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Neisseria gonorrhoeae Penicillin-Binding Protein 3 Demonstrates a Pronounced Preference for *N*^ε-Acylated Substrates[†]

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Abstract

Penicillin-binding proteins (PBPs) are bacterial enzymes involved in the final stages of cell wall biosynthesis, and are the lethal targets of β-lactam antibiotics. Despite their importance, their roles in cell wall biosynthesis remain enigmatic. A series of eight substrates, based on variation of the pentapeptide Boc-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala, were synthesized to test specificity for three features of PBP substrates: 1) the presence or absence of an *N*^ε-acyl group, 2) the presence of D-IsoGln in place of γ-D-Glu, and 3) the presence or absence of the *N*-terminal L-Ala residue. The capacity of these peptides to serve as substrates for *Neisseria gonorrhoeae* (NG) PBP3 was assessed. NG PBP3 demonstrated good catalytic efficiency ($2.5 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$) with the best of these substrates, with a pronounced preference (50-fold) for *N*^ε-acylated substrates over *N*^ε-nonacylated substrates. This observation suggests that NG PBP3 is specific for the ~D-Ala-D-Ala moiety of pentapeptides engaged in cross-links in the bacterial cell wall, such that NG PBP3 would act after transpeptidase-catalyzed reactions generate the acylated amino group required for its specificity. NG PBP3 demonstrated low selectivity for γ-D-Glu vs D-IsoGln, and for the presence or absence of the terminal L-Ala residue. The implications of this substrate specificity of NG PBP3 with respect to its possible role in cell wall biosynthesis, and for understanding the substrate specificity of the LMM PBPs in general, are discussed.

Keywords

penicillin-binding protein; peptidoglycan; bacterial cell wall; substrate specificity; peptide substrate; *Neisseria gonorrhoeae*

Penicillin-binding proteins (PBPs) are bacterial enzymes that catalyze the final steps in cell wall biosynthesis, and are the lethal targets of the β-lactam antibiotics (reviewed in (1-6)). The bacterial cell wall (peptidoglycan or murein) is composed of parallel glycan strands consisting of a repeating disaccharide, *N*-acetylglucosamine-β-1,4-*N*-acetylmuramic acid (NAG-NAM), in which the *N*-acetylmuramic acid residues are substituted with a pentapeptide chain. Some of these peptide chains are cross-linked to other peptide chains from adjacent glycan strands,

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Supporting Information Available: Supporting information on the synthesis and characterization of substrates used in this study is available free of charge via the Internet at <http://pubs.acs.org>.

which confer rigidity to the cell wall necessary for cell viability. The structure of the key pentapeptide building block varies amongst different bacteria, but in *Escherichia coli*, *Neisseria gonorrhoeae* and most other Gram-negative bacteria, the peptide is L-Ala- γ -D-Glu-*m*-DAP-D-Ala-D-Ala (*m*-DAP = *meso*-diaminopimelic acid). In Gram-negative bacteria, PBPs catalyze the reactions shown in Figure 1.

Every bacterial species has multiple PBPs, which are generally labeled in order of decreasing molecular mass. For example, *E. coli* has eight classically known PBPs, labeled 1A, 1B, and 2-7, as well as several recent additions including PBP1C (7) and PBP6B (8) (recently reviewed in (9)). PBPs have molecular masses of 20-120 kDa and can be broadly divided into two groups, the low molecular mass (LMM) PBPs and the high molecular mass (HMM) PBPs. Each of these groups can be further subdivided into three classes, A, B, and C, based on sequence analysis (3,10). LMM PBPs are monofunctional enzymes, whereas HMM PBPs possess an additional domain *N*-terminal to the PBP domain that in HMM Class A enzymes is a penicillin-insensitive transglycosylase involved in glycan polymerization of the cell wall. HMM Class A, B, and C enzymes, as well as LMM Class A and C enzymes, all possess three highly conserved active site sequence motifs (SXXK, SXN and K(T/S)G), while LMM Class B enzymes have a YXN in place of the SXN motif. NG PBP3, the subject of the current report, is a LMM Class C PBP (11).

Different PBPs have different propensities for catalyzing the transpeptidase, hydrolase (DD-carboxypeptidase), and endopeptidase reactions required for cell wall biosynthesis and modulation (Fig. 1). For example, HMM PBPs catalyze exclusively transpeptidation reactions, whereas LMM PBPs catalyze carboxypeptidase (reviewed in (4,5,9,12-14)), endopeptidase (e.g. EC PBP4 (15-18) and NG PBP3 (11)), and in one case (*Streptomyces* K15 enzyme) transpeptidase reactions (19). HMM PBPs are essential for bacterial viability and are the lethal targets for β -lactam antibiotics, whereas LMM PBPs are non-essential for cell viability. A particularly enigmatic feature of the PBPs is that LMM PBPs have readily detectible activity against peptide substrates, whereas purified HMM PBPs have either low or undetectable activity against natural or synthetic cell wall-related peptide substrates (reviewed in (20)). Recent studies have made progress in detecting and characterizing the transpeptidase activities of a few HMM PBPs, such as *E. coli* PBPs 1A and 1B ((21,22), and references therein), but these activities are still much lower than those observed with LMM PBPs. The low or undetectable activity of purified HMM PBPs has been attributed to the regulation of HMM PBP activity through interactions in macromolecular complexes within the cell wall environment (5,22-25).

The roles of individual PBPs in bacterial cell wall biosynthesis from a number of bacterial species have been elucidated by mutagenesis and knockout studies (reviewed in (9,13,26)). These studies have revealed that HMM PBPs are involved in cell elongation, cell morphology, and cell division (27). While these studies also show that LMM PBPs are not essential for cell viability, these PBPs often play important roles in normal bacterial cell morphology (9,26). Examples of LMM PBPs important for cell morphology include *Streptococcus pneumoniae* PBP3 (28,29), *Staphylococcus aureus* PBP4 (30), *E. coli* (EC) PBP5 Nelson, 2000 #322; Nelson, 2001 #206; Nelson, 2002 #205; Ghosh, 2003 #360}, and *Neisseria gonorrhoeae* (NG) PBPs 3 and 4 (11). Despite these advances, there remains a significant knowledge gap in understanding the role of individual PBPs in the cell wall biosynthetic process.

Given their readily detectible activity, most *in vitro* studies have necessarily focused on LMM PBPs. Different LMM PBPs show a large range of intrinsic activities against natural and synthetic cell wall-related substrates (recently reviewed in (14)), with activities ranging from very weak to nearing the diffusion limit. Despite extensive study, features required for substrates specificity have been identified only for a few LMM PBPs. For example, the

Actinomadura R39 enzyme shows a preference for non- N^{ϵ} -acylated substrates (31,32), and the *Streptomyces* R61 LMM PBP has demonstrated a high degree of specificity for a glycine substituted pimelic acid side chain, with an ϵ -COOH group analogous to that found in its natural DAP-containing substrates ((32,33), recently reviewed in (14)).

Neisseria gonorrhoeae are Gram-negative diplococci that are notable for having a relatively small complement of PBPs (PBPs 1-4), which in principle makes them good models for studies of PBP function (34-37). NG PBP1 is the gonococcal homolog of *E. coli* PBP1A and likely catalyzes both glycan polymerization and transpeptidation during cell growth and elongation (35), while PBP2 is the gonococcal homolog of *E. coli* PBP3 and likely functions during cell division (38). Whereas PBPs 1 and 2 are essential, the LMM PBPs 3 and 4 are not essential for bacterial survival but do play a role in normal cell morphology (11). NG PBP3 demonstrates exceptionally high activity (k_{cat}/K_m up to $1.8 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$) against simple L-Lys-D-Ala-D-Ala-based tripeptide substrates (11). Further knowledge of the substrate specificity of NG PBP3 could provide clues as to its role in gonococcal cell wall biosynthesis, and of the specificity and role of LMM PBPs in general. To further investigate the substrate specificity of NG PBP3, a series of eight L-Lys-containing tetra- and pentapeptide substrates were synthesized and characterized against this enzyme. Our results reveal that NG PBP3 displays a strong preference for N^{ϵ} -acylated substrates. The implications of this specificity for the possible role of NG PBP3 in cell wall biosynthesis, and for understanding the substrate specificity of the LMM PBPs in general, are discussed.

EXPERIMENTAL PROCEDURES

NG PBP3

NG PBP3 was prepared as described previously (11). Briefly, NG PBP 3 was purified from *E. coli* as a fusion protein to maltose-binding protein, digested with TEV protease to cleave the two proteins, and NG PBP 3 was repurified from the digest. Aliquots were stored at -80°C .

Synthesis of substrates

The synthesis and chemical characterization of substrates was performed as described in Supplemental Data.

DD-Carboxypeptidase activity

PBP DD-carboxypeptidase activity was determined by fluorescence detection of D-Ala using the Amplex Red-based assay described previously (39,40). PBP assays (50 μL) were performed in 100 mM pyrophosphate, 100 mM NaCl, 0.5 mg/mL alkylated BSA (Alk-BSA), at pH 8.5. NG PBP 3 was diluted in assay buffer and added to reactions to start the assays. All assays were performed at 25°C in blackwalled microtiter plates. PBP reactions were stopped by the addition of the detection reagent (150 μL) containing 66.7 $\mu\text{g}/\text{mL}$ ampicillin (50 $\mu\text{g}/\text{mL}$ in 200 μL final assay volume), 13.3 μM AR (10 μM in 200 μL final assay volume), 0.5 units of HRP, 1.67 $\mu\text{g}/\text{mL}$ FAD (1.25 $\mu\text{g}/\text{mL}$ in 200 μL final assay volume), and 0.015 units DAO in 0.1 M Tris, pH 8.5. Fluorescence was detected after 90 min development with an excitation wavelength of 546 nm and emission of 595 nm. Fluorescence was read in a Tecan SpectraFluor Plus microtiter plate reader (Research Triangle Park, NC). D-Ala standards were included in each experiment, and blanks were also performed with PBP in the absence of substrate, and substrate in the absence of PBP.

Data Analysis

Data was processed to obtain v/E_T values, and then analyzed by fitting with the appropriate equation by non-linear regression using SPSS for Windows (Chicago, IL). The form of the Michaelis-Menten equation shown in Eqn. 1 was used to obtain values and standard errors (SEs) for k_{cat} and K_m , and the form of this equation shown in Eqn. 2 to obtain values and SEs for k_{cat}/K_m .

$$v/E_T = \frac{k_{cat} * [S]}{K_m + [S]} \quad \text{Eqn. 1}$$

$$v/E_T = \frac{(k_{cat}/K_m) * K_m * [S]}{K_m + [S]} \quad \text{Eqn. 2}$$

For substrates that showed substrate inhibition, only those data points up to the maximally observed velocity were used for analysis. In such cases the k_{cat}/K_m value will be accurate, but the apparent K_m and k_{cat} values will be less than their true values. For substrates that were non-saturating, linear regression analysis using Eqn. 3 was performed to obtain values and SEs for k_{cat}/K_m .

$$v/E_T = (k_{cat}/K_m) * [S] \quad \text{Eqn. 3}$$

RESULTS and DISCUSSION

This study was designed to probe the substrate specificity of NG PBP3 for L-Lys-containing tetra-peptide (TP) and penta-peptide (PP) substrates. A set of eight substrates were synthesized and characterized by LCMS and amino acid composition (Supplemental Data). These substrates were designed to test three features of PBP substrates: 1) the presence or absence of an N^{ϵ} -acyl group, 2) the presence of D-IsoGln in place of the γ -D-Glu residue, and 3) the presence or absence of the N -terminal L-Ala residue. N^{ϵ} -acylation was considered an important point of variation, since within the cell wall a given peptidoglycan chain will either be engaged in a cross-link or not, and this is potentially a key distinguishing feature of PBP specificity. Another important feature of synthetic substrates is the length of the peptide chain, and therefore both tetrapeptide and pentapeptide substrates were synthesized as substrates. Finally, most bacteria use γ -D-Glu in their peptidoglycan, including *Neisseria* (41, 42), but some bacterial species use D-IsoGln (or other modified Glu residues) in this position (43), and some specificity for this feature might be anticipated. γ -D-Glu and D-IsoGln residues were therefore included in this panel of substrates.

The eight substrates designed to test these three potential sites of NG PBP3 substrate specificity are illustrated in Figure 2. Note that this set of substrates can be considered as the eight corners of a cube, with adjacent corners differing by a single substrate feature.

This design was intended to determine if a specific substrate feature gave changes in reactivity independent of other features, or if there was covariance (interaction) between features. These eight substrates were examined for their capacity to serve as substrates for NG PBP3, with the results summarized in Table 1 (note that the compound numbering scheme is based on the synthetic design, as given in Supplemental Data).

Since the activity of enzymes frequently varies from one experiment or experimenter to another, commercially available Ac-L-Lys(Ac)-D-Ala-D-Ala was included as a reference substrate. In a previous study, a set of Lys N^α - and N^ϵ -substituted L-Lys-D-Ala-D-Ala tripeptide substrates were characterized against NG PBP3 (11), and the results from this study are summarized in Table 2 for comparison. Substrate **(V)** in Table 2 is the reference substrate Ac-L-Lys(Ac)-D-Ala-D-Ala in Table 1. The variation in enzyme reactivity against Ac-L-Lys(Ac)-D-Ala-D-Ala (the reference substrate) between Tables 1 and 2 is modest.

NG PBP3 exhibited a pronounced preference for N^ϵ -acylated (+Cbz) substrates (**(7-8, 13-14)**, Table 1). Peptides lacking an N^ϵ -acyl-capping group (**(9-10, 15-16)**, Table 1) had substantially less affinity and lower turnover, with substrate **(15)** showing no turnover. It is important to note that the specificity of NG PBP3 for N^ϵ -acylated substrates is observed not only with N^ϵ -Cbz-capped substrates, but also with N^ϵ -acetyl-capped substrates (compare the effect of acetylation in the tripeptide substrate **III** in Table 2 vs unacylated tripeptide substrate **IV** in Table 2 (11)). Therefore, the observed specificity of NG PBP3 for an N^ϵ -acyl group is not an artifact of the Cbz capping group. NG PBP3 also demonstrated modestly lower K_m and higher k_{cat}/K_m values against pentapeptide substrates (**13-16**) than against the corresponding tetrapeptide substrates (**7-10**). This observation suggests that NG PBP3 forms contacts with the full length of pentapeptide substrates, but that these contacts contribute only weakly to binding and turnover. Finally, a slight preference for D-IsoGln peptides (**8, 14, 10, 16**) over γ -D-Glu peptides (**7, 9, 13, 15**) was observed. Within the set of four acylated substrates in Table 1, the presence of γ -D-Glu (**7-8**) gave substrates without apparent substrate inhibition, whereas the presence of an IsoGln residue (**13-14**) was associated with substrate inhibition. It is possible to quantify these effects by taking the average of the effect of different changes within pairs of homologous substrates. Since no turnover was observed with substrate **(15)**, pairs with substrate **(15)** were excluded from this analysis. For the effect of acylation the k_{cat}/K_m ratios for the substrates were used in the following formula;

$$\begin{aligned} R_{Ac/non-Ac} &= [(7)/(9) + (8)/(10) + (14)/(16)] / 3 \\ &= [121,000/2,500 + 192,000/2,700 + 256,000/10,000] / 3 \\ &= 48 \text{ (SE=13)} \end{aligned}$$

Thus, on average, acylation increases catalytic efficiency by a factor of 48-fold. Ratios can also be calculated for the effect of a longer vs shorter chain (PP vs TP), and of a γ -D-Glu residue vs a D-IsoGln residue

$$\begin{aligned} R_{PP/TP} &= 2.3 \text{ (SE=0.7)} \\ R_{Gln/Glu} &= 1.3 \text{ (SE=0.2)}. \end{aligned}$$

It is clear that acylation of the N^ϵ -amino group has a pronounced effect on enzyme activity, while variations in the Gln/Glu residue and the presence or absence of the terminal D-Ala (PP vs TP) have only slight or modest effects on catalytic efficiency with NG PBP3.

These results can be compared to the result from the study of Pratt and coworkers on the activity of NG PBP3 against a substrate (**JWA3**, Fig. 3) designed to include the ϵ -COOH group of the DAP residue in natural cell wall substrates of Gram-negative bacteria (Fig. 1) (32,33).

For NG PBP3, a k_{cat}/K_m of $10,000 \text{ M}^{-1} \text{ sec}^{-1}$ against the *m*-DAP-based substrate (**JWA3**) (Fig. 3) was reported (32), which is consistent with the activities observed in this study for the comparable non- N^ϵ -acylated substrates (Table 1). This observation indicates that NG PBP3 does not require the presence of the ϵ -COOH group of *m*-DAP for high activity.

The substantially higher activity of NG PBP3 with N^{ϵ} -acylated substrates, which was observed with both N^{ϵ} -Cbz-capped substrates (Table 1) and N^{ϵ} -acetyl-capped substrates from our previous study of this enzyme (Table 2) (11), is an intriguing result. Within the pool of cell wall peptides in *N. gonorrhoeae*, the presence or absence of N^{ϵ} -acylation is associated with participation in a cross-link (Fig. 1). The observation of higher activity against N^{ϵ} -acylated substrates therefore strongly suggests that NG PBP3 is selective for cross-linked cell wall pentapeptides. In contrast to N^{ϵ} -acylation, NG PBP3 shows a lack of specificity for the γ -D-Glu and L-Ala residues of the pentapeptide backbone, which are static features of the gonococcal cell wall. In comparison, the *Actinomadura* R39 PBP instead shows selectivity for substrates containing a free N^{ϵ} -amino group (i.e. non- N^{ϵ} -acylated peptides) (31). Therefore, for NG PBP3 and also for *Actinomadura* R39 PBP (both LMM Class C PBPs), enzyme specificity appears focused on acylation or non-acylation respectively of the N^{ϵ} -amino group of their substrates, which reflects participation or lack of participation in a cross-link (Fig. 1). Engagement or lack of engagement in a cross-link is the predominate characteristic that distinguishes subsets of peptidoglycan chains within bacterial cell walls, and it is logical that some PBPs would have selectivity for this feature of their natural substrates. It will be of interest for understanding PBP substrate specificity to determine if similar specificity for acylation status – analogous to participation in a cross-link – will be apparent in other LMM PBPs such as EC PBP5 (A LMM Class A PBP), the *Streptomyces* R61 enzyme (a LMM Class B PBP), or *Bacillus subtilis* PBP4a (a LMM Class C PBP). Our own observations with EC PBP5 revealed a lack of specificity with the Lys-containing substrates shown in Table 1 (unpublished observations), consistent with the general lack of specificity observed for this enzyme in other studies (reviewed in (14)). Given that some LMM PBPs require an ϵ -COOH, most notably the *Streptomyces* R61 PBP (32,33), it will likely be necessary to test all combinations of these features (e.g. +/- an amine acceptor acyl group and +/- an ϵ -COOH group) to obtain a complete picture of the specificity of a given PBP for this portion of their substrates.

The substrate specificity of NG PBP3 observed here has implications for the possible role of NG PBP3 in cell wall biosynthesis. A high degree of specificity for N^{ϵ} -acylated substrates indicates that NG PBP3 would act after transpeptidation (cross-linking) had occurred (Fig. 4). Such specificity could serve one or more functions. Transpeptidase reactions, in which one amide bond is broken and one amide bond is formed (Figure 1), are essentially isoenergetic. A general principle of metabolism is that essential metabolic processes are driven in the desired direction by coupling to an exergonic reaction (e.g., hydrolysis of pyrophosphate for ATP \rightarrow AMP + PPi coupled reactions). Although the concentration of D-Ala in the growth environment is generally expected to be small due to diffusion and recycling of D-Ala, the specificity of NG PBP3 could provide an additional energetic driving force for transpeptidase reactions by hydrolyzing the D-Ala-D-Ala bond only on those peptides that had been previously cross-linked (Fig. 4).

Such an energetic role has previously been suggested for the *Streptomyces albus* G_{DD}-carboxypeptidase (a zinc enzyme without sequence homology to the PBP superfamily (44)), which also has a preference for N^{ϵ} -acylated substrates (45). Alternatively, the activity of NG PBP3 could serve to limit the degree of cross-linking within the subset of peptidoglycan peptide chains upon which it is most active. Deletion mutants of NG PBP3 alone have only a slight effect on Nesserial cell morphology, although double knockout mutants of both NG PBP3 and NG PBP4 demonstrate loss of uniformity in cell shape and size and significantly increased doubling times for growth (11). NG PBP3 therefore does play a role in normal cell shape, and it seems likely that the specificity of NG PBP3 observed here is somehow involved in this function.

Summary

The substrate specificity results obtained in this study demonstrate that NG PBP3 exhibits a pronounced preference for *N*^ε-acylated substrates, and very little preference for features of the extended pentapeptide chain (*L*-Ala and γ -*D*-Glu residues). Comparison with previous studies indicates that at least one other LMM PBP, the *Actinomadura* R39 PBP, displays specificity for *N*^ε-acylation status, although in contrast to NG PBP3, the *Actinomadura* R39 PBP shows a preference for non-*N*^ε-acylated substrates. For other LMM PBPs, the presence of the ϵ -COOH group of their natural substrates may be required, as demonstrated by Pratt and coworkers for the *Streptomyces* R61 enzyme (32,33). These two different features, the presence or absence of an amine acceptor acyl group and the presence or absence of an ϵ -COOH group, are clustered around the acceptor amino group of the Lys/DAP residue in natural bacterial peptidoglycan (Fig. 1), and this region appears to be the key region for substrate specificity for NG PBP3, R39, and R61 enzymes, and possibly other LMM PBPs as well. An improved knowledge of PBP substrate specificity, especially towards specific subsets of natural cell wall peptides, will be useful for further understanding the role of PBPs in bacterial cell wall biosynthesis, and for the development of new inhibitors of the PBPs as potential new antibacterial agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

1 Abbreviations

Bzl, benzyl
EC, *E. coli*
LMM, low molecular mass
HMM, high molecular mass
IsoGln, $\text{NH}_2\text{-CH}(\text{CONH}_2)\text{CH}_2\text{CH}_2\text{COOH}$
m-DAP, meso-diaminopimelic acid
NAG, *N*-acetylglucosamine
NAM, *N*-acetylmuramic acid
NG, *Neisseria gonorrhoeae*
PBP, penicillin-binding protein
PP, pentapeptide
TP, tetrapeptide
UDP, uridine diphosphate

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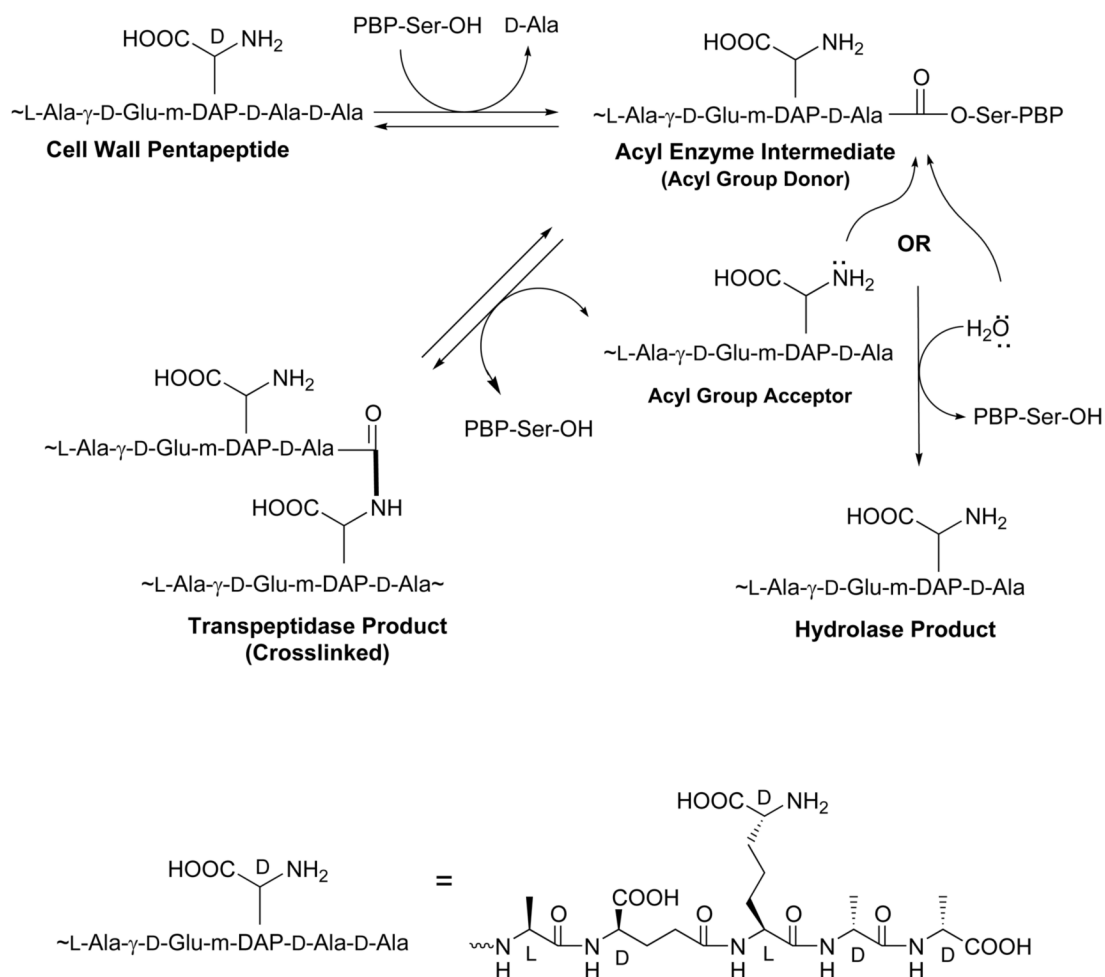


Figure 1.

Cell wall biosynthesis reactions catalyzed by the PBPs in most Gram-negative bacteria, including *Neisseria gonorrhoeae* (41,42) and *Actinomadura* R39 (46). Cell wall peptides are attached to a repeating NAG-NAM polysaccharide. Variations in the peptide substrate, such as substitution of L-Lys and other diamine acids in place of $m\text{-DAP}$, are often observed in Gram-positive bacteria (reviewed in (43)). In Gram-negative bacteria a cross-link is formed directly between the N^ϵ of the acyl group acceptor and the D-Ala carboxyl group of the acyl group donor, whereas in many Gram-positive bacteria a bridging amino acid or short peptide (interpeptide bridge) is found. For example, *Streptomyces* R61 has a Gly interpeptide bridge attached to the N^ϵ -amino group of L,L-DAP (47). PBPs attack the cell wall peptide to release D-Ala and form an acyl-enzyme intermediate (the acyl group donor). The acyl group can either be hydrolyzed ($+\text{H}_2\text{O}$) (hydrolase/ DD -carboxypeptidase activity) or transferred to the acceptor amino group on an adjacent cell wall peptide (right to left down diagonal arrow) to form a cross-link (transpeptidase activity). Cross-linked peptides are also cleaved by PBPs (endopeptidase activity, left to right up diagonal arrow).

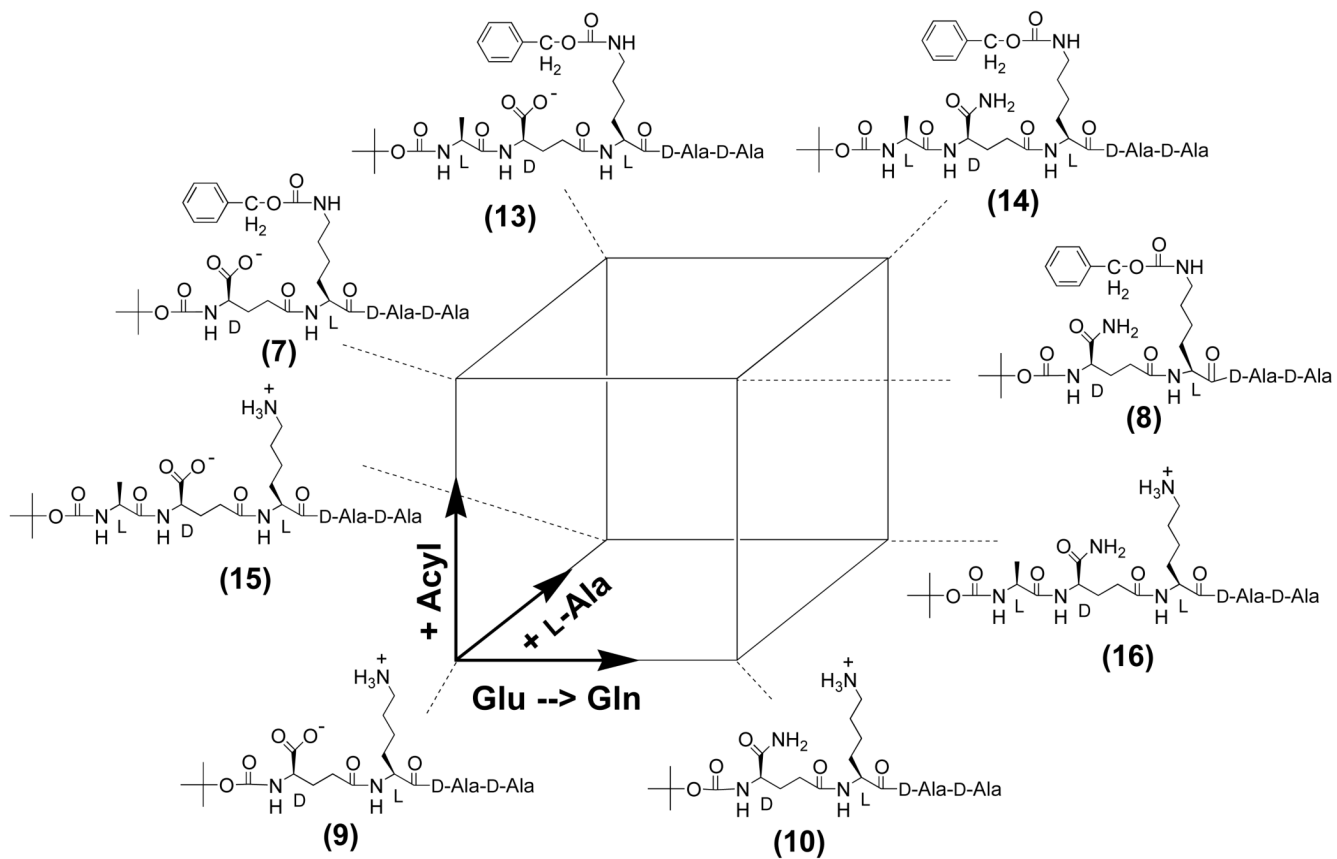
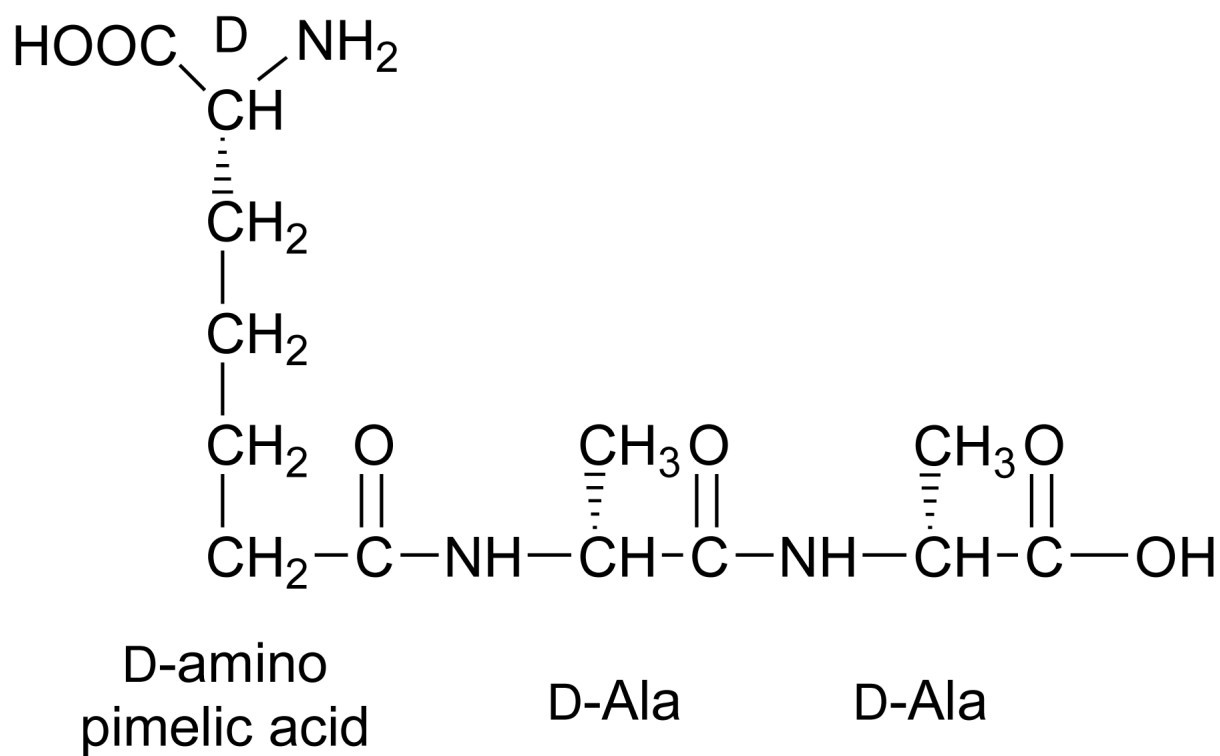


Figure 2.
Structures of and relationships between the substrates used in this study.



JWA3

Figure 3. Structure of the pimelic acid-based substrate used by Anderson *et al.* with NG PBP3 (32).

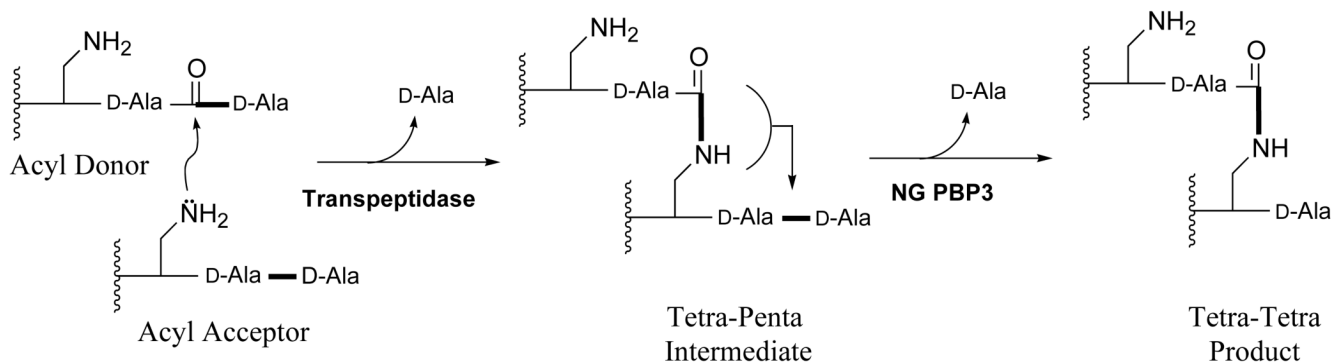


Figure 4.

A possible role for NG PBP3 in peptidoglycan biosynthesis based on its specificity for acylated pentapeptide substrates. NG PBP3 catalyzed reactions would follow transpeptidase catalyzed cross-linking reactions, which generate a substrate suitable for NG PBP3's substrate specificity. A transpeptidase reaction is essentially isoenergetic, whereas a carboxypeptidase reaction releases the energy of a peptide bond. If appropriately coupled to the peptidoglycan biosynthesis process, NG PBP3 could conceivably energetically drive gonococcal peptidoglycan biosynthesis.

Table 1
Kinetic Properties of NG PBPP3 against Tetra- and Pentapeptide Substrates.

	Substrate (R-D-Ala-D-Ala) R =	K_m (SE) mM	k_{cat} (SE) sec ⁻¹	k_{cat}/K_m (SE) M ⁻¹ sec ⁻¹
	Boc-γ-D-Glu-L- Lys(Cbz)-	1.11 (0.06)	134 (3)	121,000 (6,000)
	Boc-L-Ala-γ-D-Glu- L-Lys(Cbz)-	0.36 (0.03)	79 (2)	220,000 (20,000)
+ Acyl (Cbz)	Boc-D-IsoGln-L- Lys(Cbz)-	0.10 (0.01)*	19.2 (0.1)*	192,000 (1,000)
	Boc-L-Ala-D-IsoGln- L-Lys(Cbz)-	0.14 (0.01)*	35.9 (0.1)*	256,000 (1,000)
	Boc-γ-D-Glu-L-Lys-	8.3 (0.7)	21 (1)	2,500 (800)
	Boc-L-Ala-γ-D-Glu-L- Lys-		--NR --	
-Acyl	Boc-D-IsoGln- L-Lys-		--NS --	2,700 (300)
	Boc-L-Ala-D-IsoGln- L-Lys-		--NS --	10,000 (2,000)
Reference Substrate	Ac-L-Lys(Ac)-		--ND --	18,800 (30)

TP - tetrapeptide, PP - pentapeptide.

* Substrate inhibition observed. NR - No reaction observed. NS - Non-saturating substrate ($K_m > 15$ mM), ND - Not determined.

Table 2

Previous results for NG PBP3 activity against tripeptide \sim -D-Ala-D-Ala substrates and Ac-L-Lys(Ac)-D-Ala-D-Lac (11).

Substrate		K_m (SE) mM	k_{cat} (SE) sec ⁻¹	k_{cat}/K_m (SE) M ⁻¹ sec ⁻¹
Boc-L-Lys(Cbz)-D-Ala-D-Ala	(I)	3.3 (0.8) *	580 (60) *	180,000 (30,000)
Ac-L-Lys(Cbz)-D-Ala-D-Ala	(II)	3.7 (0.2) *	530 (10) *	142,000 (6,000)
Boc-L-Lys(Ac)-D-Ala-D-Ala	(III)	6.5 (0.8)	400 (10)	62,000 (6,000)
Boc-L-Lys-D-Ala-D-Ala	(IV)	32 (3) *	260 (10) *	8,300 (400)
Ac-L-Lys(Ac)-D-Ala-D-Ala	(V)	19 (2)	550 (20)	29,000 (2,000)
Ac-L-Lys(Ac)-D-Ala-D-Lac	(VI)	28 (3)	340 (20)	12,300 (800)

* Substrate inhibition observed.