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Analysis of Glycan Polymers Produced by Peptidoglycan Glycosyltransferases

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Bacterial cells are surrounded by a cross-linked polymer called peptidoglycan, the integrity of which is necessary for cell survival. The carbohydrate chains that form the backbone of peptidoglycan are made by peptidoglycan glycosyltransferases (PGTs), highly conserved membrane-bound enzymes that are thought to be excellent targets for the development of new antibacterials. Although structural information on these enzymes recently became available, their mechanism is not well understood because of a dearth of methods to monitor PGT activity. Here we describe a direct, sensitive, and quantitative SDS-PAGE method to analyze PGT reactions. We apply this method to characterize the substrate specificity and product length profile for two different PGT domains, PBP1A from Aquifex aeolicus and PBP1A from Escherichia coli. We show that both disaccharide and tetrasaccharide diphospholipids (Lipid II and Lipid IV) serve as substrates for these PGTs, but the product distributions differ significantly depending on which substrate is used as the starting material. Reactions using the disaccharide substrate are more processive and yield much longer glycan products than reactions using the tetrasaccharide substrate. We also show that the SDS-PAGE method can be applied to provide information on the roles of invariant residues in catalysis. A comprehensive mutational analysis shows that the biggest contributor to turnover of 14 mutated residues is an invariant glutamate located in the center of the active site cleft. The assay and results described provide new information about the process by which PGTs assemble bacterial cell walls.

The characteristic shape of a bacterial cell is a function of the three-dimensional architecture of the cell wall (1). The major rigid component of the cell wall is peptidoglycan, a mesh-like polymer comprising linear glycan chains held together by peptide cross-links (2). The glycan chains are assembled from a diphospholipid-linked disaccharide-peptide precursor, Lipid II, by peptidoglycan glycosyltransferases (PGTs)³ (Fig. 1). These glycan chains are then cross-linked via the attached peptides by transpeptidases (TPs) (3, 4).

The transpeptidases are targets for several families of clinically important antibiotics, including the penicillins, cephalosporins, and imipenems (5, 6), and the PGTs are thought to be promising antibacterial targets as well. There is a potent family of natural products, represented by the phosphoglycolipid moenomycin, which inhibit the PGTs with lethal cellular effects (7–10). However, there are still no drugs in clinical use that operate by binding directly to the PGTs. A better understanding of the PGTs and how they can be inhibited is required to exploit them as antibiotic targets and may also shed light on their cellular functions.

PGTs are membrane-anchored polymerases that exist naturally in two forms, as N-terminal domains in bifunctional penicillin-binding proteins (PBPs), called class A high molecular weight PBPs, which contain C-terminal TP domains, and as monofunctional enzymes that do not contain transpeptidase domains (Fig. 1A) (2, 11–13). All PGTs contain five conserved motifs comprised of a number of invariant residues (14). Notable progress toward understanding PGTs has been made recently. For example, structures of two PGTs, one with moenomycin bound in the active site cleft, are now available and provide a starting point for inhibitor design (11, 15). However, we still lack answers to fundamental questions about the reactions catalyzed by PGTs, which limits our ability to think about their roles in bacterial cell growth and division. Questions that remain unanswered include the following. How long are the glycan chains made by particular PGTs? Do different PGTs within the same organism show significant differences in glycan product distributions? Are the glycan chain length distributions compatible with current models for the three-dimensional structure of the bacterial cell wall? Do other cellular factors affect chain length distributions? One of our goals is to develop facile in vitro methods to analyze PGT reactions so that these and other fundamental questions about their behavior can be addressed.

PGTs catalyze the conversion of the peptidoglycan precursor, Lipid II, into glycan chains. The process involves the coupling of Lipid II units to form a tetrasaccharide, Lipid IV (Fig. 1, n = 1), followed by successive addition of Lipid II units to the

³ The abbreviations used are: PGT, peptidoglycan glycosyltransferase; TP, transpeptidase; PBP, penicillin-binding protein; TEMED, N,N',N'-tetramethylethylenediamine; PG, peptidoglycan; NAG, N-acetylglucosaminyl; NAM, N-acetylmuramyl.
reducing end of the growing polymer (16). Understanding the PGT reaction requires having both Lipid II and Lipid IV substrates, combined with an assay that reports on glycan chain lengths. We and others have developed methods to obtain useful quantities of Lipid II and Lipid IV substrates (17–21); therefore, the necessary starting materials to study PGTs are now available (Fig. 2). In this paper, we describe a facile assay to analyze PGT reactions that provides information on reaction rates as well as glycan chain lengths. This assay has enabled us to compare two PGT domains from different organisms, PBP1A from *Aquifex aeolicus* and PBP1A from *Escherichia coli*, with respect to their ability to utilize disaccharide and tetrasaccharide substrates. For the *A. aeolicus* PGT domain, for which we recently reported the structure (15), we have also carried out a comprehensive mutational analysis of the conserved residues in the five signature motifs that typify PGT domains (14, 22). We identify the most important residues for activity, which is pertinent to interpreting the crystal structures of PGTs, and we also show that the gel electrophoresis assay provides information about the products of mutant enzymes that cannot be obtained using other types of assays.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Vectors and expression hosts were obtained from Novagen (EMD Biosciences). Primers were synthesized by Integrated DNA Technologies (IDT). DNA sequencing was performed at the Dana-Farber/Harvard Cancer Center DNA Resource Core. Radiolabeled heptaprenyl-Lipid II was synthesized as described by Ye et al. (20) and radiolabeled heptaprenyl-Lipid IV was synthesized as described by Zhang et al. (21). Moenomycin A was extracted and purified from the feedstock flavomycin and purified as described (23). Non-stick PCR tubes used for enzymatic reactions were obtained from VWR. An electrophoresis grade acrylamide stock solution (30% (w/v) acrylamide:0.8% (w/v) bisacrylamide) was purchased from National Diagnostics. TEMED was purchased from American Bioanalytical. The QuikChange site-directed mutagenesis kit was obtained from Stratagene. All other reagents and buffer components were purchased from Sigma-Aldrich.

**Cloning, Expression, and Purification of PGT Constructs**—The cloning, expression, and purification of the PGT domain of *A. aeolicus* ΔPBP1A(Asn29–Lys243) and full-length *E. coli* PBP1A(Met1–Phe559) have been described previously (15, 21). The isolated PGT domain of *E. coli* PBP1A was obtained as follows. The gene encoding the truncated *E. coli* PBP1A (Met1–Asn251) was PCR amplified from pET22b::ponA containing full-length *E. coli* PBP1A using the appropriate primer pairs (restriction sites are underlined) (see Table 1 in supplemental data). The PCR product was then digested and inserted into the
NdeI and NotI restriction sites of pET22b(+) vector to produce pWTS12. The inserted ponA(Met'-Asn257) gene was confirmed by sequencing. Expression and purification followed procedures in Ref. 21 with changes described in the supplemental data.

Site-directed Mutagenesis of ΔPBP1A[Asn29-Lys243]—QuikChange site-directed mutagenesis kit was used to make all the mutants from the parent plasmid, ΔPBP1A(Asn29-Lys243), using the primer pairs given in the supplemental data, Table 1. Expression and purification of mutants was carried out as reported in Ref. 15.

Gel Analysis Conditions for the Separation of Glycan Chains—Gels were prepared as described by Lesse et al. (24) and Schägger et al. (25) with the following modifications: anode buffer (0.1 M Tris, adjusted to pH 8.8 with HCl), cathode buffer (0.1 M Tris-base, 0.1 M tricine, 0.1% SDS, pH 8.25) and 3× gel buffer (1.5 M Tris, 0.4% SDS, pH 8.45). Gels were prepared using the Protean xi system (Bio-Rad). Separating gels, 200 × 200 × 1 mm, were prepared (stacking gel omitted) in a final concentration of 9% T, 2.6% C in 1× gel buffer, where T is the total percentage concentration of both acrylamide and bisacrylamide, and C represents the percentage of bisacrylamide (cross-linker) relative to T. Gel solutions were filtered and degassed before the addition of TEMED.

Samples were prepared by vacuum centrifuging the reaction mixtures to 1 μl or dryness and redissolving them in 2 μl of sample buffer (60 mM Tris-HCl, pH 8.8, 25% glycerol, 2% SDS, 10% saturated solution of bromphenol blue). Samples were then loaded under the cathode buffer as thin bands with filling height less than 3 mm. Electrophoresis was performed on a Protean II xi vertical gel apparatus set at 30 mA constant current and a maximum voltage of 200 V. Gels were run for ∼4 h, or until the bromphenol blue dye front was 0.5 cm from the end of the gel. Gels were dried overnight without fixing and imaged by autoradiography using a tritium storage phosphor screen and scanner (Typhoon 9400, GE Healthcare). Densitometric band quantitation of the bromphenol blue dye front was 0.5 cm from the end of the gel.

**RESULTS**

Establishing a Gel Electrophoresis Assay for the Separation of Glycan Chains—Most assays to monitor PGT activity rely on...
detecting the presence of polymeric products (18, 27–30), but cannot distinguish different glycan chain lengths, which greatly limits the information they provide about the reaction. Because the glycan products of a PGT reaction are composed of repeating disaccharide units that have a net negative charge, we investigated gel electrophoresis methods to analyze glycan chain length distributions. Radiolabeled Lipid II (Fig. 2, 1b and 1c) and Lipid IV (Fig. 2, 2b) substrates were prepared as described (20, 21), and radiolabels were either incorporated enzymatically or via acetylation. Previous work has established that substrates with substituents at the third position of the pentapeptide chain, such as acetyl groups on the lysine amine (e.g. 1c and 2b) have comparable kinetic parameters to unmodified substrates (e.g. 1b) (21, 30). At high substrate:enzyme (~1:3) ratios, the A. aeolicus PBPIA PGT domain converts the substrates into mixtures of products representing a range of glycan chain lengths. Optimal gel conditions for separating PGT products utilize a modified tricine-SDS-PAGE system (24, 25). The bromphenol blue dye migrates ahead of Lipid II under the standard running conditions, allowing the progress of the runs to be visually tracked. A 9% gel was optimal for separating uncross-linked glycan strands containing from one to thirty repeat units as determined from the size standards generated using the substrates (Fig. 3; see also supplemental data, Fig. S1). The separation of glycan chains is indifferent to the presence of proteins, salts at the concentrations used in typical reaction buffers, and other additives. Moreover, because only labeled glycan chains are detected, sample cleanup is unnecessary. The use of radiolabeled substrates in the reaction obviates the need for post-reaction labeling to detect products. Finally, it should be possible to adapt this assay to detect products of reactions carried out with substrates labeled in other ways, e.g. with fluorophores or biotin moieties on the peptide side chains.

**Product Distributions with Lipid II as the Substrate**—With an assay in place to separate glycan chains, we examined the product distributions for the PGT domains of A. aeolicus PBPIA (Fig. 4A) and E. coli PBPIA (Fig. 4B) using the radiolabeled Lipid II substrate 1b and an enzyme:substrate ratio of 1:100. There is a delay in the formation of products, which is particularly pronounced for the PGT domain of E. coli PBPIA. This lag phase has also been observed for E. coli PBPIB using other assays (26, 30) and has been attributed either to a slow initial coupling step to form a “primer” or to a slow rearrangement to the active conformation. Following the lag phase, relatively long glycan chains appear without significant accumulation of short products, consistent with a processive mechanism in which coupling of Lipid II subunits can occur without release of the elongating product. The distribution of glycan chains is narrower for the E. coli PBPIA PGT domain than for the A. aeolicus PGT domain. Furthermore, shorter products are less evident early in the reaction for the E. coli enzyme than for the A. aeolicus PGT domain. These results suggest that the E. coli PGT domain is more processive than the A. aeolicus PGT domain. The SDS-PAGE method described here should make it possible to establish whether differences in processivity, and thus glycan chain length distribution, exist among different PGTs encoded in the genome of the same organism.

**Product Distributions Using Lipid IV**—We recently reported that E. coli PBPIA can self-couple Lipid IV (2b) to generate elongated products (21). This result was surprising because it was previously thought that only the disaccharide substrate...
PBP1A (Fig. 5) generates products appeared, a pattern consistent with a distributive tetrasaccharide substrate, short products accumulated before elongation of the main enzyme concentration. In the case of the A. aeolicus PBP1A PGT (1.65 μM) or B. coli PBP1A PGT domain (0.8 μM) quenched at the indicated time points as described under “Experimental Procedures.”

**TABLE 1**

Specific activities* of the PGTs for substrates 1b and 2b

|          | 1b  | 2b  |
|----------|-----|-----|
| E. coli PBP1A | 11.6| 0.2 |
| A. aeolicus PBP1A | 11.5| 0.4 |

*μmol/(min × mg protein).

(Lipid II) was capable of reacting with the growing glycan polymer. Because the paper chromatography assay used previously did not provide information about glycan chain length, we have used the newly developed SDS-PAGE assay to examine the product lengths for the A. aeolicus PBP1A (Fig. 5A) and E. coli PBP1A (Fig. 5B) PGT domains with the tetrasaccharide substrate 2b. For both enzymes, this substrate reacts much more slowly than Lipid II (Table 1) even at high enzyme concentrations. In the case of the tetrasaccharide substrate, short products accumulated before longer products appeared, a pattern consistent with a distributive mechanism in which products are released after each coupling cycle. In contrast, even at 1:10 (enzymesubstrate) ratios, Lipid II reacts to form mainly long polymers (see Fig. 6C).

**Elongation of Exogenously Added Oligomers**—The finding that PGT domains can homopolymerize Lipid IV as the sole substrate raised the possibility that PGTs may couple oligomers longer than Lipid IV. To address this possibility, we enzymatically generated a mixture of radiolabeled oligomers from 14C-Lipid IV (2b) to use as starting materials (Fig. 6, A and B, lane 1). The oligomer mixture was then treated with the PGT domain from A. aeolicus PBP1A (Fig. 6A, lane 2) or with E. coli PBP1A (lane 2). No change in the distribution of oligomers was observed in the presence of either PGT (Fig. 6, A and B, compare lanes 1 and 2), indicating negligible self-coupling of glycan chains longer than Lipid IV. However, the oligomers were chased into longer products when cold Lipid II (1a) was added to the reactions (Fig. 6, A and B, lane 3), as indicated by the appearance of new bands between oligomeric starting materials (see arrows in Fig. 6, A and B) and a smear of radioactivity above the highest band of starting material. These results show that glycan strands containing multiple N-acetylgalcosaminyl-N-acetyl muramyl (NAG-NAM) repeat units are capable of binding to PGTs and undergoing elongation. Because the radiolabeled oligomers are extended by only a few disaccharide units under reaction conditions in which Lipid II used as the sole substrate produces very long glycan chains (Fig. 6C), we have concluded that rebinding and extension of long glycan strands is slower than initiation and processive elongation of new chains.

**Gel Electrophoretic Analysis of Selected Mutants**—PGTs show modest structural homology to lambda lysozyme (11, 15), an enzyme that cleaves the NAM-β-(1,4)-NAG glycosidic linkages of peptidoglycan. The coupling reaction is proposed to involve general acid-base catalysis. There are two conserved glutamate residues in PGTs, one in motif 1 (Glu83 in the A. aeolicus PGT domain) and the other in motif 3 (Glu130 in the A. aeolicus PGT domain). Both have been proposed to play key roles in catalysis (11, 22, 28, 30, 31), although evidence for the importance of the motif 3 glutamate is contradictory. There is also a conserved aspartate (Asp84) and a conserved histidine (His87). Perhaps because most PGTs tend to be difficult to handle only a small number of the conserved residues in the five invariant domains have been mutated to examine their relative importance in catalysis. Because the isolated PGT domain of A. aeolicus PBP1A can be easily overexpressed and purified in active form and remains stable for a prolonged period of time, it is a good candidate for mutational analysis (15). Therefore, we replaced fourteen of the conserved residues in the A. aeolicus PGT domain with alanine or with more conservative substitutions. All mutants could be expressed in soluble form and purified at levels comparable with the wild-type truncated con-
The activity of the mutant enzymes was compared with that of the wild-type enzyme using a paper chromatography assay that relied on separation of polymeric products from Lipid II of the wild-type enzyme using a paper chromatography assay (15, 26). The substrate (NAG-NAM), which is just below the Km (12C[GlcNAc], Lipid II (1b)), 55 °C, buffer A, 30 min (gray bar) and 60 min (black bar) reactions. Reactions were quenched at 30, 90, and 600 min. The asterisk reaction was carried out at using 4 μM ([14C][GlcNAc]-Lipid II (1b) and 4 μM ([14C][GlcNAc]-Lipid II (1a). Lane L, ladder of oligomers.

FIGURE 7. Activity profile for PBP1A point mutants. A, standard assay conditions: 60 nM enzyme, 4 μM radiolabeled Lipid II (1b), 55 °C, buffer A, 30 min (gray bar) and 60 min (black bar) reactions. Residue numbers and substitutions are given along the x axis. The % conversion was determined using a paper chromatography assay as described in (28). Under these assay conditions enzyme activity ≤2% conversion is not distinguished over background. B, product distribution analysis by SDS-PAGE of low activity mutants. Reactions performed at higher concentrations of enzyme: 2 μM enzyme, 4 μM radiolabeled Lipid II (1b) under standard assay conditions (as described under "Experimental Procedures"). C, reaction of E83Q with Lipid II (1b) was re-examined by SDS-PAGE using an 8–16% Tris-HCl gradient gel under the conditions described in B. Reactions were quenched at 30, 90, and 600 min. The asterisk reaction was carried out at using 4 μM ([14C][GlcNAc]-Lipid II (1b) and 4 μM ([14C][GlcNAc]-Lipid II (1a). Lane L, ladder of oligomers.

Based on the mutational studies, we have concluded that four of the fourteen mutated residues (Glu83, Asp84, Arg136, and Arg218) play especially important roles in enzymatic function because even conservative mutations (e.g. Arg to Lys; Asp to Asn; Glu to Gln) decrease enzymatic activity under standard assay conditions by >20-fold. One of these four residues, Glu83, is particularly important because almost no turnover is observed even for the conservative glutamine mutation under highly forcing conditions (Fig. 7C). We conclude from the above experiments that Glu83 is the single most important residue for catalysis, and that the carboxylate side chain is critical.

The sensitivity and size resolution of the gel electrophoresis assay makes it possible to detect small amounts of short coupling products and provides far more information than the traditional paper chromatography assay. Therefore, this SDS-PAGE assay is capable of providing detailed information about individual mutants.

DISCUSSION

Knowing what an enzyme is capable of doing in vitro is fundamental to thinking about what it may do in cells. Therefore, we have established an SDS-PAGE method that allows us to
monitor the formation of the glycan chain products of in vitro PGT reactions. Separation of the glycan strands by gel electrophoresis allows for the analysis of the product distributions of PGTs.

Using this SDS-PAGE assay, we have compared the products formed by two different PGTs with two different substrates, Lipid II and Lipid IV. Lipid II is the preferred substrate, with a catalytic efficiency twenty to forty times greater than Lipid IV. Lipid II also reacts processively, i.e., without release of the growing polymer chain, to yield long glycan products with minimal accumulation of short intermediates. In contrast, Lipid IV reacts in a distributive manner. A crystal structure of the A. aeolicus PGT domain suggests that a mobile flap may cover part of the active site during the reaction, and we have speculated that this flap functions to hinder release of the coupled product from the active site (15). Following the chemical coupling step, the product can either translocate in the active site and undergo another round of coupling or it can dissociate. Translocation of the product of Lipid IV coupling to position the new reactive terminus for another round of reaction may be slower than for Lipid II. Alternatively, or in addition, the chemical-coupling step of Lipid IV with the growing polymer may be slower than for Lipid II. A decrease in the relative rate of translocation or coupling compared with the rate of dissociation would make dissociation more likely to occur prior to elongation, explaining the observed distributive pattern of products when Lipid IV is the substrate. The finding that Lipid IV can function as a sole substrate, but longer polymers cannot, suggests that PGTs recognize the diphospholipid moiety of the acceptor as well as the donor. Recognition of both the non-reducing and the reducing end of the acceptor may explain the preference for Lipid II over Lipid IV as a substrate.

The results reported above have implications for the incorporation of new disaccharide subunits into the cell wall. Two models for how this process may occur have been proposed (2). In one model, nascent chains synthesized by PGTs are handed off to partner transpeptidases for cross-linking into the fabric of the cell wall; in the other, the disaccharide units themselves are directly integrated into the fabric of the cell wall through PGT-mediated glycosyltransfer. Our in vitro results show that PGTs couple Lipid II to give long oligomers, but are also capable of rebinding and elongating relatively long oligomers, showing that both models are possible. Nevertheless, the in vitro studies reported here show that the processive synthesis of long glycan chains from Lipid II is faster than the addition of Lipid II subunits to long oligomers, suggesting that the bulk of peptidoglycan (PG) synthesis involves the incorporation of nascent chains into the existing framework of the cell wall.

In some organisms different PGTs appear to be required at different points in the cell cycle, although it is not known whether there are differences in their enzymatic properties. The results presented here show that there are differences in processivity in PGTs from different organisms. It is possible that there are also differences in processivity in PGTs from the same organism. If so, there may be differences in the distribution of product lengths, with more processive PGTs producing longer products. The assay described here will make it possible to determine whether the different PGTs show significant differences in processivity; this may, in combination with information on the spatial and temporal localization of the enzymes, help explain their different cellular roles. This assay also enables detailed studies of the factors that affect product lengths. For example, it will be possible to investigate the effects of other proteins or domains that are involved in peptidoglycan synthesis on the length of the glycan chains that are produced. It will also be possible to assess how enzymes to substrate ratios influence product lengths. These types of studies are important because glycan polymer length has profound implications for the structure of bacterial peptidoglycan. At the moment, two competing models are being actively considered, one in which the PG chains are perpendicular to the cell membrane (the scaffold model) and one in which the PG chains are parallel to the cell membrane (32). The product lengths observed in this study, using Lipid II as a substrate, are too long to be compatible with the scaffold model. Therefore, either the scaffold model is incorrect or limited to small regions of the bacterial cell (e.g., the poles) or there are other factors operative in cells that restrict the length of the glycan polymers. This assay provides a systematic way to evaluate how sensitive PGT product length distributions are to other factors, including variations in substrate availability and the presence of other components of the biosynthetic machinery.

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