INCORPORATION OF GLYCINE INTO THE CELL WALL GLYCOPEPTIDE IN STAPHYLOCOCCUS AUREUS: ROLE OF sRNA AND LIPID INTERMEDIATES

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The synthesis of the cell wall glycopeptide in Staphylococcus aureus and Micrococcus lysodeikticus from its precursors, UDP-acetylglicosamine (UDP-GlcNAc) and UDP-acetylmuramyl-L-alα-D-glu­L-lys·D-ala·D-ala (UDP-MurNAc-pentapeptide), has been under investigation in several laboratories.1–4 The product of this reaction is a linear polymer consisting of alternating GlcNAc- and MurNAc-pentapeptide residues. A unique feature of this reaction mechanism is the fact that UMP and Pi are formed from one of the substrates while UDP is formed from the other.2–8 This finding led to the observation that MurNAc(-pentapeptide)-P-lipid and GlcNAc-MurNAc(-pentapeptide)-P-lipid are intermediates in the reaction.3 This sequence is presumably a means by which the intracellular nucleotides are transported through the membrane for the synthesis of an extracellular product, the cell wall. It also ensures the alternation of the sugars in the glycopeptide product.

The biosynthesis of the cell wall of S. aureus also requires the formation of polyglycine cross bridges which link glycopeptide chains in a two- or three-dimensional network, attached at one end to the ε-amino groups of lysine and at the other to D-alanine, the carboxyl terminus of the cell wall tetrapeptide.2–7 An important clue to the mechanism of formation of these bridges was the observation that an ATP and uridine nucleotide-requiring system which incorporated glycine into an acid-precipitable product was RNase-sensitive.1 The product was incompletely characterized, however, and appeared to be lysozyme-insensitive.

With enzyme particles prepared after disintegration of cells of S. aureus with alumina and by incubation at low temperature, an extremely efficient synthesis of glycopeptide is obtained.2 Under these conditions polyglycine chains can also be added to the ε-amino groups of lysine in the glycopeptide. In the present paper the mechanism of this reaction will be described. Glycine is activated as glycyl-sRNA which then transfers glycine to the recently described lipid intermediates in
cell wall synthesis. The polyglycine-containing lipid intermediates are then used for cell wall synthesis.

**Materials and Methods.**—Substrates and enzymes from *S. aureus* strain H were prepared and assays were carried out as described previously1 with changes described in figures or tables. C14-glycine (80 mc/mmole) and H3-glycine (200 mc/mmole) were obtained from the New England Nuclear Corporation. Products were characterized using the specificity of enzymes which catalyze the hydrolysis of cell walls.2,3,4,5 Free amino groups in products were measured using dinitrofluorobenzene (DNFB). C14-glycine, dinitrophenyl (DNP)-C14-glycine, C14-L-lysine, and C-DNP-C14-L-lysine, formed after acid hydrolysis, were readily separated by thin layer chromatography. Similarly, C-terminal C14-glycine was measured after hydrazinolysis. From these data, N-terminal, internal, and C-terminal amino acids could be calculated. Details will be presented in a full publication.

**Results.**—Utilization of glycine for glycopeptide synthesis: The incorporation of glycine with *S. aureus* particles was dependent on particles, ATP, supernatant, and both of the uridine nucleotides required for glycopeptide synthesis. It was insensitive to chloramphenicol, puromycin, the glycyl analog of puromycin, and was totally inhibited by RNase (Table 1). These results largely confirm the results of Chatterjee and Park,6 except that, contrary to their report, no requirement for or stimulation by ribosomes has been observed.

**Role of sRNA and activating enzyme:** These data suggested that glycine might be activated as glycyl-sRNA. sRNA prepared from *S. aureus* by phenol extraction greatly stimulated the incorporation (Table 2).8 The supernatant also contained a glycine-activating enzyme which was purified 15-fold.9 This purified enzyme, together with sRNA, substituted for the requirement for supernatant (Table 2). This enzyme preparation catalyzed formation of glycine hydroxamate and a glycine-dependent ATP-PP exchange. The mechanism of glycine activation was, therefore, the same as that reported for glycine-activating enzyme in rat liver.10 sRNA from *E. coli* and its homologous glycine-activating enzyme substituted for *S. aureus* sRNA and activating enzyme.

**TABLE 1**

**Requirements for Incorporation of Glycine and MurNAC-pentapeptide into Glycopeptide**

<table>
<thead>
<tr>
<th>System</th>
<th>H3-gly</th>
<th>C14-D-alanine, C14-D-alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>400</td>
<td>142</td>
</tr>
<tr>
<td>- UDP-MurNAC-pentapeptide</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>- UDP-GlcNAC</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>- Chloramphenicol</td>
<td>430</td>
<td>162</td>
</tr>
<tr>
<td>- ATP</td>
<td>39</td>
<td>38*</td>
</tr>
<tr>
<td>+ RNase</td>
<td>3</td>
<td>91*</td>
</tr>
<tr>
<td>+ Puromycin</td>
<td>392</td>
<td>152</td>
</tr>
<tr>
<td>+ &quot;Glycyl-puromycin&quot;†</td>
<td>393</td>
<td>150</td>
</tr>
</tbody>
</table>

The complete reaction mixture contained in a final volume of 30 μl: 3 μmole of Tris-HCl, pH 7.8, 0.1 μmole of MgCl2, 1.3 μmole of KCl, 2.7 μmole of H3-glycine (200 mc/μmole), 2 μmole of UDP-MurNAC-L-ala-D-glu-L-lys-C14-D-ala-C14-D-ala (12 mc/μmole), 5 μmole of UDP-GlcNAC, 0.02 μmole of ATP, 0.05 μmole of K-phosphoenolpyruvate, 2.05 μg of chloramphenicol, 5 μg of pyruvate kinase (Boehringer and Soehne), 0.025 μmole of 2-mercaptoethanol, 50 μg of staphylococcal sRNA, 4 μg (as protein) of supernatant fraction (filtered through Sephadex G-25) containing glycine-activating enzyme, 371 μg (as protein) of once-washed membrane particle fraction, and, as indicated, 0.024 μg of pancreatic RNase or 10-3 M puromycin or "glycyl-puromycin." Incubation was carried out for 60 min at 30°. The mixtures were then heated at 100° for 1 min. Traces of remaining glycyl-sRNA were then destroyed by incubation with 1 μg of RNase for 5 min at 60°. For methods of measurements, see preceding paper.1

* The diminution of glycopeptide synthesis on omission of ATP or addition of RNase is reproducible and has not been explained.

† "Glycyl-puromycin" is the analog of puromycin in which the amino acid, 3-O-methyl-L-tyrosine, has been replaced by glycine.
TABLE 2  
REQUIREMENT FOR sRNA PLUS EITHER SUPERNATANT FRACTION OR PURIFIED ACTIVATING ENZYME IN GLYCINE INCORPORATION INTO GLYCOPEPTIDE

<table>
<thead>
<tr>
<th>Additions</th>
<th>H\textsuperscript{+}-gly (\textmu{}mole incorporated)</th>
<th>C\textsuperscript{14}-D-ala·C\textsuperscript{14}-D-ala (\textmu{}mole incorporated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
<td>56</td>
</tr>
<tr>
<td>sRNA</td>
<td>5</td>
<td>55</td>
</tr>
<tr>
<td>Sup.</td>
<td>3</td>
<td>53</td>
</tr>
<tr>
<td>Act. enz.</td>
<td>3</td>
<td>41</td>
</tr>
<tr>
<td>sRNA + sup.</td>
<td>42</td>
<td>41</td>
</tr>
<tr>
<td>sRNA + act. enz.</td>
<td>57</td>
<td>41</td>
</tr>
</tbody>
</table>

The conditions are essentially the same as in Table 1 except that 38 \mu{}g (as protein) of twice-washed membrane particle enzyme and, where indicated, either 1.5 \mu{}g of supernatant fraction or 0.5 \mu{}g of glycine-activating enzyme (specific activity: 27 \mu{}mole glycyl-sRNA per \mu{}g per 10 min) or 50 \mu{}g of staphylococcal sRNA were used. In these experiments the amount of activating enzyme was limiting so that the ratio of glycine to MurNAc-pentapeptide incorporated was relatively low.

Glycine is attached to a terminal adenosine group in the sRNA from \textit{S. aureus}. C\textsuperscript{14}-glycyl-adenosine, liberated by pancreatic RNase digestion of C\textsuperscript{14}-glycyl-sRNA, was purified by the method of Wolfenden, Rammel, and Lipmann.\textsuperscript{11} On paper chromatography (isobutyric acid:1 N ammonia, 5:3, \textit{R}_{\text{adenosine}} = 1.0; butanol: formic acid:water, 77:10:13, \textit{R}_{\text{adenosine}} = 0.7) and paper electrophoresis at pH 3.2, it had the same mobilities as glycyl-adenosine prepared from \textit{E. coli} glycyl-sRNA. After treatment with dilute alkali, an ultraviolet-absorbing material with the mobility of adenosine was obtained. The ultraviolet absorption spectrum of this purified material was identical to that of adenosine.

The glycyl-sRNA was purified by chromatography on DEAE-Sephadex A-50 buffered with 7 M urea, 0.03 M ammonium acetate with a NaCl gradient from 0.52 M to 0.75 M (final pH 4.5).\textsuperscript{12} Two major peaks of glycyl-sRNA were found (Fig. 1). Glycyl-sRNA from peak \textit{A} substituted for glycine, sRNA, and ATP in glycine incorporation; a maximum of 50 per cent of added glycyl-sRNA was utilized (Fig. 2). Glycine from this glycyl-sRNA appeared first in the lipid fraction previously described,\textsuperscript{8} i.e., the material with \textit{R}_{\text{f}} = 0.9 in isobutyric acid:1 N NH\textsubscript{4}OH (5:3). After a lag, radioactivity appeared in glycopeptide. These same kinetics have previously been reported for the formation of glycopeptide from UDP-Glc\textsubscript{N}Ac and UDP-Mur\textsubscript{N}Ac-pentapeptide, the lag being due to the formation of lipid intermediates.\textsuperscript{8} Glycyl-sRNA from peak \textit{B} was inactive (Fig. 2). Both of the glycyl-sRNA's were active in protein synthesis with an \textit{E. coli} ribosomal system.

\textit{Transfer of glycine to a lipid intermediate:} Four prominent possibilities existed for the acceptor of glycine, viz., UDP-acetylmyramyl-pentapeptide, Mur\textsubscript{N}Ac(-pentapeptide)-P-lipid, Glc\textsubscript{N}Ac-Mur\textsubscript{N}Ac(-pentapeptide)-P-lipid, and the incomplete glycopeptide itself. The lipid intermediates from \textit{S. aureus}\textsuperscript{8} were found to incorporate glycine rapidly, and the glycine-containing product could be observed at the same position on chromatography in isobutyric acid:NH\textsubscript{4}OH (5:3), \textit{R}_{\text{f}} = 0.9, as the lipids without glycine.

When UDP-Glc\textsubscript{N}Ac was omitted, under which condition Mur\textsubscript{N}Ac(-pentapeptide)-P-lipid accumulated, some glycine was incorporated into the lipid fraction. After formation of this lipid intermediate containing glycine linked to Mur\textsubscript{N}Ac(-pentapeptide)-P-lipid (Fig. 3), further glycine addition was prevented by addition of RNase. UDP-Glc\textsubscript{N}Ac was then added and the sample was again incubated. Under these conditions the accumulated lipid intermediate disappeared and
glycopeptide was formed (Fig. 3). It is clear that GlcNAc can be added to MurNAc (-pentapeptide-polyglycine)-P-lipid and that the product can be used for glycopeptide synthesis.

Similarly, GlcNAc-MurNAc(-pentapeptide)-P-lipid was accumulated in the particles by incubation with both nucleotide substrates. When the glycine incorporation system (glycine, ATP, sRNA, and activating enzyme) was added to these particles, the lipid intermediate was rapidly and extensively labeled by glycine (Fig. 4). Both the extent and the velocity of labeling were much greater with GlcNAc-MurNAc(-pentapeptide)-P-lipid as the glycine acceptor than with MurNAc(-pentapeptide)-P-lipid (compare Figs. 3 and 4). After short incubation (15 min) lipid accumulation was maximum and only a small amount of glycopeptide was formed. If RNase was added at this time, further addition of glycine to the lipid intermediate was prevented. The intermediate then disappeared, and glycopeptide was formed from it. These and other experiments establish that both lipid intermediates can accept glycine from glycyl-sRNA in the S. aureus system as well as transfer the product to glycopeptide but that GlcNAc-MurNAc(-pentapeptide)-P-lipid may be a more adequate acceptor.

UDP-acetylmuramyl-pentapeptide or a similar nucleotide was not labeled by the

FIG. 1.—Fractionation of glycyl-sRNA. Forty mg of sRNA from S. aureus (10 optical density units at 260 μg per mg) was charged with C14-glycine in the presence of ATP and homologous supernatant fraction in the presence of all other C14-amino acids and then extracted with phenol-water. Charged sRNA (25,000 cpm per mg) was then chromatographed on a DEAE-Sephadex column (100 cm X 1.8 cm). Fractions of 6 ml were collected. Glycyl-sRNA (peak A) is at tube 108 and glycyl-sRNA (peak B) is at tube 126. The materials at tubes 34 and 48 are C14-glycine and ATP, respectively.

FIG. 2.—Utilization of glycyl-sRNA from peak A for lipid and glycopeptide synthesis. Conditions are essentially the same as in Table 1, except that C14-glycyl-sRNA fractions replaced glycine-activating enzyme, ATP, and the ATP-generating system. 8000 cpm of C14-glycyl-sRNA from peak A or 3000 cpm of C14-glycyl-sRNA from peak B and 28 μg (as protein) of once-washed membrane particles were used. At 60 min virtually all of the glycyl-sRNA’s not utilized had decomposed.
glycine incorporation system. Glycopeptide was allowed to form in the absence of the glycine-incorporating system. Residual substrates were washed out of the particles, and then the glycine-incorporating system was added. Subsequent labeling of the preformed glycopeptide under these conditions was very low, and labeling which did occur was at least partially accounted for by the lipid intermediates which these particles contained.

It has not been established whether glycine polymerization occurs on the sRNA or on the lipid. However, polymerization by sequential transfer of glycine to the lipid is suggested by the failure of sensitive methods (C¹⁴-glycine of high specific activity and thin layer chromatography of DNP-amino acids and peptides) to reveal even traces of di-, tri-, tetra-, or penta-glycine in glycy1-sRNA during the reaction.

Effects of inhibitors on glycine addition: Among a variety of substances tested, octanol (3 mg/ml), deoxycholate (1.7 mg/ml), vancomycin (50 μg/ml), and ristocetin (50 μg/ml) have been found to uncouple glycopeptide synthesis. At the low concentrations indicated, all of these substances inhibited utilization of the lipid intermediates containing glycine for glycopeptide synthesis but did not inhibit formation of these glycine-containing lipid intermediates. Several other antibiotics which inhibit cell wall synthesis, penicillin, bacitracin, and novobiocin, had no
effect on either the formation or the utilization of the lipid intermediates.

Location of the glycine residues in lipid intermediates and glycopeptide: An average of 1.9 glycine residues was transferred to the ε-amino groups of lysine in the lipid intermediates. Some of the lysine residues were not substituted, however, so that the number of glycines transferred per substituted lysine was about 5. The N-terminal end of the glycine chain in the lipid intermediate reacted poorly with DNFB. Only one N-terminal glycine was found per 10–20 residues (Table 3). At the present time we are uncertain whether this represents a technical problem or whether the amino end of the glycine chain is substituted in some unknown manner. In the presence of octanol, virtually all of the ε-amino groups of the lysine residues in the lipid intermediates became substituted with an average of 4.9 glycine residues/lysine.

The glycopeptide product contained 2.9 glycine residues/lysine. Again some of the ε-amino groups of the lysine residues were unsubstituted, so that the actual size of the glycine chains was 4–6 in various experiments. Similarly, the amount of DNP-glycine found was 1 per 4–6 total glycines (Table 3). The reaction of the N-terminal end of the glycine chains with DNFB indicates that these chains are open and that they remain to be closed to form bridges in some subsequent reaction in cell wall synthesis. Neither the lipid intermediates nor the glycopeptide product contained any C-terminal glycine.

A bacteriolytic enzyme from Cytophaga hydrolyzes glycine bridges in the cell wall with the formation of both tri- and tetraglycine. Both the C14-lipid intermediates and the C-14-glycopeptide product were treated with this enzyme and in both cases C14-triglycine and C14-tetraglycine were formed.

GlcNAc-MurNAc(-pentapeptide-pentaglycine)-P-lipid was insensitive to lysozyme but disaccharide-decapeptide was recovered from it by mild acid hydrolysis. By contrast, the glycopeptide product was unaffected by mild acid but lysozyme hydrolyzed it to several products including disaccharide-decapeptide and tetrasaccharide-bis(decapeptide). Characterization of these materials will be reported in a full paper.

Discussion.—The data reported support the following scheme for the synthesis of the cell wall glycopeptide:
TABLE 3
ANALYSIS WITH DNFB OF PRODUCTS CONTAINING C14-Gly AND C14-Lys

<table>
<thead>
<tr>
<th>Glycopeptide</th>
<th>Lys</th>
<th>DNP-Gly</th>
<th>Gly</th>
</tr>
</thead>
<tbody>
<tr>
<td>e-DNP-Lys</td>
<td></td>
<td></td>
<td>5.9</td>
</tr>
<tr>
<td>Lipid</td>
<td>21.4</td>
<td>21.2</td>
<td>160.3</td>
</tr>
</tbody>
</table>

The reaction mixture contained C14-glycine and UDP-MurNAc-pentapeptide containing C14-lysine. C14-glycine was omitted from a control reaction mixture. After incubation, lipid intermediate and glycopeptide were isolated and reacted with DNFB. The Δ values were obtained by subtracting the values obtained for the control from the values obtained for the experimental reaction mixture. Lys and Gly refer to amino acids with no free amino groups.

This scheme is an expansion of the lipid cycle proposed recently.2 In the cell wall, at least 98 per cent of the ε-aminogroups of lysine are substituted by polyglycine bridges, averaging 5 glycines in length, which connect glycopeptide sheets.3–7 Although a glycopeptide can be synthesized without addition of glycine in vitro, the reaction cycle apparently operates in vivo only with glycine addition. On addition in vitro of the components of the glycine incorporation reaction, chains averaging about 5 glycine residues in length are transferred to the ε-aminogroups of lysine in MurNAc(-pentapeptide)-P-lipid or GlcNAc-MurNAc(-pentapeptide)-P-lipid. Although no information is available as to which or whether both lipids function as acceptors in vivo, the latter lipid appears to be a more adequate acceptor under the in vitro conditions employed here. These membrane-bound lipids appear to serve as intermediates by means of which intracellular precursors, to which the cell membrane is impermeable, can be utilized for the synthesis of the cell wall, an extracellular product. That is, these lipids are presumably membrane transport intermediates. Isolation and characterization of the lipid intermediates as well as studies of utilization of the isolated materials are in progress.

Glycyl-sRNA is an intermediate in the process and this is so far the only example of the participation of sRNA in a process other than protein synthesis. The significance of the phenomenon is not completely clear and many of its features still remain to be established. There is presently no evidence for the participation of ribosomes or of a coding mechanism. However, without extensive purification of the components of the system, it is difficult to exclude unequivocally participation of ribosomes bound to the heavy particles (presumed to be derived from cell membrane), even though these particles were prepared in the absence of Mg++. Further purification will also be required to establish whether the glycine-activating enzyme and sRNA involved in cell wall synthesis function uniquely in this system or whether they may also participate in protein synthesis.

Finally, the system reported here may be compared to that employed by Chatterjee and Park.1 The major product of the RNase-sensitive glycine incorporation which they observed was insensitive to lysozyme, and other of its features suggest that they in fact were measuring the lipid intermediates described here. These intermediates are precipitable by acid, as is the glycopeptide. Moreover, although it can be difficult to compare experiments of this kind carried out in different laboratories, particles, prepared after disintegration with glass beads in the Aliclone apparatus and incubated as they described, in our hands catalyze formation of the lipid intermediates but are relatively inefficient in catalyzing glycopeptide synthesis.

Summary.—In S. aureus glycine is activated for incorporation into cell wall gly-
copptide as glycyl-sRNA. Both the glycine-activating enzyme and the glycyl-sRNA involved in this reaction have been partially purified. Only one of the two glycyl-sRNA's found in S. aureus was active in cell wall synthesis. This glycyl-sRNA transferred about five glycine residues, apparently sequentially, to a free $\varepsilon$-amino group of lysine in the previously described intermediate, GlcNAc-MurNAc-(-pentapeptide)-P-lipid. The GlcNAc-MurNAc(-pentapeptide-pentaglycine)-P-lipid transferred disaccharide-decapeptide to a cell wall acceptor. The glycine chains in this product were open and their closure is required as a further step in cell wall assembly. MurNAc(-pentapeptide)-P-lipid appeared to be a less adequate acceptor of glycine in the in vitro system. The attachment of these glycine residues to lipid intermediates, which are found in the cell membrane, serves as a means by which intracellular precursors are utilized for the synthesis of the cell wall, an essentially extracellular product, and thus these compounds appear to be transport intermediates.

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† International postdoctoral fellow of the U.S. Public Health Service.
1 Chatterjee, A. N., and J. T. Park, these PROCEEDINGS, 51, 9 (1964).
8 Stimulation of the reaction by added sRNA has also been reported orally by J. T. Park at the Sixth International Congress of Biochemistry in New York, 1964.
9 The work on glycine-activating enzyme was carried out by Mr. J. M. Gilbert and will be reported subsequently.
13 However, formation of MurNAc(-pentapeptide-polyglycine)-P-lipid in the absence of UDP-GlcNAc can be reversed on addition of UMP with formation of UDP-MurNAc-pentapeptide-polyglycine. The latter nucleotide is, however, a poor substrate in the forward reaction.
14 The amount of unsubstituted lysine was measured by reaction with DNFB. The number of glycines per lysine was measured using both H$^4$-glycine with UDP-MurNAc-pentapeptide containing C$^{14}$L-lysine and C$^{14}$-glycine with uridine nucleotide containing H$^3$L-lysine. Such careful reverse double labeling experiments were essential to establish stoichiometry because of the difficulties of measuring specific activities and of establishing the exact efficiencies of counting under various conditions.
16 Tipper, D. J., unpublished observations.