Articles

Intramembranal Events in the Biosynthesis of Peptidoglycan in *Gaffkya homari*†

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ABSTRACT: In order to monitor the intermediates involved in nascent peptidoglycan (PG) assembly in *Gaffkya homari*, a pulse/chase assay utilizing UDP–MurNAc-Ala-DGlu-Lys(N^{ϵ}-Dns)-DAla-DAla [Dns (dansyl) = 5-(dimethylamino)naphthalene-1-sulfonyl] was devised. The perturbation introduced by the dansyl group provided a means for separating the synthesis of nascent PG into discrete stages. Together with paramagnetic quenching of the fluorophore by *n*-doxylstearic acids (n = 5, 7, 12, 16; doxyl = *N*-oxy-4′,4′-dimethyloxazolidine), this assay allows one to observe the synthesis of undecaprenyl diphosphate–MurNAc-((N^{ϵ}-Dns)pentapeptide)-GlcNAc and its utilization for the formation of dansyl-labeled PG by fluorescence emission and by change in specific positional quenching. The utilization of the dansylated lipid disaccharide–pentapeptide occurs without a lag, whereas the formation of the chromatographically immobile dansylated PG occurs with a lag of 4–6 min. Membrane-associated undecaprenyl diphosphate–MurNAc-(N^{ϵ}-Dns)-pentapeptide was quenched primarily by 7-doxylstearate. In contrast, the fluorophore of the undecaprenyl diphosphate–MurNAc-((N^{ϵ}-Dns)pentapeptide)-GlcNAc was quenched primarily by 5-doxyl- and 16-doxylstearates. In the chase phase of the assay, quenching by 16-doxylstearate decreased at a faster rate than that by 5-doxylstearate during the formation of dansyl-labeled PG.

The assembly of peptidoglycan (PG),[1] one of the major structural polymers of the bacterial cell wall, requires the concerted action of a series of membrane-associated enzymes. These enzymes utilize intermediates linked to undecaprenyl phosphate for the synthesis of nascent PG. This glycan is cross-linked to the preexisting PG matrix of the wall by a series of penicillin-sensitive processing enzymes.

In the synthesis of nascent PG, phospho-MurNAc-pentapeptide translocase catalyzes the transfer of phospho-MurNAc-pentapeptide from UDP–MurNAc-pentapeptide to undecaprenyl phosphate (Anderson et al., 1966; Higashi et al., 1967; Neuhaus, 1971). The product of this reaction undecaprenyl diphosphate–MurNAc-pentapeptide is the acceptor for GlcNAc derived from UDP–GlcNAc in a reaction catalyzed by GlcNAc transferase (Anderson et al., 1966). In the final step of nascent PG synthesis, transglycosylase catalyzes the $\beta 1 \rightarrow 4$ addition of disaccharide units at the reducing end of the growing chain attached to undecaprenyl diphosphate (Ward & Perkins, 1973). This mechanism predicts the existence of a series of lipid-linked oligomers of disaccharide–pentapeptide. With only the exception of undecaprenyl diphosphate–(MurNAc-(pentapeptide)-GlcNAc)$_{12}$ (Fuchs-Cleveland & Gilvarg, 1976), no smaller lipid-linked intermediates have been detected between undecaprenyl diphosphate–disaccharide–pentapeptide and the nascent PG, which is measured as chromatographically immobile glycan. Thus, the precise mechanism for the assembly of nascent PG is not fully understood.

To monitor the undecaprenyl-linked intermediates in this cycle of reactions, a pulse/chase assay utilizing UDP–MurNAc-Ala-DGlu-Lys(N^{ϵ}-Dns)-DAla-DAla was devised. Weppner and Neuhaus (1977, 1978) described the use of the dansyl moiety linked to this nucleotide for incorporating a reporter group into the site of PG synthesis in *Staphylococcus aureus* Copenhagen. They observed a large value of anisotropy (0.32), a high rotational relaxation time (>100 ns), and a high quantum yield for the dansyl group of the membrane-associated undecaprenyl diphosphate–MurNAc-(N^{ϵ}-Dns)pentapeptide and concluded that the fluorescently labeled lipid intermediate experiences a hydrophobic or structured environment within the membrane. This methodology, along with the paramagnetic quenching of the lipid monosaccharide–pentapeptide by spin-labeled fatty acids (Weppner & Neuhaus, 1978), provided an experimental approach to monitor the lipid intermediates in PG assembly. However, since purified membranes from *S. aureus* Copenhagen have poor activity for the synthesis of PG, a new membrane system from *Gaffkya homari* was developed. It is our goal to understand the intramembranal events associated with nascent PG assembly in this system. This paper describes the use of fluorescence spectroscopy and paramagnetic quenching to monitor the synthesis of undecaprenyl diphosphate–MurNAc-((N^{ϵ}-Dns)-pentapeptide)-GlcNAc and its utilization for the formation of nascent PG.

Materials and Methods

Materials. UDP-*N*-[U-^{14}C]acetylglucosamine (GlcNAc) (345 mCi/mmol) and L-[^{14}C]lysine (342 mCi/mmol) were

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[1] Abbreviations: PG, peptidoglycan; MurNAc, *N*-acetylmuramyl; GlcNAc, *N*-acetylglucosaminyl; UDP, uridine 5′-diphosphate; dansyl (Dns), 5-(dimethylamino)naphthalene-1-sulfonyl; UMP, uridine 5′-monophosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, DL-dithiothreitol; doxyl, *N*-oxy-4′,4′-dimethyloxazolidine; DMHTP, 4-(*N*,*N*-dimethyl-*N*-hexadecylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl iodide. Unless stated, all abbreviations of residues denote the L configuration.

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purchased from Amersham Corp., Arlington Heights, IL, and International Chemical and Nuclear, Irving, CA, respectively. *N*-Oxy-4′,4′-dimethyloxazolidine derivatives (doxyl) of 5-ketostearic acid, 7-ketostearic acid, 12-ketostearic acid, and 16-ketostearic acid, 4-[*N*-(2-hydroxyethyl)-*N*,*N*-dimethylammonio]-2,2,6,6-tetramethylpiperidine-1-oxyl chloride (tempocholine), 5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride), and 4-(*N*,*N*-dimethyl-*N*-hexadecylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (DMHTP) were purchased from Molecular Probes, Junction City, OR.

UDP-MurNAc-Ala-DGlu-Lys-DAla-DAla and UDP-MurNAc-Ala-DGlu-[^{14}C]Lys-DAla-DAla were prepared as described by Hammes and Neuhaus (1974b). UDP-MurNAc-Ala-DGlu-Lys(N^{ϵ}-Dns)-DAla-DAla and UDP-MurNAc-Ala-DGlu-[^{14}C]Lys(N^{ϵ}-Dns)-DAla-DAla were prepared by the procedure of Weppner and Neuhaus (1977).

Preparation of Membranes from G. homari for Fluorescence Measurements. *Gaffkya homari*, properly named *Aerococcus viridans* subsp. *homarus* (Kelly & Evans, 1974), was obtained from the American Type Culture Collection as *Pediococcus homari* 10400. Membranes were isolated from this organism by the procedures of Kalomiris et al. (1982). After removal of the membrane-wall fraction by centrifugation at 30000*g* at 4 °C for 15 min (2 times), the supernatant fraction containing membranes was incubated with 2 mM UMP according to the procedure of Weppner and Neuhaus (1977) to generate the highest concentration of membrane-associated undecaprenyl phosphate.

The UMP-treated membranes in 20 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, and 1 mM DTT were subjected to sucrose density step gradient [25% (w/w)–65% (w/w)] centrifugation at 150000*g* for 90 min at 4 °C. It was established that the membranes sedimenting between 32% (w/w) and 45% (w/w) sucrose had the highest specific activity for PG synthesis, the lowest specific activity for UDP-GlcNAc pyrophosphatase, a higher capacity to synthesize lipid intermediates, and lower levels of an intrinsic membrane fluorophore. To ensure the removal of low-density membranes [27–32% (w/w)] that contain high levels of this fluorophore, the above sucrose density gradient centrifugation was repeated. The membranes in sucrose were frozen at −196 °C and stored at −20 °C.

Fluorescence Measurements. Fluorescence measurements were made in a double-beam differential Farrand Mark I spectrofluorometer in the difference mode. For the measurement of lipid intermediates, this instrumentation allowed us to correct for unreacted UDP-MurNAc-(N^{ϵ}-Dns)pentapeptide, Rayleigh scattering, solvent Raman fluorescence, and intrinsic membrane fluorescence. The temperature in the cuvettes was regulated by constant-temperature circulating water through the cell block. Band-passes of 2.5 nm in the excitation beam and 10 nm in the emission beam were used. To keep the membranes from settling during the fluorescence measurements, all reaction mixtures contained 40% (w/w) (1.54 M) sucrose.

Membranes (4–8 μg of protein/μL) were suspended in 20 mM Tris-HCl buffer (pH 7.8), 20 mM $Mg(OAc)_2$, 1.54 M sucrose, and 50 mM DTT. This mixture was subjected to seven cycles of freezing (−196 °C) and thawing (25 °C) to reactivate the activity for PG synthesis (Kalomiris et al., 1982). The freeze-thawed reaction mixture was made 20 μM in UDP-MurNAc-(N^{ϵ}-Dns)pentapeptide and 10 mM in KCl, NH_4OAc, and ATP in a total volume of 450 μL. It was divided into two equal portions, one for the sample cuvette and one for the reference cuvette. In the assays for measuring the incorporation of radiolabel into PG and the lipid intermediates, the freeze-thaw procedure described above was also used.

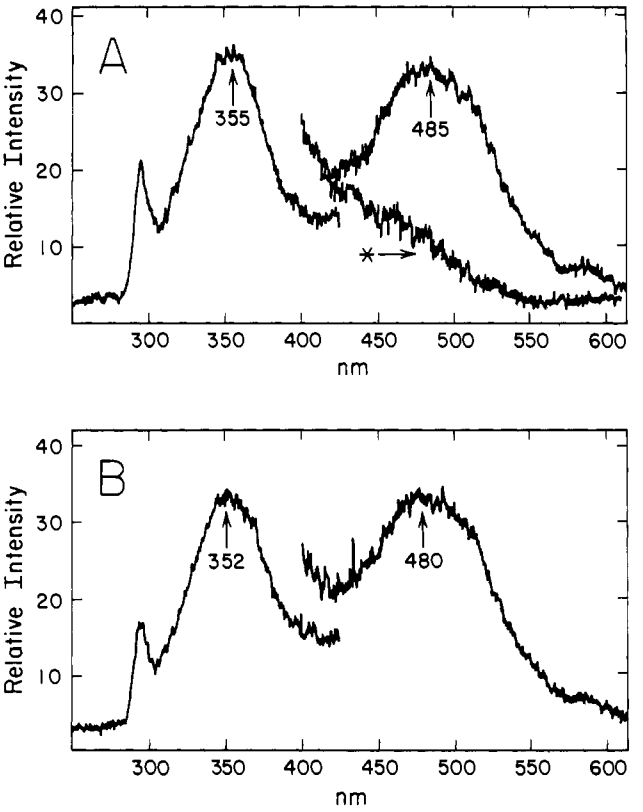

FIGURE 1: Fluorescence emission and excitation spectra of membrane-associated undecaprenyl diphosphate-MurNAc-(N^{ϵ}-Dns)-pentapeptide (A) and undecaprenyl diphosphate-MurNAc-((N^{ϵ}-Dns)pentapeptide)-GlcNAc (B). The uncorrected spectra were determined by the procedure in the text. The differential emission spectrum before the addition of UMP to the reference cuvette in A (*) reflects some loss of detectable emission from the reference cuvette in the region of 400–550 nm by the spectrofluorometer.

To determine the fluorescence emission of undecaprenyl diphosphate-MurNAc-(N^{ϵ}-Dns)pentapeptide, 2 mM UMP was added to the reference cuvette to reverse the reaction catalyzed by phospho-MurNAc-pentapeptide translocase. Because the quantum efficiency of the UDP-MurNAc-(N^{ϵ}-Dns)pentapeptide is 18% of that of the dansylated lipid intermediate (Weppner & Neuhaus, 1977), the difference in fluorescence intensity between the reference and sample cuvettes reflects that of the dansylated lipid monosaccharide–pentapeptide. For this lipid intermediate, the excitation maximum was 355 nm, and the emission maximum was 485 nm (Figure 1A). For the dansylated lipid disaccharide–pentapeptide, the excitation maximum was 352 nm, and the emission maximum was 480 nm (Figure 1B). The quantum efficiency of isolated dansylated lipid disaccharide–pentapeptide has not been established. In aqueous solution, UDP-MurNAc-(N^{ϵ}-Dns)pentapeptide has uncorrected excitation and emission maxima of 340 and 525 nm, respectively (Weppner & Neuhaus, 1977).

Paramagnetic Quenching. The doxyl-labeled stearic acids, tempocholine, and DMHTP were used as paramagnetic quenchers of the fluorescence of the membrane-associated dansyl-labeled lipid monosaccharide–pentapeptide (Weppner & Neuhaus, 1978). These compounds were dissolved in $CHCl_3$–MeOH (1:2) at a concentration of 25 mM and stored at −20 °C. The spin-labeled compounds were incorporated into the membranes by evaporating the appropriate amount

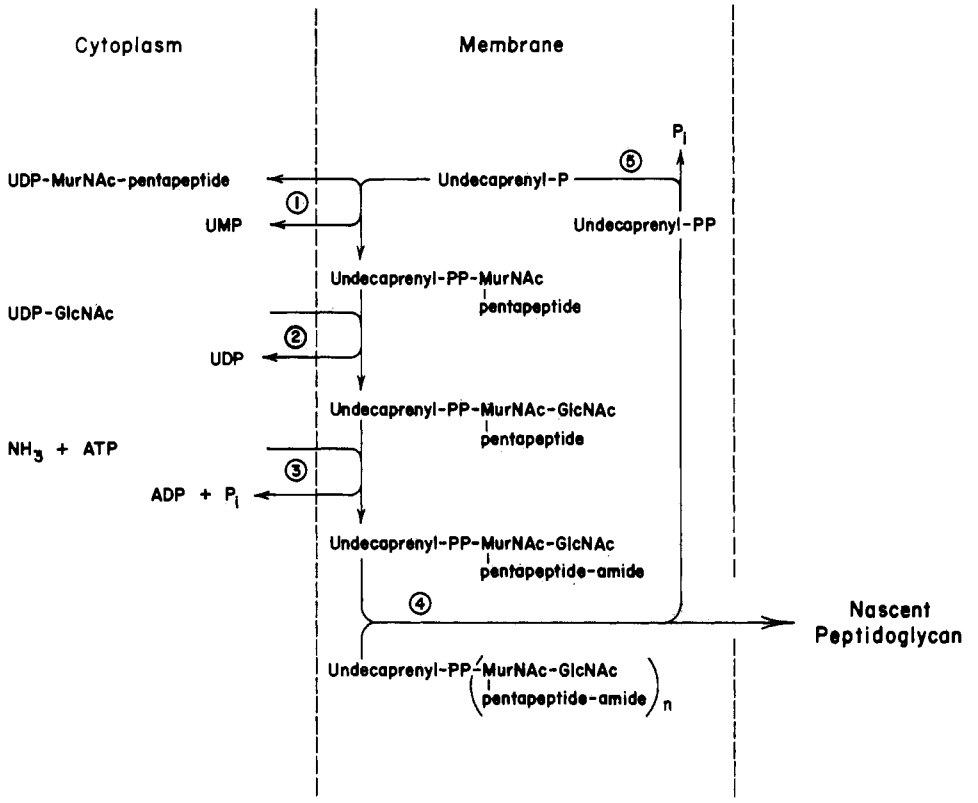

FIGURE 2: Assembly of nascent PG in *G. homari*. Enzyme 1 is phospho-MurNAc-pentapeptide translocase (transferase), enzyme 2 is *N*-acetylglucosaminyl transferase, enzyme 3 is D-isoglutamyl amidase, enzyme 4 is PG transglycosylase (PG polymerase), and enzyme 5 is undecaprenyl pyrophosphate phosphatase. Neither topological organization nor mechanism is implied in this figure.

to dryness under N_2 prior to the addition of 450 μL of membranes (4–8 μg/μL) suspended in 20 mM Tris–HCl (pH 7.8), 20 mM $Mg(OAc)_2$, and 1.54 M sucrose. The final concentration of spin-labeled compound added was 250 μM and was based on experiments previously reported with membranes from *S. aureus* Copenhagen (Weppner & Neuhaus, 1978). This mixture was subjected to seven cycles of alternate freezing (−196 °C) and thawing (25 °C). Electron paramagnetic resonance (ESR) measurements with membranes from *S. aureus* Copenhagen indicated that the *n*-doxylstearates (n = 5, 7, 12, 16) at this concentration partitioned into the membrane to the same extent (Weppner & Neuhaus, 1978). When [^{14}C]stearate replaced the spin-labeled stearic acid in this procedure, the amount that partitioned into membranes was 35% of that added to the membrane mixture.

Formation of Undecaprenyl Diphosphate–MurNAc-((N$^{\epsilon}$-Dns)pentapeptide)-[^{14}C]GlcNAc and (N$^{\epsilon}$-Dns)[^{14}C]PG in Pulse/Chase Assay. The synthesis of radiolabeled (N$^{\epsilon}$-Dns)PG was measured by the incorporation of [^{14}C]GlcNAc from UDP–[^{14}C]GlcNAc in the pulse/chase assay. Membranes (8 μg/μL) were preincubated with 15 μM UDP–MurNAc-(N$^{\epsilon}$-Dns)pentapeptide to form undecaprenyl diphosphate–MurNAc-(N$^{\epsilon}$-Dns)pentapeptide in a reaction mixture that contained 1.54 M sucrose, 20 mM Tris-HCl (pH 7.8), 20 mM $Mg(OAc)_2$, 10 mM KCl, 10 mM NH_4OAc, 10 mM ATP, and 50 mM DTT in a total volume of 300 μL. The time course was started with the addition of UDP–[^{14}C]-GlcNAc (final concentration = 3.3 μM, 345 μCi/μmol) to the reaction mixture. After 20 min, the mixture was made 250 μM in UDP-MurNAc-pentapeptide. The reactions were terminated by the addition of 2 volumes of chromatographic solvent A [isobutyric acid–concentrated NH_4OH–water (66:2:33 v/v/v)]. The amounts of (N$^{\epsilon}$-Dns)[^{14}C]PG (chromatographically immobile material) and the double-labeled lipid intermediate (R_f 0.55–1.0) were measured on descending chromatograms (Whatman 3MM) developed in solvent A.

RESULTS

In the biosynthesis of nascent PG outlined in Figure 2, the ratio of V_{max}/K_m for UDP–MurNAc-(N$^{\epsilon}$-Dns)pentapeptide is 0.05 times that of UDP–MurNAc-pentapeptide (Weppner & Neuhaus, 1977). In contrast, the ratio of V_{max}/K_m for UDP–MurNAc-(N$^{\epsilon}$-Dns)pentapeptide is 5.3 times that of UDP–MurNAc-pentapeptide for the synthesis of undecaprenyl diphosphate–MurNAc-(N$^{\epsilon}$-Dns)pentapeptide catalyzed by phospho-MurNAc-pentapeptide translocase (enzyme 1) (Figure 2) (Weppner & Neuhaus, 1977). This unique perturbation by the dansyl group on these syntheses provided a feature that was exploited in separating the synthesis of nascent PG into discrete stages in a pulse/chase assay. In the first phase of our experiments, the effect of the dansyl group as a perturbant on the incorporation of UDP–MurNAc–(N$^{\epsilon}$-Dns)pentapeptide into nascent PG was assessed. In the second phase, the dansyl group was used both as a perturbant and as a reporter group for its incorporation into the lipid intermediates and nascent (N$^{\epsilon}$-Dns)PG.

Synthesis of [Lys-^{14}C](N$^{\epsilon}$-Dns)PG in Pulse/Chase Assay. The time course of radiolabeled (N$^{\epsilon}$-Dns)PG synthesis in a pulse/chase assay is shown in Figure 3. In this assay, membranes were preincubated with UDP–MurNAc-(N$^{\epsilon}$-Dns)-pentapeptide to form undecaprenyl diphosphate–MurNAc-(N$^{\epsilon}$-Dns)pentapeptide. These charged membranes were incubated with UDP–[^{14}C]GlcNAc so that the fluorescently labeled lipid monosaccharide was converted to undecaprenyl diphosphate–MurNAc-((N$^{\epsilon}$-Dns)pentapeptide)-[^{14}C]GlcNAc. The reduced velocity of PG synthesis from UDP–MurNAc-(N$^{\epsilon}$-Dns)pentapeptide and the concomitant formation of undecaprenyl diphosphate–MurNAc-((N$^{\epsilon}$-Dns)pentapeptide)-[^{14}C]GlcNAc provided the experimental system to generate a pool ("pulse") of dansyl-labeled lipid di-

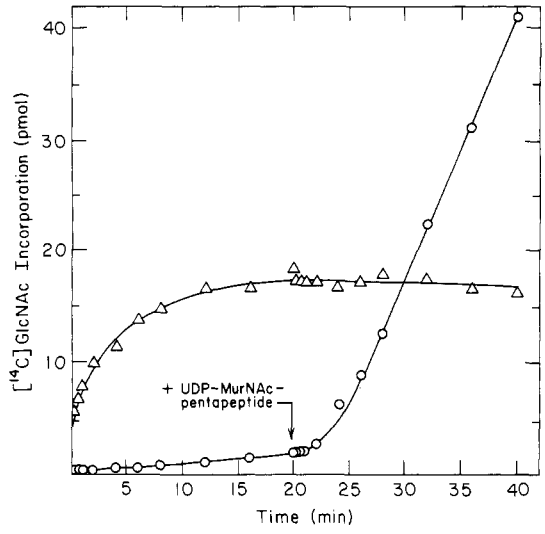

FIGURE 3: Time course of incorporation of [^{14}C]GlcNAc into undecaprenyl diphosphate–MurNAc-((N^{ϵ}-Dns)pentapeptide)-[^{14}C]-GlcNAc and (N^{ϵ}-Dns)[*GlcNAc*-^{14}C]PG during the pulse/chase assay. The syntheses of the radiolabeled lipid disaccharide–pentapeptide (△) and radiolabeled nascent PG (○) from undecaprenyl diphosphate–MurNAc-(N^{ϵ}-Dns)pentapeptide are described in the text. The time course was started with the addition of 3.3 μM (final concentration) UDP-[^{14}C]GlcNAc. The chase was started at 20 min with 250 μM (final concentration) UDP–MurNAc-pentapeptide.

saccharide–pentapeptide in the absence of significant PG formation.

In the chase stage of the assay (Figure 3, 20 min), the addition of high levels of non-dansylated UDP–MurNAc-pentapeptide (17-fold molar ratio) resulted in a change in rate of PG synthesis from 0.11 to 2.22 pmol/min. This 20-fold change in velocity was attained by 4–6 min. Thus, a high molar ratio of UDP–MurNAc-pentapeptide competed effectively with the fluorescently labeled nucleotide for the formation of lipid-linked intermediates and allowed us to design a pulse/chase assay for the assembly of (N^{ϵ}-Dns)PG in which each of the lipid intermediates can be independently studied.

To establish that the fluorescently labeled lipid disaccharide–pentapeptide was converted to (N^{ϵ}-Dns)PG during the second stage of the assay, membranes were charged with undecaprenyl diphosphate–MurNAc-([*Lys*-^{14}C](N^{ϵ}-Dns)-pentapeptide)-GlcNAc and separated from reactants. These double-labeled membranes were incubated with nonradiolabeled UDP–MurNAc-pentapeptide and UDP–GlcNAc (Figure 4), and the amounts of [*Lys*-^{14}C](N^{ϵ}-Dns)PG and undecaprenyl diphosphate–MurNAc-([*Lys*-^{14}C](N^{ϵ}-Dns)-pentapeptide)-GlcNAc as a function of time were measured. By 20 min, 67% of the double-labeled lipid disaccharide–pentapeptide was used. Although there was a 4–6-min lag in the formation of radiolabeled PG, there was no lag in the utilization of the fluorescently labeled lipid disaccharide–pentapeptide. The observation that 33% of the undecaprenyl diphosphate–MurNAc-([*Lys*-^{14}C](N^{ϵ}-Dns)pentapeptide)-GlcNAc did not participate in this synthesis will be important for the interpretation of the fluorescence measurements in the pulse/chase assay. The synthesis of nascent PG in *G. homari* is stimulated by NH_4^+ and ATP (Hammes & Neuhaus, 1974a). Omission of either NH_4^+ or ATP in the present system gave 53% and 20%, respectively, of the velocity of PG synthesis (Manteuffel, 1984). Thus, D-isoglutamyl amidase (Figure 2) plays an important role in the synthesis of nascent PG.

Time Course of Fluorescence Intensity during Pulse/Chase Assay. An advantage of the pulse/chase assay is the ability

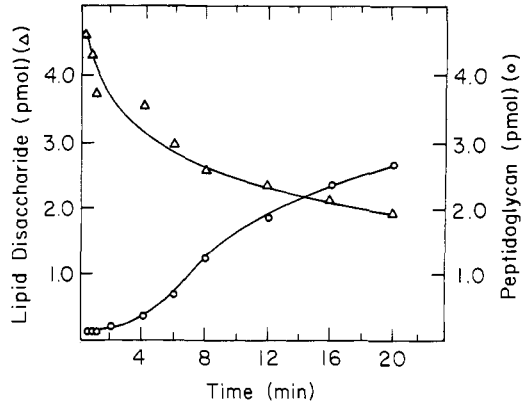

FIGURE 4: Conversion of double-labeled undecaprenyl diphosphate–MurNAc-([*Lys*-^{14}C](N^{ϵ}-Dns)pentapeptide)-GlcNAc (△) to [*Lys*-^{14}C](N^{ϵ}-Dns)PG (○). *G. homari* membranes (10 μg/μL) were charged with undecaprenyl diphosphate–MurNAc-([*Lys*-^{14}C](N^{ϵ}-Dns)pentapeptide)-GlcNAc by incubation with 25 μM UDP–MurNAc-[*Lys*-^{14}C](N^{ϵ}-Dns)pentapeptide (345 μCi/μmol) and 50 μM UDP–GlcNAc in a reaction mixture that contained 20 mM Tris-HCl (pH 7.8), 20 mM $Mg(OAc)_2$, 10 mM KCl, 10 mM NH_4OAc, 10 mM ATP, and 5 mM DTT in a total volume of 150 μL. The mixture was incubated at 25 °C for 5 min and then chilled to 4 °C. These double-labeled membranes were separated from reactants on a sucrose density gradient (0–50%, w/w) at 4 °C. After removal from the gradient, the membranes (12 μg/μL) were incubated with 125 μM UDP–MurNAc-pentapeptide and 50 μM UDP–GlcNAc in the reaction mixture described above. Double-labeled lipid disaccharide–pentapeptide and nascent PG were measured as described in the text.

to discern undecaprenyl diphosphate–MurNAc-((N^{ϵ}-Dns)-pentapeptide)-GlcNAc in the absence of both the lipid monosaccharide–pentapeptide and (N^{ϵ}-Dns)PG. Alternatively, in the absence of UDP–GlcNAc, undecaprenyl diphosphate–MurNAc-(N^{ϵ}-Dns)pentapeptide accumulated (Weppner & Neuhaus, 1978). Thus, we can examine the fluorescence properties of the individual lipid intermediates in the assembly of nascent (N^{ϵ}-Dns)PG. Because of the high concentration of UDP–MurNAc-pentapeptide added in the chase phase of the assay, the released undecaprenyl pyrophosphate is utilized by the system for the synthesis of non-dansylated PG (Figure 2).

The changes in fluorescence intensity during a pulse/chase assay are illustrated in Figure 5. As described under Materials and Methods, the preformed undecaprenyl diphosphate–MurNAc-(N^{ϵ}-Dns)pentapeptide was determined from the difference in intensities between the reference and sample cuvettes upon the addition of UMP to the reference cuvette. Because of the reversibility of the reaction catalyzed by phospho-MurNAc-pentapeptide translocase (Struve et al., 1966), greater than 95% of the phospho-MurNAc-(N^{ϵ}-Dns)-pentapeptide of the lipid monosaccharide–pentapeptide in the reference cuvette was converted to UDP–MurNAc-(N^{ϵ}-Dns)pentapeptide within 5 min. Thus, the difference in the fluorescence intensities between the sample and reference cuvettes reflected the difference in quantum yield of the two species and, hence, the amount of undecaprenyl diphosphate–MurNAc-(N^{ϵ}-Dns)pentapeptide in the sample cuvette (Figure 5, part A). In the pulse phase of the assay, the addition of UDP–GlcNAc was associated with a time-dependent increase (35%) in fluorescence intensity (Figure 5, part B). Maximal levels of lipid disaccharide–pentapeptide accumulated in 12 min. The emission maximum appears to be blue shifted by 5 nm (Figure 1). In contrast to the sensitivity of undecaprenyl diphosphate–MurNAc-pentapeptide to reversal in reaction 1 (Figure 2) by UMP, the addition of UMP to the reaction mixture after 20 min in part B (data not shown) did not decrease the fluorescence intensity and, thus,

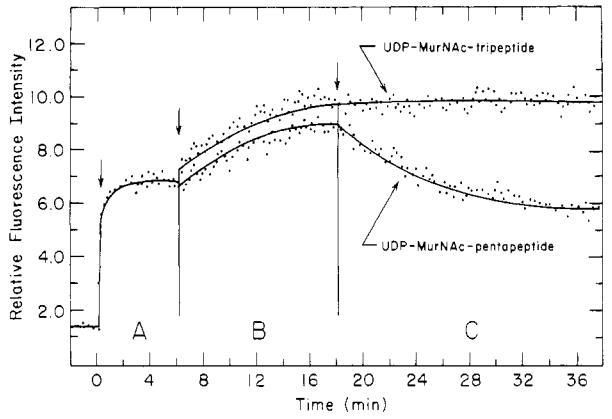

FIGURE 5: Time course of fluorescence intensity during the pulse/chase assay. The pulse/chase assay was carried out and monitored for fluorescence intensity at 485 nm as described in the text. In (A), the differential fluorescence intensity of undecaprenyl diphosphate–MurNAc-(N^{ϵ}-Dns)pentapeptide was measured after adding 2 mM UMP (↓) to the reference cell. In (B), the formation of undecaprenyl diphosphate–MurNAc-((N^{ϵ}-Dns)pentapeptide)-GlcNAc was monitored after adding 50 μM UDP-GlcNAc (↓). In (C), the utilization of undecaprenyl diphosphate–MurNAc-((N^{ϵ}-Dns)pentapeptide)-GlcNAc for (N^{ϵ}-Dns)PG synthesis was observed. The synthesis of (N^{ϵ}-Dns)PG was initiated by the addition of 200 μM (final concentration) UDP–MurNAc-pentapeptide (↓). Replacement of this nucleotide by 200 μM UDP–MurNAc-Ala-DGlu-Lys did not result in a decrease of fluorescence intensity.

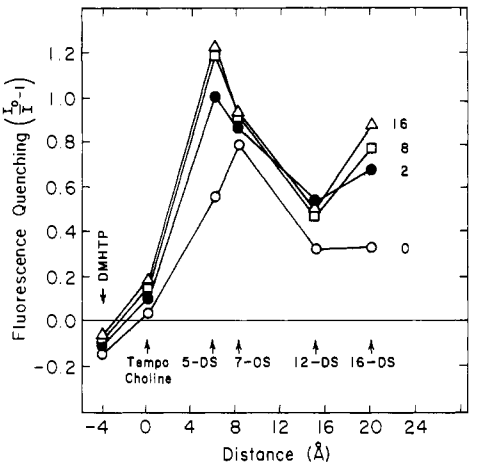

FIGURE 6: Fluorescence quenching profile of undecaprenyl diphosphate–MurNAc-(N^{ϵ}-Dns)pentapeptide and undecaprenyl diphosphate–MurNAc-((N^{ϵ}-Dns)pentapeptide)-GlcNAc. Quenching measurements were made according to the procedure described in the text. After the quenched intensity of the lipid monosaccharide–pentapeptide (○) was established, 50 μM (final concentration) UDP–GlcNAc was added, and the intensities were measured at the designated times: (●) 2, (□) 8, and (△) 16 min. The data were plotted by using the Stern–Volmer parameter ($I_0/I - 1$) as a function of the location of the paramagnetic center from the surface of the membrane. The distance (Å) of the paramagnetic center from the surface assumes an extended conformation of the doxylstearate.

is consistent with the formation of dansylated lipid disaccharide–pentapeptide.

The addition of UDP–MurNAc-pentapeptide in part C resulted in the fluorescently labeled lipid disaccharide–pentapeptide being utilized for the synthesis of PG in the chase (Figure 5, part C). The major portion of the decrease in fluorescence intensity occurred within 10–12 min (without a lag) after the addition of this nucleotide. The fluorescence intensity did not return to base line within 20 min after the addition of UDP–MurNAc-pentapeptide. This intensity reflected in part the population of fluorescently labeled lipid disaccharide–pentapeptide (33%) that was not utilized in this time interval (Figure 5) and in part the quantum efficiency of the fluorophore in (N^{ϵ}-Dns)PG.

The specificity of this system for UDP–MurNAc-pentapeptide is illustrated in Figure 5 (part C). For example, the addition of high levels of UDP–MurNAc-tripeptide did not yield a decrease in fluorescence intensity. This observation reflects the specificity of phospho-MurNAc-pentapeptide translocase (Figure 2, reaction 1) for UDP–MurNAc-pentapeptide. It has been established that UDP–MurNAc-tripeptide is not utilized by this enzyme for the synthesis of undecaprenyl diphosphate–MurNAc-peptide (Hammes & Neuhaus, 1974b).

A comparison of the fluorescence intensity (Figure 5) with the incorporation of radiolabeled precursors (Figure 3) supports the interpretation that the emission change in part A reflects the presence of undecaprenyl diphosphate–MurNAc-(N^{ϵ}-Dns)pentapeptide, in part B reflects the formation of undecaprenyl diphosphate–MurNAc-((N^{ϵ}-Dns)pentapeptide)-GlcNAc, and in part C reflects the utilization of the dansylated lipid disaacharide–pentapeptide for the formation of nascent (N^{ϵ}-Dns)PG.

Paramagnetic Quenching of Dansylated Lipid Intermediates in Pulse/Chase Assay. Paramagnetic quenching by *n*-doxylstearic acids (n = 5, 7, 12, 16) provides a method for locating the fluorophore in the transverse dimension of the membrane. Quenching of the fluorescence occurs when the fluorophore and the doxyl groups are within 4–6 Å (Bieri & Wallach, 1975; Blatt & Sawyer, 1985). This approach was used to locate the fluorophore of undecaprenyl diphosphate–MurNAc-(N^{ϵ}-Dns)pentapeptide in membranes from *S. aureus* Copenhagen (Weppner & Neuhaus, 1977, 1978). Maximal quenching in these membranes was achieved with 5-doxylstearate, suggesting that the fluorophore of this lipid monosaccharide–pentapeptide is near the depth monitored by this paramagnetic center.

The fluorescence quenching profile for undecaprenyl diphosphate–MurNAc-(N^{ϵ}-Dns)pentapeptide from *G. homari* is shown in Figure 6. Maximal quenching was observed with 7-doxylstearate followed by (in decreasing order) 5-doxylstearate and 12-doxyl- and 16-doxylstearates. The fluorescence of the lipid monosaccharide–(N^{ϵ}-Dns)pentapeptide was not quenched by the amphipathic surface spin-label DMHTP and by the hydrophilic spin-label tempocholine. These data suggested that the fluorophore of the lipid monosaccharide-(N^{ϵ}-Dns)pentapeptide is not equally accessible to all of the paramagnetic centers of the different spin-labeled fatty acids and that the fluorophore is primarily at the depth monitored by 7-doxylstearate.

The profile of quenching changed when undecaprenyl diphosphate–MurNAc-(N^{ϵ}-Dns)pentapeptide was converted to undecaprenyl diphosphate–MurNAc-((N^{ϵ}-Dns)pentapeptide)-GlcNAc. After the addition of UDP–GlcNAc, an increase in quenching by 16-doxylstearate was observed (Figure 6). Quenching by this paramagnetic center continued to increase to a level that was 3-fold higher than the dansylated lipid monosaccharide–(N^{ϵ}-Dns)pentapeptide. In addition, a fraction of this fluorophore population is quenched more effectively by 5-doxylstearate than by 7-doxylstearate. Because the efficiency of quenching in situ by these doxyl-labeled fatty acids is not known, the relative fractions of the fluorophore of undecaprenyl diphosphate–MurNAc-((N^{ϵ}-Dns)pentapeptide)-GlcNAc quenched by 5-doxyl- and 16-doxylstearates cannot be established from these experiments.

Quenching Profile of Undecaprenyl Diphosphate–MurNAc-((N^{ϵ}-Dns)pentapeptide)-GlcNAc during Chase into (N^{ϵ}-Dns)PG. The changes in the profile of quenching by spin-labeled fatty acids during the conversion of undecaprenyl

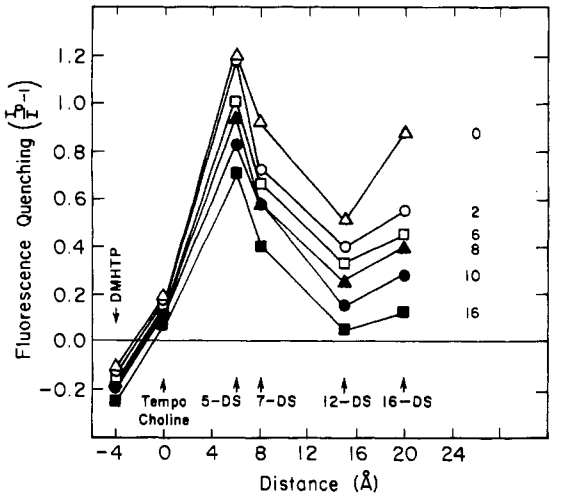

FIGURE 7: Fluorescence quenching profile of undecaprenyl diphosphate–MurNAc-((N^{ϵ}-Dns)pentapeptide)-GlcNAc during its utilization for the synthesis of (N^{ϵ}-Dns)PG. Quenching measurements were made according to the procedure described in the text. After the quenched intensity of lipid disaccharide–pentapeptide (△) (Figure 6) was measured, 200 μM (final concentration) UDP–MurNAc-pentapeptide was added, and the quenched intensities were measured at the designated times, (○) 2, (□) 6, (▲) 8, (●) 10, and (■) 16 min, and plotted as a function of the location of the paramagnetic center from the surface of the membrane.

diphosphate–MurNAc-((N^{ϵ}-Dns)pentapeptide)-GlcNAc to (N^{ϵ}-Dns)PG are shown in Figure 7. Quenching by 16-doxylstearate decreased at a faster rate than that by 5-doxylstearate. For example, 12 min after the addition of UDP–MurNAc-pentapeptide, quenching with 16-doxylstearate had been reduced by 67% of the initial value whereas quenching with 5-doxylstearate was reduced by only 30%.

Discussion

The perturbation introduced by the dansyl group has provided a means to separate the stages of PG assembly in a pulse/chase assay. The changes in fluorescence intensities during this assay were correlated with the formation of fluorescently labeled undecaprenyl diphosphate–MurNAc-((N^{ϵ}-Dns)pentapeptide)-GlcNAc and its subsequent utilization during the chase for the synthesis of fluorescently labeled nascent PG. Not only will this methodology allow one to monitor spectroscopically the formation of lipid disaccharide–pentapeptide and its utilization for nascent PG synthesis, but it will eventually allow one to examine the microenvironments and mobilities that the lipid intermediates experience.

With UDP–MurNAc-(N^{ϵ}-Dns)pentapeptide as substrate, the rate of (N^{ϵ}-Dns)PG synthesis is only 0.05 of that observed for the non-dansylated substrate. Addition of a high molar ratio of UDP–MurNAc-pentapeptide to the reaction mixture increased the rate of nascent PG synthesis to that of the non-dansylated nucleotide while incorporating a large fraction (67%, 20 min) of the preexisting dansylated lipid disaccharide–pentapeptide into nascent PG. Thus, the chase of dansylated disaccharide into nascent PG is accomplished by the addition of a large pool of UDP–MurNAc-pentapeptide in the presence of UDP–GlcNAc.

The assay of PG transglycosylase in membranes has routinely been measured by the incorporation of radiolabel from UDP–GlcNAc and UDP–MurNAc-pentapeptide into muramidase-sensitive, chromatographically immobile polymer (Anderson et al., 1966). In *G. homari* the incorporation is characterized by a lag period of approximately 4–6 min (Hammes & Neuhaus, 1974a; Weppner & Neuhaus, 1977). This period reflects the time that is required for the radiolabeled nascent PG to acquire the physical property of solvent immobility. In contrast, the utilization of dansylated and undansylated lipid disaccharide–pentapeptide for nascent PG synthesis occurs without a lag. The discrepancy between the amount of lipid disaccharide–pentapeptide that was utilized and the amount of nascent PG in the early part of the time courses may reflect the presence of "lipid oligomers" that have not acquired the characteristic solvent immobility. Undecaprenyl diphosphate linked oligomeric intermediates have been reported in a number of organisms (Ward, 1983). The smallest of these is undecaprenyl diphosphate–(MurNAc-(pentapeptide)-GlcNAc)$_{12}$ isolated from *Bacillus megaterium* by Fuchs-Cleveland and Gilvarg (1977). Larger lipid-linked intermediates have been detected in *Escherichia coli* and in *Micrococcus luteus* (Mett et al., 1980; Thorpe & Perkins, 1979).

The time course of fluorescence emission correlates with the formation of dansylated lipid disaccharide–pentapeptide and its utilization by PG transglycosylase during the chase. The increase in emission associated with the formation of the lipid disaccharide–pentapeptide suggests that it may be incorporated into a different microenvironment of the membrane. Initiation of nascent PG synthesis by UDP–MurNAc-pentapeptide in the presence of UDP–GlcNAc results in an immediate decrease in emission of the fluorophore from dansylated lipid disaccharide–pentapeptide. This time course is similar to that observed for the utilization of radiolabeled, dansylated lipid disaccharide–pentapeptide for the synthesis of dansylated nascent PG. Since the decrease in fluorescence emission in the chase is specific for UDP–MurNAc-pentapeptide, it is concluded that this change is associated with the utilization of dansylated lipid disaccharide–pentapeptide for the formation of nascent PG.

The preferential quenching of undecaprenyl diphosphate–MurNAc-(N^{ϵ}-Dns)pentapeptide by 5-doxylstearate has revealed the fluorophore's position in the transverse dimension of the membrane from *S. aureus* (Weppner & Neuhaus, 1978). In membranes from *G. homari*, the fluorophore is quenched to a greater extent by 7-doxylstearate than by 5-doxylstearate. It is not understood why 4-(*N*,*N*-dimethyl-*N*-hexadecylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl bromide is an excellent quencher of dansylated lipid monosaccharide–pentapeptide in *S. aureus* and 4-(*N*,*N*-dimethyl-*N*-hexadecylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl iodide is a poor quencher in membranes from *G. homari*. The addition of UDP–GlcNAc to membranes containing undecaprenyl diphosphate–MurNAc-(N^{ϵ}-Dns)pentapeptide results in a time-dependent increase in the quenching by 16-doxylstearate and a shift in quenching from 7-doxyl- to 5-doxylstearate. The time course of quenching by 5-doxyl- and 16-doxylstearate is consistent with the time course of formation of the fluorescently labeled lipid disaccharide–pentapeptide. These results suggest that the depth of the fluorophore in a fraction of the dansylated lipid disaccharide–pentapeptide is significantly different from that of the dansylated lipid monosaccharide–pentapeptide. At this stage of our investigation it is not known why the quenching profile for the disaccharide is bimodal. This profile may represent either two conformations of the fluorescently labeled peptide moiety or two locations of the fluorescently labeled lipid intermediate.

In the chase phase of the pulse/chase assay, there is an immediate time-dependent decrease in the fluorescence emission of the fluorophore population and a time-dependent

decrease in quenching, both of which are correlated with the utilization of the dansylated lipid disaccharide–pentapeptide. The fluorescence quenching with 16-doxylstearate decreased at a faster rate than the fluorescence quenching with 5-doxylstearate. On the basis of the assumption that the efficiencies of quenching are identical for each doxylstearate, it is suggested that the lipid disaccharide–pentapeptide with the fluorophore in the deep location is utilized for nascent PG synthesis at a higher rate than the lipid disaccharide–pentapeptide that has the fluorophore in the shallow location.

The methodology described in this paper permits one to monitor the synthesis of the lipid intermediates as well as the utilization of the lipid disaccharide–pentapeptide for nascent PG synthesis. The use of the dansyl group allows one to accumulate undecaprenyl diphosphate–MurNAc-((N^{ϵ}-Dns)-pentapeptide)-GlcNAc in the presence of only a small amount of (N^{ϵ}-Dns)PG. The addition of UDP–MurNAc-pentapeptide together with UDP–GlcNAc to the precharged membranes generates a pulse of fluorescently labeled disaccharide–peptide that is chased into nascent PG. The use of the doxyl-labeled stearic acids as reference points in the transverse dimension of the membrane permits one to follow the progression of the MurNAc-(N^{ϵ}-Dns)pentapeptide moiety through the subsequent stages of nascent PG synthesis. The microenvironments experienced by these lipid intermediates in this system can be further characterized by manipulating the physical properties of the membrane (Weppner & Neuhaus, 1979; Lee et al., 1980). This methodology will contribute to both a characterization of the PG-synthesizing system and the mechanism of intramembranal translocation of precursors.

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