th>
<th>$T_{\text{main}}^{1/2}$ (°C)</th>
<th>$C_p^{m}_{\text{main}}$ (kJ/mol/°C)</th>
<th>$\Delta H^o$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPG</td>
<td>33.0 ± 0.3</td>
<td>1.7 ± 0.1</td>
<td>39.0 ± 0.1</td>
<td>0.47 ± 0.05</td>
<td>51.5 ± 0.1</td>
<td>46.26 ± 0.3</td>
</tr>
<tr>
<td>DPPG-PG (9:1)</td>
<td>33.5 ± 0.3</td>
<td>0.8 ± 0.1</td>
<td>38.5 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>14.2 ± 0.2</td>
<td>41.28 ± 0.4</td>
</tr>
<tr>
<td>DPPG-DGDG (9:1)</td>
<td>40.6 ± 0.1</td>
<td>4.8 ± 0.1</td>
<td>46.5 ± 1.0</td>
<td>2.9 ± 0.2</td>
<td>5.8 ± 0.2</td>
<td>38.05 ± 0.4</td>
</tr>
<tr>
<td>DPPG-LTA (9.82:0.18)</td>
<td>39.5 ± 0.2</td>
<td>3.2 ± 0.1</td>
<td>42.8 ± 0.5</td>
<td>3.7 ± 0.2</td>
<td>8.4 ± 0.2</td>
<td>36.38 ± 0.4</td>
</tr>
<tr>
<td>DPPG-LTA (9.57:0.43)</td>
<td>42.8 ± 0.5</td>
<td>3.7 ± 0.2</td>
<td>46.5 ± 1.0</td>
<td>2.9 ± 0.2</td>
<td>5.8 ± 0.2</td>
<td>38.05 ± 0.4</td>
</tr>
<tr>
<td>DPPG-LTA (9.25:0.75)</td>
<td>43.5 ± 0.8</td>
<td>4.4 ± 0.3</td>
<td>47.4 ± 0.6</td>
<td>2.6 ± 0.2</td>
<td>4.1 ± 0.2</td>
<td>27.25 ± 0.4</td>
</tr>
<tr>
<td>DPPG-LTA (9:1)</td>
<td>43.2 ± 0.6</td>
<td>3.8 ± 0.2</td>
<td>45.3 ± 0.4</td>
<td>2.0 ± 0.2</td>
<td>5.8 ± 0.2</td>
<td>37.97 ± 0.5</td>
</tr>
<tr>
<td>DPPG-LTA (8.5:1.5)</td>
<td>44.9 ± 0.5</td>
<td>4.0 ± 0.3</td>
<td>46.2 ± 0.5</td>
<td>2.4 ± 0.2</td>
<td>4.1 ± 0.2</td>
<td>25.62 ± 0.4</td>
</tr>
</tbody>
</table>

$^a$ $T_{\text{pre}}$, pretransition temperature; $C_p^{m}_{\text{pre}}$, $C_p^{m}$ at the pretransition temperature; $C_p^{m}_{\text{main}}$, $C_p^{m}$ at $T_{\text{main}}$; $T_{\text{main}}^{1/2}$, full width at half height of main transition peak. All values are means ± standard deviations.

$^b$ Numbers in parentheses are molar ratios. For DPPG-LTA mixtures, two main transition peaks were found (see the text).

$^c$ $\Delta H^o$ was calculated for the whole transition profile.
(Fig. 2). Compared with that of PG, the broadening of the main transition peak was more than doubled ($T_{\text{main}}^{1/2} = 4.8 \pm 0.1^\circ\text{C}$). The main transition temperature shifted upward to $40.6 \pm 0.1^\circ\text{C}$, and $C_p^{m}$ decreased more drastically to $4.6 \pm 0.2$ kJ/mol (Table 2).

Since the fatty acid compositions of PG and DGDG are almost identical (Table 1), the reason for the different values for $C_p^{m}$ and $T_{\text{main}}$ must reside in the glycosidic head group. Increased transition temperatures which were ascribed to hydrogen bonding between the sugar hydroxyls of neighboring molecules have been described for glycolipids (1, 3, 4, 14). The stretched conformation of the (β1→6)-interlinked glucopyranosyl residues in $S.\ aureus$ DGDG (19, 22) may be particularly prone to hydrogen bonding, which could occur between the sugar residues of the glycolipid and the glycerol moieties of DPPG.

Although DGDG is the hydrophobic anchor of LTA, the effect of LTA on the DPPG matrix was strikingly different. As little as 1.8 mol% of LTA in the vesicles caused an increase of $T_{\text{main}}$ to $39.5 \pm 0.2^\circ\text{C}$, a significant broadening of the peak ($T_{\text{main}}^{1/2} = 3.2 \pm 0.1^\circ\text{C}$), and $C_p^{m}$ decreased to $8.4 \pm 0.2$ kJ/mol/°C. Two shoulders in the ascending part and a smaller and a larger shoulder in the descending part of the main transition peak point to heterogeneity. This heterogeneity became evident at higher LTA concentrations, where essentially two main transition peaks were observed (Fig. 3). They differed for $T_{\text{main}}$ up to $4^\circ\text{C}$, and in each pair, the peak with the higher $T_{\text{main}}$ displayed a lower $C_p^{m}$ value (Table 2). The higher $T_{\text{main}}$ was fairly constant at $46.4 \pm 0.9^\circ\text{C}$ over the LTA concentrations tested, whereas the lower gradually increased, presumably reflecting an increasing LTA concentration in the DPPG matrix.

Monolayer experiments revealed that DPPG and LTA form stable layers up to LTA concentrations of 20 mol% (12). The LTA concentrations used in the present experiments were far below this limit; thus, both amphiphiles are expected to be present in mixed layers. We therefore suggest that the double peaks observed at LTA concentrations higher than 2 mol% may represent vesicle populations of lower and higher LTA content, possibly due to curvature effects in small and large vesicles which are present in sonicated dispersions (1).

It is unlikely that the effect of less than 2 mol% LTA on the whole DPPG system is brought about by the interaction of the LTA anchor with neighboring DPPG molecules. In this context, one must consider the fact that LTA contains a mobile chain of 25 glycerophosphate residues on average, which measures 8.5 nm in coiled conformation and 18.7 nm in stretched conformation (18). The increase by up to $7^\circ\text{C}$ of the $T_{\text{main}}$ compared with that for DGDG may therefore reflect stronger long-distance polar interactions between the DPPG head groups and the LTA chain, suggesting stabilizing effects on the surface of the vesicle.

In vivo, these interactions might be a regulated process: a limited amount of highly negatively charged LTA would stabilize the bacterial membrane surface, and LTA concentrations higher than 20 mol% would form stable monolayers and destabilize the lamellar aggregation. LTA segregates into the subphase, presumably in the form of micelles, the supramolecular structure dictated by the small cross-sectional diameter of the diacylglycerol moiety and the resulting conical molecular shape of LTAs (Fig. 4). The physicochemical properties of LTA differ significantly from those of lipopolysaccharides (LPS) in gram-negative bacteria, where the lipid anchor lipid A carries up to seven fatty acids and a noncharged or moderately negatively charged O-chain resulting in a cylindrical molecular shape that induces a lamellar arrangement of the molecules (Fig. 4) (17, 20, 21). LPS, therefore, forms the stable outer layer of the outer membrane of gram-negative bacteria and determines rather than modifies its physicochemical properties.

**Conclusions.** PG, DGDG, and LTA isolated from $S.\ aureus$ possess similar fatty acid compositions in their diacylglycerol moieties. The natural fatty acids of different lengths increase
the fluidity in the hydrocarbon layer of the DPPG vesicles, as indicated by the observed decrease in \( C_p'' \). The upshift of the \( T_{main} \) seen with DGDG and more pronounced with LTA in the form of two main transition peaks, indicates stabilizing effects within the head group region. We hypothesize that they are exerted in the case of DGDG by hydrogen bonding to the form of two main transition peaks, indicates stabilizing effects within the head group region. We hypothesize that they are exerted in the case of DGDG by hydrogen bonding to the hydrophilic phospholipid neighbors but that they are exerted in the case of LTA by dynamic long-distance interactions of the hydrophilic poly(glycerophosphate) chain with the phospholipids of the LTA.

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