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In Vitro Murein (Peptidoglycan) Synthesis by Dimers of the Bifunctional Transglycosylase-Transpeptidase PBP1B from *Escherichia coli**

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PBP1B is a major bifunctional murein (peptidoglycan) synthase catalyzing transglycosylation and transpeptidation reactions in *Escherichia coli*. PBP1B has been shown to form dimers *in vivo*. The K_D value for PBP1B dimerization was determined by surface plasmon resonance. The effect of the dimerization of PBP1B on its activities was studied with a newly developed *in vitro* murein synthesis assay with radioactively labeled lipid II precursor as substrate. Under conditions at which PBP1B dimerizes, the enzyme synthesized murein with long glycan strands (>25 disaccharide units) and with almost 50% of the peptides being part of cross-links. PBP1B was also capable of synthesizing trimeric muropeptide structures. Tri-, tetra-, and pentapeptide compounds could serve as acceptors in the PBP1B-catalyzed transpeptidation reaction.

Most bacteria contain a murein (peptidoglycan) sacculus, an exoskeleton that is essential for the osmotic stability of the cell (1). Murein has a net-like structure and is composed of glycan strands of alternating β1,4-linked *N*-acetylmuramic acid and *N*-acetylglucosamine residues that are cross-linked by short peptides (2). *Escherichia coli* is rod-shaped and has a simple cell cycle; a newborn cell elongates with a constant diameter, and when the length of the cell has doubled, the cell divides at mid-cell, forming two new polar caps. Cell growth requires a well coordinated incorporation of the murein precursor, lipid II, into the existing sacculus (3).

Two reactions are required for murein synthesis. First, disaccharide units of lipid II are oligomerized to murein glycan strands by transglycosylation. Second, peptide cross-links are formed by transpeptidation, the latter reaction being the target of β-lactam antibiotics. Murein synthases have a modular domain structure (4). *E. coli* has three bifunctional transglycosylases-transpeptidases, the class A penicillin-binding proteins (PBPs)[3] 1A, 1B, and 1C (4–6), two monofunctional transpeptidases, the class B PBPs 2 and 3, which contain a non-catalytic domain of unknown function (7), and one monofunctional transglycosylase, MtgA (8). Different molecular interactions between certain murein synthases and between murein synthases and hydrolases have been identified, indicating that the enlargement of the sacculus might be achieved by murein synthesis holoenzymes (9).

PBP1B exists in three isoforms (α, β, and γ) that are encoded by the same gene (*ponB*) and is a major murein synthase in *E. coli* (10–12). The protein has a short cytoplasmic part and is anchored in the cytoplasmic membrane via the amino acids 64–88. Most of the enzyme is located in the periplasm (amino acids 89–844), and the periplasmic part contains the transglycosylase (amino acids 198–435) and transpeptidase (amino acids 447–780) domains (13). PBP1B was shown to form dimers *in vivo* (14, 15), and dimerization does not involve disulfide bridges (16). Moreover, PBP1B interacts with PBP3 and with the MltA-interacting protein A (MipA), that itself interacts with the murein hydrolase MltA (17). Overproduction of inactive PBP1B variants but not of the active enzyme results in lysis only in the presence of lytic transglycosylases, indicating the existence of *in vivo* interactions of PBP1B with lytic transglycosylases (18).

Previous studies have demonstrated the catalytic transglycosylase and transpeptidase activities of PBP1B from *E. coli* with lipid II or with artificial substrates (13, 19, 20), have determined the kinetic parameters of the transglycosylation reaction (21), and have described high throughput screens for inhibitors for both activities (22, 23). However, the published assays do not directly quantify the muropeptide reaction products by high-resolution techniques such as HPLC, and the yield of the transpeptidation reaction was generally low in the previously published methods (13, 19, 20). In this work, we have established a new HPLC-based assay to determine the reaction products of transglycosylation and transpeptidation of lipid II with PBP1B. We found reaction conditions under which PBP1B dimers produced murein with glycan strands of an average length of more than 25 disaccharide units and with almost 50% cross-linked peptides. PBP1B could also form trimeric structures. Tri-, tetra-, and pentapeptide compounds (L-Ala-D-iGlu-m-A_2pm, L-Ala-D-iGlu-m-A_2pm-D-Ala, and L-Ala-D-iGlu-m-A_2pm-D-Ala-D-Ala, respectively, where iGlu is isoglutamate and A_2pm is diaminopimelic acid) could be used as acceptors in PBP1B-catalyzed transpeptidation reactions.

MATERIALS AND METHODS

Purification of PBP1B—The short γ-form of PBP1B with an N-terminal extension of six amino acids, $GSHMASM^{46}$-N^{844}, was purified in two chromatography steps. For this, the His-tagged form of PBP1B was isolated from the overproduction strain BL21(DE3) pDML924 (13) grown at 37 °C in 10 liters of LB medium with 50 μg/ml kanamycin. When the optical density at 578 nm reached 0.7, 1 mM isopropyl 1-thio-β-D-galactopyranoside was added to the culture, and the cells were grown for 5 more hours. The cells were harvested by centrifugation at 4200 × *g* for 15 min at 4 °C, and the membrane extract was prepared as described previously (17), with the exception that the membrane was

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[3] The abbreviations used are: PBP, penicillin-binding protein; MurNAc, *N*-acetylmuramic acid; HPLC, high-pressure liquid chromatography.

extracted with 30 ml of 5 mM Tris/HCl, 1 M NaCl, 10 mM $MgCl_2$, 20% glycerol, 2% Triton X-100, pH 7.5, for 1 h at 6 °C. The sample was centrifuged at 150,000 × *g* for 60 min at 4 °C. The supernatant was taken, and 15 mM imidazole (pH 7.5) and 8 ml of an aqueous suspension of nickel-nitrilotriacetic acid beads (Superflow, Qiagen) were added. After an incubation for 18 h at 6 °C, the beads were washed seven times with extraction buffer containing 15 mM imidazole. Then PBP1B was eluted twice by 2 bead volumes of extraction buffer containing 400 mM imidazole. Both elution fractions were combined and dialyzed six times against 1 liter of buffer I (25 mM Tris/HCl, 1 M NaCl, 10 mM $MgCl_2$, 10% glycerol, 0.5 mM EGTA, pH 7.5). Then 2 units/ml thrombin (Novagen) were added to the dialysis tube, and dialysis continued for 20 h against buffer I, for 2.5 h against 3 liters of buffer II (10 mM sodium acetate, 10 mM $MgCl_2$, 10% glycerol, 0.02% NaN_3, pH 5.0) containing 1 M NaCl, and for 17 h against 5 liters of buffer II containing 300 mM NaCl. The dialyzed protein was diluted 2-fold in buffer II containing 100 mM NaCl, dialyzed for 5 h against this buffer and purified on a 5-ml HiTrap SP HP MonoS cation exchange column using an Äkta purifier (Amersham Biosciences) in a 65-ml gradient from buffer II containing 100 mM NaCl and 0.25% Triton X-100 to buffer II containing 2 M NaCl and 0.25% Triton X-100. The fractions containing PBP1B were dialyzed against 10 mM sodium acetate, 500 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 0.02% NaN_3, pH 5.0, and stored at −20 °C. The purified PBP1B was active and bound biotinylated ampicillin (24).

Surface Plasmon Resonance Studies—A BIAcore™ 2000 (BIAcore AB, Uppsala, Sweden) machine and the BIAcore control software version 1.3 were used for surface plasmon resonance studies. PBPs were immobilized on the surface of ampicillin-coated CM5 sensor chips as described previously (17), with the exception that after protein coupling the excess of ampicillin was digested by an injection of 120 μl of 50 units/ml type II penicillinase from *Bacillus cereus* (Sigma) at a flow rate of 5 μl/min. Routinely, one control surface was prepared that was treated in the same way but did not contain immobilized protein. Interaction studies were performed in HBS running buffer (10 mM HEPES/NaOH, 10 mM $MgCl_2$, 150 mM NaCl, 0.05% Triton X-100, pH 7.4) at a flow rate of 10 μl/min. The analyte was diluted into HBS at different concentrations and was injected with simultaneous recording of the association signal. Then, HBS was injected, and the dissociation was measured. The sensorgrams were evaluated with the software package of the BIAcore™ system for the determination of the kinetic parameters (BIAevaluation version 2.1). A graphical representation of R_{eq} (response at equilibrium) divided by the concentration of the injected protein against R_{eq} (Scatchard plot) yields a straight line with a slope of $-K_a$, with $K_a = 1/K_D$ (K_D, dissociation constant). K_D values were determined in seven independent experiments, each with series of injections with certain concentration intervals.

Preparation of [^{14}C]GlcNAc-labeled Lipid II—First, lipid I was synthesized and purified essentially as described for lipid II (25), with the exception that during the synthesis no UDP-GlcNAc was present and that UDP-MurNAc pentapeptide, which was isolated from *Bacillus cereus* as described (26), was used. Lipid I was converted to lipid II using the MurG enzyme that was purified as described previously (27). Briefly, 2.5 μmol of lipid I was dissolved in 20 ml of a buffer containing 100 mM Tris/HCl, pH 8.0, 1 mM $MgCl_2$, 1% Triton X-100. Then 10 μCi of [^{14}C]UDP-GlcNAc (10.5 GBq/mmol, Amersham Biosciences) was added followed by 70 μg of MurG. The mixture was incubated for 30 min at room temperature. Then 5 μmol of "cold" UDP-GlcNAc and another 70 μg of MurG was added, and the mixture was incubated for another 30 min at room temperature, which resulted in a complete conversion of lipid I to lipid II, as was determined by TLC (not shown).

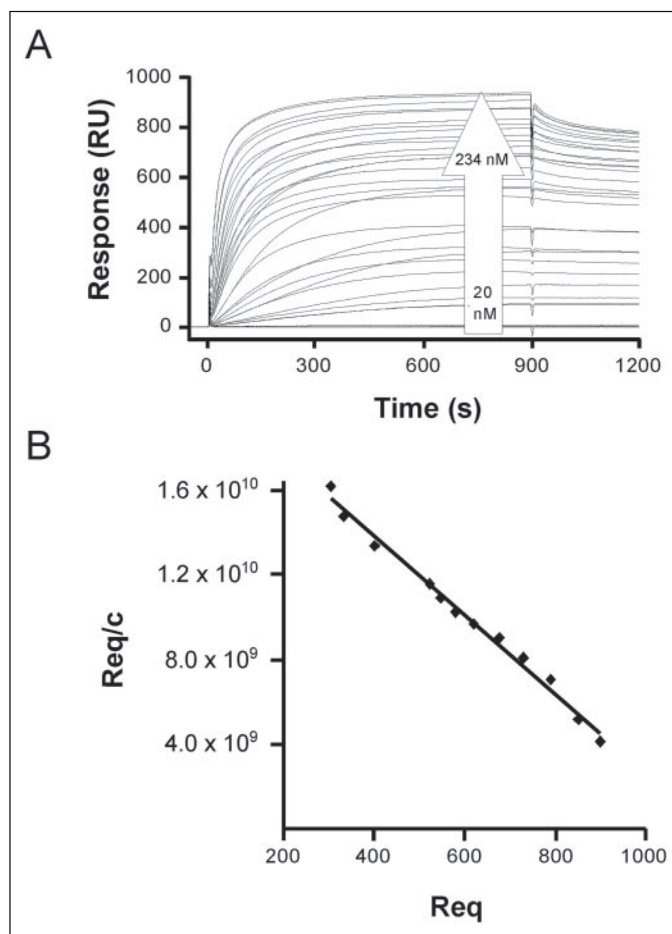

FIGURE 1. **Dimerization of PBP1B by surface plasmon resonance.** PBP1B was applied at concentrations of 20–426 nM to a surface containing immobilized PBP1B and to a control surface without PBP1B followed by injection of running buffer. Association and dissociation were monitored as change in resonance units (*RU*). *A*, signals of the series of PBP1B injections to the PBP1B surface after subtraction of the signals from the control surface. *B*, Scatchard plot (R_{eq}/c *versus* R_{eq}) of the data from *A*.

The reaction was stopped by the addition of 30 ml of butanol, 6 M pyridine acetate (pH 4.2), and lipid II was purified as described previously (25). The final pure product had a specific activity of 9180 dpm/nmol and was stored in chloroform/methanol 1:1 (v/v) at a concentration of 0.24 mM at −20 °C until use.

Transglycosylation and Transpeptidation Reaction of PBP1B with Lipid II—Lipid II (1.2 nmol, 11,000 dpm) was vacuum-dried and dissolved on ice in 5 μl of 1% Triton X-100 for 10 min. The reactions were performed in buffer containing 10 mM HEPES/NaOH, pH 7.5, 3 mM $MgCl_2$, 3.4% glycerol, 0.18% Triton X-100, and 150 mM NaCl. Unless otherwise stated, PBP1B was present at a concentration of 940 nM, and the total reaction volume was 50 μl. In different experiments, 0.2 mg/ml penicillin G, 0.2 mg/ml moenomycin, 1 mM UDP-MurNAc tripeptide, 1 mM UDP-MurNAc tetrapeptide, or 1 mM UDP-MurNAc pentapeptide was added. The reaction mixture was incubated at 30 °C for 60 min. Then the pH was adjusted to 4.8 by the addition of 0.1 N HCl, and 10 μg of cellosyl (kindly provided by Hoechst AG, Frankfurt, Germany) was added followed by an incubation at 37 °C for 3 h. The sample was then boiled for 10 min and either directly analyzed by HPLC or reduced prior to HPLC analysis. For reduction, 60 μl of 0.5 M sodium borate buffer, pH 9.0, containing 10 mg/ml $NaBH_4$ was added, and the sample was incubated at room temperature for 30 min. Excess of $NaBH_4$ was destroyed by adjustment of the pH to 3–4 with 20% phosphoric acid.

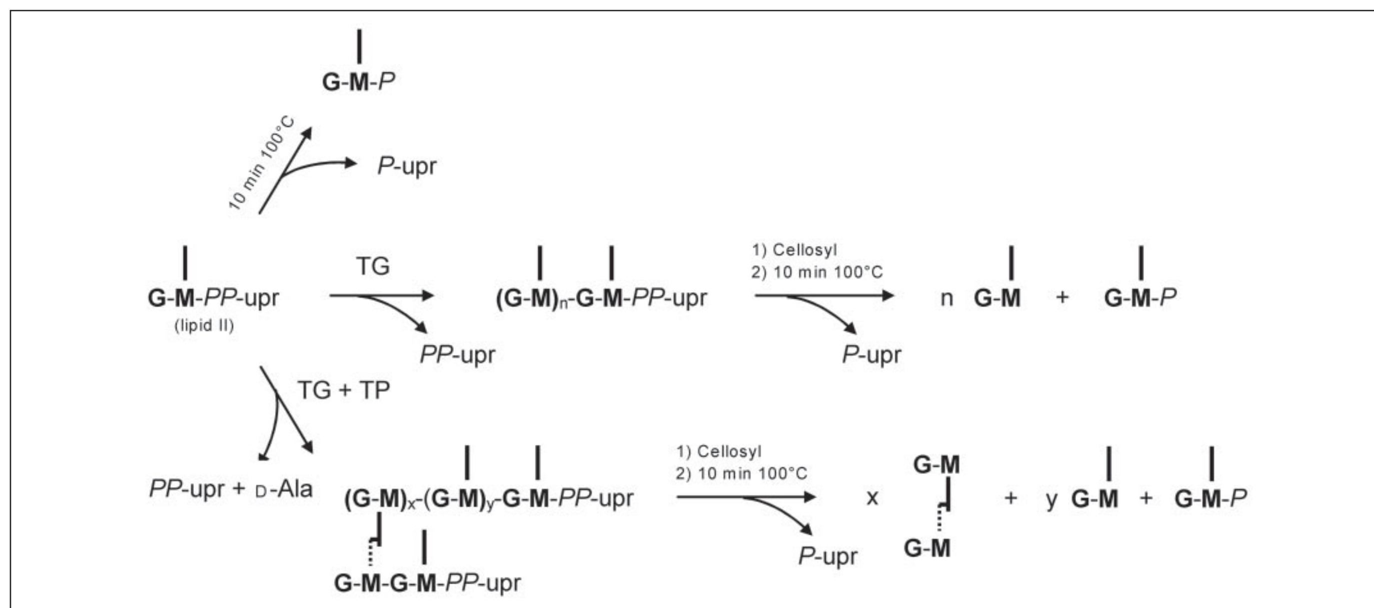

FIGURE 2. **Scheme for the reaction of lipid II with transglycosylases and transpeptidases and for the analysis of the reaction products.** Incubation of lipid II at 100 °C yields the monophosphate of the disaccharide pentapeptide. By transglycosylation of lipid II, the murein glycan strands are formed. Incubation with cellosyl and boiling for 10 min yields disaccharide pentapeptide and phosphorylated disaccharide pentapeptide that originates from chain ends. By transglycosylation and transpeptidation of lipid II, cross-linked murein glycan strands are formed. Incubation with cellosyl and boiling for 10 min yields, next to disaccharide pentapeptide and phosphorylated disaccharide pentapeptide, the dimeric bis-disaccharide tetrapentapeptide. *TG*, transglycosylation, *TP*, transpeptidation; *G*, GlcNAc; *M*, MurNAc; *P*, phosphate; *upr*, undecaprenyl; *PP*, diphosphate; *solid line* at *M*, pentapeptide; *dashed line* at *M*, tetrapeptide; *n*, *x*, *y*, stoichiometric factors.

Analysis of the Reaction Products—The reduced reaction products were analyzed by HPLC according to a published method (28, 29). Reduction at pH 9.0 resulted in a loss of ~5–15% of the phosphates from the monophosphorylated compounds. Therefore, the reduction step was omitted in experiments for quantification of the reaction products. In contrast to the reduced samples, non-reduced muropeptides were separated in a 70-min gradient from 0 to 30% methanol.

RESULTS

We aimed to test the effect of dimerization of PBP1B on its *in vitro* activities. For this, we first determined the K_D value for dimerization by surface plasmon resonance. PBP1B was immobilized to the surface of a sensor chip via its active site as described under "Materials and Methods." The amount of immobilized PBP1B was 1000–1200 resonance units (1 resonance unit corresponds to ~1 pg of protein/mm^2). We chose β-lactoglobulin A from cow milk that has a theoretical pI value of 5.1 as a control protein for the binding to immobilized PBP1B (pI 9.5). Although both proteins have opposite charges at the pH of the running buffer (7.4), there was no binding of β-lactoglobulin A to the PBP1B surface, even if injected at a very high concentration of 1 mM (not shown).

When PBP1B was applied to the sensor chip, we could detect a signal for its specific binding to immobilized PBP1B. To determine the affinity constant for the dimerization of PBP1B, a series of PBP1B solutions with concentrations from 20 to 426 nM were applied in HBS buffer. The Scatchard plot (R_{eq}/c over R_{eq}) gave a straight line with the slope of $-1/K_D$ (Fig. 1*B*). Seven independent experiments resulted in K_D values of $(1.27 \pm 0.96) \cdot 10^{-7}$ M for the dimerization of PBP1B.

To test whether the dimerization of PBP1B has an effect on its enzymatic activities, we developed a new method to monitor *in vitro* murein synthesis from radioactively labeled lipid II precursor (Fig. 2). Boiling of lipid II for 10 min at a mild acidic pH resulted in the removal of undecaprenyl phosphate and the formation of monophosphorylated disaccharide pentapeptide. The monophosphorylated compound can be converted by extended boiling at acidic pH or by treatment with acidic phosphatase from wheat to dephosphorylated disaccharide pentapeptide that coelutes (after reduction with $NaBH_4$) in HPLC with authentic disaccharide pentapeptide isolated from *E. coli* murein (not shown). By reaction of a transglycosylase with lipid II, murein glycan strands are oligomerized with concomitant removal of undecaprenyl pyrophosphate, presumably leaving one undecaprenyl pyrophosphate residue at the terminal MurNAc residue of the new glycan strand. Treatment with the muramidase cellosyl followed by boiling for 10 min resulted in the dephosphorylated disaccharide pentapeptide from subunits within the glycan chain that were released by cellosyl, the monophosphorylated disaccharide pentapeptide from the subunits from the chain end and/or from lipid II molecules that had not been oligomerized, and undecaprenyl phosphate. If transglycosylation is accompanied by transpeptidation, the above treatment additionally yields bis-disaccharide tetrapentapeptide. The murein synthesis reaction products were separated by HPLC and detected by a radioactivity flow-through detector. As expected and because of the presence of the different anomers (29), the non-reduced samples gave rise to broad peaks that nonetheless were well separated (Fig. 3).

To test the effects of dimerization on the activity of PBP1B and on the product formation, we applied different PBP1B concentrations to the reaction mixture. Strikingly, the extent of product formation depended strongly on the concentration of PBP1B (Fig. 3). At a concentration corresponding to one-third of the determined K_D for dimerization, only about 50% of the lipid II was used in the transglycosylation reaction, and there was no transpeptidation product. At a concentration equal to the K_D for dimerization, lipid II was converted to both transglycosylation and transpeptidation products. If PBP1B was present at a concentration of 7.5-fold the K_D for dimerization, lipid II was converted to murein with glycan strands of at least 27.8 disaccharide units, as can be calculated from the fraction of 3.6 $\pm$ 0.9% of monophosphorylated disaccharide pentapeptide (average from three experiments), that represent glycan chain ends plus non-reacted lipid II. In addition, the *in vitro* synthesized murein was highly cross-linked; 48.0 $\pm$ 2.5% of the material consisted of

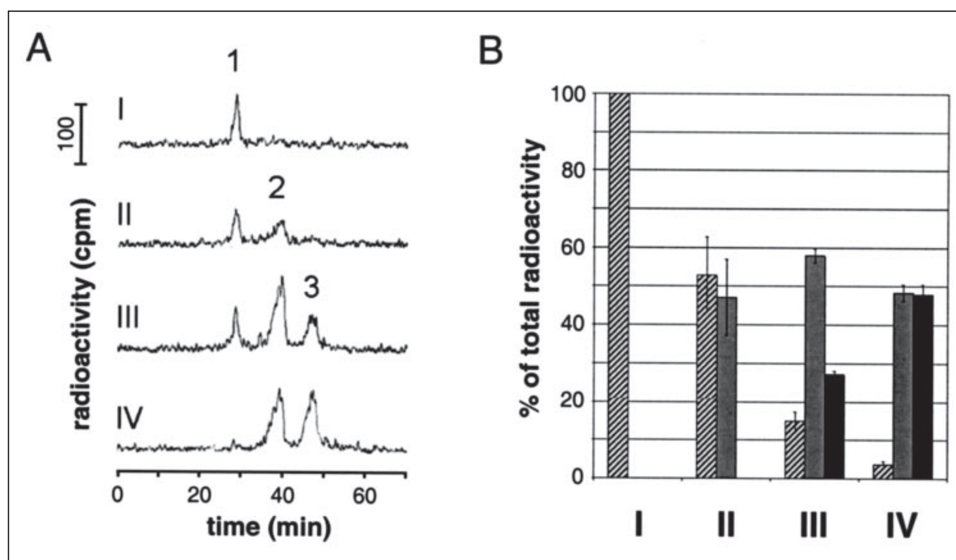

FIGURE 3. **Dependence of product formation on concentration of PBP1B.** *A*, lipid II was incubated with PBP1B followed by digestion with cellosyl, boiling for 10 min, and HPLC analysis of the reaction products. The concentrations of PBP1B were 42 nM (corresponds to one-third of the K_D value for dimerization, *chromatogram II*), 127 nM (corresponds to the K_D value for dimerization, *chromatogram III*), or 940 nM (7.5-fold the K_D value for dimerization, *chromatogram IV*). *Chromatogram I* shows the control sample without PBP1B. *Peak 1*, phosphorylated disaccharide pentapeptide; *peak 2*, disaccharide pentapeptide (transglycosylation product); *peak 3*, bis-disaccharide tetrapentapeptide (transglycosylation-transpeptidation product). *B*, three independent experiments were performed with different concentrations of PBP1B, and the peaks 1–3 were quantified. *Striped bars*, peak 1; *gray bars*, peak 2; *black bars*, peak 3. The sample *numbering I–IV* corresponds to the chromatograms I–IV in *A*. The yields of both reactions, transglycosylation and transpeptidation, were highest at the highest PBP1B concentration at which PBP1B dimers prevail over monomers. At the highest concentration of PBP1B, the *in vitro* synthesized murein had an average length of the glycan strands of about 20 disaccharide units with close to 50% of the peptides being part of cross-links.

cross-linked structures that were formed by transpeptidation (average from three experiments). The percentage of cross-linked product obtained in our *in vitro* murein synthesis reactions showed some day-to-day variation depending (in part) on the batch and on the aging of the PBP1B preparation, but cross-linked products were always produced at percentages between 30 and 50% of the total material at this concentration of PBP1B.

To further examine the reaction products of the *in vitro* murein synthesis assay in more detail, we treated the reaction mixture with $NaBH_4$, resulting in sharper peaks. The reduction step allowed the detection of an additional minor cross-linked trimeric product (Fig. 4*A*, *chromatogram II*, *peak 4*) that presumably represents the trimeric transpeptidation product of PBP1B, the tris-disaccharide tetratetrapentapeptide because (i) it is not present if transpeptidation is blocked by penicillin and (ii) it has the same retention time as the tris-disaccharide tetratetrapenta compound present in isolated *E. coli* murein (not shown). We have confirmed that the non-phosphorylated, reduced products of the reaction (disaccharide pentapeptide, bis-disaccharide tetrapentapeptide, and tris-disaccharide tetratetrapentapeptide) coeluted with authentic muropeptides isolated from murein of a pentapeptide-rich *E. coli* strain (not shown). Addition of 200 μg/ml penicillin G completely inhibited the transpeptidation (cross-linking) reaction but had no effect on transglycosylation, whereas the addition of 200 μg/ml moenomycin, a known transglycosylase inhibitor (30, 31), completely inhibited both reactions (not shown), thus validating our results.

The specificity of the transpeptidation reaction catalyzed by PBP1B was further studied by adding a 42-fold molar excess (over lipid II) of UDP-MurNAc tripeptide, UDP-MurNAc tetrapeptide, or UDP-MurNAc pentapeptide to the reaction (Fig. 4*A*, *chromatograms III–V*). All three compounds could act as acceptors in the transpeptidation reaction, as indicated by the formation of novel cross-linked compounds that were separated by HPLC from the normal bis-disaccharide tetrapenta cross-linking product. The proposed structures of the "mixed" dimers (*structures 5–7*) are shown in Fig. 4*C*.

We also aimed to test whether the transglycosylation and transpeptidation reaction products are produced simultaneously from lipid II or if one reaction precedes the other. The reactions were too fast to measure a time-dependent increase in transglycosylation-transpeptidation products at our standard conditions at the high PBP1B concentration (940 nM) that favors dimerization. Therefore, this experiment was performed at 20 °C (Fig. 5). The reaction was started by the addition of PBP1B, and samples were taken after different reaction times, boiled for 10 min, and digested with cellosyl. Then the samples were reduced with $NaBH_4$ and analyzed by HPLC. The first sample was taken prior to the addition of PBP1B and was used to correct for the dephosphorylation during the reduction step. Within the first 10 min, lipid II was consumed at a rate of ~2.23 mol/(mol PBP1B·min), which represents the rate of transglycosylation. The monomeric transglycosylation product increased with a rate of ~1.48 mol/(mol PBP1B·min). The dimeric transglycosylation-transpeptidation product increased with a rate of 0.67 mol/(mol PBP1B·min). In total, the cross-linking reaction occurred at a rate of 0.38 mol/(mol PBP1B·min) (transpeptidation rate). After 10 min of reaction time, the monomeric transglycosylation product did not further increase. In contrast, the dimeric (cross-linked) product continued to increase between 15 and 60 min, albeit with a much lower rate of $3.3 \cdot 10^{-2}$ mol/(mol PBP1B·min).

DISCUSSION

The bifunctional murein synthase PBP1B was shown to form dimers *in vivo* (14, 32), and the dimerization does not depend on intramolecular disulfide bonds between cysteine residues (16). In this work, we have studied the dimerization of isolated PBP1B by surface plasmon resonance. At a pH of 7.5, a NaCl concentration of 150 mM, and a Triton X-100 concentration of 0.05%, the dissociation constant was determined as $(1.27 \pm 0.96) \cdot 10^{-7}$ M. It is indeed possible that the anchoring of the enzyme in the cytoplasmic membrane contributes to its dimerization *in vivo*.

Purified murein precursor (lipid II) with a radioactive label in the GlcNAc residue was used as substrate for *in vitro* murein synthesis reactions by PBP1B. For analysis of the transglycosylation and transpeptidation reaction products, we have established an assay that is based on the HPLC method for *E. coli* muropeptide analysis (28, 29). The advantage of the new assay is that transglycosylation and transpeptidation products are analyzed directly and simultaneously with high resolution, as compared with previous assays employing paper chromatography (13, 20, 33). The buffer conditions used for surface plasmon resonance studies and for *in vitro* murein synthesis assays were similar with respect to the concentrations of NaCl, Triton X-100, and $MgCl_2$ and to the pH value.

The isolated PBP1B showed a significantly higher activity at condi-

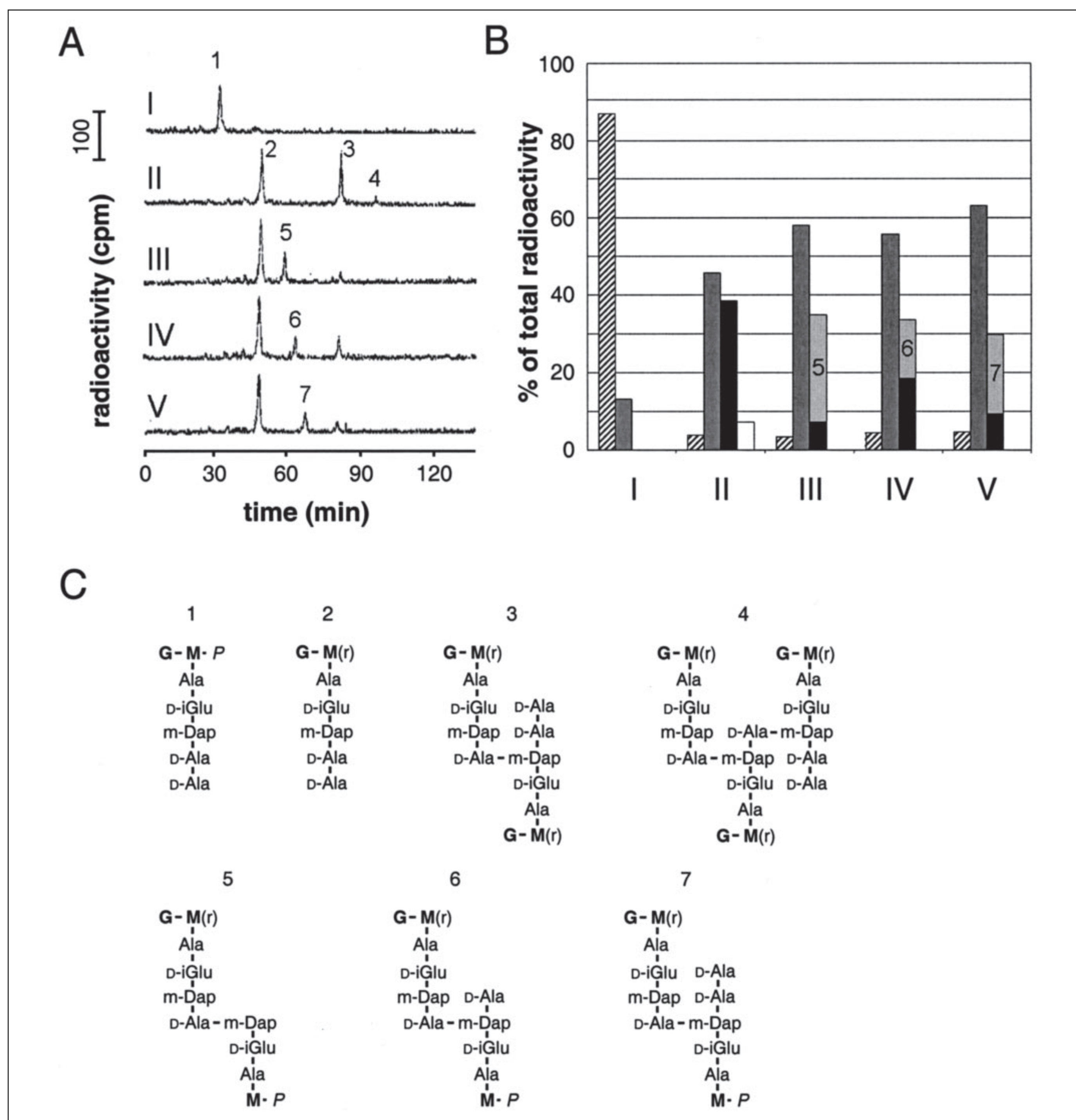

FIGURE 4. **Specificity of the transpeptidation reaction catalyzed by PBP1B.** PBP1B was incubated with lipid II in the absence or in the presence of different soluble murein precursors followed by cellosyl digestion, boiling for 10 min, and reduction with $NaBH_4$. The reaction products were analyzed by HPLC. *A*, chromatograms obtained from the sample with lipid II alone (*II*), lipid II and UDP-MurNAc tripeptide (*III*), lipid II and UDP-MurNAc tetrapeptide (*IV*), and lipid II and UDP-MurNAc pentapeptide (*V*). One control sample contained only lipid II and no PBP1B (*I*). The different peaks are *numbered*. *B*, quantification of the reaction products. Numbering *I–V* corresponds to the chromatograms *I–V* in *A*. *Striped bars*, peak 1; *gray bars*, peak 2; *black bars*, peak 3; *white bar*, peak 4. The *numbered gray bars* represent the percentage of the compounds 5, 6, or 7. *C*, proposed structures of the peaks 1–7. *G*, GlcNAc; *M*(*r*), reduced MurNAc (*N*-acetylmuramitol); *P*, phosphate; *iGlu*, *iso*-glutamate; *m-Dap*, *meso*-diaminopimelic acid. Note that compounds 1 and 2 are monomeric, compounds 3, 5, 6, and 7 are dimeric, and compound 4 is a trimeric muropeptide. PBP1B was able to transfer the disaccharide tetrapeptide to tri-, tetra-, or pentapeptide structures, yielding tetratri-, tetratetra-, or tetrapenta products. Furthermore, with lipid II, a small fraction of the trimeric muropeptide was produced.

tions that favor dimerization (Fig. 3). In particular the transpeptidation reaction is strongly enhanced at PBP1B concentrations well above the determined K_D value for dimerization. Such a concentration effect has not been reported before, and PBP1B has been used at lower concentrations in most previous *in vitro* murein synthesis studies. In contrast to most earlier studies, we have omitted 1-octanol and Me_2SO in the assay buffer and used only a non-ionic detergent, Triton X-100, to solubilize PBP1B and lipid II. At high concentrations of PBP1B, almost 50% of the peptides became part of cross-links, *i.e.* substantially higher than in previous studies. The low percentage (3.6%) of the phosphorylated compounds present after the reaction indicates that the synthesized glycan strands are on average longer than 25 disaccharide units. The transgly-

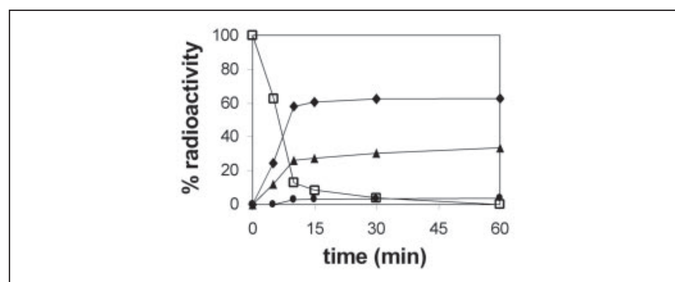

FIGURE 5. **Time course of the PBP1B-catalyzed transglycosylation and transpeptidation reactions.** Radioactively labeled lipid II (24 μM) was incubated with PBP1B (940 nM) in a total volume of 325 μl at 20 °C. At different time points, 50-μl aliquots were withdrawn, boiled for 10 min, digested with cellosyl, reduced by $NaBH_4$, and analyzed by HPLC. *Open squares*, compound 1 (Fig. 4*C*), substrate plus chain ends; *closed diamonds*, compound 2 (Fig. 4*C*), transglycosylation product; *closed triangles*, compound 3 (Fig. 4*C*), dimeric transglycosylation-transpeptidation product; *closed circles*, compound 4 (Fig. 4*C*), trimeric transglycosylation-transpeptidation product.

cosylation and transpeptidation products simultaneously increased within the first 10 min of the reaction (Fig. 5), with the rate of transpeptidation being approximately one-sixth of the rate of transglycosylation, yielding a murein with approximately one-third of the peptides being part of cross-links. At a 12-fold lower rate (as compared with the initial rate), secondary transpeptidation reactions occurred, resulting in a slow increase in transpeptidation products between 15 and 60 min of the reaction. Such a delayed transpeptidation activity has also been observed with recombinant PBP1B from *Streptococcus pneumoniae* that has been overproduced and purified from *E. coli* (34). These results suggest that the initial cross-linking reaction occurs simultaneously with the transglycosylation of lipid II, whereas the secondary cross-linking occurs at the already oligomerized glycan strands.

The new assay allowed the detection of a minor trimeric transpeptidation product, the tris-disaccharide tetratetrapenta muropeptide. In different experiments with high concentrations of PBP1B, the yield of this compound was between 4 and 7%, and it appeared only after 10 min of reaction time (Fig. 5). Therefore, it is likely that transpeptidation occurs to the already formed dimeric compound, yielding the trimer.

We have studied the specificity of the transpeptidation reaction by using different artificial substrates, the soluble murein precursors UDP-MurNAc tripeptide, UDP-MurNAc tetrapeptide, and UDP-MurNAc pentapeptide. The normal and mixed dimers could be separated by HPLC. PBP1B could use all UDP-linked substrates as acceptors for the transpeptidation reaction, indicating that the enzyme is not specific for pentapeptides as an acceptor for the cross-linking reaction but can utilize tripeptides and tetrapeptides as well.

Interestingly, PBP1B synthesized *in vitro* murein, that is, with respect to certain structural features, similar to murein formed *in vivo* and isolated from *E. coli* cells. *In vitro* synthesized murein consists of glycan strands of more than 25 disaccharide units (*in vivo*, 25–35 disaccharide units). *In vitro* synthesized murein had almost 50% of peptides being part of cross-links (*in vivo*, 40–50%). *In vitro* synthesized murein had a small percentage of trimeric compounds (*in vivo*, about 5%). Despite these similarities, there is a major difference between *in vitro* murein synthesis by PBP1B and *in vivo* murein synthesis. *In vivo*, new material (lipid II or newly synthesized larger murein fragments) is attached to the existing sacculus and is incorporated into the stress-bearing layer, presumably by well controlled activities of bifunctional and monofunctional murein synthases and murein hydrolases, resulting in the enlargement of the existing murein sacculus (2, 3). Our results of the *in vitro* activity of PBP1B point to the possibility that *in vivo* dimers of the bifunctional murein synthases (PBP1B or PBP1A) produce fragments of cross-linked murein that are then attached to and inserted into the existing murein layer by monofunctional transpeptidases and murein hydrolases. Until now, a murein sacculus enlargement reaction (as it occurs *in vivo*) could not be observed *in vitro*. The characterization of the *in vitro* activities of the different murein synthases, as studied here for PBP1B, is a step forward in understanding their possible roles in murein synthesis *in vivo*.

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