JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Primer Preactivation of Peptidoglycan Polymerases

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Supporting Information

ABSTRACT: Peptidoglycan glycosyltransferases are highly conserved bacterial enzymes that catalyze glycan strand polymerization to build the cell wall. Because the cell wall is essential for bacterial cell survival, these glycosyltransferases are potential antibiotic targets, but a detailed understanding of their mechanisms is lacking. Here we show that a synthetic peptidoglycan fragment that mimics the elongating polymer chain activates peptidoglycan glycosyltransferases by bypassing the rate-limiting initiation step.

Peptidoglycan (PG) is a cross-linked polymer that surrounds bacterial cell membranes and prevents them from rupturing at high internal osmotic pressures. The PG matrix is assembled on the surface of bacterial membranes from a diphospholipid-linked peptidyl disaccharide known as Lipid II (**1**, Figure 1).[1] This disaccharide, composed of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNac), is polymerized via the intermediate Lipid IV to form linear glycan chains that are cross-linked through the peptides attached to the MurNac residues. Since PG is essential for bacterial survival, its biosynthesis is a target for many clinically used antibiotics.[2]

The enzymes that form the PG chains (peptidoglycan glycosyltransferases, or PGTs) are potential antibacterial targets; however, no PGT inhibitors are in the clinic, and a detailed understanding of how these enzymes function is lacking.[3] PGTs are polymerases that convert a single substrate into a long polymer, so dissecting their mechanisms requires the development of approaches to characterize individual steps in the polymerization process. Here we report the use of a modified oligosaccharide substrate to show that the formation of Lipid IV is the rate-limiting step in PG synthesis. We conclude that Lipid IV reorganizes the PGT active site to enable rapid glycan chain polymerization. Substrate analogues such as the one described here may be useful in characterizing the structures of "activated" PGT complexes, which can guide new approaches to inhibitor design.

We have previously shown that PGTs catalyze PG polymer extension by adding disaccharide subunits to the reducing end of the growing chain.[4] The reaction is processive,[5a−d] meaning that elongation occurs *without* release of the product of the previous coupling.[5e,f] Reaction time courses of different PGTs revealed a prolonged lag phase[6] that could be due to a slow conformational rearrangement of the enzyme to an active form[7a,b] and/or a slow first coupling step.[7c−e] We reasoned that if the formation of Lipid IV, the product of the first coupling step, is rate-limiting, then the addition of Lipid IV should accelerate the reaction. In order to test this prediction, we synthesized Lipid IV (**3**, Figure 1b) but found that PGTs utilize it as a substrate even in the absence of Lipid II.[5a,8] Therefore, we developed an approach to block the nonreducing end of **3** through enzymatic attachment of galactose by GalT, producing **4**.[4,9]

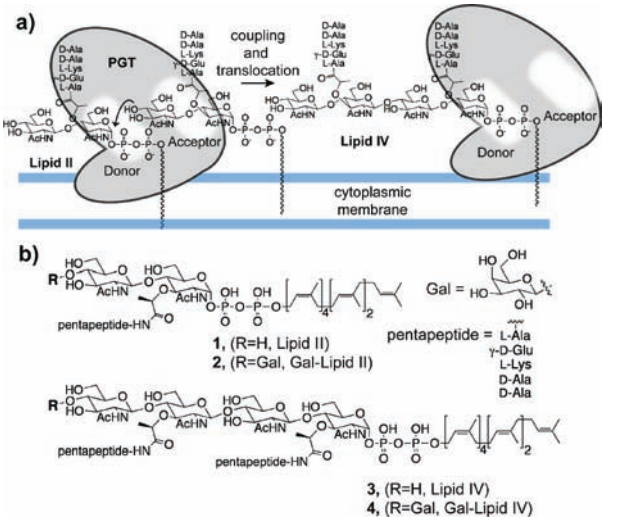

Figure 1. Lipid II polymerization by peptidoglycan glycosyltransferases (PGTs). (a) Schematic depicting the two PGT binding sites. The acceptor site binds the monomer Lipid II, and after the initial coupling of two monomers, the donor site binds the elongating polymer. (b) Chemical structures of synthetic Lipid II and Lipid IV derivatives.

Compound **4** is incapable of reacting with itself but is incorporated into nascent (non-cross-linked) PG at the nonreducing terminus.[4] Since **4** functions as a "donor-only" substrate that mimics the product of the first coupling step of PGTs, we tested its effect on the reaction rate of *Escherichia coli* PBP1A, which contains an N-terminal PGT domain and a C-terminal transpeptidase (TP) domain.[5a,8] After the enzyme was incubated for 20 min with compound **4** and radiolabeled **1**[10] was added, the reaction mixtures were incubated for varying periods of time and then analyzed by paper chromatography, which separates the polymeric product from **1** and short oligosaccharides.[6b,11] Unlike the control reaction, there was no lag phase in the presence of **4**, and the reaction rate was ∼4-fold higher (Figure 2a).

Received: March 29, 2011
Published: May 13, 2011

dx.doi.org/10.1021/ja2028712 | *J. Am. Chem. Soc.* 2011, 133, 8528–8530

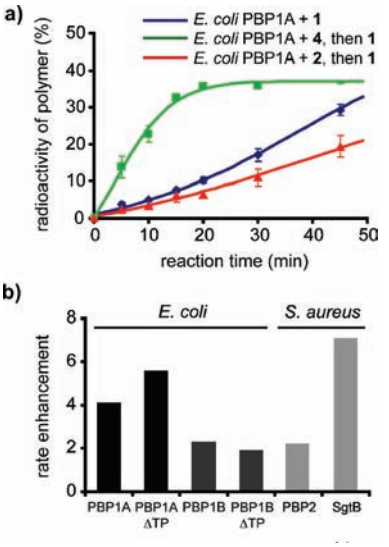

Figure 2. Activation of PGTs using a PG fragment. (a) Time course of polymerization of radiolabeled Lipid II (4 μM) by *E. coli* PBP1A (20 nM) without (blue) and with preincubation with blocked substrates Gal-Lipid II (red) and Gal-Lipid IV (green, 1.2 μM each). (b) Comparison of rate enhancements in PGT activity due to Gal-Lipid IV preincubation with various PGTs (10−200 nM; see Figure S3).

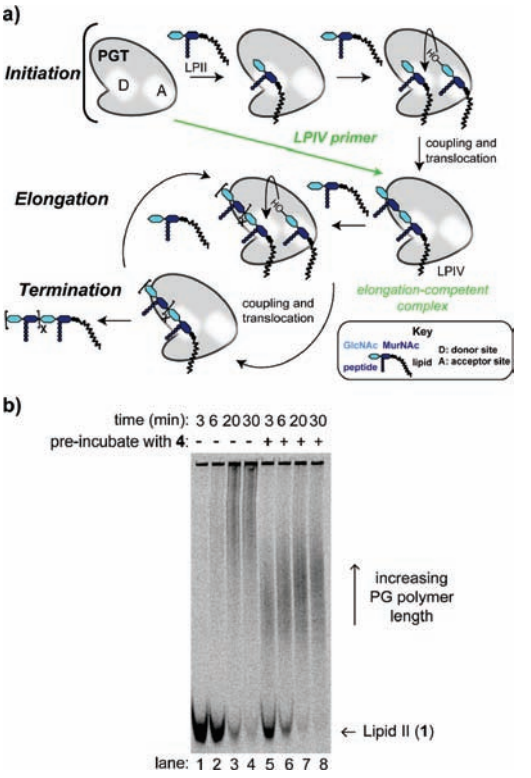

Figure 3. Blocked PG fragments bypass the initiation step of the PGTs. (a) Mechanistic model depicting the three steps involved in the process of Lipid II (LPII) polymerization. Gal-Lipid IV (LPIV primer) binding to PGTs changes the rate-limiting step by directly accessing the elongation-competent complex, leading to immediate processive polymerization. (b) SDS-PAGE of PG polymers produced by *E. coli* PBP1A (600 nM) showing that relative to the control lanes (1−4), preincubation of Gal-Lipid IV (1.2 μM) with the enzyme results in shorter polymers and faster consumption of substrate Lipid II (4 μM) over time (lanes 5−8).

To probe whether PGT activity would occur with a "donor-only" substrate containing a disaccharide rather than a tetrasaccharide, we prepared Gal-Lipid II (**2**), whose nonreducing end is blocked in the same manner as in **4**.[4] Like Gal-Lipid IV, Gal-Lipid II was incorporated into PG at the nonreducing end of the polymer. Gal-Lipid II was preincubated with *E. coli* PBP1A prior to initiating the reaction with Lipid II, but its presence did not accelerate the reaction (Figure 2a). Hence, activation does not depend simply on having a functional "donor-only" substrate. Instead, the donor substrate must contain a tetrasaccharide or longer fragment of the elongating chain.

We compared the initial reaction rates of several other PGTs from *E. coli* and *Staphylococcus aureus*[5a,6c,8,12] (Figures S1 and S2 in the Supporting Information) in the presence and absence of compound **4**. Similar results were observed in all cases (Figure 2b and Figure S3). Therefore, the activating effect of **4** was general, although the magnitude of the rate enhancement varied depending on the enzyme (Figure 2b). The largest rate enhancement was observed for *S. aureus* SgtB, a naturally occurring monofunctional PGT lacking a TP domain.[12b] Two full-length bifunctional PGTs were compared with their truncated variants lacking the TP domains (PBP1AΔTP and PBP1BΔTP), but there were no differences in the rate enhancements due to **4**. Therefore, we concluded that the presence of TP domains does not affect the activation of the PGT domains.

The concentration of **4** required for maximal activation of *E. coli* PBP1A was evaluated by carrying out a titration series in which the activator concentration was varied from 0 to 1.2 μM, the latter being the concentration used in the activation experiments reported above. Initial reaction rates were measured following addition of Lipid II, and the rate enhancement relative to the control was plotted as a function of the Gal-Lipid IV concentration (Figure S4). The results showed that the activation is saturable, indicating a specific site of interaction. A dissociation constant of 0.3 μM was calculated by treating the activation plot as a binding curve.

The reported results are consistent with the model shown in Figure 3a, in which there are three distinct processes involved in polymerization: initiation, elongation, and polymer release (termination).[13] Gal-Lipid IV (**4**) accelerates polymerization, implying that it bypasses the rate-limiting step. Since **4** mimics the first coupling product, the rate-limiting step must be initiation. Lag-phase kinetics were not observed upon preincubation with **4** because the enzyme was already in an "elongation-competent" complex when Lipid II was added.

The proposed model leads to a testable prediction about how to alter the polymer length. We previously reported that PGTs produce long glycan polymers even at a 1:1 enzyme:substrate ratio.[5c] This remarkable insensitivity of PGT product lengths to the enzyme:substrate ratio can be explained if initiation is so much slower than elongation that only a small fraction of the available enzyme makes polymer before the substrate runs out.[7c−e,14] If **4** does indeed increase the fraction of active enzyme, then its addition would be expected to decrease the glycan chain lengths produced at a given enzyme:substrate ratio, since more enzyme molecules would be competing for substrate.[15] To test this prediction, we incubated *E. coli* PBP1A in the presence or absence of **4** and then added a 7-fold excess of Lipid II. The product distribution was evaluated using an SDS-PAGE method that separates products with single-disaccharide resolution (Figure 3b).[5a]

dx.doi.org/10.1021/ja2028712 |*J. Am. Chem. Soc.* 2011, 133, 8528–8530

Consistent with the prediction, the addition of Gal-Lipid IV resulted in a decrease in product length.

We have shown that the addition of Gal-Lipid IV bypasses the slow initiation step of PG polymerization. Since PGT activation requires a glycan strand containing at least four sugars bound in the donor site, distal portions of extended donor substrates likely contact the enzyme and help organize the binding site for processive elongation. It has recently been proposed that outer membrane lipoproteins (Lpo) in *E. coli* activate PGTs in cells. It is possible that these proteins play a role in influencing the initiation step in vivo. In any event, the use of **4** to bypass initiation may enable more accurate measurements of the steady-state rate of elongation. Perhaps more importantly, it should be possible to obtain complexes of "activated" PGTs with **4** or a similar substrate. Information on different possible conformational states of PGTs is important for understanding both their mechanisms and biological regulation.[12c]

■ ASSOCIATED CONTENT

Supporting Information. Protein cloning, overexpression, and purification; SDS-PAGE of purified proteins; preparation of substrates; activator titration curve; and time courses of PGTs with and without activator. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

This research was supported by the National Institutes of Health (R01 GM076710 to S.W. and D.E.K; R01 GM066174 to D.E.K.; P01A108321Y to Y.R.). We acknowledge Pfizer for their donation of the overexpression plasmid for *S. aureus* SgtB and the Bernhardt lab (Harvard Medical School) for the overexpression plasmid for full length *E. coli* PBP1B.

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