Kinetic Characterization of the Monofunctional Glycosyltransferase from *Staphylococcus aureus*

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Received 28 September 2005/Accepted 9 January 2006

Bacterial wall peptidoglycan (PG) is a net-like macromolecule consisting of glycan strands made of alternating β-1,4-linked N-acetylmuramic acid (GlcNAc) and N-acetylmuramylpentapeptide (MurNAc-pentapeptide) to synthesize the glycan chain of the bacterial wall peptidoglycan. MGTs appear to be dispensable for growth of some bacteria in vitro. However, new evidence shows that they may be essential for the infection process and development of pathogenic bacteria in their hosts. Only a small number of class A PBPs have been characterized so far, and no kinetic data are available on MGTs. In this study, we present the principal enzymatic properties of the *Staphylococcus aureus* MGT. The enzyme catalyzes glycan chain polymerization with an efficiency of $\sim 5,800 \text{ M}^{-1} \text{s}^{-1}$ and has a pH optimum of 7.5, and its activity requires metal ions with a maximum observed in the presence of Mn$^{2+}$. The properties of *S. aureus* MGT are distinct from those of *S. aureus* PBP2 and *Escherichia coli* MGT, but they are similar to those of *E. coli* PBP1b. We examined the role of the conserved Glu100 of *S. aureus* MGT (equivalent to the proposed catalytic Glu233 of *E. coli* PBP1b) by site-directed mutagenesis. The Glu100Gln mutation results in a drastic loss of GT activity. This shows that Glu100 is also critical for catalysis in *S. aureus* MGT and confirms that the conserved glutamate of the first motif EdxxfxH(N/9)xG/A is likely the key catalytic residue in the GT51 active site.

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The glycosyltransferase (GT) module of class A penicillin-binding proteins (PBPs) and monofunctional GTs (MGTs) belong to the GT51 family in the sequence-based classification of GTs. They both possess five conserved motifs and use lipid II precursor (undecaprenyl-pyrophosphate-N-acetylglucosaminyl-N-acetylmuramoyl-pentapeptide) to synthesize the glycan chain of the bacterial wall peptidoglycan. MGTs are likely to play a key catalytic role in the GT reaction (20). *S. aureus* MGT was found to be sensitive to moenomycin, whereas *E. coli* MGT was not. Deletion of the *mgt* gene in *E. coli*, *H. influenzae*, and *B. abortus* has a slight effect on cell growth in broth culture (5, 19, 24). However, *B. abortus* cells depleted of MGT were found to be less effective in the initial phase of infection in mice than were wild-type cells (5). Therefore, MGTs seem to play a role in the pathogenicity process of infectious bacteria. Similarly, *Pasteurella multocida* depleted of class A PBP1c (homologue of *E. coli* PBP1c) was viable in vitro but showed significant attenuation of pathogenicity in vivo (13). Taken together, these observations suggest that nonessential genes in laboratory conditions and the apparent redundant PBPs involved in PG biosynthesis are probably essential for bacterial development in host environments.

*S. aureus* possesses only one class A PBP (PBP2). In the presence of penicillin, PBP2 can be replaced by the class B low-affinity PBP2a in the methicillin-resistant *S. aureus* strain (18), suggesting a cooperation between the transpeptidase module of PBP2a and the GT module of class A PBP2. In addition, inactivation by site-directed mutagenesis of the GT activity of PBP2 gives rise to a viable mutant susceptible to methicillin. Glycan chain elongation in the mutant is presumably catalyzed by the MGT (17, 18). This enzyme thus seems to play a role in cell wall assembly.

The GTs of *E. coli* PBP1b, *Streptococcus pneumoniae* PBP2a, and *S. aureus* PBP2 have been characterized (3, 9, 21). From site-directed mutagenesis experiments, it was shown that Glu233 in motif 1, which is conserved in all class A PBPs and MGTs, is the key element of the GT catalytic center of *E. coli* PBP1b (21). Ca$^{2+}$ or Mg$^{2+}$ ions are required for the activity and thus appear to play a catalytic role in the GT reaction (20). Moenomycin and vancomycin derivatives inhibit the GT step.
of PG polymerization (2, 6). Owing to its location outside of the cytoplasmic membrane and its specificity, the glycosyltransferase is an interesting drug target that has not been fully explored yet. The development of new inhibitors requires the characterization of these enzymes at structural and mechanistic levels.

Various constructions have been made to isolate the GT module of class A PBP1b. In each case, the activity of the GT module was ~20% of that of the full-size PBP (4, 21). MGTs appear to be of great interest for GT structure determination because of their smaller size compared to class A PBPs. However, no kinetic data are available on any MGT.

The objective of this work was to determine the kinetic parameters of the S. aureus MGT and analyze the role of the putative catalytic Glu100 (equivalent to the catalytic Glu233 of E. coli PBP1b) by site-directed mutagenesis. The enzymatic properties of S. aureus MGT were compared to those of S. aureus PBP2, E. coli PBP1b, and E. coli MGT.

MATERIALS AND METHODS

Materials. The plasmid pNJ2DmpSa, carrying S. aureus mgt gene (UniProt Q93Q23), was a gift from M. Arthur (1). The lipid II, undecaprenyl-pyrophosphate (D68-R268) MGT. The recombinant protein was called -(5')-CTAC-3

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QuikChange site-directed mutagenesis kit (Stratagene), the template plasmid VOL. 188, 2006 MONOFUNCTIONAL GLYCOSYLTRANSFERASE OF S. AUREUS

Results: Protein preparation and GT assay optimization. A soluble form of S. aureus MGT devoid of its membrane anchor, called SauH2-MGT (D68-R268), was expressed in the cytoplasm of E. coli C41 (DE3) strain (15) and purified on a HisTrap Ni-Septahose HP column (see Materials and Methods) (Fig. 1). The protein was 85% pure, and the final yield was about 5 mg/liter of culture. Gel filtration analysis in the presence of 25 mM Tris-HCl (pH 7.5)–0.3 M NaCl–10% glycerol (buffer G) showed that the protein was eluted with an apparent molecular mass of ~400 kDa, indicating an aggregation of the protein as described previously (24). When gel filtration was carried out in buffer G containing 0.7% CHAPS, most of the protein (80%) was eluted with the expected molecular mass of 25 kDa (data not shown).

SauH2-MGT (D68-R268) catalyzed glycogen chain polymerization when the protein (64 nM) was incubated in the presence of meso-[14C]A2pm-labeled C55 lipid II (2.24 mM) in 50 mM Tris-HCl (pH 7.5) buffer containing 10 mM MnCl2, 0.5% decyl PEG, 12.5% 1-octanol, and 12.5% DMSO. We verified that the polymerized product was completely digested with lysozyme. Moenomycin, used at a concentration of 75 nM and an antibiotic/MGT ratio of 12.1, inhibited the GT activity by 50%. SauH2-MGT (D68-R268) was fivefold less sensitive to moenomycin than E. coli PBP1b (75 nM versus 15 nM) (21).

The optimal conditions for the activity of SauH2-MGT (D68-R268) were determined by using variable concentrations of 1-octanol, DMSO, or decyl PEG. Omitting one of these reagents in the reaction mixture resulted in very low GT activity. The optimal conditions are probably a compromise between C25-lipid II solubility and enzyme integrity in the pres-
mine the kinetic parameters of SauH₂-MGT, respectively, indicating that Mn²⁺
SauH₂-l-octanol, 64 nM

The effect of metal ions on the activity of SauH₂-MGT (D68-R268) activity. The effect of metal ions on the activity of SauH₂-MGT was tested at pH 7.5. The results show that SauH₂-MGT was almost inactive in the presence of EDTA or in the absence of metal ions (0.8 and 0.65 nmol of disaccharide U/min/mg, respectively). The GT activity of the enzyme was strongly stimulated in the presence of Mn²⁺ (18 nmol/min/mg), Ca²⁺ (13 nmol/min/mg), or Mg²⁺ (12 nmol/min/mg) at a concentration of 10 mM. Effects of various concentrations of Mn²⁺ and Mg²⁺ on the enzyme activity were also determined (Fig. 2A). The results show that the concentrations of Mn²⁺ or Mg²⁺ needed for optimal SauH₂-MGT activity were 10 mM and 50 mM, respectively, indicating that Mn²⁺ was a better cofactor for the enzyme activity than Mg²⁺.

The pH dependence study was carried out at various pHs ranging from 5 to 8.5 in the presence of 10 mM MnCl₂. Figure 2B shows that the activity of SauH₂-MGT was optimal at pH 7.5.

Kinetic parameters of SauH₂-MGT (D68-R268). To determine the kinetic parameters of SauH₂-MGT (D68-R268), the enzyme (64 nM) was incubated in the presence of meso-[^14]C]A₂pm-lipid II substrate at concentrations varying from 0.25 to 5 μM, at pH 6.0 with Mn²⁺ and at pH 7.5 with Mn²⁺ or Mg²⁺. Analysis of the initial rate measurements of radioactive peptidoglycan synthesis and application of the Michaelis-Menten equation permitted determination of the kinetic parameters of the reaction (Table 1). At pH 7.5 and in the presence of Mn²⁺ (10 mM or 50 mM), the Kₘ values were 1.9 ± 0.8 and 1.5 ± 0.5 μM and the k₅ values were (8 ± 2.0) × 10⁻³ s⁻¹ and (7.6 ± 1.0) × 10⁻³ s⁻¹, respectively. Therefore, the k₅/Kₘ efficiencies were ~4,400 M⁻¹ s⁻¹ at 10 mM and 5,100 M⁻¹ s⁻¹ at 50 mM of Mn²⁺. At pH 7.5 and in the presence of Mn²⁺ (10 mM), the reaction proceeded with a K₅ value of 2.2 ± 1.4 μM, a k₅ value of (13 ± 4) × 10⁻³ s⁻¹, and a k₅/Kₘ of ~5,800 M⁻¹ s⁻¹.

At pH 6.0 and in the presence of Mn²⁺, the Kₘ value was 2.8 ± 2.0 μM, the k₅ value was (6.3 ± 2.6) × 10⁻³ s⁻¹, and the k₅/Kₘ efficiency was ~2,100 M⁻¹ s⁻¹. This result shows that the activity of the enzyme is higher at pH 7.5 than at pH 6.0. The effect of pH 7.5 versus pH 6.0 seems to affect mainly the k₅ value, which increased about twofold.

Role of the conserved Glu100 amino acid residue. The Glu residue in motif 1 is strictly conserved in all class A PBPss and MGTs. We have previously shown that Glu233 is essential for the GT activity of E. coli PBP1b (21). In order to test the function of Glu100 in motif 1 of S. aureus MGT, this residue was replaced by Gln. The SauH₂-MGT Glu100Gln mutant was

![FIG. 2. Effects of Mn²⁺ and Mg²⁺ concentrations on the GT activity of SauH₂-MGT (A) and pH dependence (B). The reaction was carried out at 30°C with SauH₂-MGT (64 nM), 0.5% decyl PEG, 12.5% DMSO, 12.5% octanol, and 2.24 μM meso-[^14]C]A₂pm-labeled C₅₅ lipid II. In addition, the assay mixtures contained 50 mM buffer and metal ions. The assay conditions were 50 mM Tris-HCl, pH 7.5, with variable concentrations of MnCl₂ and MgCl₂ (0.1 to 150 mM) (A) and 10 mM MnCl₂ with different buffer conditions: sodium acetate (pH 5.0), imidazole-N,N'-bis(2-ethanesulfonic acid) or morpholineethanesulfonic acid (pH 6.0), HEPES (pH 7.0 to 8.0), and Tris-HCl (pH 7.5 to 8.5) (B).](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>10 mM Mn²⁺ (pH 6.0)</th>
<th>10 mM Mn²⁺ (pH 7.5)</th>
<th>10 mM Mg²⁺ (pH 7.5)</th>
<th>50 mM Mg²⁺ (pH 7.5)</th>
<th>E. coli PBP1bᵇ</th>
<th>S. aureus PBP2ᶜ</th>
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<tbody>
<tr>
<td>k₅ (10⁻³ s⁻¹)</td>
<td>6.3 ± 2.6</td>
<td>13.0 ± 4.0</td>
<td>8.0 ± 2.0</td>
<td>7.6 ± 1.0</td>
<td>70 ± 13</td>
<td>15.0 ± 1.0</td>
</tr>
<tr>
<td>Kₘ (μM)</td>
<td>2.8 ± 2.0</td>
<td>2.2 ± 1.4</td>
<td>1.9 ± 0.8</td>
<td>1.5 ± 0.5</td>
<td>1.8 ± 0.8</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>k₅/Kₘ (10⁻³ s⁻¹)</td>
<td>2,100</td>
<td>5,800</td>
<td>4,400</td>
<td>5,100</td>
<td>39,000</td>
<td>3,400</td>
</tr>
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ᵃ The reactions were carried out in 50 mM PIPES (pH 6.0) or 50 mM Tris-HCl (pH 7.5) containing 10 or 50 mM metal ion, 0.5% decyl PEG, 12.5% DMSO, 12.5% l-octanol, 64 nM SauH₂-MGT, and 0.25 to 5 μM meso-[^14]C]A₂pm-labeled C₅₅ lipid II.
ᵇ Data are from reference 21.
ᶜ Data are from reference 3.
expressed and purified as described for the wild-type \textit{SauH}$\_5^{-}$-MGT without a detectable difference in the behavior of the two proteins in terms of stability, except that the expression level of the mutant was four times higher than that of the wild type, presumably because the mutant has a less deleterious effect on bacterial growth than the nonmutated protein. The glycosyltransferase activity of the \textit{SauH}$\_5^{-}$-MGT Glu100Gln mutant was reduced 500-fold compared to the wild type (23.3 nmol of lipid II used/mg of enzyme/min versus 11.6 \times 10^{3} \text{ nmol/mg/min}). This result is similar to that obtained with the \textit{E. coli} PBP1b E233Q mutant, which in addition was not active in vivo (21). Therefore, although the Glu100Gln mutant retains very low activity, the Glu100 residue may also be essential for the physiological activity of \textit{S. aureus} MGT in vivo.

**DISCUSSION**

A very limited number of GTs have been purified and characterized (\textit{E. coli} PBP1b, \textit{S. aureus} PBP2, and \textit{S. pneumoniae} PBP2a) and among them no MGTs. To our knowledge this is the first detailed characterization (kinetic parameters, metal ion, and pH effects) of an MGT using purified enzyme and purified lipid II substrate. We produced a soluble form of the \textit{S. aureus} MGT devoid of its membrane anchor similar to that previously reported (24); both constructs contain the polypeptide D68-R268 but differ slightly in the added N-terminal poly-His-containing segments (H$_{6}$ and H$_{10}$, respectively). H$_{10}$-MGT was expressed in \textit{E. coli} BL21(DE3)/pLysS, whereas \textit{SauH}$\_5^{-}$-MGT could not be overexpressed in this strain but was highly expressed in \textit{E. coli} C41 BL21(DE3) (15).

As determined in the in vitro GT assay developed for \textit{E. coli} PBP1b and purified \textit{meso-}[\textsuperscript{14}C]A$_{pm}$-labeled lipid II as substrate, \textit{SauH}$\_5^{-}$-MGT was able to catalyze glycan chain polymerization. This precursor contains A$_{2pm}$ in the third position of the peptide moiety instead of penta-Gly-substituted Lys, the natural substrate in \textit{S. aureus}. These results show that the integrity of the peptide sequence is not an absolute requirement for the GT activity, but we cannot rule out the possibility that the peptide may have a role in interaction with the enzyme. These questions can be answered by using lipid II containing different peptide moieties. In contrast to \textit{E. coli} MGT, which was insensitive to moenomycin (8), the GT activity catalyzed by \textit{SauH}$\_5^{-}$-MGT was inhibited by this inhibitor, as previously shown (24). The inhibition efficiency was about fivefold lower than for \textit{E. coli} PBP1b (75 nM versus 15 nM) (21). It is possible that moenomycin makes interactions beyond the GT domain of PBP1b (the additional N-terminal insertion or transpeptidase domain), increasing its affinity for this protein.

In a previous study, the enzymatic activity of the purified \textit{S. aureus} H$_{10}$-MGT was measured on the basis of the incorporation of \textit{[\textsuperscript{14}C]A$_{pm}$-labeled N-acetylglucosamine using the membrane fraction of \textit{Aerococcus viridans} (24). On the other hand, the enzymatic activity of \textit{E. coli} MGT was measured directly with \textit{[\textsuperscript{14}C]A$_{pm}$-labeled lipid II substrate but the enzyme was not purified (8). \textit{S. aureus} H$_{10}$-MGT and \textit{E. coli} MGT have optimal activity at pH 6.0. Compared to our GT reaction, where both lipid II and the \textit{SauH}$\_5^{-}$-MGT enzyme were purified and the GT assay was optimized, the previous studies used a complex mixture; this may explain the differences in the enzyme activities in pH function in the two studies (see below). \textit{S. aureus} PBP2 also had a low pH optimum of 5.0 (3). Furthermore, \textit{S. aureus} PBP2 was active in the absence of metal ions, and the addition of ions had a moderate effect on activity (3). In contrast, we found that \textit{SauH}$\_5^{-}$-MGT had a pH optimum of 7.5 and that the protein was inactive in the absence of metal ions; addition of Mg$^{2+}$, Mn$^{2+}$, or Ca$^{2+}$ drastically enhanced the activity of the enzyme. The \textit{SauH}$\_5^{-}$-MGT properties determined here are similar to those of \textit{E. coli} PBP1b in terms of pH optimum and metal ion requirement, although PBP1b has a preference for Ca$^{2+}$ (20) whereas \textit{SauH}$\_5^{-}$-MGT seems to prefer Mn$^{2+}$.

The enzymatic efficiency of \textit{SauH}$\_5^{-}$-MGT, the $k_{cat}/K_{m}$ value, is about 5,800 M$^{-1}$ s$^{-1}$ (with a $K_{m}$ value of 2.2 $\mu$M and a $k_{cat}$ value of 13 \times 10^{-3} s^{-1})$. The catalytic efficiency is 7.5-fold lower than that of \textit{E. coli} PBP1b and similar to that of \textit{S. aureus} PBP2 (Table 1). The $k_{cat}$ value of \textit{S. aureus} MGT was close to that of \textit{S. aureus} PBP2 (13 \times 10^{-3} versus 70 \times 10^{-3} s^{-1}). The catalytic constant $k_{cat}$ with Mn$^{2+}$ was 1.6-fold that with Mg$^{2+}$, suggesting that the metal ion may play a catalytic role in the reaction catalyzed by \textit{SauH}$\_5^{-}$-MGT, as has been shown for \textit{E. coli} PBP1b (20). The $K_{m}$ value of \textit{SauH}$\_5^{-}$-MGT was comparable to those of PBP1b (2.2 versus 1.8 $\mu$M) and \textit{S. aureus} PBP2 (2.2 versus 4 $\mu$M). This observation may suggest that the contribution of the peptide moiety of lipid II in overall binding between the substrate and the bifunctional enzymes is probably weak.

The glutamate residue of the first conserved motif, EDXXFXX(H/N)(X/G/A), is highly conserved in all known GT51 sequences (P. M. Coutinho and B.Henrissat, Carbohydrate-Active Enzymes server [http://afmb.cnrs-mrs.fr/CAZY/GT_51.html]). In \textit{E. coli} PBP1b, E233 in this position was found by site-directed mutagenesis to be essential for catalysis, and a model based on the glycosidase mechanism was proposed (21). Further investigations of PBP1b GT activity dependence on pH and metal ions allowed the proposition of a model wherein Glu233 catalyzes deprotonation of the 4-OH nucleophile of the growing glycan chain while a metal ion stabilizes the leaving group (20). In the present study, we found that \textit{S. aureus} MGT presents a pH optimum and metal ion requirement similar to that of \textit{E. coli} PBP1b. In addition, the conserved Glu100 was important for the activity of \textit{S. aureus} MGT, confirming the essential role of the glutamate residue of the first motif, EDXXFXX(H/N)(X/G/A), and suggesting that it is likely the key catalytic residue of the GT51 active site.

For more than two decades, \textit{E. coli} PBP1b, a multimodular class A PBP, has been the source of knowledge on the GT reaction (16, 20, 21, 23). While this protein is a useful tool for biochemical studies, its properties, which are probably common to many other class A PBPs (10), have until now prevented the growth of useful crystal and determination of its structure. The \textit{SauH}$\_5^{-}$-MGT characterized in this work displays relatively high GT activity, indicating that the native GT domain is properly folded. Its properties, in terms of solubility and small size, could make it an appropriate candidate for structural and mechanistic studies.

**ACKNOWLEDGMENTS**

This work was supported by Return Grant to M.T. from the Belgian Science Policy and was partially supported by the Belgian Program on Interuniversity Poles of Attraction, initiated by the Belgian State,
Prime Minister’s Office, Science Policy Programming (IAP P5/33); the Actions de Recherche Concertées (grant 03/08-297); and the European Commission (grant LSMH-CT-2003-503335). We thank J.-M. Frère for reading the manuscript.

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