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The Bimodular G57-V577 Polypeptide Chain of the Class B Penicillin-Binding Protein 3 of *Escherichia coli* Catalyzes Peptide Bond Formation from Thiolesters and Does Not Catalyze Glycan Chain Polymerization from the Lipid II Intermediate

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Abstract

Because the specificity profile of the membrane anchor-free G57-V577 penicillin-binding protein 3 (PBP3) of *Escherichia coli* for a large series of β-lactam antibiotics is similar to that of the full-size membrane-bound PBP, the truncated PBP is expected to adopt the native folded conformation. The truncated PBP3 functions as a thiolesterase. In aqueous media and in the presence of millimolar concentrations of a properly structured amino compound, it catalyzes the aminolysis of the thiolester until completion, suggesting that the penicillin-binding module of PBP3 is designed to catalyze transpeptidation reactions. In contrast, the truncated PBP3 is devoid of glycan polymerization activity on the *E. coli* lipid II intermediate, suggesting that the non-penicillin-binding module of PBP3 is not a transglycosylase.

The multimodular class B penicillin-binding protein 3 (PBP3) is a key element of the cell septation network in *Escherichia coli* (30). This tripartite protein consists of an M1-E56 membrane anchor-containing module that is fused to a G57-1237 non-penicillin-binding (n-PB) module that is fused to the D238-V577 PB module (7, 12, 16). The membrane anchor-free G57-V577 polypeptide chain of PBP3 which comprises the n-PB and PB modules has been overproduced in the periplasm of *E. coli* as an autonomous folding entity (9, 16). With "p" denoting "periplasmic," this truncated PBP3 is called PBP3p.

Indirect experimental evidence suggests that the PB module of PBP3 is involved, one way or another, in peptide cross-linking during synthesis of the septal peptidoglycan (3, 26). In contrast, the role of the n-PB module of PBP3 is a matter of controversy. According to Ishino and Matsuhashi (19), this module is a transglycosylase catalyzing glycan chain elongation from disaccharide (peptide) lipid II intermediates. According to van Heijenoort et al. (29), it is not.

Because the thermostability and affinity for benzylpenicillin and cephalexin of the folded, membrane anchor-free polypeptide are unchanged in comparison with those of the membrane-bound PBP3, one can reasonably postulate that the conformation adopted by the truncated PBP3 reflects faithfully that of the native-state structure (9). β-Lactams and thiolester carbonyl donors and the disaccharide (peptide) lipid II intermediate were used to probe the enzymatic activities of the G57-V577 PBP3 and thus shed light on the possible functions of the protein in cell septation. The results are presented below.

(Some of the work described in this paper is part of a dissertation presented by M.A. in partial fulfilment of a Ph.D. degree at the University of Liège.)

MATERIALS AND METHODS

PBP3p and carbonyl donor substrates. PBP3p was purified as described previously (9). It was stored frozen at -20°C in 10 mM Tris-HCl (pH 8.0)-10% (vol/vol) glycerol-10% (vol/vol) ethylene glycol-0.5 M NaCl. The esters and thiolesters were described previously (1, 2). [^{14}C]benzylpenicillin (54 Ci/mol) was from Amersham International (Buckinghamshire, United Kingdom). 5'-Fluoresceyl-glycyl-6-aminopenicillanate (5'-Flu-Gly-6-

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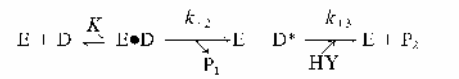
APA), 5'-fluoresceyl-ampicillin, 6'-fluoresceyl-ampicillin, and dansyl-6-APA were described previously (4, 23). Nonlabelled β-lactams were gifts from pharmaceutical companies.

Kinetic parameters. The interactions between PBP3p (E, for enzyme) and the thiolester and β-lactam compounds (D, for carbonyl donor) were interpreted on the basis of the following three-step reaction:



K is the dissociation constant of the Henri-Michaelis complex E · D, k_{+2}/K ($M^{-1}\,s^{-1}$) is the second-order rate constant of acyl enzyme (E - D*) formation, k_{+3} (s^{-1}) is the first-order rate constant of acyl enzyme breakdown, P_1 is the leaving group, HY is the attacking nucleophile (H_2O or amino compound), and P_2 is the second reaction product.

Hydrolysis of properly structured thiolesters proceeded to completion. D-Alanine and the tetrapeptide L-Ala-γ-D-Glu(amide)-(L)*m*A_2pm(L)-D-alanine (where *m*A_2pm is *meso*-diaminopimelic acid [15]) were used as amino acceptors (HY) of the transfer reactions. With the β-lactam antibiotics, the leaving group, P_1, remained part of the acyl enzyme, E - D*, and the reaction stopped at this level, at least for a long time (3 to 5 h).

Inactivation of PBP3p by β-lactam antibiotics, (i) Determination of the k_{+3} values. PBP3p samples were quantitatively inactivated by the β-lactam, the β-lactam in excess was hydrolyzed by treatment with the β-lactamase of *Bacillus licheniformis* (or that of the *Actinomadura* sp. strain R39 in the case of aztreonam), the β-lactamase was inactivated by treatment with 5 μM β-iodopenicillanate, and the samples were maintained at 37°C for increasing time intervals. The PBP3p released in an active form during the latter incubation was labelled with 2×10^{-5} M [^{14}C]benzylpenicillin, the acyl PBP3p was precipitated with trichloroacetic acid as described previously (25), the pellet was dissolved in 1% sodium dodecyl sulfate, and the radioactivity was measured. One may note that the k_{+3} values are related to the half-lives of the acyl PBP3ps by the equation $0.69/k_{+3}$ = half-life.

(ii) Determination of the k_{+2}/K values. PBP3p samples were incubated with increasing β-lactam concentrations during a given time at 37°C, and the extents of PBP labelling were estimated. The k_{+2}/K value for cephalexin was determined with the thiolester benzyl-D-alanyl-thioglycolate as the reporter substrate (6). On the basis of this value (45 $M^{-1}\,s^{-1}$), the k_{+2}/K value for benzylpenicillin was determined by competition experiments between cephalexin and [^{14}C]benzylpenicillin (10). On the basis of this value (4,000 $M^{-1}\,s^{-1}$), the k_{+2}/K values for the other β-lactams were determined by competition with [^{14}C]benzylpenicillin. The k_{+2}/K values for cephalexin and benzylpenicillin were confirmed by competition with dansyl-6-APA. Acylation of PBP3p by dansyl-6-APA caused fluorescence transfer to the dansyl group (excitation at 280 nm and maximum emission at 535 nm).

PBP3p-catalyzed acyl transfer reactions on esters and thiolesters. PBP3p samples were dialyzed against 10 mM Tris-HCl (pH 7.8) containing 10% (vol/ vol) dimethyl sulfoxide and 0.5 M NaCl (to eliminate the glycerol and ethylene glycol present in the enzyme preparation, which were susceptible to function as nucleophiles in transfer reactions). PBP3p and the esters and thiolesters were incubated at 37°C in 0.5 mM Tris-HCl-10 mM sodium phosphate (pH 7.4) containing 10% (vol/vol) dimethyl sulfoxide and 0.5 M NaCl. The k_{cat}/K_m values (equivalent to the k_{+2}/K values) were determined under first-order conditions at substrate concentrations lower than the K_m values. The increase of A_{254} ($\Delta\varepsilon$ = 500 $M^{-1}\,cm^{-1}$ [esterase activity]) and the decrease of A_{250} ($\Delta\varepsilon$ = 2,200 $M^{-1}\,cm^{-1}$ [thiolesterase activity]) were monitored with a UVIKON 860 spectrophotometer coupled to a microcomputer via an RJ322 interface (20-22). In those experiments in which D-alanine and L-Ala-γ-D-Glu(NH_2)-*m*A_2pm-D-Ala were used as alternative acceptors of the transfer reactions, the carbonyl donor (D) and the hydrolysis (H) and aminolysis (T [transpeptidation]) products were separated by high-performance liquid chromatography with an ET 250/8/4 Nucleosil 5C_{18} column (Macherey and Nagel) under the conditions used previously (2). With the cosubstrates benzoyl-D-Ala-thioglycolate and tetrapeptide, the retention times for D, H, and T were 14.89, 12.94, and 11.13 min, respectively (solvent A, 10 mM sodium acetate [pH 3.5] solvent B, acetonitrile gradient [0 to 100%] for 30 min). The T/H ratios were measured as described previously (2, 20). The product of the transfer reaction to the tetrapeptide was identified by electrospray mass spectrometry (8).

Polymerization of disaccharide-peptide units by transglycosylation from the lipid II intermediate. The *E. coli* lipid II intermediate *N*-acetylglucosaminyl-*N*-acetylmuramyl [L-Ala-γ-D-Glu-(L)*m*A_2pm-(L)-D-Ala-D-Ala] pyrophosphate-undecaprenol labelled with D-[^{14}C]alanine residues in its peptide moiety was prepared as described previously (29). Lipid II was used in two different in vitro polymerization assays (19-29). PBP3p was used at a final protein concentration of 5.9 μM in both assays.

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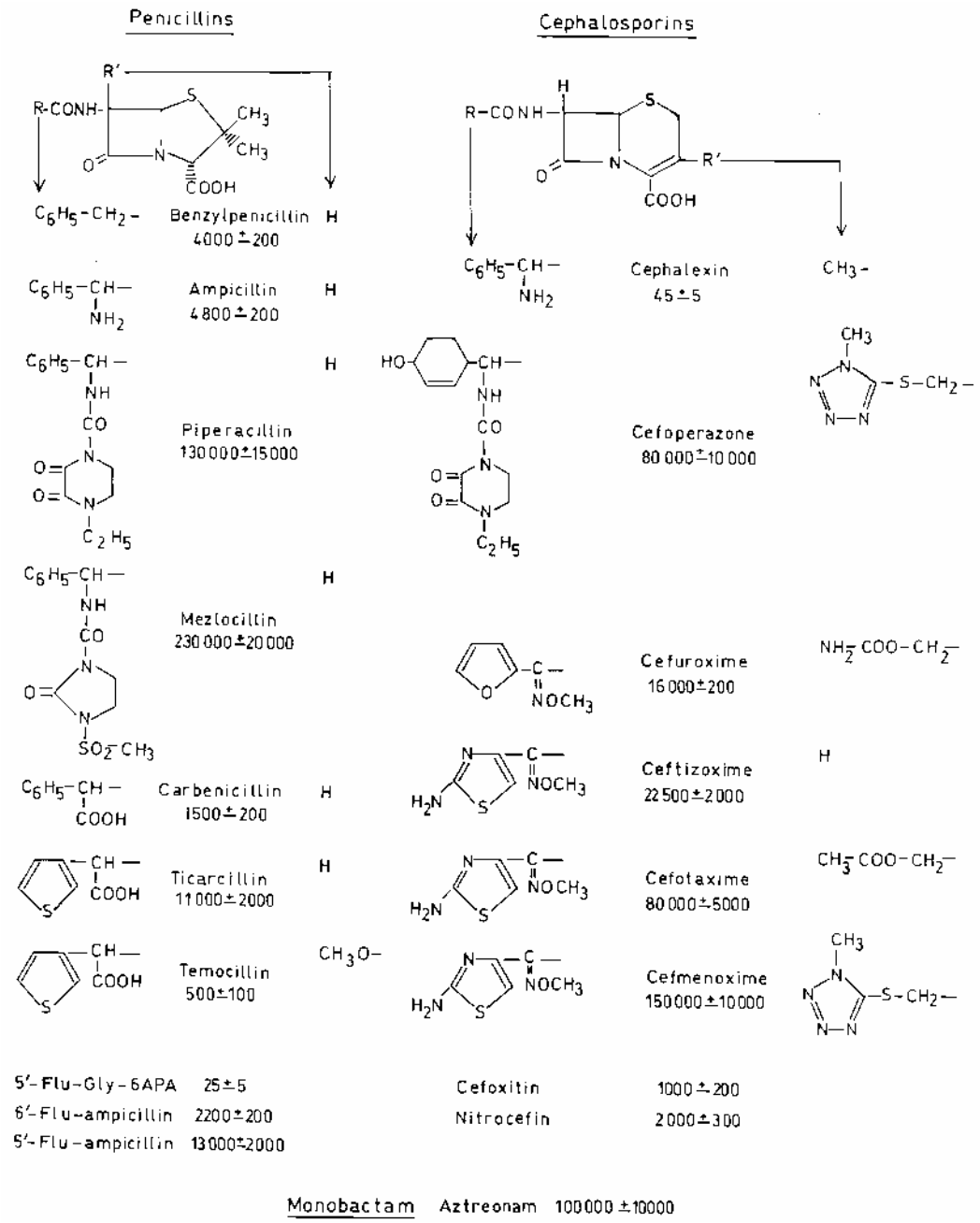
FIG. 1. *Second-order rate constant (k_{+2}/K) of acylation of E. coli PBP3p by β-lactam antibiotics. The constant values are expressed in molar concentrations per second. Fully developed formulas highlight structure-activity relationships.*



RESULTS

Inactivation of PBP3p by β-lactam antibiotics. In all cases tested, the acyl enzymes were very stable. Very low k_{+3} values (at 37°C) were obtained with cephalexin (1.5×10^{-6} s^{-1}), benzylpenicillin (4×10^{-5} s^{-1}), carbenicillin, ampicillin, mezlocillin, and aztreonam (from 3×10^{-6} s^{-1} to 5×10^{-6} s^{-1}). Hence, variations in the inactivation potency of the β-lactams for PBP3p depended on their efficacy as acylating agents.

The data shown in Fig. 1 led to the following conclusions, (i) The k_{+2}/K values (at 37°C) varied greatly, depending on the structure of the exocyclic substituents of the β-lactams, from 45 M^{-1} s^{-1} for cephalexin to 230,000 M^{-1} s^{-1} for mezlocillin. (ii) Mezlocillin, piperacillin, cefmenoxime, cefotaxime, cefoperazone, and aztreonam, which each cause filamentation of the *E. coli* cells at low concentrations, were the most potent

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acylating agents of PBP3p *(k_{+2}/K* values of ≥80,000 M^{-1} s^{-1}). (iii) Four groups of structurally related β-lactams highlighted the effects of the side chains on the rate of protein acylation, namely, the group benzylpenicillin ≤ ampicillin < piperacillin < mezlocillin; the group benzylpenicillin > carbenicillin < ticarcillin > temocillin; the diad piperacillin ≥ cefoperazone; and the triad ceftezoxime, cefotaxime, and cefmenoxime.

The k_{+2}/K values obtained with PBP3p (at 37°C) were compared to the corresponding k_{+2}/K values for the membrane-bound PBP3 (at 30°C). The latter values were calculated from the published ID_{50} (or ID_{90}) values, *i.e.* the antibiotic concentrations which inactivate the PBP by 50 or 90% after a given time of incubation (5, 11), by the equation $[ID_{50}] \cdot t = 0.69\ K/k_{+2}$ or $[ID_{90}] \cdot t = 4.6\ K/k_{+2}$ (14). As shown by the data in Table 1, all of the β-lactam antibiotics tested fell in the same order of decreasing acylating potency toward the two PBP forms showing unambiguously that PBP3p adopted the same folded conformation as the native PBP3. Given the different temperatures used in these experiments, PBP3ρ was expected to be more susceptible to inactivation (at 37°C) than the membrane-bound PBP3 (at 30°C). However, the observed ratios of k_{+2}/K (for PBP3p) versus k_{+2}/K (for PBP3) varied from 3 to 11, depending on the antibiotics, except for cephalexin, which was a very weak acylating agent. Note that binding to isolated membranes involves a competition between multiple PBPs. Moreover, not all of the ID_{50} (ID_{90}) values may have been determined under the conditions under which the use of the aforementioned conversion equations was fully justified.

TABLE 1. *Second-order rate constant (k_{+2}/K) of acylation of E. coli PBP3p and membrane-bound PBP3 by β-lactam antibiotics*

	k_{+2}/K (M^{-1} s^{-1})[a]		
Antibiotic	PBP3p at 37°C (A)	Wild-type PBP3 at 30°C (B)	A/B ratio
Mezlocillin	230,000 ± 20,000	26,700	8
Aztreonam	100,000 ± 10,000	33,000	3
Cefoperazone	80,000 ± 10,000	15,000	5
Cefotaxime	80,000 ± 5,000	>11,500	<7
Cefuroxime	16,000 ± 2,000	5,750	3
Ampicillin	4,800 ± 200	480	10
Benzylpenicillin	4,000 ± 200	450	8
Carbenicillin	1,500 ± 200	230	6
Cefoxitin	1,000 ± 200	90	11
Cephalexin	45 ± 5	50	1

[a] The k_{+2}/K values for the wild-type PBP3 were derived from the ID_{90} value for aztreonam (10); in all the other cases, they were derived from ID_{50} values (5).

PBP3p-catalyzed transfer reactions on acyclic carbonyl donors. PBP3p hydrolyzed properly structured thiolesters of the type C_6H_5-CONH-CH(*R*)-COS-CH(*R*')COOH (Table 2) but not the corresponding esters. Hydrolysis proceeded to completion with release of the leaving group, HS-CH(*R*')COOH. The K_m values were high, and only the k_{cat}/K_m values were computed by analysis of the complete time courses (6). The k_{cat}/K_m values are equivalent to the second-order rate constant *(k_{+2}/K)* values of protein acylation. Table 2 also compares the k_{cat}/K_m (or k_{+2}/K) values obtained with PBP3p with those obtained with a soluble form of the *Streptococcus pneumoniae* class B PBP2x (21), the *Streptomyces* sp. strain K15 monofunctional DD-transpeptidase/PBP (17), and the *Streptomyces* sp. strain R61 monofunctional DD-carboxypeptidase/PBP (22). With this limited series of thiolesters, the activity of PBP3p was comparable to that of the *Streptomyces* sp. strain K15 transferase (17).

In the presence of D-alanine, the PBP3p-catalyzed conversion of the thiolester benzoyl-D-alanyl-thiolglycolate (thiolester 1 in Table 2) underwent partitioning into hydrolysis (H reaction products, benzoyl-D-alanine plus thiolglycolate) and aminolysis (T [transpeptidation] reaction products, benzoyl-D-alanyl-D-alanine plus thiolglycolate). Because the presence of D-alanine did not modify the k_{cat}/K_m values of the catalyzed reactions, it was concluded that D-alanine did not influence the rate of enzyme acylation by the thiolester. D-Alanine behaved as simple alternative nucleophile at the level of the acyl enzyme leading to the formation of a ternary acyl enzyme-D-alanine complex that was productive only toward the formation of a peptide bond. The data in Table 3 show that (i) at a fixed 1 mM concentration of D-alanine, the T/H ratio was independent of the thiolester concentration from 50 to 200 μM; (ii) at a fixed 1.1 mM concentration of thiolester, the T/H ratios increased proportionally to the D-alanine concentration from 0.5 to 4 mM; and (iii) at D-alanine concentrations larger than 4 mM, the thiolester was quantitatively converted into benzoyl-D-alanyl-D-alanine. One may note that this

reaction product was not a substrate of PBP3p.

PBP3p also utilized the tetrapeptide L-Ala-γ-D-Glu(NH_2)-*m*A_2pm-D-Ala (a close analog of the tetrapeptide unit of the *E. coli* peptidoglycan) as an amino acceptor for the catalyzed aminolysis of benzoyl-D-alanyl-thiolglycolate. The amino group on the D-center of the diaminopimelic acid residue presumably served as the required nucleophile. Electrospray mass spectrometry showed that the reaction product had the expected M_r of 636.

Transglycosylation. The possible transglycosylation activity of PBP3ρ was tested with radiolabelled lipid II as a substrate according to the assay developed for PBP1b (29). Under these conditions, purified PBP1b catalyzed glycan polymerization (Fig. 2), whereas none was detectable with PBP3p as previously observed with complete PBP3 (29). Moreover, when the assays were carried out under the conditions initially proposed for PBP3 (18), no glycan polymerization was detectable with PBP3p, whereas PBP1b was fully active.

TABLE 2. *Second-order rate constant (k_{cat}/K_m [equivalent to k_{+2}/K]) of acylation of the E. coli PBP3p, S. pneumoniae PBP2x, Streptomyces sp. strain K15 DD-transpeptidase-PBP, and Streptomyces sp. strain R61 DD-carboxypeptidase-PBP by thiolesters C_6H_5-CONH-CH(R)-COS-CH(R')COOH in the hydrolysis pathway*

Thiolester[a]	*R*	*R'*	k_{cat}/K_m (M^{-1} s^{-1})[b]			
			PBP3p	PBP2x	K15 PBP	R61 PBP
1	CH_3	H	100 ± 20	5,000	75	700,000
2	H	H	20 ± 5	600	8	100,000
3	CH_3	CH_3	40 ± 10	4,900	3	125,000
4	H	CH_3	30 ± 7	3,200	75	100,000
5	$(CH_3)_2$-CH	H	NA	ND	NA	8,000

[a] In thiolesters 1, 2, and 3, the asymmetric carbon atoms have the D configuration. Thiolesters 4 and 5 are racemic mixtures.
[b] ND, not determined; NA, no detectable activity. For details about PBP2s, *Streptomyces* sp. strain K15 PBP, and *Streptomyces* species strain R61 PBP, see references 20, 16, and 21, respectively.

TABLE 3. *E. coli PBP3p-catalyzed hydrolysis (H) and aminolysis (T) of the thiolester benzoyl-D-alanyl-thioglycolate*[a]

Reaction condition	Concn (mM) of:		T/H ratio[b]
	Thiolester 3	D-Ala	
A	0.05	1	2.2 ± 0.4
	0.10	1	3.0 ± 0.3
	0.15	1	2.7 ± 0.1
	0.20	1	2.5 ± 0.4
B	1.10	0.5	1.4 ± 0.1
	1.10	1	2.9 ± 0.2
	1.10	2	5.7 ± 0.3
	1.10	4	10.3 ± 0.8

[a] Thiolester 1 in Table 2. Data represent the effects of increasing concentrations of thiolester at a fixed concentration of D-alanine (reaction condition A) and of increasing concentrations of D-alanine at a fixed concentration of thiolester (reaction condition B). The enzyme concentration was 1.56 μM. Incubation was for 20 min at 37°C.
[b] Reaction products: benzoyl-D-alanine (H) or benzoyl-D-alanyl-D-alanine (T) plus thiolglycolate.

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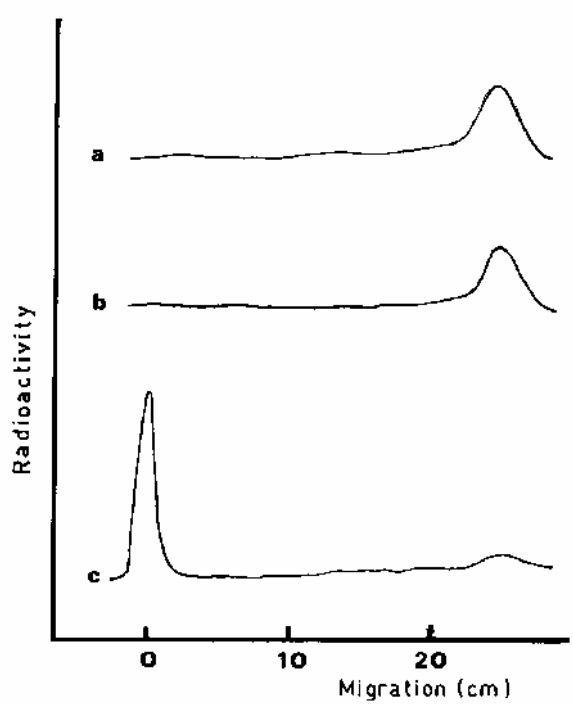
FIG. 2. *Separation of the products of the polymerization reaction by paper chromatography. The in vitro glycan polymerization was assayed as described previously (29). Reaction products were separated by chromatography on Whatman no. 1 filter paper run overnight in isobutyric acid-1 M ammonia (5:3). Radioactive compounds (polymerized peptidoglycan material at the start, and lipid II at an R_f of 0.85) were detected with a Berthold scanner model LB283. a, assay without any enzyme; b, assay with 8 µg of PBP3p; c, assay with 8 µg of PBP1b.*



DISCUSSION

The essential feature of the lipid II precursor is that the lipid-transported disaccharide peptide units can undergo polymerization into peptidoglycan at extracellular sites without any input of energy. Two enzymatic activities, a transglycosylase and a transpeptidase, are required. Assay systems leading to the in vitro PBP-catalyzed conversion of lipid II into polymeric peptidoglycan have been developed, and on this basis, bifunctionality with transglycosylase (the n-PB module) and transpeptidase (the PB module) activities has been assigned to the class A PBP1a and PBP1b of *E. coli.*

Serine-assisted peptidoglycan cross-linking implies that an amino acceptor prevents water from performing a nucleophilic attack on the peptidyl enzyme formed by reaction of the transpeptidase with the D-alanyl-D-alanine-terminated carbonyl donor. The 262-amino-acid residue monofunctional DD-transpeptidase-PBP of *Streptomyces* sp. strain K15 fulfills this criterion (26). The enzyme catalyzes the rupture of the carboxy-terminal peptide bond of $(acetyl)_2$-L-lysyl-D-alanyl-D-alanine. However, in aqueous media at 55.5 M H_2O, the enzyme is seemingly inert, because D-alanine that is released during the enzyme acylation step is reutilized as an amino acceptor for the ensuing enzyme deacylation step, so that the original carbonyl donor is continuously regenerated. Glycyl-glycine has a much greater acceptor activity than water. Millimolar concentrations of this dipeptide led to the enzyme-catalyzed conversion of the tripeptide into the tetrapeptide $(acetyl)_2$-L-lysyl-D-alanyl-glycyl-glycine (26).

PBP3p is a truncated, membrane anchor-free form of the class B PBP3 of *E. coli.* Its specificity profile for a large series of β-lactam antibiotics is identical, within the limits of experimental errors, to that of the wild-type PBP. As a corollary, the folded conformation adopted by PBP3ρ is expected to be identical or at least very similar to that of the native structure, and the enzymatic properties of PBP3p on acyclic carbonyl donors should reflect faithfully those for the membrane-bound PBP.

PBP3p has no detectable activity on the tripeptide $(acetyl)_2$-L-lysyl-D-alanyl-D-alanine, but in analogy with the *Streptomyces* sp. strain K15 DD-transpeptidase-PBP, aqueous assay systems containing a millimolar

concentration of D-alanine or a peptide related to the *E. coli* wall peptidoglycan led to the PBP3p-catalyzed conversion of properly structured thiolesters into the corresponding aminolytic products, showing that the acyl serine transferase-PB module of PBP3 can catalyze the formation of a new peptide bond at the expense of a preformed thiolester bond. Because the substrate requirements conferring optimal substrate activity to a carbonyl donor are not the same for a β-lactam, peptide, ester, or thiolester, PBP3 may well be capable of catalyzing the formation of a new peptide bond at the expense of a preformed peptide bond. Arising from this view, the peptide carbonyl donor and amino acceptor that PBP3 utilizes for the synthesis of the septal peptidoglycan remain to be characterized biochemically.

PBP3p has no detectable glycan chain polymerization activity on the *E. coli* lipid II intermediate under conditions under which the class A PBP1b functions as a transglycosylase. That the n-PB module of the class B PBP3 is not a transglycosylase is consistent with the fact that it has a different amino acid signature from that of the n-PB module of the bienzymatic (transglycosylase-transpeptidase) PBP1a and PBP1b of class A and of the monofunctional transglycosylases (12, 13, 28). In addition to its assisted folding function (16), the n-PB module of PBP3 may serve as a "wiring" element connecting the associated acyl serine transferase module to the transglycosylase module of the class A PBP1a and/or PBP1b and to other cell cycle proteins. Experimental evidence suggests that PBP3, PBP1b, the monofunctional (endopeptidase) PBP7, and the lytic transglycosylase Slt70 interact with each other (18). Also note that the M1-E56 membrane-spanning module of PBP3, which is not involved in polypeptide folding, is essential for the cell septation activity of the protein in exponentially growing cells (16). Positioning of PBP3 at the septum site might be the result of interactions between the intracellular segment of its membrane-anchoring module and the FtsZ ring (24).

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