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Primer Preactivation of Peptidoglycan Polymerases

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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 crosslinked polymer that surrounds bacterial cell membranes and prevents them from rupturing under 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-acetyl glucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc), is polymerized via the intermediate Lipid IV to form linear glycan chains that are crosslinked 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 developing 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 analogs 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 have revealed a prolonged lag phase,⁶ which could be due to a slow conformational rearrangement of the enzyme to an active form^{7a–b} and/or to a slow first coupling step.^{7c–e} We reasoned that if the formation of Lipid

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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>.

IV, the product of the first coupling step, was 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 non-reducing end of **3** through the enzymatic attachment of galactose by GalT to produce **4**.^{4,9}

Compound **4** is incapable of reacting with itself, but is incorporated into nascent (uncrosslinked) peptidoglycan at the non-reducing 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 *E. coli* PBP1A, which contains an N-terminal PGT domain and a C-terminal transpeptidase (TP) domain.^{5a,8} The enzyme was incubated for twenty minutes with compound **4** and then radio-labeled Lipid II¹⁰ (**1**) was added and reactions were incubated for varying periods of time. Reaction mixtures were analyzed by paper chromatography, which separates polymeric product from Lipid II and short oligosaccharides.^{6b,11} Unlike the control reaction, there was no lag phase in the presence of **4** and the reaction rate was approximately four-fold higher (Figure 2a).

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**), which has its non-reducing end blocked in the same manner as **4**.⁴ Like Gal-Lipid IV, Gal-Lipid II is incorporated into peptidoglycan at the non-reducing 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 simply depend 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 *S. aureus*^{5a,6c,8,12} (Figures S1, S2) in the presence and absence of compound **4**. Similar results were observed in all cases (Figure 2b, 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 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 compared with the control was plotted as a function of the Gal-Lipid IV concentration (Figure S4). The results show that 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 results reported 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 are not observed upon preincubation with **4** because the enzyme is already in an “elongation-competent” complex when Lipid II is added.

The proposed model leads to a testable prediction about how to alter polymer length. We have 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 enzyme:substrate ratio can be explained if initiation is so much slower than elongation that only a small fraction of available enzyme makes polymer before 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 are competing for substrate.¹⁵ To test this prediction, we incubated *E. coli* PBP1A in the presence or absence of **4** and then added a seven-fold excess of Lipid II. The product distribution was evaluated using a SDS-PAGE method that separates products to single disaccharide resolution (Figure 3b).^{5a} Consistent with the prediction, the addition of Gal-Lipid IV results in a decrease in product length.

We have shown that the addition of Gal-Lipid IV bypasses the slow initiation step of peptidoglycan 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 important, 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}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. (a) Holtje JV. Microbiol Mol Biol Rev. 1998; 62:181–203. [PubMed: 9529891] (b) Vollmer W, Bertsche U. Biochim Biophys Acta. 2008; 1778:1714–1734. [PubMed: 17658458] (c) Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P. FEMS Microbiol Rev. 2008; 32:234–258. [PubMed: 18266856]
2. Walsh, C. Antibiotics: actions, origins, resistance. ASM Press; Washington D. C.: 2003. Introduction to Antibiotics; p. 1-9.
3. (a) Walker S, Chen L, Hu Y, Rew Y, Shin D, Boger DL. Chem Rev. 2005; 105:449–476. [PubMed: 15700952] (b) Kahne D, Leimkuhler C, Lu W, Walsh C. Chem Rev. 2005; 105:425–448. [PubMed: 15700951] (c) Halliday J, McKeveney D, Muldoon C, Rajaratnam P, Meutermans W. Biochem Pharmacol. 2006; 71:957–967. [PubMed: 16298347] (d) Ostash B, Walker S. Nat Prod Rep. 2010; 27:1594–1617. [PubMed: 20730219]
4. Perlstein DL, Zhang Y, Wang TS, Kahne DE, Walker S. J Am Chem Soc. 2007; 129:12674–12675. [PubMed: 17914829]
5. (a) Barrett D, Wang TS, Yuan Y, Zhang Y, Kahne D, Walker S. J Biol Chem. 2007; 282:31964–31971. [PubMed: 17704540] (b) Yuan Y, Barrett D, Zhang Y, Kahne D, Sliz P, Walker S. Proc Natl Acad Sci USA. 2007; 104:5348–5353. [PubMed: 17360321] (c) Wang TS, Manning SA, Walker S, Kahne D. J Am Chem Soc. 2008; 130:14068–14069. [PubMed: 18834124] (d) Perlstein DL, Wang

- TS, Doud EH, Kahne D, Walker S. *J Am Chem Soc.* 2010; 132:48–49. [PubMed: 20017480] (e) Breyer WA, Matthews BW. *Protein Sci.* 2001; 10:1699–1711. [PubMed: 11514661] (f) May JF, Splain RA, Brotschi C, Kiessling LL. *Proc Natl Acad Sci.* 2009; 106:11851–11856. [PubMed: 19571009]
6. (a) Schwartz B, Markwalder JA, Seitz SP, Wang Y, Stein RL. *Biochemistry.* 2002; 41:12552–12561. [PubMed: 12369847] (b) Chen L, Walker D, Sun B, Hu Y, Walker S, Kahne D. *Proc Natl Acad Sci USA.* 2003; 100:5658–5663. [PubMed: 12714684] (c) Barrett D, Leimkuhler C, Chen L, Walker D, Kahne D, Walker S. *J Bacteriol.* 2005; 187:2215–2217. [PubMed: 15743972]
 7. (a) Wodzinska J, Snell KD, Rhomberg A, Sinskey AJ, Biemann K, Stubbe J. *J Am Chem Soc.* 1996; 118:6319–6320. (b) Yin YW, Steitz TA. *Science.* 2002; 298:1387–1395. [PubMed: 12242451] (c) Peng L, Kawagoe Y, Hogan P, Delmer D. *Science.* 2002; 295:147–150. [PubMed: 11778054] (d) DeAngelis PL. *J Biol Chem.* 1999; 274:26557–26562. [PubMed: 10473619] (e) Cartee RT, Forsee WT, Yother J. *J Bacteriol.* 2005; 187:4470–4479. [PubMed: 15968057]
 8. Zhang Y, Fechter EJ, Wang TS, Barrett D, Walker S, Kahne DE. *J Am Chem Soc.* 2007; 129:3080–3081. [PubMed: 17323951]
 9. (a) Schindler M, Mirelman D, Schwarz U. *Eur J Biochem.* 1976; 71:131–134. [PubMed: 1009945] (b) Masibay AS, Qasba PK. *Proc Natl Acad Sci USA.* 1989; 86:5733–5737. [PubMed: 2503823] (c) Roquemore EP, Chou TY, Hart GW. *Methods Enzymol.* 1994; 230:443–460. [PubMed: 8139512] (d) Boeggeman E, Ramakrishnan B, Kilgore C, Khidekel N, Hsieh-Wilson LC, Simpson JT, Qasba PK. *Bioconjug Chem.* 2007; 18:806–814. [PubMed: 17370997] (e) Khidekel N, Ficarro SB, Clark PM, Bryan MC, Swaney DL, Rexach JE, Sun YE, Coon JJ, Peters EC, Hsieh-Wilson LC. *Nat Chem Biol.* 2007; 3:339–348. [PubMed: 17496889]
 10. (a) Men H, Park P, Ge M, Walker S. *J Am Chem Soc.* 1998; 120:2484–2485. (b) Ha S, Chang E, Lo MC, Men H, Park P, Ge M, Walker S. *J Am Chem Soc.* 1999; 121:8415–8426. (c) Ye XY, Lo MC, Brunner L, Walker D, Kahne D, Walker S. *J Am Chem Soc.* 2001; 123:3155–3156. [PubMed: 11457035] (d) Chen L, Men H, Ha S, Ye XY, Brunner L, Hu Y, Walker S. *Biochemistry.* 2002; 41:6824–6833. [PubMed: 12022887]
 11. Anderson JS, Matsushashi M, Haskin MA, Strominger JL. *Proc Natl Acad Sci USA.* 1965; 53:881–889. [PubMed: 14324547]
 12. See SI and the indicated reports for descriptions of constructs used: (a) Barrett DS, Chen L, Litterman NK, Walker S. *Biochemistry.* 2004; 43:12375–12381. [PubMed: 15379577] (b) Heaslet H, Shaw B, Mistry A, Miller AA. *J Struct Biol.* 2009; 167:129–135. [PubMed: 19416756] (c) Paradis-Bleau C, Markovski M, Uehara T, Lupoli TJ, Walker S, Kahne DE, Bernhardt TG. *Cell.* 2010; 143:1110–1120. [PubMed: 21183074]
 13. For a review on nontemplate-dependent polymerase processes, see: Stubbe J, Tian J, He A, Sinskey AJ, Lawrence AG, Liu P. *Annu Rev Biochem.* 2005; 74:433–480. [PubMed: 15952894]
 14. Product lengths depend on the enzyme and are presumed to reflect processivity.
 15. (a) Tomsho JW, Moran RG, Coward JK. *Biochemistry.* 2008; 47:9040–9050. [PubMed: 18672898] (b) Jing W, DeAngelis PL. *J Biol Chem.* 2004; 279:42345–42349. [PubMed: 15299014]

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.

Figure 2.

Activation of PGTs using a PG fragment. (a) Time course of radiolabeled Lipid II (4 μM) polymerization 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, 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) shows that compared to the control lanes (1–4), Gal-Lipid IV (1.2 μM) preincubation with enzyme results in shorter polymers and faster consumption of substrate Lipid II (4 μM) over time (lanes 5–8).