On the Specificity of Phospho-N-acetylmuramyl-pentapeptide Translocase

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SUMMARY

Phospho-N-acetylmuramyl-pentapeptide translocase (UDP-MurNAc-Ala-γD-Glu-Lys-DAla-DAla; undecaprenylphosphate phospho-MurNAc-pentapeptide transferase) catalyzes the initial membrane reaction in the biosynthesis of peptidoglycan. In addition, the enzyme catalyzes the exchange of [3H]uridine monophosphate with the uridine monophosphate moiety of UDP-MurNAc-pentapeptide. A specificity profile towards the peptide subunit of UDP-MurNAc-pentapeptide has been established for the exchange and transfer reactions catalyzed by the translocase from Staphylococcus aureus Copenhagen. A good correlation between Vmax/Km in the transfer reaction and Rmax/Km in the exchange reaction was observed. On the basis of glycine substitution, the enzyme has a high specificity for L-alanine in position 1 (R1) and D-alanine in position 4 (R4) and a low specificity for D-alanine in position 5 (R5). For example, when compared with UDP-MurNAc-Ala-γD-Glu-Lys-DAla-DAla, glycine replacement in either R1 or R4 results in a 9- and 15-fold decrease in Vmax/Km, respectively. In contrast, glycine substitution in R5 results in only a 1.5-fold decrease in Vmax/Km. These results are further supported by comparing UDP-MurNAc-Ala-γD-Glu-Lys-DAla-DAla - Ala-γD-Glu-Lys-DAla, and -Ala-γD-Glu-Lys. For UDP-MurNAc-tetrapeptide a 4-fold decrease in Vmax/Km was observed whereas a 7-fold decrease was found for the -tripeptide. Replacement of both R1 and R4 by glycine reduces Rmax/Km 135-fold. The translocase has a low specificity for the diamino acid in R5. Substitution of lysine with meso-diaminopimelic acid results in a 1.5-fold decrease in Rmax/Km and with ornithine in no change.

The established specificity profile of the translocase is consistent with the in vivo effects of elevated concentrations of glycine on the growth of S. aureus Copenhagen (Hammers, W., Schleifer, K. H., and Kandler, O. (1973) J. Bacteriol. 116, 1029-1053). Thus, it is suggested that the translocase has a key role in selecting analogs of UDP-MurNAc-Ala-γD-Glu-Lys-DAla-DAla for peptidoglycan synthesis.

The biosynthesis of peptidoglycan, the major structural polymer of most bacterial cell walls, is catalyzed by a series of cytoplasmic and membrane-associated enzymes. The inhibition of one of these enzymes by either D-cycloserine (1, 2), O-carbamyl-D-serine (3, 4), bacitracin (5, 6) vancomycin (7-9), or penicillin (10-13) results in an inhibition of peptidoglycan synthesis and in the accumulation of nucleotide-activated peptidoglycan precursors. These nucleotides are either UDP-MurNAc-Ala-γD-Glu-Lys-DAla-DAla or precursors of this compound.

A second class of inhibitors includes analogs of components of UDP-MurNAc-pentapeptide that are incorporated into this nucleotide. For example, in Staphylococcus aureus Copenhagen the inhibition of peptidoglycan synthesis by 5-fluorouracil is correlated with the accumulation of fluorouridine diphosphate MurNAc-pentapeptide (14, 15), an analog which is only poorly utilized for the synthesis of this polymer (16). In addition, the synthesis of modified peptidoglycan in the presence of glycine is accompanied by the accumulation of a family of UDP-MurNAc-pentapeptides with partial replacement of both stereoisomers of alanine by glycine (17). One feature of the modified peptidoglycan is a decrease in cross-linking when compared with the control (17). Thus, incorporation of an analog into peptidoglycan may result in specific effects on the final assembly of the cross-linked polymer.

It is the purpose of this paper to define some of the specificity requirements of the phospho-MurNAc-pentapeptide translocase for the peptide subunit of UDP-MurNAc-pentapeptide. This enzyme catalyzes the transfer of phospho-MurNAc-pentapeptide from UMP to undecaprenyl-phosphate according to Reaction 1 (8, 18, 19).

UDP-MurNAc-pentapeptide + undecaprenyl-phosphate

K+ Mg2+ →

undecaprenyl-diphosphate-MurNAc-pentapeptide + UMP

The reaction is reversible and has an equilibrium constant of

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1 Unless stated all abbreviations of residues denote the L configuration. The omission of the hyphen, i.e., d-Ala for D-Ala, conforms with the suggestion cited in Biochemistry 5, 2485 (1966). Although not stated, all n-glutamic residues are linked through the γ-carboxyl group to the diamino acid. The abbreviations used are: mDap, meso-α, ε-diaminopimelic acid; MurNAc, N-acetylmuramyl.
The specificity profile of the enzyme obtained from investigations of Reactions 1 and 2 defines features of the peptide subunit that provide for the preferential discrimination against certain UDP-MurNAc-peptides. In addition, these experiments define features of the peptide subunit that do not significantly influence the activity of phospho-MurNAc-pentapeptide translocase and, therefore, allow the incorporation of these peptidoglycan precursors in the polymer. The correlation of the specificity profile of this enzyme with the in vivo effects of glycine on bacterial growth establishes the translocase as one of the key enzymes for discriminating analogs of UDP-MurNAc-pentapeptide for peptidoglycan synthesis. A preliminary report of this work has been presented (21).

**EXPERIMENTAL PROCEDURE**

**Materials**

L-[U-14C]Lysine monohydrochloride with a specific activity of 312 mCi per mmole was purchased from ICN Isotope and Nuclear Division. ν-[U-14C]Alanine with a specific activity of 30 mCi per mmole and [β-H]thymidine 5'-monophosphate (37.3 Ci per mmole) were obtained from Amersham-Searle and Schwarz-Mann, respectively. Bacterial alkaline phosphatase (chromatographically purified) was the product of Worthington Biochemical Corp. Gly-pAla, nAla-Gly, and dAla-dAla were purchased from Ceylon Chemical Corp. Membrane filters (MF type, 0.45 μm pore size) and filter holder apparatus were the products of Millipore. The sources of other chemicals have been previously described (22).

**Bacteria and Media**

Staphylococcus aureus, Bacillus licheniformis ATCC 9945, Streptococcus faecalis (faecium) ATCC 8045, Staphylococcus epidermidis ATCC 11739 were grown in a medium described by Heydenek et al. (23). Lactobacillus cellulosus ATCC 11739 was grown in a medium described by Reusch and Neuhaus (24), and Streptococcus R2 (kindly provided by Jean-Marie Ghysen, Université de Liège, Liège, Belgium) was grown in a medium described by Leyh-Bouille et al. (25). Medium A for nucleotide accumulation contained per liter: n-cycloserine, 20.6 mg; t-alanine, 200 mg; v-glutamic acid, 200 mg; t-lysine, 200 mg; uracil, 100 mg; glucose, 10 g; K₂HPO₄, 5 g. Medium B for nucleotide accumulation contained per liter: v-alanine, 400 mg; v-glutamic acid, 200 mg; t-lysine, 200 mg; uracil, 150 mg; glucose, 10 g; K₂HPO₄, 5 g; penicillin G, 250 μg.

**Preparation of UDP-MurNAc-peptides**

The sequence of the nucleotide-activated precursors, the bacteria from which they were isolated, and the method of their enzymatic preparation are summarized in Table I. Isolation of UDP-MurNAc-peptides from Cells Inhibited with Antibiotics—For accumulation in Medium A (n-cycloserine) or B (Penicillin G), bacteria were grown to 0.5× maximal growth, harvested, and transferred to the accumulation medium. After 2 hours incubation at growth temperature (S. aureus, 37°; B. licheniformis, 37°; S. faecalis, 37°; M. luteus, 34°; L. cellulosus, 30°), the cells were harvested and the UDP-MurNAc-peptides were isolated by extraction with trichloroacetic acid and by Sephadex gel filtration as described by Stickgold and Neuhaus (16). The UDP-MurNAc-peptides were purified by descending paper chromatography in Solvent A. The separation of UDP-MurNAc-pentapeptide from UDP MurNAc-dipeptide, which do not separate well in the known paper chromatography systems (26), was achieved by column chromatography on DEAE-cellulose (Whatman DE52). The samples were applied to a column pre-equilibrated with 0.1 M NH₄HCO₃, pH 8.4, and eluted with a gradient of NH₄HCO₃ from 0.1 M to 0.5 M, pH 8.4. In the elution profile, two peaks were obtained with absorbance ratios (290:290 and 250:250) characteristic of uridine. The compounds giving rise to the first and second peak were identified as UDP-MurNAc-tripeptide and UDP-MurNAc-dipeptide, respectively.

In preliminary tests it was found that some bacteria yielded higher amounts of UDP-MurNAc-peptides when the antibacterial was added to the growing culture. Where indicated in Table I, the inhibition in the growth medium was achieved with 2 mg n-cycloserine or vancomycin (25 mg per liter). After incubation for 2 hours, the UDP-MurNAc-peptides were isolated as described above. When L. cellulosus was inhibited with n-cycloserine in the growth medium, a mixture of two UDP-MurNAc-peptides accumulated which could be separated by paper chromatography (Rᶠᵉᵗₑ = 0.53 and 0.67 in Solvent A). The amino acid analysis showed that the component with the higher Rᶠᵉᵗₑ contained alanine, glutamic acid, and lysine whereas that with the lower Rᶠᵉᵗₑ contained alanine, glutamic acid, and ornithine in equimolar ratios. These ratios are consistent with an accumulation in this culture of UDP-MurNAc-Ala-DGlu-Lys and UDP-MurNAc-Ala-DGlu-Lys. From 4 liters of culture, 8.0 and 5.1 μmoles of UDP-MurNAc-tripeptide containing either lysine or ornithine, respectively, were obtained. UDP-MurNAc-pentapeptide containing only the amino acid (27).

**Enzymatic Synthesis of UDP-MurNAc-pentapeptides**—The enzymatic syntheses of UDP-MurNAc-pentapeptides were performed with UDP-MurNAc-Ala-DGlu-Lys:αLAla-αLAla ligase (ADP) from S. faecalis. The reaction mixture contained: 3 μmoles of UDP-MurNAc-tripeptide (Compounds 2 and 7 in Table I) and 222 μmoles of either Gly-αLAla or DαLa-Gly, or 30 μmoles of Gly-αLAla, 30 μmoles of Tris-HCl, pH 7.8; 190 μmoles of MgCl₂; 12 μmoles of ATP, and 2.4 mg of the partially purified ligase (Step 3 in the procedure described by Neuhaus and Struve (28)) in a total volume of 2.22 ml. The reaction was terminated by heating in a boiling water bath for 2 min. The denatured protein was removed by centrifugation and the supernatant fraction was filtered through a Sephadex G-25. The fraction containing UDP-MurNAc-peptides was concentrated, and the UDP-MurNAc-pentapeptide was separated from unreacted UDP-MurNAc-tripeptide by paper chromatography. After desalting the eluate from paper chromatograms by gel filtration on Sephadex G-25, the amino acid composition was established. The molar ratios of the amino acids for the synthesized products are consistent with the theoretical values and are summarized in Table II.

**Preparation of UDP-MurNAc-Ala-DGlu-Lys:αLAla-αLAla ligase**—The enzyme preparations were performed (Compound I in Table I) with a n-carboxypeptidase from Streptomyces R30 (Step 2 in the preparation described by Leyh-Bouille et al. (25)). The incubation mixture contained: 3 μmoles of UDP-MurNAc-Ala-DGlu-Lys:αLAla-αLAla, 30 μmoles of Tris-HCl, pH 7.5; 9.5 μmoles of MgCl₂; and enzyme (5% of protein) in a total volume of 1.35 ml. The reaction mixture was incubated at 37° for 30 min and the reaction was terminated by boiling in a water bath for 2 min. The purification of UDP-MurNAc-Ala-DGlu-Lys:αLAla was the same as for the enzyme eluted from Sephadex G-25. The enzyme for the enzymatically synthesized UDP-MurNAc-pentapeptides. Separation of UDP-MurNAc-Ala-DGlu-Lys:αLAla from the UDP-MurNAc-Ala-DGlu-Lys:αLAla was achieved by paper chromatography (120 hours) (Reagents and procedures are described in Table II). This chromatographic behavior is different from the reference observations for UDP-MurNAc-tetra- and -pentapeptides containing diaminopimelic acid which do not separate in this system (20).

**Preparation of Labeled UDP-MurNAc-peptides**—UDP-MurNAc-peptides labeled in the αLAla moiety (Compounds 4, 10, and 14 in Table I) were prepared from UDP-MurNAc-tripeptide (Compounds 2, 7, or 13 in Table I) and [14C]Ala-[314C]Ala as previously described (28). In preliminary tests it was found that the UDP-MurNAc-Ala-DGlu-Lys:αLAla-αLAla ligase from S. faecalis does not significantly differentiate between these UDP-MurNAc-tripeptides as substrates. This observation agrees with the finding that other bacteria also uses several UDP-MurNAc-tripeptides as substrates. UDP-MurNAc-Ala-DGlu-[14C]Lys was prepared from UDP-MurNAc-Ala-DGlu-Lys (Compound 1 in Table I) and [14C]Lysine according to Stickgold and Neuhaus (16) with UDP-MurNAc-Ala-DGlu-Lys:αLAla-αLAla ligase from S. faecalis. The modified labeled UDP-MurNAc-tripeptides (Compounds 5 and 6 in Table I) were prepared from UDP-MurNAc-Ala-DGlu-[14C]Lys and αLAla-Gly or Gly-αLAla with αLAla.
<table>
<thead>
<tr>
<th>Nucleotide*</th>
<th>Bacterium used</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. UDP-MurNAc-Ala-DGlu</td>
<td>S. aureus</td>
<td>Accumulation in medium A without lysine</td>
</tr>
<tr>
<td>2. UDP-MurNAc-Ala-DGlu-Lys</td>
<td>S. aureus</td>
<td>Accumulation in medium A with D-cycloserine</td>
</tr>
<tr>
<td>3. UDP-MurNAc-Ala-DGlu-Lys-DAla</td>
<td>S. aureus</td>
<td>Degradation of compound 4 with D-D-carboxypeptidase</td>
</tr>
<tr>
<td>4. UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla</td>
<td>S. aureus</td>
<td>Accumulation in medium B</td>
</tr>
<tr>
<td>5. UDP-MurNAc-Ala-DGlu-Lys-DAla-Gly</td>
<td>M. lacticum</td>
<td>Enzymatic addition of DAla-Gly to nucleotide 2</td>
</tr>
<tr>
<td>7. UDP-MurNAc-Gly-DGlu-Lys</td>
<td>L. celllobiosus</td>
<td>Inhibition with D-cycloserine in the growth medium</td>
</tr>
<tr>
<td>8. UDP-MurNAc-Gly-DGlu-Lys-DAla-DAla</td>
<td>M. lacticum</td>
<td>Accumulation in medium B (plus 200 mg glycine per liter)</td>
</tr>
<tr>
<td>11. UDP-MurNAc-Ala-DGlu-Orn</td>
<td>B. licheniformis</td>
<td>Inhibition with D-cycloserine in the growth medium</td>
</tr>
<tr>
<td>12. UDP-MurNAc-Ala-DGlu-Orn-DAla-DAla</td>
<td>B. licheniformis</td>
<td>Enzymatic addition of DAla-DAla to nucleotide 11</td>
</tr>
<tr>
<td>13. UDP-MurNAc-Ala-DGlu-DAp</td>
<td>B. licheniformis</td>
<td>Inhibition with D-cycloserine in the growth medium</td>
</tr>
<tr>
<td>14. UDP-MurNAc-Ala-DGlu-DAp-DAla-DAla</td>
<td>B. licheniformis</td>
<td>Inhibition with Vancomycin in the growth medium</td>
</tr>
</tbody>
</table>

*As described in the text, nucleotides 2, 3, 5 and 6 were also prepared with [14C]lysine, and nucleotides 4, 8 and 14 were prepared with D-[14C]Ala-D-[14C]Ala.
UDP-MurNAc-Ala-oGlu-Lys:Ala-nGlu-ligase as described above. The preparation of the lysine-labeled UDP-MurNAc-tetrapeptide was identical with that described for the unlabeled compound. The specific activities of the compounds labeled in the lysine moiety and for UDP-MurNAc-Ala-nGlu-mDap-n\(^{14}\text{C}\)Ala were 16 cpm per pmole, those for UDP-MurNAc-Ala-nGlu-Lys-n\(^{14}\text{C}\)Ala and for UDP-MurNAc-Gly-nGlu-Lys-n\(^{14}\text{C}\)Ala were 60 and 20 cpm per pmole, respectively.

**TABLE II**

*Analysis of enzymatically synthesized UDP-MurNAc-peptides*

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Ala</th>
<th>Lys</th>
<th>Gly</th>
<th>Orn</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-MurNAc-Ala-gGlu-Lys-Gly-DAla</td>
<td>2.01</td>
<td>0.95</td>
<td>1.05</td>
<td>-</td>
</tr>
<tr>
<td>UDP-MurNAc-Ala-gGlu-Lys-DAla-Gly</td>
<td>1.95</td>
<td>0.97</td>
<td>1.01</td>
<td>-</td>
</tr>
<tr>
<td>UDP-MurNAc-Gly-gGlu-Lys-Gly-DAla</td>
<td>1.02</td>
<td>0.95</td>
<td>1.89</td>
<td>-</td>
</tr>
<tr>
<td>UDP-MurNAc-Gly-gGlu-Lys-DAla-Gly</td>
<td>1.04</td>
<td>1.06</td>
<td>1.84</td>
<td>-</td>
</tr>
<tr>
<td>UDP-MurNAc-Ala-gGlu-Orn-DAla-DAla</td>
<td>2.90</td>
<td>-</td>
<td>-</td>
<td>0.94</td>
</tr>
</tbody>
</table>

*ratio to glutamic acid.

**TABLE III**

*Synthesis of undecaprenyl-diphosphate-MurNAc-peptides*

The reaction mixture contained: 0.21 m KCl; 0.042 m MgCl\(_2\); 0.16 unit of bacterial alkaline phosphatase; 0.05 m Tris-HCl, pH 7.5; membrane fragments (103 pg of protein) and the indicated concentrations of labeled UDP-MurNAc-peptide in a total volume of 60 l. After incubation for 12 hours at 25°, the reaction was terminated by the addition of 0.05 ml of cold 0.3 M HClO\(_4\). The precipitate was isolated on a membrane filter, and the tube and filter were washed twice with 1.0 ml of cold 0.3 M HClO\(_4\). The filter was washed with an additional 5.0 ml of cold 0.3 M HClO\(_4\). The damp filter was dissolved in the Triton-toluene scintillation fluid and radioactivity was determined.

For the isolation of undecaprenyl-diphosphate-MurNAc-peptides, a parallel series was performed. The mixtures were analyzed by paper chromatography in Solvent A. The amount of radioactivity in the undecaprenyl-diphosphate-MurNAc-peptide band \((R_f = 0.81)\) corresponds to the amount of radioactivity transferred.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(\mu\text{M}^1)</th>
<th>pmoles/12 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. UDP-MurNAc-Ala-gGlu-Lys-D(^{14}\text{C})Ala-D(^{14}\text{C})Ala</td>
<td>15</td>
<td>250</td>
</tr>
<tr>
<td>2. UDP-MurNAc-Ala-gGlu-[^{14}\text{C}]Lys</td>
<td>570</td>
<td>250</td>
</tr>
<tr>
<td>3. UDP-MurNAc-Ala-gGlu-[^{14}\text{C}]Lys-DAla</td>
<td>30</td>
<td>240</td>
</tr>
<tr>
<td>4. UDP-MurNAc-Ala-gGlu-[^{14}\text{C}]Lys-DAla-Gly</td>
<td>17</td>
<td>230</td>
</tr>
<tr>
<td>5. UDP-MurNAc-Ala-gGlu-[^{14}\text{C}]Lys-Gly-DAla</td>
<td>127</td>
<td>240</td>
</tr>
<tr>
<td>6. UDP-MurNAc-Gly-gGlu-Lys-D(^{14}\text{C})Ala-D(^{14}\text{C})Ala</td>
<td>94</td>
<td>240</td>
</tr>
<tr>
<td>7. UDP-MurNAc-Ala-gGlu-mDap-D(^{14}\text{C})Ala</td>
<td>21</td>
<td>250</td>
</tr>
</tbody>
</table>

\(^1\) The nucleotide concentrations in the reaction mixture were calculated from the Lineweaver–Burk plots so that the initial velocity of undecaprenyl-diphosphate-MurNAc-peptide formation was >5 pmoles per min.
Preparation of Membrane Fragments

Membrane fragments from *S. aureus* Copenhagen were prepared as described by Struve et al. (18). Since endogenous undecaprenyl-diphosphate-MurNAc-pentapeptide gives high backgrounds in the exchange assay, phospho-MurNAc-pentapeptide was removed from the membranes used for this assay by prior incubation with UMP. The incubation mixture contained: 0.21 M KCl; 0.042 M MgCl₂; 0.05 M Tris-HCl, pH 7.8; 0.012 M MgCl₂; 4 × 10⁻⁴ M UMP; and membranes (50 mg of protein) in a total volume of 13.1 ml. After 10 min at 25°, the membranes were sedimented by centrifugation for 60 min at 105,000 × g, washed four times in 0.02 M Tris-HCl, pH 7.8 containing 1 M KCl, and resuspended in the same buffer. The membranes were stored in liquid nitrogen and used for the "Exchange Assay." For the "Exchange Assay." The transfer of phospho-MurNAc-³²P-peptide from UDP-MurNAc-³²P-peptide to undecaprenyl-phosphate (Reaction 1) was assayed as described under "Experimental Procedure." The reaction mixture contained: 0.21 M KCl; 0.042 M MgCl₂; 0.16 units of bacterial alkaline phosphatase; 0.05 M Tris-HCl, pH 7.8; UDP-MurNAc-³²P-peptide; and membrane fragments (410 µg of protein) in a total volume of 1.0 ml. After 10 min at 25°, the membranes were sedimented by centrifugation for 45 min at 105,000 × g, washed four times in 0.02 M Tris-HCl, pH 7.8 containing 1 M KCl, and resuspended in the same buffer. The membranes were stored in liquid nitrogen and used for the "Exchange Assay."

Transfer Assay

The transfer of phospho-MurNAc-³²P-peptide from UDP-MurNAc-³²P-peptide to undecaprenyl-phosphate (Reaction 1) was assayed as described under "Experimental Procedure." The reaction mixture contained: 0.21 M KCl; 0.042 M MgCl₂; 0.16 units of bacterial alkaline phosphatase; 0.05 M Tris-HCl, pH 7.8; UDP-MurNAc-³²P-peptide; and membrane fragments (410 µg of protein) in a total volume of 1.0 ml. After 10 min at 25°, the membranes were sedimented by centrifugation for 45 min at 105,000 × g, washed four times in 0.02 M Tris-HCl, pH 7.8 containing 1 M KCl, and resuspended in the same buffer. The membranes were stored in liquid nitrogen and used for the "Exchange Assay."

Exchange Assay

For the determination of the exchange rate, the exchange of [³²P]UMP with the unlabeled UMP moiety of UDP-MurNAc-peptides (Reaction 2) was measured by a modification (31) of the method described by Heydanek et al. (32). The reaction mixture contained: 0.21 M KCl; 0.05 M Tris-HCl, pH 7.8; 0.042 M MgCl₂; 3.33 × 10⁻⁴ M [³²P]UMP (20 cpm per pmole); pre-incubated membranes (50 µg of protein) and UDP-MurNAc-peptides. The incubation was carried out at 25° for 10 min. After terminating the reaction by boiling for 2 min, 0.16 unit of bacterial alkaline phosphatase was added and the mixture was incubated for 10 min at 25°. The second stage of the reaction was terminated by dilution with 0.5 ml of 0.002 M Tris-HCl, pH 7.8 containing 0.01 M KCl. The contents of the tube were quantitated as described under "Experimental Procedure." The product, [³²P]UDP-MurNAc-peptide, was eluted with three 0.5-ml aliquots of 0.02 M Tris-HCl, pH 7.8 containing 0.5 M KCl. The eluates were combined for the determination of radioactivity.

Exchange activity was calculated according to the first order rate equation reported by Struve et al. (18). The rate of exchange, R, is reported as moles exchanged per liter per min.

Analytical Procedures

Protein was determined according to the method of Lowry et al. (33) using bovine serum albumin as standard. Radioactivity was measured for aqueous samples in a scintillation fluid described by Patterson and Greene (34) and for paper chromatograms in toluene containing 0.3% 2,5-diphenyloxazole. Descending paper chromatography was performed with Whatman No. 3MM paper using the isobutyric acid solvent system (isobutyric acid-concentrated NH₄OH-H₂O, 66:2:33) (Solvent A). Amino acids were analyzed on a Durrum amino acid analyzer model D-500 after hydrolysis of the samples (6 × HCl, 12 hours, 100°). The presence of the uridine was determined by measuring the absorbance ratios at 320:260 and 260:250.

RESULTS

The transfer and exchange activities catalyzed by phospho-MurNAc-pentapeptide translocase have been interpreted with the following model (22):

\[ E + C_{15}P \rightleftharpoons E \cdot C_{15}P \]  
\[ E \cdot C_{15}P + UMPPMp \rightleftharpoons UMPPMp \cdot E \cdot C_{15}P \]  
\[ UMPPMp \cdot E \cdot C_{15}P \rightleftharpoons UMP \cdot E \cdot C_{15}PPMP \]  
\[ UMP \cdot E \cdot C_{15}PPMP \rightleftharpoons PMP \]  
\[ E \cdot C_{15}PPMP \rightleftharpoons E + C_{15}PPMP \]

The transfer of phospho-MurNAc-pentapeptide (PMP) from UDP-MurNAc-pentapeptide (UMPPMp) to undecaprenyl-phosphate (C₁₅P) requires Reactions 3 through 7 and access to the substrate pool of undecaprenyl-phosphate. Exchange activity would require the initial formation of a complex between the translocase and undecaprenyl-phosphate and requires Reactions 4 through 6. This reaction sequence is used to interpret the data presented in this communication.

Although lipid-dependent membrane preparations have been described for the translocase (22), the study of intact membranes was considered to reflect the in situ status better than...
### Table IV

**Specificity of translocase (transfer assay)**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{\text{max}}$</th>
<th>$K_{\text{m}}$</th>
<th>Ratio $^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Series A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP-MurNAc-Ala-$\delta$Glu-Lys-$\delta$Ala-($\delta$Ala-$\delta$Ala)</td>
<td>170</td>
<td>5.5</td>
<td>1.0</td>
</tr>
<tr>
<td>UDP-MurNAc-Ala-$\delta$Glu-Lys-$\delta$Ala</td>
<td>290</td>
<td>40</td>
<td>7.3</td>
</tr>
<tr>
<td>UDP-MurNAc-Ala-$\delta$Glu-Lys</td>
<td>170</td>
<td>440</td>
<td>80.0</td>
</tr>
<tr>
<td>Series B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP-MurNAc-Ala-$\delta$Glu-Lys-$\delta$Ala-Gly</td>
<td>170</td>
<td>8.3</td>
<td>1.5</td>
</tr>
<tr>
<td>UDP MurNAc-Ala-$\delta$Glu-Lys-$\delta$Ala-Gly</td>
<td>89</td>
<td>44</td>
<td>8.0</td>
</tr>
<tr>
<td>UDP-MurNAc-Gly-$\delta$Glu-Lys-($\delta$Ala-$\delta$Ala)</td>
<td>170</td>
<td>50</td>
<td>9.1</td>
</tr>
<tr>
<td>UDP-MurNAc-Ala-$\delta$Glu-$\delta$Dap-$\delta$Ala-$\delta$Ala</td>
<td>170</td>
<td>10</td>
<td>1.8</td>
</tr>
</tbody>
</table>

$^{a}$ The values for $K_{\text{m}}$ and $V_{\text{max}}$ were established from the Lineweaver-Burk plots presented in Figure 1 A, B.

$^{b}$ Ratio = $K_{\text{m}}$ UDP-MurNAc peptide/$K_{\text{m}}$ UDP-MurNAc-Ala-$\delta$Glu-Lys-$\delta$Ala-$\delta$Ala

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The initial rates of formation of undecaprenyl-diphosphate-MurNAc-peptides were measured at varying concentrations of nucleotide analogs and compared with the normal substrate UDP-MurNAc-Ala-$\delta$Glu-Lys-$\delta$Ala-$\delta$Ala. The Lineweaver-Burk plots for UDP-MurNAc-tripeptide, -tetrapeptide, and -pentapeptide are illustrated in Fig. 1A. The Michaelis constants ($K_{\text{m}}$) and the maximum velocities ($V_{\text{max}}$) are summarized in Table IV. Although the values for $V_{\text{max}}$ are similar, the values for $K_{\text{m}}$ decrease 10- and 7-fold, respectively, as each residue of $\delta$-alanine is linked to the tripeptide. Thus, UDP-MurNAc-tripeptide is characterized by a high $K_{\text{m}}$ when compared with UDP-MurNAc-pentapeptide. However, at high concentrations of UDP-MurNAc-tripeptide, the transfer of the phospho-MurNAc-tripeptide proceeds at rates comparable to phospho-MurNAc-pentapeptide from UDP MurNAc pentapeptide.

As illustrated in Fig. 1B, analogs of UDP-MurNAc-pentapeptide with a single replacement of 1 residue are compared in the transfer reaction. The values for $K_{\text{m}}$ and $V_{\text{max}}$ are presented in Table IV (Series B). On the basis of values for $K_{\text{m}}$, replacement of residue-5 ($R_5$) by glycine has little effect whereas replacement of either residue-1 ($R_1$) or -4 ($R_4$) by glycine has a significantly larger effect. For example, $K_{\text{m}}R_1(\text{Gly})/K_{\text{m}}$ is 9.1 and $K_{\text{m}}R_4(\text{Gly})/K_{\text{m}}$ is 8.0 whereas $K_{\text{m}}R_5(\text{Gly})/K_{\text{m}}$ is 1.5. The values for $V_{\text{max}}$ are again similar as in the first series (Series A, Table IV).

In an analysis of the transfer assay, the similar values of $V_{\text{max}}$ (two exceptions) suggested that the analogs would compete with UDP-MurNAc-Ala-$\delta$Glu-Lys-$\delta$Ala-$\delta$Ala for a binding site on the translocase. This was tested with UDP-MurNAc-Ala-$\delta$Glu-Lys-$\delta$Ala-Gly as an inhibitor of the transfer of phospho-MurNAc-Ala-$\delta$Glu-Lys-$\delta$Ala-$\delta$Ala from UDP-MurNAc-pentapeptide. From the Lineweaver-Burk plots, it was observed that the analog is a competitive inhibitor with a $K_i$ of $8.4 \times 10^{-5}$ M. This value is similar to that observed for the $K_{\text{m}}$ of this analog.
Fig. 2. Exchange of [H]UMP with the UMP moieties of UDP-MurNAc-tripeptide, -tetrapeptide, and -pentapeptide (A) and analogs of UDP-MurNAc-pentapeptide (B) (Lineweaver-Burk plots). The exchange assay was used with the indicated UDP-MurNAc-peptides as described under “Experimental Procedure.” In B, 2 additional data points justify the extension of the plot for UDP-MurNAc-Gly-dGlu-Lys-Gly-dAla.

The results support the proposal that the translocase utilizes each of the analogs at the same binding site(s).

Specificity Profile of Phospho-MurNAc-pentapeptide Translocase in Exchange Reaction—The exchange reaction (Reaction 2) catalyzed by phospho-MurNAc-pentapeptide translocase has been employed extensively as a routine assay because of its greater sensitivity. In addition, a single labeled substrate, [H]UMP, can be used in the presence of unlabeled analogs of UDP-MurNAc-pentapeptide. Several reports describing the activity of unlabeled analogs in the exchange assay have been published (20, 36). Each of these measurements utilized a single concentration of analog and, thus, is difficult to interpret.

The Lineweaver-Burk plots for UDP-MurNAc-tripeptide, -tetrapeptide and -pentapeptide are illustrated in Fig. 2A. From these plots, the Michaelis constants and the maximum exchange rates (Rmax) have been established and are summarized in Table V. They are apparent values since it has been found that the values for UDP-MurNAc-pentapeptide in the exchange reaction are a function of the UMP concentration. Since UMP is the labeled substrate, it was not feasible to use high concentrations of labeled UMP with a high specific activity. All experiments with the “Exchange Assay” were performed with 3.3 mg M UMP. Both Km and Rmax reflect the fact that UDP-MurNAc-tripeptide is a poor substrate when compared with UDP-MurNAc-pentapeptide. With UDP-MurNAc-tetrapeptide, Rmax is similar to and the Km is 3-fold larger than the Rmax and Km values observed for UDP-MurNAc-pentapeptide. Although the values of Vmax for this series (Series A, Table IV) in the transfer assay are similar, the values of Rmax from the exchange assay vary significantly from UDP-MurNAc-tripeptide to UDP-MurNAc-pentapeptide. In Series B, nucleotides with a single replacement in the peptide subunit also show variations in both Rmax and Km.

In order to compare substrates in the exchange assay, the ratio Rmax/Km, the reciprocal slope of the Lineweaver-Burk plot, will be used as an index for comparison. This ratio has been used in several studies to establish specificity profiles (25, 37, 38) and represents the apparent first order rate constant for the reaction of substrate at very low concentration, i.e., v = (Vmax/Km) (S) at low S (39). Thus, the reciprocal slope, Rmax/Km, reflects the effectiveness of an analog in the exchange assay.

As summarized in Table V (Series A), on the basis of Rmax/Km, UDP-MurNAc-tetrapeptide and UDP-MurNAc-pentapeptide differ only by a factor of 2.5 whereas UDP-MurNAc-tripeptide and UDP-MurNAc-pentapeptide differ by 150. In Series B, the replacements of glycine in R1, R4, and R9 are compared. Substitution of R9 with glyoxyl results in no significant change in Rmax/Km whereas substitution at either R8 or R4 by glyoxyl results in a 13- and 6-fold decrease in Rmax/Km, respectively.

**Table V**  
*Specificity of translocase (exchange reaction)*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ a (moles/liter) x $10^5$</th>
<th>$R_{max}$ a (moles/liter min$^{-1}$ x $10^5$)</th>
<th>$R_{max}/K_m$ min$^{-1}$ x $10^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Series A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP-MurNAc-Ala-ΔGlu-Lys-ΔAla-ΔAla</td>
<td>2.0</td>
<td>4.9</td>
<td>25</td>
</tr>
<tr>
<td>UDP-MurNAc-Ala-ΔGlu-Lys-ΔAla</td>
<td>5.8</td>
<td>6.0</td>
<td>10</td>
</tr>
<tr>
<td>UDP-MurNAc-Ala-ΔGlu-Lys</td>
<td>10.0</td>
<td>0.3</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Series B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP-MurNAc-Ala-ΔGlu-Lys-ΔAla-Gly</td>
<td>2.9</td>
<td>6.6</td>
<td>23</td>
</tr>
<tr>
<td>UDP-MurNAc-Ala-ΔGlu-Lys-Gly-ΔAla</td>
<td>6.3</td>
<td>1.2</td>
<td>1.9</td>
</tr>
<tr>
<td>UDP-MurNAc-Gly-ΔGlu-Lys-ΔAla-ΔAla</td>
<td>4.4</td>
<td>1.9</td>
<td>4.3</td>
</tr>
<tr>
<td><strong>Series C</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP-MurNAc-Ala-ΔGlu-Dap-ΔAla-ΔAla</td>
<td>2.2</td>
<td>3.6</td>
<td>16</td>
</tr>
<tr>
<td>UDP-MurNAc-Ala-ΔGlu-Orn-ΔAla-ΔAla</td>
<td>1.6</td>
<td>4.1</td>
<td>26</td>
</tr>
<tr>
<td><strong>Series D</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDP-MurNAc-Gly-ΔGlu-Lys-ΔAla-Gly</td>
<td>8.0</td>
<td>1.9</td>
<td>2.4</td>
</tr>
<tr>
<td>UDP-MurNAc-Gly-ΔGlu-Lys-Gly-ΔAla</td>
<td>27.0</td>
<td>0.5</td>
<td>0.19</td>
</tr>
</tbody>
</table>

a. The values for $K_m$ and $R_{max}$ were calculated from the Lineweaver-Burk plots presented in Figure 2 A, B.

In Series C, replacement of R₅ with either ornithine or meso-diaminopimelic acid does not result in a significant change in $R_{max}/K_m$. In Series D, the results with the doubly modified peptide subunits reflect the combined effects observed in Series B. Replacement of R₅ in addition to R₄ by glycine results in a small decrease in $R_{max}/K_m$ (4.3 to 2.4), whereas replacement of R₄ in addition to R₅ results in a large decrease in this ratio (4.3 to 0.19). The Lineweaver-Burk plots for the glycine-substituted analogs are shown in Fig. 2B.

Comparison of Results Obtained from Transfer and Exchange Assay—With the exception of UDP-MurNAc-Ala-ΔGlu-Lys-ΔAla and UDP-MurNAc-Ala-ΔGlu-Lys-Gly-ΔAla (Table IV), the values for $V_{max}$ in the transfer assay are the same for each UDP-MurNAc-peptide tested. Thus, a comparison of these compounds as substrates in the transfer reaction can be achieved by comparing the values for $K_m$. In the exchange assay, since both $R_{max}$ and $K_m$ varied, the ratio $R_{max}/K_m$ provided a better index of specificity. For comparison of the results of both assays, the values of $V_{max}/K_m$ in the transfer assay and the $R_{max}/K_m$ in the exchange assay are normalized to those for UDP-MurNAc-Ala-ΔGlu-Lys-ΔAla-ΔAla (Table VI). Good correlation is observed between these ratios in each assay. Thus, although UDP-MurNAc-Ala-ΔGlu-Orn-ΔAla-ΔAla, UDP-MurNAc-Gly-ΔGlu-Lys-ΔAla-Gly, and UDP-MurNAc-Gly-ΔGlu-Lys-Gly-ΔAla were not tested in the transfer assay, the results ($R_{max}/K_m$) with the exchange assay provide an adequate index of specificity for comparison with the other analogs.

**Discussion**

On the basis of these results, we conclude that substitution of glycine for L-alanine in position 5 has only a minor effect on the activity of the translocase whereas substitution of glycine at either positions 1 or 4 has a major effect. This conclusion can be correlated with the growth studies recently reported by Hammes et al. (17).

Growth of *S. aureus* Copenhagen in the presence of elevated concentrations of glycine results in the accumulation of a family of peptidoglycan precursors in which both stereoisomers of alanine are partially replaced by glycine. The composition of this family is consistent with the presence of four UDP-MurNAc-peptides: -Gly-ΔGlu-Lys-Gly-ΔAla, -Gly-ΔGlu-Lys-ΔAla-Gly, -Ala-ΔGlu-Lys-ΔAla-Gly, -Ala-ΔGlu-Lys-Gly-ΔAla. In this mixture, glycine replaced L-alanine in position 1 by 66%, D-alanine in position 4 by 65%, and Δ-alanine in position 5 by 35% (17). This distribution indicated that precursors with glycine in position 5 do not accumulate to the same level as precursors with replacements at positions 1 and 4. Since phospho-MurNAc-pentapeptide translocase has a higher specificity for
Substrate Series A
UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla  1.0  1.0
UDP-MurNAc-Ala-DGlu-Lys-DAla   0.24  0.40
UDP-MurNAc-Ala-DGlu-Lys       0.013  0.0068
Series B
UDP-MurNAc-Ala-DGlu-Lys-DAla-Gly  0.67  0.92
UDP-MurNAc-Ala-DGlu-Lys-Gly-DAla  0.067  0.076
UDP-MurNAc-Gly-DGlu-Lys-DAla-DAla  0.11  0.17
Series C
UDP-MurNAc-Ala-DGlu-mDap-DAla-DAla  0.57  0.66
UDP-MurNAc-Ala-DGlu-Orn-DAla-DAla  -c  1.0
Series D
UDP-MurNAc-Gly-DGlu-Lys-DAla-Gly  -  0.096
UDP-MurNAc-Gly-DGlu-Lys-Gly-DAla  -  0.0074

a. Ratio of $V_{\text{max}}/K_m$ of UDP-MurNAc-peptide to $V_{\text{max}}/K_m$ of UDP-MurNAc-Ala-\text{DGlu-Lys-DAla-DAla}

b. Ratio of $R_{\text{max}}/K_m$ of UDP-MurNAc-peptide to $R_{\text{max}}/K_m$ of UDP-MurNAc-Ala-\text{DGlu-Lys-DAla-DAla}

c. Blank: labeled nucleotide not available

alanine as opposed to glycine in positions 1 and 4 than in position 5, the above distribution is consistent with the preferential utilization of the UDP-MurNAc-peptide with glycine in position 5. Thus, the poorer substrates, e.g. UDP-MurNAc-Gly-DGlu-Lys-Gly-nAla, UDP-MurNAc-Ala-DGlu-Lys-Gly-nAla, UDP-MurNAc-Gly-DGlu-Lys-nAla-Gly, will accumulate to a larger extent than UDP-MurNAc-Ala-DGlu-Lys-nAla-Gly.

In addition to the action of the translocase, the composition of the above family of nucleotides may also be influenced by the specificity requirements of n-alanine:n-alanine ligase (ADP) and UDP-MurNAc-tripeptide:nAla-nAla ligase (ADP) (28). However, in studies with enzyme preparations from *S. aureus* Copenhagen, a similar rate of synthesis of UDP-MurNAc-Ala-DGlu-Lys-nAla-Gly and -Ala-nGlu-Lys-Gly-nAla was observed in the presence of 5 mm n-alanine and 50 to 500 mm glycine.

Our results are also consistent with the selective incorporation of peptide subunits into peptidoglycan in vivo (17). In *S. aureus* grown in the presence of elevated concentrations of glycine only 17% and 10% of the total peptide subunits of peptidoglycan contained glycine in positions 1 and 4, whereas the family of accumulated precursors contained 66 and 65% in these positions, respectively. However, 31% of the peptide subunits contained glycine in position 5 of the peptidoglycan compared with 35% in the precursors. Thus, the preferential accumulation of several modified precursors and the preferential incorporation of certain modified peptide subunits into the peptidoglycan from the family of precursors can be related to the specificity of the translocase.

The high amount of COOH-terminal glycine in the peptide subunit of the peptidoglycan and its decreased cross-linking in the presence of glycine suggested that the peptide subunit with glycine in position 5 might be a poor substrate for the transpeptidase. Thus, the lower specificity of the translocase for position 5 of the UDP-MurNAc-pentapeptide may allow the incorporation of a variety of residues into the substrate of the
transpeptidase. Some of these, however, may not be substrates in the cross-linking reaction and, thus, result in decreased cross-linking. The low specificity of the translocase toward position 5 is not restricted to glycine substitution. In defining the action of O-carbamyl-d-serine, the analog UDP-MurNAc-Ala-d-Glu-Lys-d-Ala-O-carbamyl-d-serine was found to be as effective as UDP-MurNAc-Ala-d-Glu-Lys-d-Ala-d-Ala in the exchange reaction catalyzed by the translocase (4). This result further supports the present studies which indicate that the translocase does not have a high specificity for the residue in position 5.

The results with the incomplete peptidoglycan precursors further support the above consideration for positions 4 and 5. For example, with UDP-MurNAc-Ala-d-Glu-Lys-d-Ala, 24% of the activity observed for UDP-MurNAc-pentapeptide was found, whereas this enzyme activity was only 1.6% with UDP-MurNAc-Ala-d-Glu-Lys (Table VI, Transfer). In comparison to the UDP-MurNAc-tripeptide, UDP-MurNAc-tetrapeptide is an effective substrate. Thus, the presence of the d-alanine residue in position 4 would appear to be more critical than a residue in position 5. The observation that UDP-MurNAc-tripeptide has the same $V_{max}$ as UDP-MurNAc-pentapeptide suggests that at high concentrations of UDP-MurNAc-tripeptide in in vitro systems for peptidoglycan synthesis the rate of synthesis may be similar to that for UDP-MurNAc-pentapeptide. This presupposes that the other enzymes involved in the synthesis of the cross linked polymer do not have a high specificity requirement for the peptide subunit. In the case of membrane preparations from *M. lysodeikticus* significant incorporation of the tripeptide subunit was observed at a rate comparable to the rate of exchange catalyzed by the translocase (30).

An additional feature of the specificity profile concerns Residue 3. Replacement of the lysine residue by either ornithine or meso-diaminopimelic acid results in no change with ornithine and only a small decrease with meso-diaminopimelic acid. Thus, the translocase does not appear to have a high specificity for the diamino acid in position 3. The low specificity in position 3 had previously been indicated in the peptidoglycan synthesizing systems from *Escherichia coli* (40, 41) and *Bacillus megaterium* (42).

In our studies, good correlation was observed between $V_{max}/K_m$ in the transfer and $R_{max}/K_m$ in the exchange assay. These ratios are the apparent first order reaction rate constants at very low substrate concentrations. However, no such correlation was observed between the individual parameters, i.e., $K_m$ and $V_{max}$ in the transfer with $K_m$ and $R_{max}$ in the exchange reaction. This lack of correlation might be expected on the basis of the reaction model proposed by Plees and Neuhaus (22) (Reactions 3 to 7). In this model the association and the dissociation of the lipid substrate and the lipid product are reflected in the transfer reaction and not in the exchange reaction. Thus, the association and dissociation steps could affect the $K_m$ and $V_{max}$ of the transfer reaction without influencing $K_m$ and $R_{max}$ in the exchange reaction. The results with the various UDP-MurNAc-peptides obtained in both the transfer and exchange reactions may provide additional clues to the reaction sequence of the phospho-MurNAc-pentapeptide translocase.

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On the Specificity of Phospho-N-acetylmuramyl-pentapeptide Translocase: THE PEPTIDE SUBUNIT OF URIDINE DIPHOSPHATE-N-ACETYLMURAMYLPENTAPEPTIDE
Walter P. Hammes and Francis C. Neuhaus


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