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JOURNAL OF BACTERIOLOGY, Mar. 2005, p. 2215–2217 Vol. 187, No. 6
0021-9193/05/$08.00+0 doi:10.1128/JB.187.6.2215–2217.2005

Kinetic Characterization of the Glycosyltransferase Module of *Staphylococcus aureus* PBP2

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Received 27 September 2004/Accepted 20 December 2004

We report the heterologous overexpression and purification of *Staphylococcus aureus* PBP2 and demonstrate efficient glycan polymerization from lipid II in vitro. *S. aureus* PBP2 is the first purified gram-positive class A penicillin-binding protein to show good transglycosylase activity. This enables further studies on this important class of enzymes.

Bacterial cells are surrounded by layers of peptidoglycan, a cross-linked carbohydrate polymer that protects the cells from lysing under high internal osmotic pressures (2). The glycan chains of peptidoglycan are assembled from repeating β-1,4-linked *N*-acetylglucosaminyl-*N*-acetylmuramyl units by periplasmic glycosyltransferases (Fig. 1). Glycosyltransferases (GTs) that synthesize peptidoglycan come in two forms: as N-terminal modules in bifunctional proteins that also contain C-terminal transpeptidase modules (called class A penicillin-binding proteins [PBPs]) and as monofunctional enzymes (called monofunctional glycosyltransferases) (6, 14). These periplasmic GTs are perhaps the least understood of any of the enzymes involved in the biosynthetic pathway to peptidoglycan.

The paucity of information about the kinetic behavior of peptidoglycan GTs is due primarily to the fact that lipid II, the substrate needed to monitor enzymatic activity, is present in only a few thousand copies per bacterial cell and cannot be isolated readily from natural sources (15). We (18) and others (1a, 13, 16) have developed approaches to the synthesis of lipid II and analogues and have used these synthetic substrates to characterize *Escherichia coli* PBP1b (3, 12). Until now, however, efforts to characterize the enzymatic activity of periplasmic GTs from gram-positive organisms have not been successful (4). Here we describe the expression, purification, and characterization of *Staphylococcus aureus* PBP2. We chose this enzyme because resistant *S. aureus* strains have become a major global health care problem (7, 8), and in resistant *S. aureus* strains the glycosyltransferase domain of PBP2 functions together with PBP2a, an acquired methicillin-resistant transpeptidase, to make peptidoglycan (10). The glycosyltransferase module of PBP2 is thus an important antibacterial target in a major pathogen.

The *pbp2* gene was PCR amplified from *S. aureus* (209P) purified genomic DNA by using the following primer pair: 5′-GCGCTAGCATGACGGAAAACAAAGGATCT-3′ and 5′-GCGGATCCTTACTCGAGGTTGAATATACCTGTTAATCC-3′. The PCR product was subcloned into pET21b(+) (Novagen) for expression in *E. coli* BL21(DE3) (Novagen) as a C-terminal His_6 fusion. PBP2 was expressed after induction of a log-phase culture with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h at 37°C. The cells were lysed with a French pressure cell at 16,000 lb/in^2 in buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) supplemented with 360 kU of rLysozyme (Novagen). Freely soluble proteins were decanted, and the pellet was resuspended in buffer containing 0.5% Sarkosyl to release membrane-anchored PBP2. The solubilized fusion protein was then loaded onto a Ni^{2+} column, which was washed with an imidazole gradient (5 to 60 mM) using detergent-free buffer A (20 mM Tris-HCl, 500 mM NaCl, pH 7.9) and then eluted with buffer A containing 100 to 200 mM imidazole. The protein was more than 85% pure, and the yield was estimated to be 20 mg/liter.

Enzymatic activity of the purified protein was evaluated by monitoring the incorporation of [^{14}C]GlcNAc-labeled C_{35} lipid II, prepared as previously described (14), into peptidoglycan in the presence of penicillin G (1,000 U/ml), which inactivates the transpeptidase domain (3). Reactions were carried out at substrate concentrations varying from 0.2 to 16 μM in a wide-range pH buffer containing 50 mM CHES (2-[cyclohexylamino]ethanesulfonic acid), 50 mM HEPES, 50 mM acetic acid, 50 mM MES (2-morpholineethanesulfonic acid), 10 mM $CaCl_2$, and 20% dimethyl sulfoxide. Reactions were initiated by adding enzyme to a final concentration of 90 nM as determined by titration with moenomycin, which is presumed to be a stoichiometric inhibitor of active peptidoglycan glycosyltransferases (3). The reactions were then quenched and analyzed as previously described (3, 18). Michaelis-Menten analysis (Fig. 2) established the catalytic parameters of *S. aureus* PBP2 under optimal conditions (pH 5.0) to be the following: $K_m = (4.0 \pm 1.0) \times 10^{-6}$ M, $k_{cat} = (1.5 \pm 0.1) \times 10^{-2}$ s^{-1}, and $k_{cat}/K_m = 3.4 \times 10^3$ M^{-1} s^{-1}. The catalytic efficiency of *S. aureus* PBP2 is within 10-fold that of *E. coli* PBP1b ($k_{cat}/K_m = 3.5 \times 10^4$ M^{-1} s^{-1}) (3) and is more than 6 orders of magnitude higher than that of the only other gram-positive GT for which any kinetics have been reported, a truncated *Streptococcus pneumoniae* construct studied by Di Guilmi et al. (4). It should be

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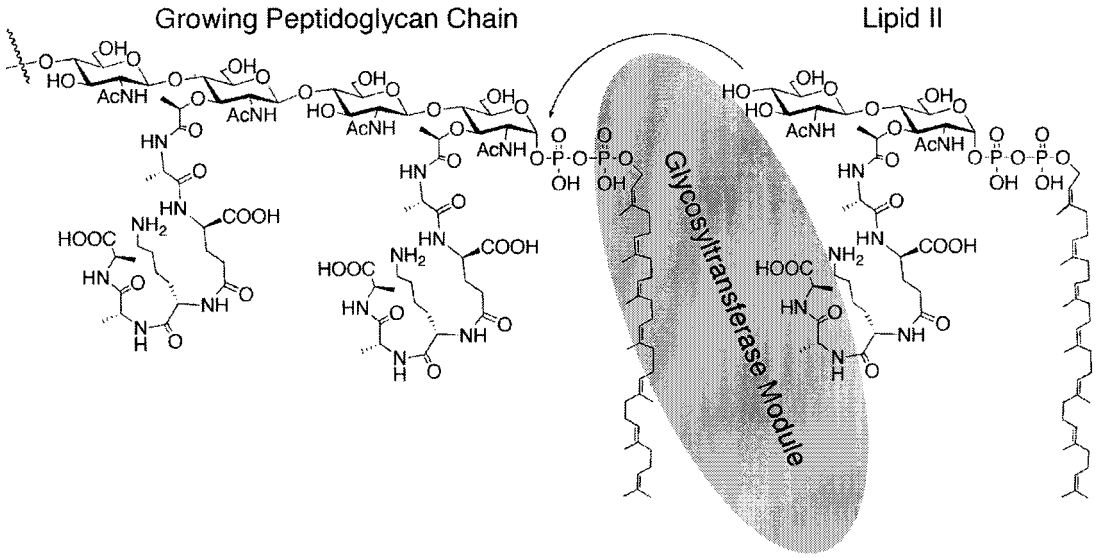

FIG. 1. Chemical reaction proposed to be catalyzed by peptidoglycan glycosyltransferases. The lipid II monomeric unit is believed to add to the reducing end of the growing glycan chain (5, 14, 17). For the enzymatic reactions described in this paper, the lipid II substrate contains a heptaprenyl chain and functions as both the donor and the primer in the initial reaction cycle (3).

noted that the lipid II analogue used in the present studies lacks the pentaglycine branch characteristic of *S. aureus* peptidoglycan precursors (11), and it is possible that the catalytic activity would be even higher in the presence of this pentaglycine substrate.

There are several differences between the *S. aureus* enzyme and *E. coli* PBP1b, which is the most well-studied periplasmic GT. Notably, the two enzymes have considerably different pH optima (4.5 to 5.5 for *S. aureus* PBP2 versus 7.5 to 8.0 for *E. coli* PBP1b [12]). Second, *E. coli* PBP1b functions optimally in the presence of the detergent octaethylene glycol monodecyl ether (3, 9, 12), whereas the activity of the *S. aureus* enzyme decreased by 5- to 10-fold in the presence of all of the detergents we examined, which included octaethylene glycol monodecyl ether (0.19 mM), CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; 8.9 mM}, Sarkosyl (17.0 mM), *n*-octyl-β-D-glucoside (27.0 mM), and Triton X-100 (0.77 mM). Since we used the same C_{35} lipid II substrate to study both PBP1b and PBP2, the different detergent requirements reflect properties of the enzymes themselves. In general, we observe that the *S. aureus* enzyme is more soluble and better behaved than *E. coli* PBP1b, which may make it a better candidate for structural and mechanistic analysis. Finally, the *S. aureus* enzyme is less sensitive to metal ions than the *E. coli* enzyme, which is strongly activated (>10-fold) by Ca^{2+} (12). It has been proposed that Ca^{2+} ions play a role in the catalytic activity of *E. coli* PBP1b. However, the enzymatic activity of *S. aureus* PBP2 is only modestly enhanced (two- to threefold) in the presence of 10 mM Ca^{2+}, Mg^{2+}, or Mn^{2+} and is not affected by the presence of 2 to 5 mM EDTA. It remains to be seen whether the significant differences in pH optima and metal ion requirements between periplasmic GTs reflect substantial differences in active site architecture that will affect the discovery of broad-spectrum GT inhibitors.

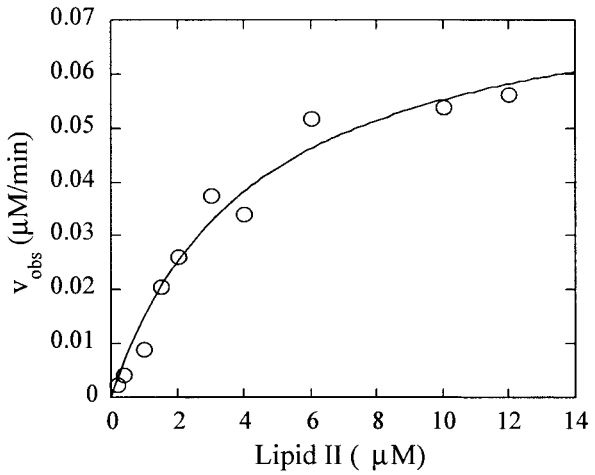

FIG. 2. Velocity versus lipid II concentration for *S. aureus* at pH 5.0. Curve fits and associated errors were generated in Kaleidagraph using the following equation: $v_{obs}/[\text{enzyme}] = k_{cat} \times [\text{lipid II}]/(K_m + [\text{lipid II}])$.

The work reported here lays the groundwork for further detailed investigations. Using the full-length *S. aureus* PBP2 construct as a standard, the expression and activity of truncated constructs lacking the transmembrane or transpeptidase domain can now be assessed to identify suitable candidates for structural analysis (1). Since it is unlikely that this *S. aureus* enzyme is unique in being well behaved, it should not be long before other active gram-positive enzymes are obtained and structures become available.

This work was supported by the National Institutes of Health (NIH grants R01AI50855 to S.W. and R01GM66174 to D.K.).

We thank Kim Specht for her efforts in the plasmid construction of pET21b::*pbp2*.

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