

A Kinetic Characterization of the Glycosyltransferase Activity of *Escherichia coli* PBP1b and Development of a Continuous Fluorescence Assay

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ABSTRACT: The bacterial cell wall is a polymer consisting of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) units, cross-linked via peptides appended to MurNAc. The final steps in the formation of cell wall, also referred to as murein, are catalyzed by high-molecular-weight, class A penicillin-binding proteins (PBPs). These bifunctional enzymes catalyze both glycosyltransfer, to form the carbohydrate backbone of murein, and transpeptidation, to form the interstrand peptide linkages. Using PBP1b from *Escherichia coli*, an in vitro kinetic characterization of the glycosyltransfer reaction was carried out. Initial studies with unlabeled substrate (Lipid II) revealed that activity is strongly influenced by DMSO, as well as metal and detergent. In addition, a continuous fluorescence assay was developed and used to determine the effect of pH on the reaction. A single basic residue was titrated, with a pK_a of 7.0. Taken together, these data suggest a mechanism for PBP1b where the glycosyltransfer reaction is catalyzed by the concerted effect of an active site base to deprotonate the glycosyl acceptor and a divalent metal to assist departure of the leaving group of the glycosyl donor.

Bacteria require the protective presence of a cell wall for viability. Also known as murein, the cell wall not only helps to maintain the proper size and shape of growing bacteria, but also prevents lysis arising from the large osmotic pressure differential that exists between the cytosol and exterior of the cell (1). Murein is a polymer consisting of repeating saccharide units cross-linked via appended peptides. The saccharide backbone is a linear array with alternating *N*-acetylglucosamine (GlcNAc)¹ and *N*-acetylmuramic acid (MurNAc) units. The peptidic side chain is linked to MurNAc by a peptide bond and varies depending on the species in which it is found (1). In general it is a pentapeptide consisting of H₂N-L-Ala- γ -D-Glu-L-X-D-Ala-D-Ala-COOH, in which X is either lysine or diaminopimelic acid.

The biosynthesis of murein occurs in three stages (Scheme 1) (1). Stage 1, which occurs in the cytosol, involves the action of soluble enzymes and substrates, resulting in the synthesis of the UDP-monosaccharide. Stage 2 occurs on the interior of the cell membrane, with the addition of undecaprenyl phosphate by MraY to form Lipid I and transformation of Lipid I to Lipid II, catalyzed by MurG. Lipid I, Lipid II, MraY, and MurG are all membrane-associated. Finally, stage 3 occurs on the exterior of the cell membrane, catalyzed by enzymes known as penicillin-

binding proteins (PBPs). These enzymes perform the final maturation of the cell wall, utilizing the cognate monomer Lipid II as substrate. In particular, class A high MW PBPs such as PBP1b from *Escherichia coli* (*E. coli*) are capable of catalyzing both reactions of stage 3, namely, the elongation of carbohydrate units (glycosyltransfer) and peptide cross-linking (transpeptidation). Because of the crucial nature of these activities, knockouts of class A high MW PBPs are lethal (3). In vivo formation of murein is thought to be a concerted process, involving a multienzyme complex known as a divisome (2). The divisome consists of lytic enzymes that hydrolyze preexisting cell wall, producing primers that can be utilized by synthetic enzymes for production of new material.

Drugs that target the late stages of cell wall biosynthesis, such as penicillin and vancomycin, are effective antibiotics. However, due to the continuing problem of resistance, the need for new antimicrobial agents is ongoing (4). In particular, inhibition of the glycosyltransferase activity of PBPs has long been regarded as an excellent target for drug discovery (5). However, a prerequisite understanding of this reaction has been hampered by the inability to obtain sufficient quantities of substrate and recombinant enzyme, as well as the need for a facile, informative assay. Recently, we (6) and others (7–9) have solved these issues, and we report here the first detailed kinetic evaluation of the glycosyltransferase activity of *E. coli* PBP1b, as well as the design of a new, continuous assay format appropriate for higher throughput screening.

MATERIALS AND METHODS

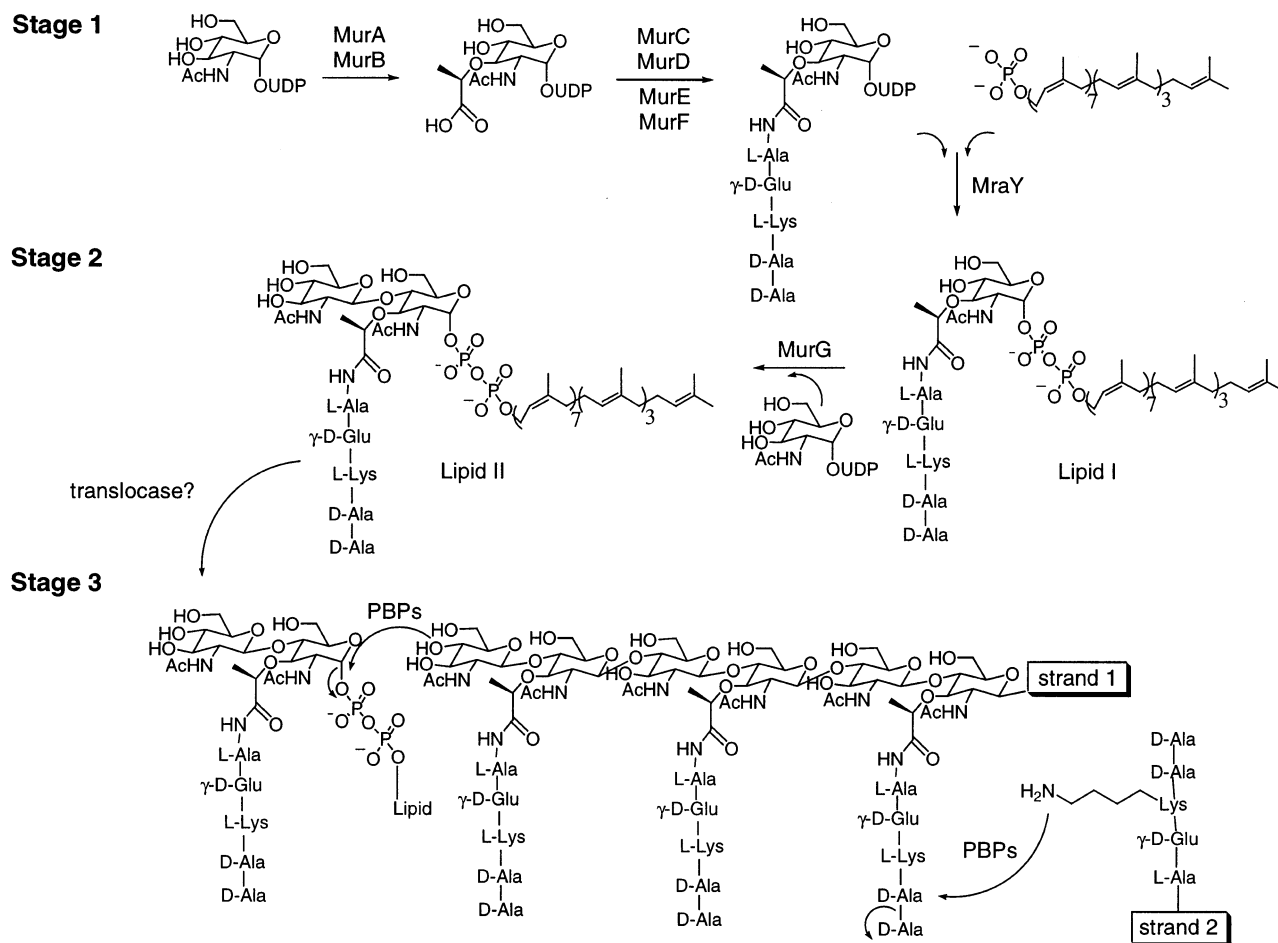
Materials and Stock Solutions. Disaccharide (compound 5, Scheme 3) was purchased from Toronto Research Chemicals Inc. Fluorescamine was purchased from Molecular

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¹ Abbreviations: GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; PBPs, penicillin-binding proteins; DMSO, dimethyl sulfoxide; CMC, critical micelle concentration; FucT V, α -(1,3)-fucosyltransferase V; TMSE, 2-(trimethylsilyl)ethyl; TBAF, tetra-(*n*-butyl)ammonium fluoride.

Scheme 1: Biosynthetic Pathway for Production of the Bacterial Cell Wall



Probes and stored as a 10 mM stock in methanol at -20°C . *E. coli* PBP1b was cloned, expressed, and purified as previously described (6) and stored at 4°C . Lipid II was synthesized as previously described (6) and stored as a 1 mM stock solution in methanol at -20°C . *N*-Acetylmuramidase from *Streptomyces globisporus* (Calbiochem) was stored as a stock at 0.2 mg/mL and diluted into reactions as needed. The SAX1 anion-exchange column was purchased from Supelco Co. (St. Louis, MO). HPLC buffers were made by dissolving ammonium acetate in methanol and filtering before use.

HPLC Assays. All reactions were carried out at 30°C . Reactions consisted of buffer (HEPES, 50 mM, pH 7.5), 0.085% decyl PEG, 10 mM metal, and 0–10% DMSO unless specifically noted otherwise. Reactions were initiated with the addition of PBP1b (final concentration 20–50 nM), and aliquots (30 μL) from the assay were removed at various times for analysis. For reactions involving unlabeled Lipid II, the pH of each aliquot was adjusted to >9 with 6 μL Bicine (1 M, pH 10.2) and then labeled with fluorescamine (3.6 μL of 10 mM stock). Samples of 20 μL were then injected onto an anion-exchange HPLC column and eluted with a linear gradient of ammonium acetate (20 mM to 0.5 M) in methanol. The eluent was monitored for fluorescence with $\lambda_{\text{ex}} = 380\text{ nm}$ and $\lambda_{\text{em}} = 470\text{ nm}$. A standard for Lipid II concentration was constructed using *N*-acetyl lysine in the range of 0.1–100 μM . For reactions with dansyl-Lipid II, aliquots were removed from the reaction and injected directly onto the anion-exchange column, using a similar elution

profile. Monitoring for these reactions was carried out with $\lambda_{\text{ex}} = 340\text{ nm}$ and $\lambda_{\text{em}} = 520\text{ nm}$.

Continuous Fluorescent Assay. All reactions were 100 μL in volume and carried out at 30°C in a Perkin-Elmer LS50B fluorimeter. Scans of dansyl-Lipid II revealed a $\lambda_{\text{ex}} = 340\text{ nm}$ and $\lambda_{\text{em}} = 520\text{ nm}$ as the maximum wavelengths for excitation and emission, respectively (data not shown). Reaction solutions contained buffer (50 mM), 0.085% decyl PEG, 10 mM CaCl_2 , 200 mM NaCl, *N*-acetylmuramidase (1 unit), and dansyl-Lipid II (1–30 μM). Buffers included MES (pH 5.5–6.5), PIPES (pH 6.5–7.0), HEPES (pH 7.0–8.0), TAPS (pH 8.0–9.0), and CHES (pH 9.0–10.0). Individual assays were initiated with the addition of enzyme (18.8 nM) to the reaction, and initial velocities were collected from the linear portion of the timecourse. Muramidase activity was determined not to be rate-limiting at the highest concentration of Lipid II at each pH by doubling the concentration of muramidase and failing to observe any effect on the observed rate.

General Synthetic Procedures. ^1H NMR spectra were recorded on dilute solutions in CDCl_3 or $\text{MeOH}-d_4$ at 300 MHz on Varian Unity instruments. Chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane. Low-resolution mass spectral analyses were performed on an Micromass Platform II (ESI) instrument. High-resolution mass spectra were obtained on a Finnigan MAT95S (ESI) instrument. Reactions were performed under an atmosphere of dry nitrogen or argon in flame-dried glassware and were monitored for completeness by thin-layer

chromatography (TLC) using silica gel 60 F-254 (0.25 mm) plates. Visualization of TLC plates was accomplished by I_2 vapor, phosphomolybdic acid in ethanol, ceric ammonium molybdate in aqueous methanol, or UV light absorption at 254 nm. Flash column chromatography was performed by the method of Still (10) using 230–400 mesh silica gel (E Merck). Tetrahydrofuran was distilled from potassium/benzophenone ketyl immediately prior to use. Other solvents and reagents were purchased from commercial sources and were used without further purification.

CbzLys(Dansyl)-D-Ala-D-AlaOTMSE (2). A solution of 525 mg(1.33 mmol) of Cbz-D-Ala-D-AlaOTMSE in 8 mL of MeOH was treated with 150 mg of 10% Pd/C and hydrogenated at 40 psi for 2 h. The mixture was diluted with 10 mL of CH_2Cl_2 , dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The crude amine was combined with 650 mg(1.27 mmol) of CbzLys(Dansyl)OH, 200 mg(1.5 mmol) of HOBT, and 780 μ L(4.5 mmol) of DIEA, dissolved in 8 mL of 1:1 CH_2Cl_2 –THF, and then treated with 620 mg(1.4 mmol) of BOP. The reaction was stirred for 1.5 h at ambient temperature and then applied to a silica gel column. Elution with 1:1 ether–hexanes and then ether afforded, after removal of solvent, 890 mg(93%) of the above protected tripeptide **2** as a pale yellow foam. 1H NMR ($CDCl_3$, 300 MHz): δ 8.50 (d, 1H, J = 8.4 Hz), 8.25 (d, 1H, J = 8.4 Hz), 8.19 (d, 1H, J = 7.3 Hz), 7.45–7.53 (m, 2H), 7.24–7.30 (m, 5H), 7.13 (d, 1H, J = 7.7 Hz), 6.76 (d, 1H, J = 7.4 Hz), 6.70 (d, 1H, J = 7.4 Hz), 5.42 (d, 1H, J = 7.7 Hz), 5.06–5.15 (m, 3H), 4.41–4.49 (m, 2H), 4.17 (t, 2H, J = 7.6 Hz), 4.00–4.05 (m, 1H), 2.78–2.85 (m, 8H), 1.20–1.58 (m, 12H), 0.96 (t, 2H, J = 8.6 Hz), 0.00 (s, 9H). HRMS for $C_{37}H_{54}N_5O_8SSi$: calcd $[M + H^+]$ 756.346, found 756.346.

Cbz- γ -D-Glu(OTMSE)Lys(Dansyl)-D-Ala-D-AlaOTMSE (3). CbzLys(Dansyl)-D-Ala-D-AlaOTMSE (756 mg, 1.0 mmol) was deprotected and coupled to Cbz-D-Glu(OH)OTMSE using the conditions described above to afford 630 mg(64%) of the protected tetrapeptide **3** as a pale yellow foam. 1H NMR ($CDCl_3$, 300 MHz): δ 8.50 (d, 1H, J = 8.4 Hz), 8.26 (d, 1H, J = 8.8 Hz), 8.19 (dd, 1H, J = 7.3, 1.1 Hz), 7.45–7.54 (m, 2H), 7.25–7.30 (m, 5H), 7.20 (d, 1H, J = 7.0 Hz), 7.15 (d, 1H, J = 7.7 Hz), 6.82 (d, 1H, J = 7.3 Hz), 6.55 (d, 1H, J = 7.3 Hz), 5.72 (d, 1H, J = 8.4 Hz), 5.34 (t, 1H, J = 7 Hz), 5.06 (s, 2H), 4.12–4.48 (m, 8H), 2.86 (s, 6H), 2.79–2.87 (m, 2H), 2.20–2.40 (m, 3H), 1.20–1.58 (m, 12H), 1.36 (d, 6H, J = 6.9 Hz), 1.34–1.91 (m, 7H), 0.92–1.01 (m, 4H), 0.00 (s, 18H). HRMS for $C_{47}H_{73}N_6O_{11}SSi_2$: calcd $[M + H^+]$ 985.460, found 985.457.

CbzAla- γ -D-Glu(OTMSE)Lys(Dansyl)-D-Ala-D-AlaOTMSE (4). Cbz- γ -D-Glu(OTMSE)Lys(Dansyl)-D-Ala-D-AlaOTMSE (600 mg, 0.61 mmol) was deprotected and coupled to CbzAlaOH using the conditions described above to afford 530 mg(82%) of the protected pentapeptide **4** as a foam. 1H NMR (CD_3OD , 300 MHz): δ 8.51 (d, 1H, J = 8.4 Hz), 8.30 (d, 1H, J = 8.4 Hz), 8.20 (d, 1H, J = 7.3 Hz), 8.10–8.14 (m, 2H), 7.89 (d, 1H, J = 6.2 Hz), 7.48–7.56 (m, 2H), 7.20–7.32 (m, 7H), 5.04 (s, 2H), 4.22–4.38 (m, 3H), 3.99–4.20 (m, 6H), 2.83 (s, 6H), 2.78 (t, 2H, J = 6.4 Hz), 2.14–2.22 (m, 3H), 1.80–1.90 (m, 1H), 1.42–1.50 (m, 2H), 1.20–1.38 (m, 13H), 0.90–0.99 (m, 4H), 0.00 (s, 18H). HRMS for $C_{50}H_{78}N_7O_{12}SSi$: calcd $[M + H^+]$ 1056.497, found 1056.495.

Peptidodisaccharide (6). CbzAla- γ -D-Glu(OTMSE)Lys(Dansyl)-D-Ala-D-AlaOTMSE (450 mg, 0.43 mmol) was deprotected and coupled to disaccharide **5** using the conditions described above to afford 549 mg(80%) of peptidodisaccharide **6**. 1H NMR (CD_3OD , 300 MHz): δ 8.50 (d, 1H, J = 8.4 Hz), 8.29 (d, 1H, J = 8.5 Hz), 8.13 (d, 1H, J = 7.3 Hz), 7.49–7.56 (m, 2H), 7.22 (d, 1H, J = 7.4 Hz), 6.06 (d, 1H, J = 3.7 Hz), 5.17 (t, 1H, J = 9.9 Hz), 4.93 (t, 1H, J = 9.7 Hz), 4.58 (d, 1H, J = 9.1 Hz), 3.72–4.46 (m, 19H), 3.62 (dd, 1H, J = 10.4, 8.3 Hz), 2.83 (s, 6H), 2.77 (t, 2H, J = 6.4 Hz), 2.26 (t, 2H, J = 6.6 Hz), 1.70–2.20 (m, 6H), 2.04 (s, 3H), 2.03 (s, 3H), 1.96 (s, 3H), 1.94 (s, 3H), 1.92 (s, 3H), 1.88 (s, 3H), 1.84 (s, 3H), 1.20–1.63 (m, 16H), 0.92–0.98 (m, 4H), 0.00 (s, 9H), –0.02 (s, 9H). HRMS for $C_{50}H_{112}N_9O_{27}SSi_2$: calcd $[M + H^+]$ 1610.693, found 1610.692.

Alcohol (7). A solution of 510 mg(0.317 mmol) of peptidodisaccharide **6** in 5 mL of THF and 0.1 mL of water was treated with 0.07 mL(0.64 mmol) of benzylamine. The solution was stirred 16 h at ambient temperature and then treated with an additional 0.07 mL of benzylamine. The solution was stirred for 5 h, concentrated under reduced pressure, and chromatographed on silica gel (elution with 1:1 toluene–acetone then 2:1 toluene–acetone) to afford 289 mg (58%) of alcohol **7** as a foam. 1H NMR (CD_3OD , 300 MHz): δ 8.55 (d, 1H, J = 8.8 Hz), 8.14–8.28 (m, 3H), 8.02 (t, 1H, J = 7.0 Hz), 7.55–7.61 (m, 2H), 7.26 (d, 1H, J = 7.4 Hz), 5.27 (t, 1H, J = 9.9 Hz), 5.13 (d, 1H, J = 3.3 Hz), 4.96 (t, 1H, J = 9.7 Hz), 4.66 (d, 1H, J = 8.4 Hz), 3.62–4.53 (m, 20H), 2.88 (s, 6H), 2.83 (t, 2H, J = 6.6 Hz), 2.30 (t, 2H, J = 6.0 Hz), 1.70–2.24 (m, 4H), 2.08 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.97 (s, 3H), 1.93 (s, 3H), 1.92 (s, 3H), 1.24–1.63 (m, 16H), 0.96–1.04 (m, 4H), 0.05 (s, 9H), 0.04 (s, 9H).

Phosphodisaccharide (8). To a stirred, cooled (0 °C) solution of 270 mg (0.17 mmol) of alcohol **7** in 5 mL of CH_2Cl_2 was added 46 mg (0.66 mmol) of tetrazole followed by 135 mL (0.43 mmol) of *N,N*-diisopropylidibenzylphosphoramidite. The solution was stirred for 1 h at 0 °C, treated with 300 mg (1.1 mmol) of *m*-CPBA, and warmed to ambient temperature with stirring over 1 h. The reaction was diluted with EtOAc, washed twice with aq. $NaHCO_3$ and once with brine, and dried over $MgSO_4$. This solution was concentrated and filtered through silica gel (elution with 10% MeOH–EtOAc) to afford the dibenzyl phosphotriester as an oil. The phosphotriester was dissolved in 5 mL of EtOH, treated with 50 mg of 10% palladium on carbon, and stirred for 1 h under an atmosphere of H_2 . The mixture was diluted with CH_2Cl_2 , treated with $MgSO_4$, and filtered. The filtrate was treated with 100 μ L (0.72 mmol) of Et_3N and concentrated to afford 210 mg (70%) of phosphodisaccharide **8** an off-white solid. 1H NMR (CD_3OD , 300 MHz): δ 8.55 (d, 1H, J = 8.8 Hz), 8.35 (d, 1H, J = 8.4 Hz), 8.18 (d, 1H, J = 7.3 Hz), 7.55–7.62 (m, 2H), 7.26 (d, 1H, J = 7.7 Hz), 5.19–5.49 (m, 4H), 3.60–4.98 (m, 20H), 3.16 (q, 6H, J = 7.3 Hz), 2.88 (s, 6H), 2.84 (t, 2H, J = 6.4 Hz), 1.80–2.47 (m, 24H), 1.26–1.77 (m, 16H), 1.30 (t, 9H, J = 7.4 Hz), 0.96–1.02 (m, 4H), 0.04 (s, 9H), 0.00 (s, 9H). HRMS for $C_{69}H_{111}N_9O_{29}SPSi$: calcd $[M + H^+]$ 1648.649, found 1648.649.

***N*-Dansyl Lipid II (9).** A suspension of 82 mg (0.045 mmol) of phosphate **8** in 5 mL of dry toluene was evaporated to dryness, dissolved in 0.4 mL of dry DMF, and treated with 38 mg(0.24 mmol) of carbonyldiimidazole. The solution

was stirred 4.5 h, treated with 17 μ L (0.42 mmol) of MeOH, and stirred an additional 30 min. To this solution was added 34 mg (0.036 of undecaprenyl phosphate triethylammonium salt in 0.4 mL of CH_2Cl_2). The solution was stirred 48 h at ambient temperature, concentrated under reduced pressure, and partially purified (some undecaprenyl phosphate could not be removed) by anion-exchange chromatography (Bio-Rad High Q, gradient elution with NH_4OAc , 0.01–2 M in MeOH). The appropriate fractions were lyophilized and dissolved in 0.5 mL of DMF. This solution was treated with 1 mL of 1 M TBAF in THF, and the reaction was allowed to stir 24 h at ambient temperature. The solution was concentrated under reduced pressure and purified from excess TBAF by filtration through anion-exchange resin. The appropriate fractions were lyophilized, dissolved in 1 mL of CH_2Cl_2 , and treated with 0.36 mL of 0.5 M NaOMe in MeOH. The solution was stirred 2 h, partially concentrated, and diluted with 10 mM methanolic NH_4OAc . Anion-exchange chromatography afforded, after lyophilization, 13 mg (17% from undecaprenyl phosphate triethylammonium salt) of *N*-dansyl Lipid II as a white powder. ^1H NMR (CD_3OD , 300 MHz): δ 8.55 (d, 1H, $J = 8.5$ Hz), 8.35 (d, 1H, $J = 8.7$ Hz), 8.18 (d, 1H, $J = 6.2$ Hz), 7.54–7.62 (m, 2H), 7.27 (d, 1H, $J = 7.7$ Hz), 5.50–5.54 (m, 1H), 5.43 (t, 1H, $J = 7.7$ Hz), 5.04–5.19 (m, 10H), 4.81–4.91 (m, 2H), 4.28–4.57 (m, 8H), 3.80–4.17 (m, 5H), 3.61–3.73 (m, 4H), 3.50 (dd, 1H, $J = 10.2$, 8.1 Hz), 3.24 (t, 1H, $J = 8.4$ Hz), 2.88 (s, 6H), 2.83 (t, 2H, $J = 6.2$ Hz), 1.79–2.38 (m, 50H), 1.58–1.72 (m, 40H), 1.28–1.55 (m, 16H). HRMS for $\text{C}_{106}\text{H}_{166}\text{N}_9\text{O}_{28}\text{SP}_2$: calcd $[M - \text{H}^-]$ 2107.104, found 2107.106.

RESULTS AND DISCUSSION

Kinetic Characterization of Glycosyltransferase Activity. Class A, high-MW PBPs such as PBP1b from *E. coli* are bifunctional enzymes that are capable of catalyzing the two ultimate steps in bacterial cell wall biosynthesis, glycosyltransfer and transpeptidation (11, 12). Because of the critical nature of these steps, both activities are considered viable targets for antimicrobial intervention. Indeed, penicillin (and all β -lactam drugs) is bactericidal because of ability to inhibit transpeptidation (13).

Natural products that inhibit the glycosyltransfer reaction are also known. Flavomycin (moenomycin, bambermycin) is a lipopolysaccharide structural analogue of Lipid II, which is believed to be a competitive inhibitor of PBP1b (14). Vancomycin is a glycopeptide antibiotic, which inhibits both enzymatic activities of PBP1b, by acting as an artificial receptor to the D-Ala-D-Ala portion of Lipid II (15). Both compounds, however, have problems associated with their clinical use; neither is orally bioavailable, and vancomycin has been met with resistance. To overcome these problems, recent attempts have been directed at preparing analogues of both flavomycin (5) and vancomycin (16, 17). Though these efforts have been met with modest successes, it is desirable to discover new chemical entities as leads for the development of antimicrobial drugs.

The discovery of new antibiotics against class A high MW PBPs has been hampered by lack of mechanistic information about these enzymes. This, in turn, is due to the difficulty in obtaining a ready supply of substrate and recombinant enzyme, as well as lack of an informative and facile assay system. Significantly, each of these issues has recently been

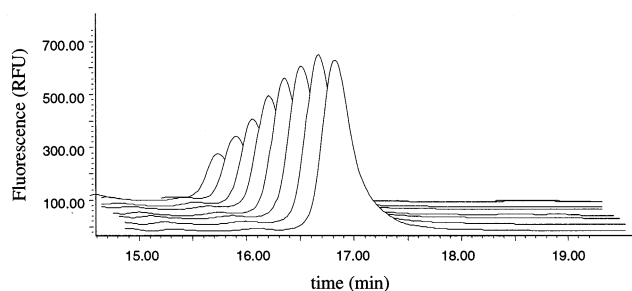


FIGURE 1: Measurement of glycosyltransferase reaction by HPLC. Lipid II ($2\mu\text{M}$) was incubated at 30 $^\circ\text{C}$ with PBP1b (60 nM) in 50 mM HEPES buffer (pH 7.5) containing 0.085% decyl PEG, 10% DMSO, and 10 mM MgCl_2 . Aliquots were removed, labeled, and injected on an anion-exchange HPLC column. Labeled Lipid II elutes at 16–17 min. Samples are at $t = 0, 5, 10, 15, 20, 30, 45$, and 60 min (time increasing front to back).

resolved. We (6) and others (9) have recently reported total chemical syntheses of Lipid II, and Walker and co-workers have achieved a chemoenzymatic (7) synthesis of the same substrate. Importantly, both of these methodologies are capable of producing milligram quantities of substrate, sufficient for mechanistic studies. The production of recombinant PBP1b was accomplished by Ghuysen and co-workers (8), and we reported an HPLC based assay to easily follow the in vitro enzymatic reaction (6). Utilizing these new tools, we report the first analysis of the glycosyltransferase activity of PBP1b.

The enzymatic consumption of substrate by PBP1b can be monitored by labeling Lipid II in aliquots removed from the in vitro reaction, followed by HPLC analysis. Lipid II is labeled with fluorescamine, which reacts specifically with the sole primary amino group of the substrate. With this methodology, the timecourse of the reaction can be easily monitored and quantitated (Figure 1). This in vitro reaction requires only buffer, detergent, enzyme, substrate, and Mg^{2+} to observe activity. This system is much simpler than those involving whole cell preparations (5, 7), making mechanistic interpretations more straightforward. To better understand the reaction catalyzed by PBP1b, each of the assay components was systematically varied and their effect on enzyme activity noted.

Previously, studies of PBP1b reported an enhancement of activity when DMSO was added to the reaction (8). To determine if this same effect could be observed in our in vitro system, the amount of DMSO was varied from 0% to 10% (Figure 2). The observed rate was found to increase linearly up to $\sim 5\%$, at which point further addition had little effect. To determine whether this effect was more prevalent on binding or catalysis, the dependence of steady-state velocities on substrate concentration was obtained in both the absence and presence of DMSO (Figure 3). The effect of DMSO appears on k_{cat} , and not K_M . With DMSO present, k_{cat} is $0.11 \pm 0.01 \text{ s}^{-1}$ (vs $0.029 \pm 0.002 \text{ s}^{-1}$ without DMSO), and k_{cat}/K_M is $(4.4 \pm 0.7) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, comparable to previous estimates of activity (8). The observed enhancement in rate may be due to the greater solubility of Lipid II in aqueous solutions containing DMSO, which in turn can result in faster exchange of the substrate into micelles containing enzyme. In previous studies with cholesterol, the exchange rate between micelles was shown to increase ~ 3 -fold in the presence of 10% (v:v) DMSO (18).

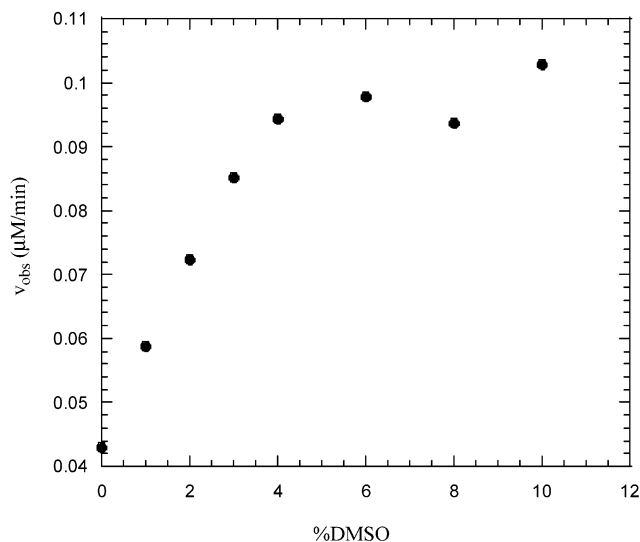


FIGURE 2: Effect of DMSO on glycosyltransferase activity. DMSO was uniformly varied between 0% and 10%, and the effect on observed velocity (v_{obs}) was measured. Reactions were carried out at 30 °C with PBP1b (20 nM) in 50 mM HEPES buffer (pH 7.5) containing 0.085% decyl PEG and 10 mM MgCl_2 .

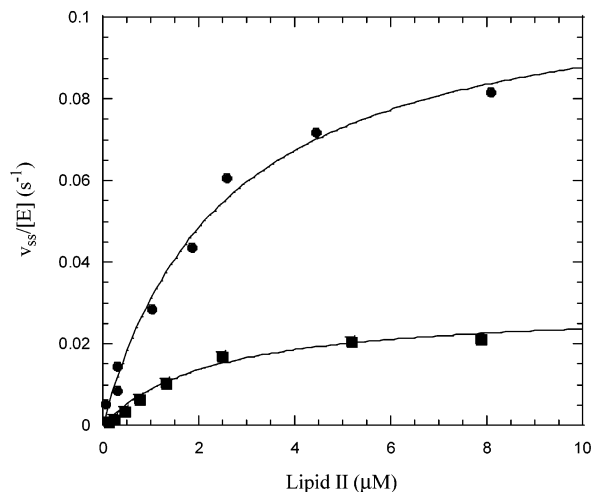


FIGURE 3: Effect of DMSO on steady-state velocities of PBP1b. The dependence of steady-state velocity on substrate concentration was determined in the absence (■) and presence of 10% DMSO (●). Reactions were carried out at 30 °C, with PBP1b (20 nM) in 50 mM HEPES buffer (pH 7.5) containing 0.085% decyl PEG, and 10 mM MgCl_2 . The concentration of Lipid II was varied between 0.1 and 8 μM. Curve fits (as well as their associated errors) were generated by fitting data to the equation: $v_{\text{ss}}/[E] = k_{\text{cat}}[\text{sub}]/(K_M + [\text{sub}])$ using Kaleidagraph.

We next investigated the effect of detergent on the glycosyltransferase activity. The concentration of detergent in the reaction was varied from zero up to several 100-fold the critical micelle concentration (CMC). Concentrations below the CMC (~ 100 μM) resulted in no observable activity, while those above the CMC increasing micelle concentration resulted in inhibition of activity (Figure 4A). The data were fit to a model for simple, competitive inhibition, resulting in a K_i of 50 μM for detergent micelle. This type of behavior has been previously observed with other detergent bound enzymes (19) and can be understood with a simple physical model. In a given assay, on average micelles are either empty or contain enzyme or substrate. As the concentration of detergent increases above the CMC,

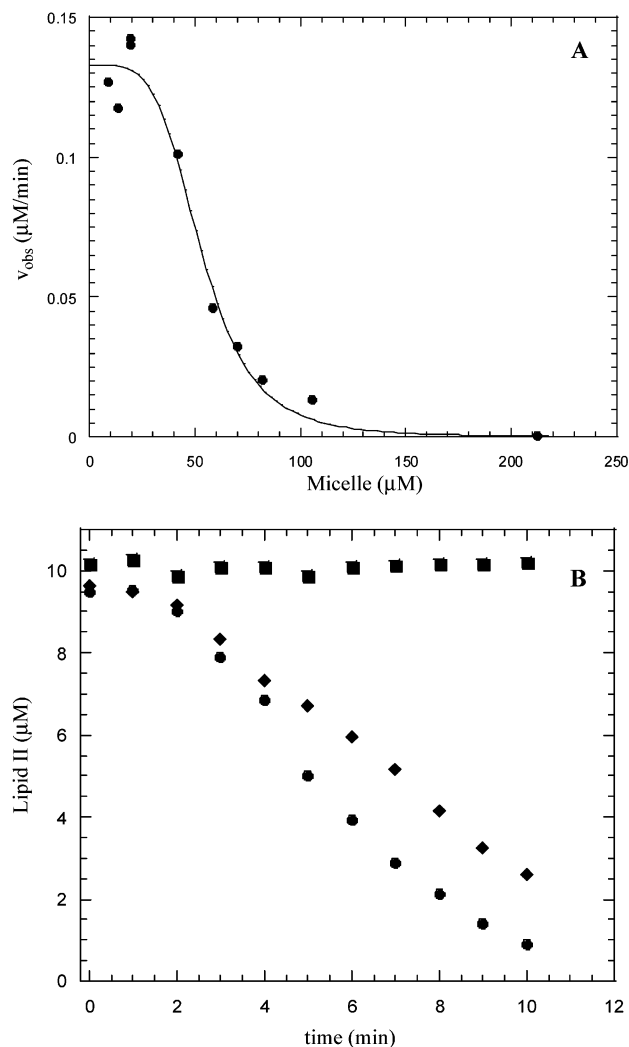


FIGURE 4: Effect of detergent on activity. (A) The concentration of decyl PEG was varied from $5\times$ to $>200\times$ CMC. The data were fit to a simple model for reversible inhibition, $v_{\text{obs}} = v_{\text{obs}}(\text{max})/(1 + ([\text{micelle}]/K_i)^n)$, where n is the Hill slope. From this fit, $K_i = 53 \pm 3$ μM, and $n = 4.4 \pm 0.8$. (B) Timecourses for glycosyltransfer reactions catalyzed by PBP1b (20 nM) were recorded at 20 (●) and 200 μM (■) detergent micelle. PBP1b (200 nM) was incubated for 10 min in 200 μM detergent micelle and then diluted 10-fold into an assay to final concentrations of 20 nM PBP1b and 20 μM micelle (◆).

the number of micelles containing enzyme and substrate remain the same, whereas empty micelles increase in number. As interfacial catalysis is proposed to involve fusion of micelles containing enzyme and substrate to allow for productive encounter, the increasing presence of empty micelles results in an increase in nonproductive fusions.

One aspect of the data in Figure 4A that is at odds with this model is the (Hill) slope of about 4, which in the simple case of competitive inhibition should be equal to unity. The greater than expected slope may be due to cooperativity involved in the inhibition process, or to a different, irreversible inhibition process (such as enzyme denaturation). To address these possibilities, the reversibility of the detergent inhibition was investigated. At 20 μM detergent micelle, enzyme activity is near its maximum value, while at 200 μM, little activity is observed (Figure 4B). PBP1b was subsequently preincubated at 200 μM micelle and then diluted into an assay mixture at a final concentration of 20

Table 1: Relative Rates of Glycosyltransfer Reaction with Various Metals^a

metal	v_{obs} ($\mu\text{M}/\text{min}$)	k_{rel}
Mg ²⁺	0.10	1
Co ²⁺	0.12	1.2
Ni ²⁺	0.28	2.8
Mn ²⁺	0.38	3.8
Ca ²⁺	0.58	5.8
None	<0.01	<0.1
Mg ²⁺ + EDTA	<0.01	<0.1

^a Reactions were carried out in 50 mM HEPES, pH 7.5, with 10% DMSO, 0.085% decyl PEG, 10 mM metal, 20 nM PBP1b, and 5 μM Lipid II.

μM . After dilution, nearly all of the original activity is regained (Figure 4B), indicating that the inhibition by detergent is reversible and that the observed inhibition is not simply due to protein denaturation.

We next turned our attention to the effect of metal on the glycosyltransferase activity. Historically, only Mg²⁺ has been used to study this reaction, though it has been reported that enzyme lacking metal has little activity (20). A range of other divalent metals was tested, and in all cases activity was either equal to or exceeded the observed rate with Mg²⁺ (Table 1). The addition of Ca²⁺ led to the greatest increase in rate, around 6-fold. Without the addition of any metals or with the addition of EDTA to Mg²⁺-containing reactions, no activity was observed. Metals were tested at 5, 10, and 20 mM, with no detectable changes in rate; thus, the observed alterations in rates are not due to relative differences in binding constants.

To further probe the effect of metal on the enzymatic reaction, the dependence of steady-state velocity on substrate concentration was determined with both Mg²⁺ and Ca²⁺ present (Figure 5). The nearly 6-fold increase in rate with Ca²⁺ appears to be manifested solely in k_{cat} , which increases from 0.11 to $0.64 \pm 0.06 \text{ s}^{-1}$, while $k_{\text{cat}}/K_{\text{M}}$ increases to (2.5

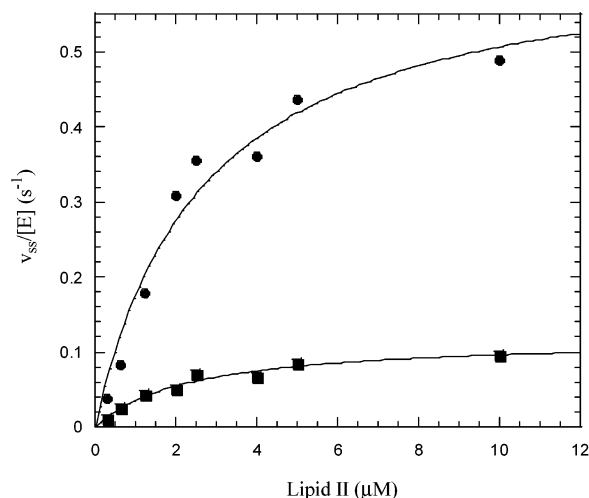
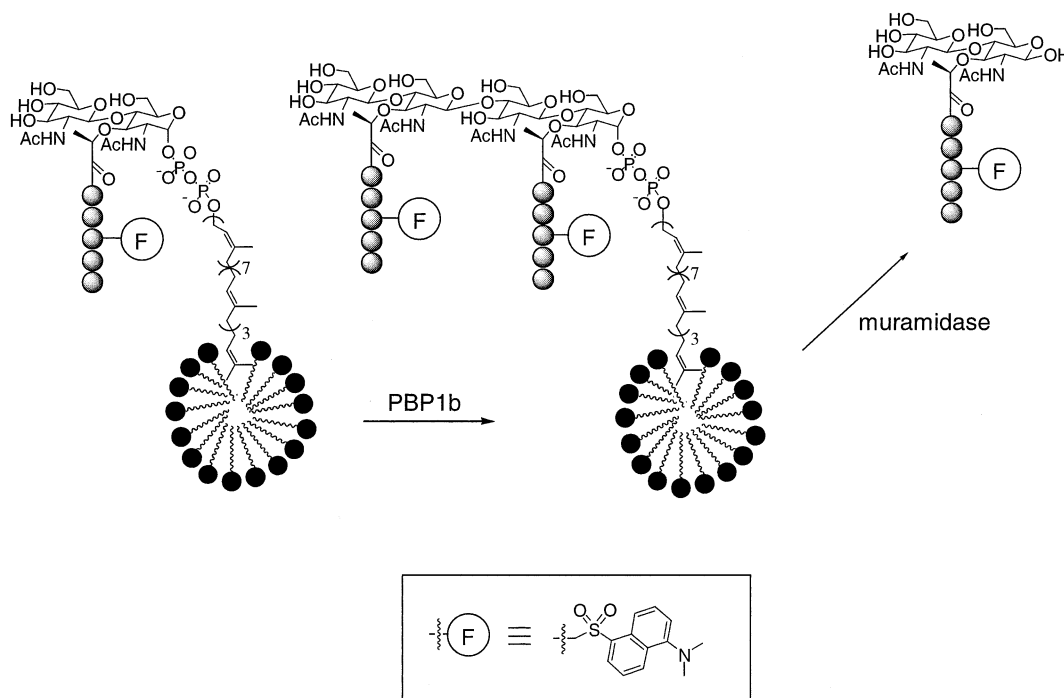


FIGURE 5: Effect of metal on steady-state velocities of PBP1b. The dependence of steady-state velocity on substrate concentration was determined with 10 mM Mg²⁺ (■) and 10 mM Ca²⁺ (●). Reactions were carried out at 30 °C, with PBP1b (20 nM) in 50 mM HEPES buffer (pH 7.5) containing 0.085% decyl PEG and 10% DMSO. The concentration of Lipid II was varied between 0.3 and 10 μM . Curve fits (as well as their associated errors) were generated by fitting data to the equation: $v_{\text{ss}}/[E] = k_{\text{cat}}*[\text{sub}]/(K_{\text{M}} + [\text{sub}])$ using Kaleidagraph.

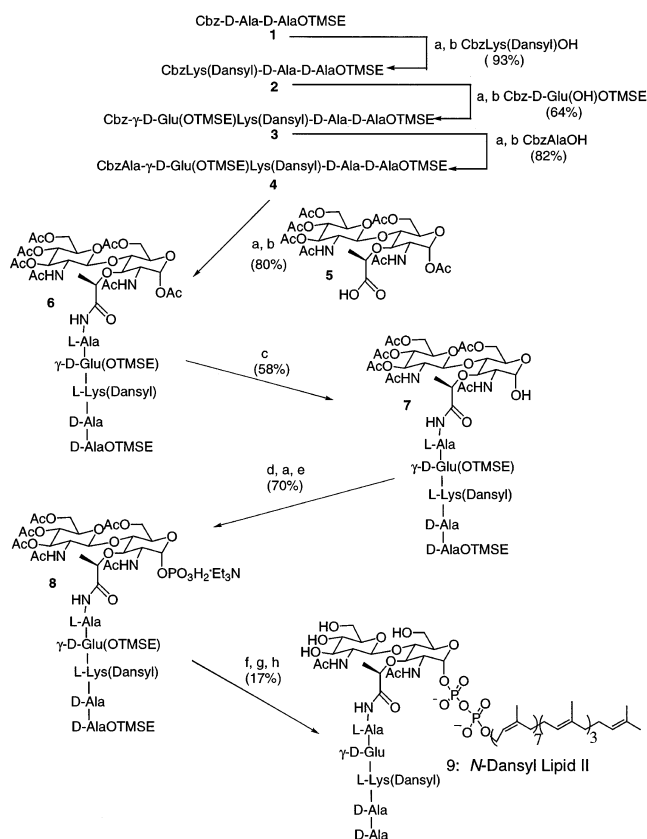
$\pm 0.6) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. Thus, the metal in PBP1b appears to play a catalytic role in the glycosyltransfer reaction.

Development of Continuous, Fluorescent Assay. While the HPLC assay proved valuable in obtaining basic kinetic parameters for the glycosyltransferase reaction, a continuous assay was desired to allow for more facile collection of data, including screens of drug libraries. It had been previously shown with farnesyl:protein transferase (FPTase) that reactions involving changes in the hydrophobic environment of specific substrates could be utilized as a basis for fluorescence assays (21), as the quantum yield of some fluorophores

Scheme 2: Continuous Fluorescence Assay for Glycosyltransferase Activity



Scheme 3: Synthesis of Dansylated Lipid II: (A) H_2 , Pd/C, and EtOH; (B) BOP, HOBT, DIEA, THF, and CH_2Cl_2 ; (C) Benzylamine, THF, and H_2O ; (D) (i) $(PhCH_2O)_2PN(i-Pr)_2$, Tetrazole, and CH_2Cl_2 and (ii) *m*-CPBA; (E) Et_3N ; (F) (i) CDI and DMF, (ii) MeOH, and (iii) Undecaprenyl phosphate and CH_2Cl_2 ; (G) TBAF and THF; (H) NaOMe, MeOH, and CH_2Cl_2



increases significantly in hydrophobic surroundings. In this vein we envisioned a coupled assay in which a fluorescently labeled Lipid II derivative, initially partitioned into detergent, would be polymerized by PBP1B into un-cross-linked cell wall. This product would subsequently be hydrolyzed by muramidase to form soluble, labeled monomer, which would result in a detectable decrease in fluorescence intensity (Scheme 2). The ability of muramidase to hydrolyze the polymeric products of the *in vitro* reactions of PBP1b and Lipid II (but not Lipid II itself) has been previously demonstrated with unlabeled material (6), and it was hoped that a similar result could be obtained with a substrate containing a label remote from the hydrolyzed polysaccharide bond. A dansyl label was initially investigated, as it had been successfully employed with the FPTase system.

The dansylated analogue was synthesized, with the fluorescent group appended via a sulfonamide linkage onto the ϵ -amino group of the lysine side chain of Lipid II (Scheme 3). When dansyl-Lipid II was incubated with enzyme in the presence of muramidase, a time-dependent decrease in fluorescence intensity was observed (Figure 6). As with unlabeled material, the addition of flavomycin, a known glycosyltransferase inhibitor (12), greatly decreased the observed activity (Figure 6). To confirm that the observed decrease in fluorescence intensity was the result of consumption of dansyl-Lipid II, the reaction was monitored by anion-exchange HPLC. An excellent correspondence (Figure 7) was

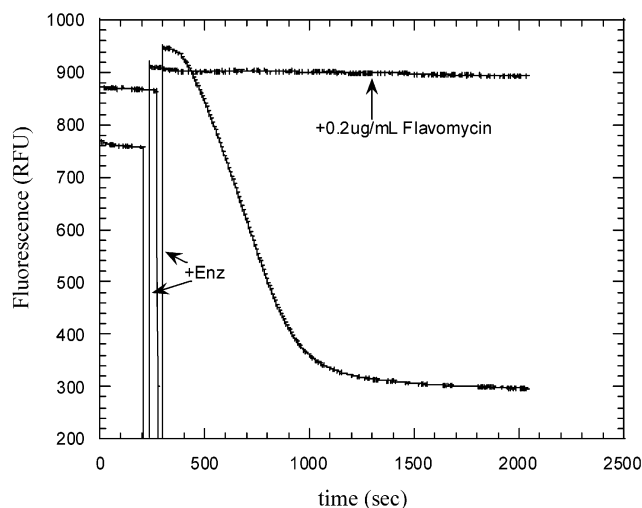


FIGURE 6: Timecourse for continuous fluorescent PBP1b assay. Dansyl-Lipid II ($10 \mu M$) was incubated in buffer (50 mM HEPES, pH 7.5, 0.085% decyl PEG) containing 10% DMSO, 10 mM $CaCl_2$, and $0.1 \mu g$ of *N*-acetylmuramidase (*S. globisporus*) at $30^\circ C$ and either without or with flavomycin ($0.2 \mu g/mL$) as indicated. After observing a stable baseline, PBP1b ($18 nM$) was added (as indicated by +Enz in figure), and the change in fluorescence was monitored for ~ 30 min.

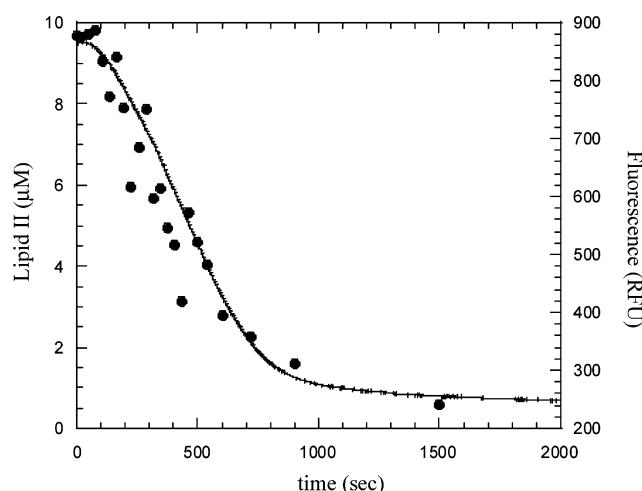


FIGURE 7: Comparison of HPLC and fluorescence assays. The consumption of dansyl-Lipid II was assayed in buffer (50 mM HEPES, pH 7.5, 0.085% decyl PEG) containing 10% DMSO, 10 mM $CaCl_2$, and $0.1 \mu g$ of *N*-acetylmuramidase (*S. globisporus*) at $30^\circ C$. The reaction was monitored for ~ 30 min, after the addition of PBP1b ($18 nM$), either by fluorescence intensity (solid line) or by anion-exchange HPLC (●).

found between the two assay formats. It is important to note that in the fluorescence assay complete turnover of substrate does not result in complete disappearance of signal, as the product of the coupled enzyme system (dansyl-labeled monomer) still retains some intrinsic fluorescence.

An interesting feature of the reaction timecourse displayed in Figures 6 and 7 is the lengthy pre-steady state lag in consumption of Lipid II. This result was found regardless of whether the reaction was initiated with the addition of either enzyme or substrate (data not shown). Because PBP1b is a polymerase, it may be imagined that during the steady state, the enzyme is acting in a processive manner, adding Lipid II units to a growing polysaccharide chain. However, when enzyme is initially added to our *in vitro* reaction, it

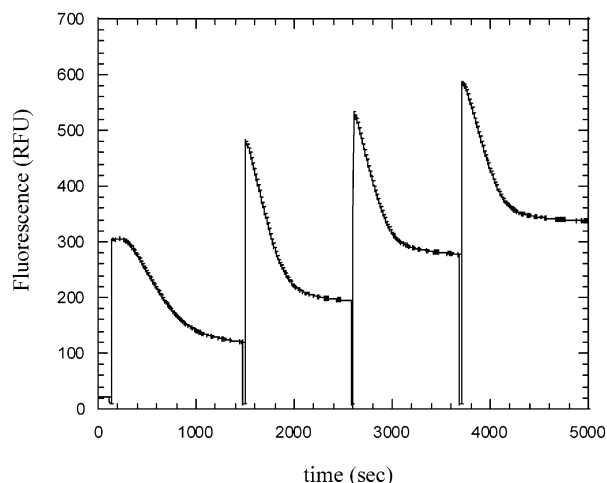


FIGURE 8: Lag kinetics of PBP1b. To an assay at 30 °C containing buffer (50 mM HEPES, pH 7.5, 0.085% decyl PEG), 10% DMSO, 10 mM CaCl_2 , 18 nM PBP1b, and 0.1 μg of *N*-acetylmuramidase was added 3 μM dansyl-Lipid II. After observing completion of the enzymatic reaction (~ 20 min), another 3 μM aliquot of substrate was added. This procedure was repeated twice more.

lacks a polymeric substrate to extend and presumably must synthesize a short oligosaccharide primer by condensing individual molecules of Lipid II. This *de novo* process of synthesis is expected to be less efficient than the steady-state process, since PBP1b is likely to have an extended cleft for binding interactions with the polysaccharide which cannot be fully utilized by Lipid II. Though no crystal structures are available for PBP1b or similar enzymes from other organisms, a recent 1.2 Å structure of a class B high MW PBP (from *Streptomyces* sp.) has been reported, revealing an extended saccharide-binding cleft (22).

To test our hypothesis about the cause of the observed pre-steady-state lag, several successive aliquots of substrate were added to a single reaction. After the first aliquot is consumed, subsequent additions of substrate should quickly lead into a steady-state kinetic regime because the presence of the polysaccharide product would obviate the need for any *de novo* synthesis. With the first added aliquot of substrate, a clear lag is observed (Figure 8). Beginning with the second addition, however, the lag disappears. This continues to be the case for each subsequent addition of substrate. The lag is not due to effects of the coupled enzyme system, as increasing the concentration of muramidase had no effect on the observed kinetics (data not shown). These results support the idea that the pre-steady-state lag is the result of slow production of a polysaccharide, capable of being extended in a more efficient, processive manner.

It is important to note that the observed lag in our *in vitro* system is not expected to have important biological implications, as most peptidoglycan biosynthesis occurs as extension of preexisting material. Indeed, it is believed that enzymes such as PBP1b act in concert with lytic enzymes as part of a larger complex known as a divisome (2). To initiate processes such as cell division, lytic enzymes hydrolyze intact cell wall, leaving efficient initiation points for new synthesis.

Utilizing the continuous fluorescent assay, we investigated the effect of pH on the activity of PBP1b. For k_{cat} a single pK_a of 6.8 ± 0.1 was found (Figure 9A), with a similar result for k_{cat}/K_M (single $\text{pK}_a = 7.0 \pm 0.1$) (Figure 9B). The limiting value at high pH for k_{cat} (0.75 s^{-1}) is similar to that found

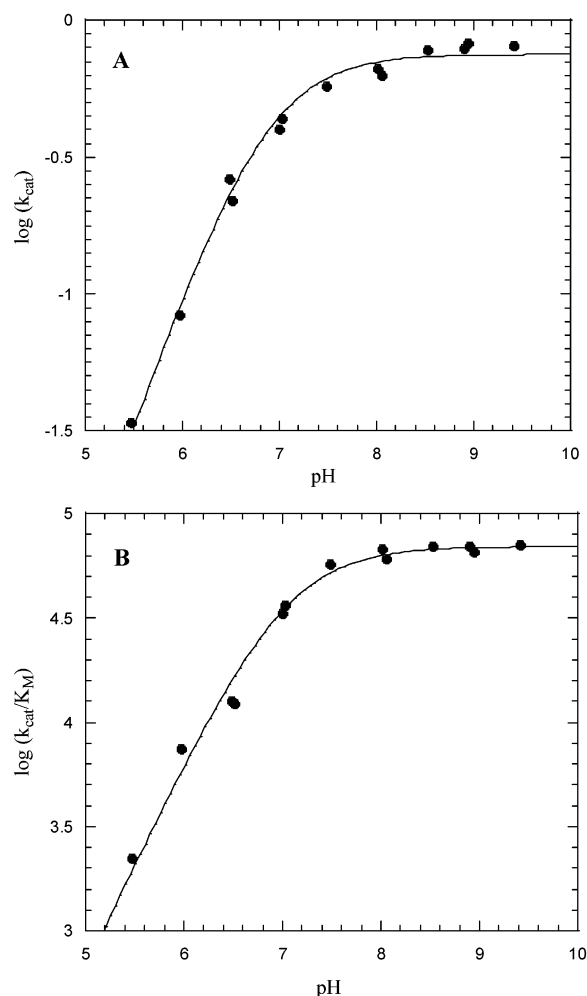
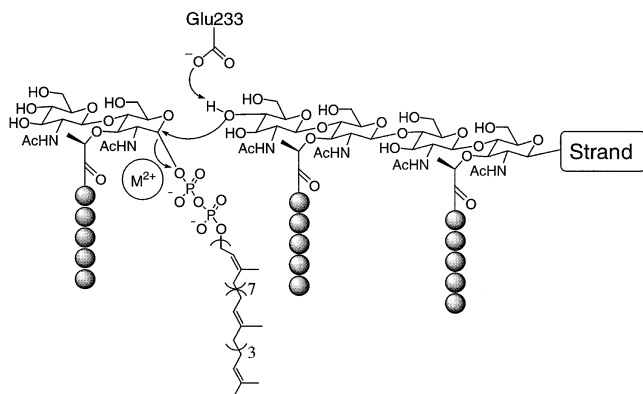


FIGURE 9: Effect of pH on glycosyltransferase activity. k_{cat} (A) and k_{cat}/K_M (B) were measured, varying the pH between 6.5 and 9.5. Assays contained 50 mM buffer, 200 mM NaCl, 0.085% decyl PEG, 10% DMSO, 10 mM CaCl_2 , 10–30 nM PBP1b, 0.1 μg of *N*-acetylmuramidase, and 1–40 μM dansyl-Lipid II. Curve fits (as well as their associated errors) were generated by fitting data to the equation $\log(k_{\text{cat}}) = \log(k_{\text{cat}})_{\text{max}} - \log(1 + 10^{\text{pK}_a - \text{pH}})$ and $\log(k_{\text{cat}}/K_M) = \log(k_{\text{cat}}/K_M)_{\text{max}} - \log(1 + 10^{\text{pK}_a - \text{pH}})$.

for unlabeled Lipid II, while the limiting value for k_{cat}/K_M ($7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$), is severalfold less than the value of $2.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ found with PBP1b containing Ca^{2+} (Figure 5). This reduction likely reflects a slightly lower affinity for the dansylated Lipid II by the enzyme.

No mechanistic details are known about PBP1b and related enzymes from other bacterial sources. Indeed, the current state of mechanistic information about glycosyltransferases in general has been characterized as “in its infancy” (23). On the basis of homology to the well studied and understood glycosidases, two mechanisms are proposed for glycosyltransferases, depending on the stereochemical outcome of the transfer reaction (24). With inverting transferases, the incoming nucleophile from the acceptor displaces the leaving group on the glycosidic donor, resulting in inversion. With retaining enzymes, a double displacement mechanism is proposed, in which an active site aspartate or glutamate is the initial nucleophile, forming a covalent substrate-enzyme complex. Then a second displacement reaction occurs, resulting in the formation of a new saccharide bond, with net retention. For both inverting and retaining glycosidases,

Scheme 4: Proposed Mechanism for PBP1b Glycosyltransferase Activity



a pair of carboxylate residues (either aspartates or glutamates) act as general acids and bases to promote these reactions.

Lipid II contains an α -configuration at the anomeric carbon, while the newly formed saccharide bond in murein is a β -(1,4)-linkage. Thus, this reaction proceeds with inversion of stereochemistry and likely involves direct attack of the growing polysaccharide on Lipid II. However, On the basis of the observed pH effects with PBP1b, it appears that only a single residue is directly involved in catalysis, acting as an active site base. Coupled with the observed effects of metal on activity, we propose the following mechanism for the glycosyltransfer reaction of PBP1b (Scheme 4). An enzyme residue (most likely an aspartate or glutamate) deprotonates the 4'-hydroxyl group of the terminal residue of the growing saccharide strand, while the active site metal stabilizes through ionic interactions the incipiently formed negative charge on the oxygen of the diphosphate leaving group. In line with other glycosyltransferases and glycosidases, this transition state is likely dissociative, with substantial oxocarbenium character on the glycosyl donor (24). The active site base, or another aspartate/glutamate, may play a role in stabilizing this positively charged species.

Recent experiments involving site directed mutants of PBP1b support this proposed mechanism (8). Three conserved carboxylate residues were identified within the glycosyltransferase domain (Glu233, Asp234, and Glu290) and mutated to their corresponding amide residues. While mutations of Asp234 and Glu290 had modest (<10-fold) effects on catalysis, the conversion of Glu233 \rightarrow Gln resulted in negligible (0.2%) activity.

There also exists precedence in other systems for the mechanism proposed in Scheme 4. α -(1,3)-fucosyltransferase V (FucT V) is an inverting glycosyltransferase, responsible for the synthesis of the ubiquitous polysaccharides Lewis x and sialyl Lewis x. FucT V, one of the few enzymes of this class for which detailed information is available, displays a pH profile in which a single, basic residue was titrated (25). In addition, a Mn^{2+} ion has been implicated in assisting catalysis by stabilizing the nucleotide diphosphate leaving group, via coordination of the phosphate oxygen (25).

This role for the active site metal is also supported by recent crystallographic work with another glycosyltransferase (SpsA), where a Mn^{2+} was found to coordinate both oxygens of the UDP-GlcNAc glycosyl donor (26). It will be interesting to determine whether the use of a divalent metal is

general for all glycosyltransferases, as is the two carboxylate motif for glycosidases.

SUMMARY

An initial kinetic characterization of PBP1b has been carried out, using a simple in vitro system of synthetic substrate and recombinant enzyme. The reaction is sensitive to every facet of the assay mixture, including detergent, metal, and pH. Detergent in excess of the CMC proved to be a reversible inhibitor of the enzyme, presumably by diluting its interactions with substrate micelles. The presence and identity of the metal also had a pronounced effect. Though Mg^{2+} , Co^{2+} , Ni^{2+} , Mn^{2+} , and Ca^{2+} all significantly increased the observed enzymatic reaction, Ca^{2+} proved to have the greatest enhancement. The differences in observed rate were related to changes in k_{cat} , indicating a catalytic role for the metal. Finally, changes in pH indicated that a single, basic residue is involved in catalysis. The effects of metal and pH together suggest a plausible mechanism for PBP1b. The direct attack of Lipid II by the growing polysaccharide chain is catalyzed by deprotonation of the hydroxylic nucleophile by an active site base (likely Glu233), concomitant with stabilization of the leaving group by the active site metal. Future experiments to further elucidate the mechanism of glycosyltransfer by PBP1b will be explored, including kinetic IEs, as well as solvent IEs and proton inventories.

In addition, a new continuous assay was developed, based on changes in the intrinsic fluorescence of dansyl-Lipid II when it is liberated from the hydrophobic micellar environment. This assay will be utilized for future mechanistic work, as well as screening compound libraries for novel chemical entities to be developed for antimicrobial therapeutics.

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