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Reactions of Peptidoglycan-Mimetic β-Lactams with Penicillin-Binding Proteins *In Vivo* and in Membranes

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Abstract

The membrane-bound bacterial DD-peptidases or penicillin-binding proteins catalyze the final transpeptidation reaction of bacterial cell wall biosynthesis and are the targets of β-lactam antibiotics. Rather surprisingly, the substrate specificity of these enzymes is not well understood. In this paper we present measurements of the reactivity of typical examples of these enzymes with peptidoglycan-mimetic β-lactams under *in vivo* conditions. The MICs of β-lactams with *Escherichia coli*-specific side chains were determined against *E. coli* cells. Analogous measurements were made with *Streptococcus pneumoniae* R6. The reactivity of the relevant β-lactams with *E. coli* PBPs in membrane preparations was also determined. The results show that under none of the above protocols were β-lactams with peptidoglycan-mimetic side chains more reactive than generic analogues. This suggests that *in vivo*, as *in vitro*, these enzymes do not specifically recognize elements of peptidoglycan structure local to the reaction center. Substrate recognition must thus involve extended structure.

Keywords

b-lactam Ring < Antibiotics, Bacteria < Biological Systems, Rational Drug Design < Biotechnology/ Drug Discovery, Biomimetic Compounds < Chemical Synthesis and Screening Technologies, Enzymology < Proteins And Peptides

The penicillin binding proteins (PBPs) or D-alanyl-D-alanine peptidases (DD-peptidases) have been studied for many years because of their role in bacterial cell wall (peptidoglycan) biosynthesis and their identification as the targets of β-lactam antibiotics (1). These investigations have included, on one hand, microbiological and genetics aspects, and, on the other, biochemical and molecular aspects of their activity as enzymes. The latter studies have shown that these enzymes, under *in vivo* and, to some degree (see below), under *in vitro* conditions, catalyze the final cross-linking step of peptidoglycan synthesis (Scheme 1). Reaction of the enzyme with the C-terminus of a D-Ala-D-Ala stem peptide generates a covalent acyl-enzyme intermediate and D-alanine. The acyl-enzyme is then attacked by an amine from an adjacent peptidoglycan strand to achieve the cross-link in an overall transpeptidase reaction. Acylation of the enzyme by β-lactams yields an inert acyl-enzyme.

The study of these enzymes was initially facilitated by the discovery by Ghuysen and coworkers that certain *Streptomyces* strains, for reasons not yet clear, produce a series of low molecular weight (< 50 kDa), fully water soluble DD-peptidases that catalyze hydrolysis and aminolysis of small D-alanyl-D-alanine peptides (2). Work on these enzymes established details of the kinetics and mechanism of the transpeptidase reaction (3). Crystal structures are available for members of the major classes of these enzymes (4–6). As one might anticipate, peptidoglycan-

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mimetic peptides are turned over rapidly. For example, the peptides **1** and **2** are excellent ($k_{cat}/K_m \geq 10^6\ s^{-1}M^{-1}$) substrates of the *Streptomyces* R61 and *Actinomadura* R39 DD-peptidases, respectively (7,8). The analogous β-lactams **3** and **4** also inactivate these enzymes rapidly ($k_{inact} > 10^6\ s^{-1}M^{-1}$) (9,10). Structural studies of the R61 DD-peptidase revealed a specific binding site, associated with the enzyme active site, for the stem peptide branch present in **1** and **3** (11,12).

In contrast to the results outlined above for soluble DD-peptidases, the enzymes centrally involved in cell wall biosynthesis in the majority of bacteria, and the killing targets of β-lactam antibiotics, are membrane bound and, *in vitro*, exhibit little or no activity against D-alanyl-D-alanine peptides (8). In many cases, these enzymes have been water-solubilized by removal of terminal membrane anchor peptides and a number of crystal structures of the resulting proteins have been obtained (13,14). These structures show the same general protein fold and conserved active site residues as those of the soluble enzymes. Their DD-peptidase activity, even against peptidoglycan-mimetic peptides, however, is almost unobservably small. For example, solubilized *E. coli* PBP2 and PBP5 have essentially no activity against **2**, which contains the signature N-terminal element of the stem peptide of *E. coli* peptidoglycan (8). They do, however, react with and are inhibited by β-lactams, both generic, benzylpenicillin and cephalothin, for example, and peptidoglycan-mimetic, **4** and **5** for example (10). Their reactivity with the latter, however, is not greater than with the former. These enzymes, therefore, do not appear, *in vitro* at least, to recognize elements of local peptidoglycan structure. We have predicted (10) that the active sites of these enzymes, unlike those of the water-soluble analogues described above, do not contain specific binding sites for local peptidoglycan structure. How then do they recognize their substrates *in vivo*?

The situation of these enzymes *in vivo* may, arguably, be very different from in homogeneous aqueous solution. There is the presence of the membrane to which the enzyme is attached, there is the presence of other proteins, including other PBPs, with which the enzyme may form complexes (15–17), and there is the presence of extended peptidoglycan structure. The measurement of substrate turnover by a specific enzyme in such a complex environment is difficult but measurement of the rates of reaction of individual PBPs with β-lactams is quite feasible (18,19). In this paper, we investigate the reactivity of the *E. coli*-specific β-lactams **4** – **6** with *E. coli* PBPs *in vivo* and in membrane extracts. We have also made analogous measurements with *Streptococcus pneumoniae* R6, using the appropriate β-lactams **7** and **8**. The stem peptides of *E. coli*, **9**, and *S. pneumoniae*, **10**, are shown for comparison.

First, we determined the MICs of the *E. coli*-specific β-lactams **4** and **5** against a wild-type *E. coli* strain (MM 294), with results shown in Table 1; also shown are the MIC values of the generic β-lactams, benzylpenicillin and cephalothin, obtained under the same conditions. The immediate conclusion is that **4** and **5** are less effective at inhibiting *E. coli* growth than the generic β-lactams. Since the outer membrane of gram negative bacteria serves a permeability barrier that can retard entry of β-lactams (24), MICs of **4** and **5** were also measured against the *E.coli* permeability mutant (DC2), known to have lower barriers to β-lactams (21). Although the MICs of benzylpenicillin, cephalothin, and **5** were reduced, the generics maintained their advantage (Table 1). Note that **3** – **8** are poorer substrates of the *E. coli* AmpC β-lactamase than benzylpenicillin and cephalothin and thus would appear to be better rather than poorer inhibitors if significant β-lactamase activity were present in our experiments.

A similar result was obtained when the MIC values of **7** and **8** were determined against *Streptococcus pneumoniae* R6 (Table 2). Again, the peptidoglycan-mimetic β-lactams were inferior to the generics. No permeability barriers against β-lactams have been observed in *S. pneumoniae* (25).

Finally, to assess whether **4** – **6** showed any sign of enhanced affinity for any particular *E. coli* PBP, IC_{50} values of these β-lactams were determined for individual PBPs in a membrane preparation by a competition method where the penicillin Bocillin FL was used as the reference and fluorescent probe (19). The generic β-lactams benzylpenicillin and cephalothin were also used, for comparison. SDS-PAGE electrophoresis gels showing the results for benzylpenicillin and **4** are shown in Figure 1. From the decrease in fluorescence intensity of the Bocillin FL label as the concentration of the test β-lactam increased, an IC_{50} value for the latter could be determined, as described in Methods. The IC_{50} values determined in this way are reported in Table 3. The results, in general, resemble those above, *viz.* the peptidoglycan-mimetics are generally poorer than the generics with all of the PBPs visualized. As reported earlier, the same outcome was obtained when solubilized PBP2 and PBP5 were tested (10).

The general conclusion from these studies appears clear. Under no conditions yet found, in cells, in membranes, or in homogeneous solution (10) do the peptidoglycan-mimetic β-lactams **4**–**8** appear to react with their cognate PBPs at rates that indicate specific binding. This seems to support the tentative conclusion reached earlier (10) that the specific enzymes studied do not have an active site that recognizes local peptidoglycan structure, i.e. the structure of their substrate adjacent to the reaction center. The results from this paper go further in showing that even under *in vivo* conditions where, in principle, the active site of the enzymes might be induced into a more reactive state by interaction with membrane, local proteins etc., there is no sign of recognition of elements of local substrate structure. It seems likely, therefore, that the level of activity of these enzymes required for bacterial growth must arise from interactions between the enzymes and extended structural elements of the peptidoglycan substrate and the local environment. Such conditions are difficult to mimic *in vitro*. There is some indication that these enzymes might respond to peptidoglycan, but not yet for activation with respect to specific substrates (26).

It is interesting to reflect on the possible reasons for this unusual and counter-intuitive result, *viz.* that these enzymes have no specific affinity for elements of their natural substrate structure directly adjacent to the reaction center. One explanation might be that it represents an elegant defense against small molecule substrate analogue inhibitors. Bacteria are thought to be engaged in chemical warfare with each other (27) and the exposed DD-peptidases of peptidoglycan synthesis must be enticing targets for small peptidomimetic inhibitors. No such potent inhibitors, however, are known. Only transition state analogs, such as β-lactams, directed at the chemical reaction center itself, are generally effective inhibitors among the natural and unnatural products yet screened. A similar situation may be present at the transglycosylase active sites of high molecular weight class A PBPs which are responsible for incorporating the disaccharide monomers into the glycan polymer of the bacterial cell wall; the only really effective inhibitors yet discovered (as natural products) are quite large very elaborate molecules such as moenomycin (28). The design of inhibitors for both transpeptidases and transglycosylases should therefore focus on small transition state analogues or quite large molecules, where design of the latter, also incorporating a broad spectrum of activity, is very challenging.

METHODS

Bacterial Strains and Reagents

The wild type *E. coli* strain MM 294 (20) and the DC2 permeability mutant of *E. coli* (21) were kindly provided by D. B. Oliver (Dept. Biochemistry and Molecular Biology, Wesleyan University) and D. P. Clark (Dept. Microbiology, Southern Illinois University, Carbondale, IL), respectively. *Streptococcus pneumoniae* R6 (ATCC BAA-255) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). BOCILLIN FL was purchased from Invitrogen. The specific β-lactams **4**–**8** were synthesized as described elsewhere (10).

They do not hydrolyze in aqueous buffer at rates significantly higher than those of benzylpenicillin and cephalothin.

Growth of *Escherichia coli* cells and preparation of membranes

Cells were grown in Difco LB broth medium at 35 °C with vigorous aeration at 210 rpm. For strain DC2, L-methionine was also added at a concentration of 50 μg/ml (D.P. Clark, personal communication). About 300 ml of *E. coli* MM 294 was grown for 13 hrs and the cells were centrifuged at 3000 g for 6 minutes and washed twice with chilled sodium phosphate, 140 mM sodium chloride buffer at pH 7.3 (50 ml). The total of 2 g wet cells were suspended in 10 ml of same buffer. The suspension was French pressed at 20,000 lb/in^2 and the debris was centrifuged at 15000 g for 20 min. The supernatant was then ultracentrifuged at 100,000 g for 60 minutes. The supernatant was discarded and membranes were washed with the same buffer (5 ml) and ultracentrifuged for another 45 min. The membranes were resuspended (7 mg protein/ml) in ice-cold phosphate buffer and stored at −70 °C. These washed cell membrane preparations consist of inner membrane, outer membrane and peptidoglycan but are referred to as membranes for brevity. Protein concentration was determined by the Bradford method (22).

Growth of *Streptococcus pneumoniae* R6

Streptococcus pneumoniae R6 was grown in a 3% soybean-casein digest broth (Difco) (pH 7.3), also containing 0.2% yeast extract. The cells were then grown in culture tubes (50% volume) at 35°C. The culture was grown broth to broth by transferring 10% culture from one tube to another tube with fresh medium.

Antimicrobial Susceptibility of *E. coli*

This was determined using the tube dilution method (23). A series dilution of each antibiotic solution in LB medium (100 μl in each) was made from wells 2 to 11 in 96 well plates. Wells 1 and 12 contained 100 μl of the same medium without any antibiotic solution. The inoculum of freshly grown bacterial culture (5 μl) was added to wells 2 to 12 and incubated at 37 °C for 20 hours. Wells 1 and 12 acted as negative and positive controls, respectively. The first well showing macroscopic inhibition of the growth was considered as containing the minimum inhibitory concentration (MIC). All tests were performed in triplicate.

Antimicrobial Susceptibility of *Streptococcus pneumoniae* (R6)

This was also determined using the tube dilution method. In this case, a series of dilutions of different antibiotics in 500 μl of culture medium was prepared in culture tubes (12 × 75 mm). To each tube, freshly grown *S. pneumoniae* culture (70μl) was added and each incubated at 35°C for 20 hrs. One tube, containing medium without culture inoculum, was taken as the blank.

Detection of PBPs in *E. coli* Membranes (19)

To 5 μl portions of membrane preparation (35 μg protein), 5 μl of phosphate buffer containing 140 mM NaCl, pH 7.4 was added. To the above mixture 3.3 μl of labeling reagent, 100 μM Bocillin Fl solution (final concentration 25 μM), was added and the resulting mixture incubated for 15 min at 35 °C. The reaction was then quenched with 12 μl SDS denaturing sample buffer and heated at 100 °C for 3 min. Then 6–11 μl (8–15 μg) of the sample was subjected to SDS-PAGE (7.5% acrylamide) analysis. The protein gels were rinsed with water immediately after electrophoresis. To visualize the proteins, gels were scanned by means of a Typhoon FluorImager (excitation 488 nm, emission 530 nm). The various bands observed were quantified by IMAGE QUANT software.

Competition experiments with other β-lactam inhibitors

To 5μl of phosphate buffer containing 140 mM NaCl pH 7.4 and 0 – 2 mg/ml of the competing β-lactam, 3.3μl of labeling reagent (100 μM Bocillin Fl, final concentration 25μM) solution was added. The membrane preparation (5 μl, 35 μg protein) was then added and the mixture incubated for 15 min at 35°C. It was then treated as described above to obtain band densities in the presence of the competing β-lactam.

These data were fitted to equation 1, which was derived from Scheme 2, where two competing pseudo-first order reactions are assumed. F and F_0 are the emission intensities of the PBP bands in the presence and absence of the competing β-lactam, respectively, I_1 and I_2 represent Bocillin Fl and the test β-lactam, respectively, and EI_1 and EI_2 their respective covalent PBP complexes. The relative reactivity of any pair of β-lactams ($r = k_1/k_2 = IC_{50}/[I_1]$) could thus be obtained. The IC_{50} values thus obtained, representing the concentrations of test β-lactams required to reduce the intensity by 50 % at a particular Bocillin Fl concentration, are therefore relative values. Their ratio for a pair of test β-lactams, however, would be the same as the ratio of absolute IC_{50} values from a direct experiment. Fitting uncertainties and reproducibility were ± 10 – 30%.

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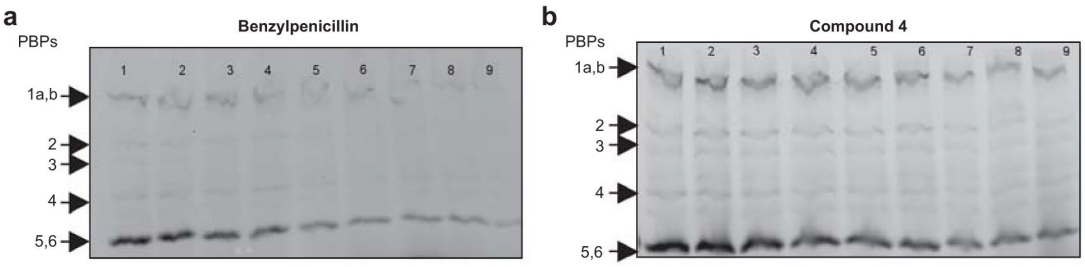
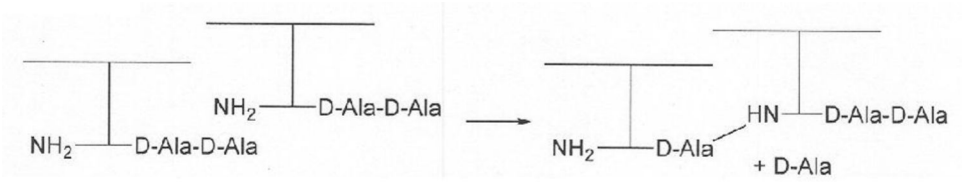
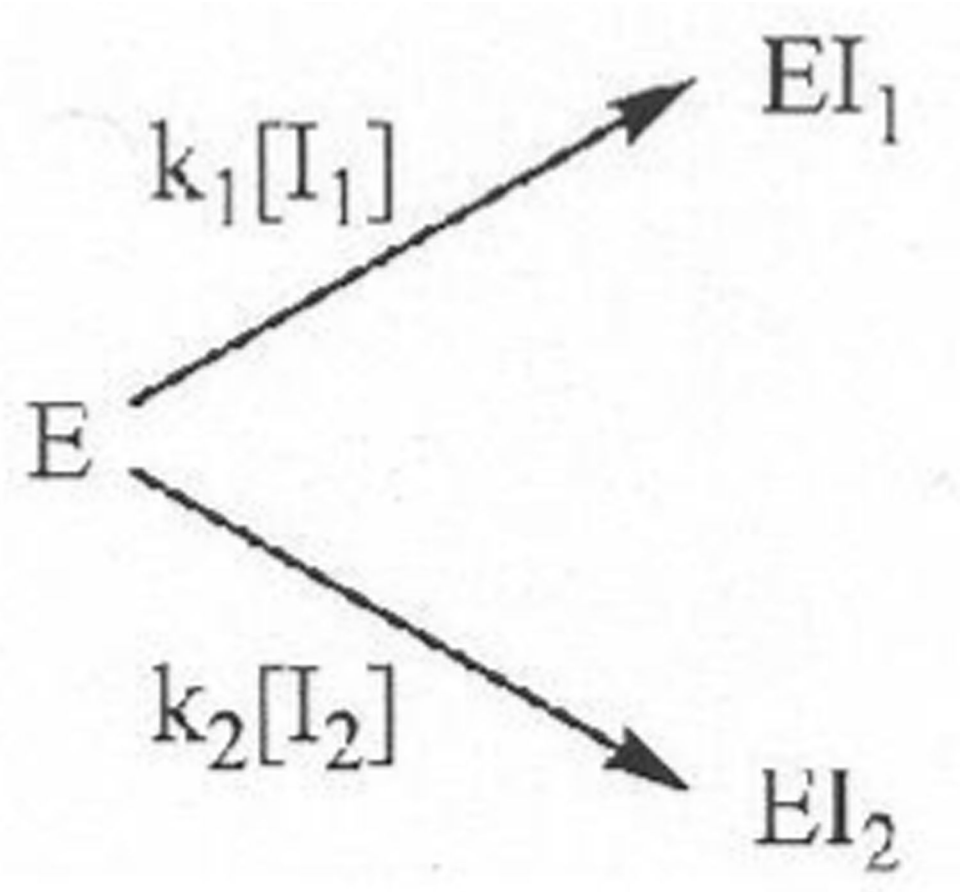


Figure 1.

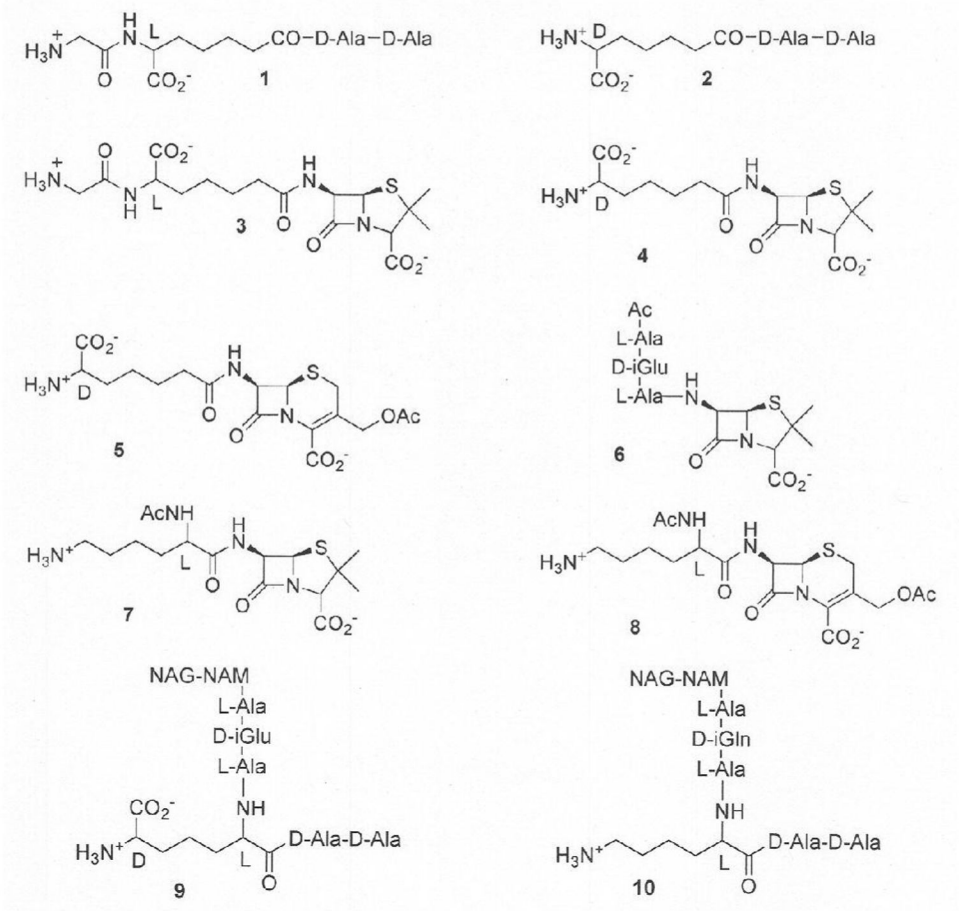
Determination of relative IC_{50} values of penicillin binding proteins of *E.coli*. (a) In competition with Bocillin FI (20 μg/ml), samples in lanes 1–9 contained benzylpenicillin (0, 2, 4, 8, 15, 23, 30, 38 and 76 μg/ml, respectively). (b) In competition with Bocillin FI (20μg/ml), samples in lanes 1–9 contained 4 (0, 6, 12, 24, 47, 95, 189, 379 and 758 μg/ml, respectively).



Scheme 1.



Scheme 2.



$$F = \left(\frac{\mathrm{r}[I_1]}{\mathrm{r}[I_1] + [I_2]} \right) F_0 \qquad (1)$$

Table 1

MIC Values of β-Lactams against *E. coli* strains

β-Lactam	MIC (μg/ml)	
	***E. coli* MM 294**	***E. coli* DC2**
Cephalothin	60	5
Benzylpenicillin	120	60
5	250	40
4	240	> 200

Table 2

MIC Values of β-Lactams against *S. pneumoniae* R6

β-Lactam	MIC (ng/ml)
Benzylpenicillin	14
Cephalothin	28
7	> 900
8	> 900

Table 3

Inactivation of *E. coli* PBPs by β-Lactams

β-Lactam	Relative IC_{50} values[a] (μg/ml) PBP1a,b	PBP2	PBP3	PBP4	PBP5,6
Benzylpenicillin	17	100	12.5	45	6.2
Cephalothin	70	330	> 700	400	200
4	120	≥ 800	≥ 1300	> 1000	80
5	> 1000	400	> 1000	> 1000	800
6	1000	> 1000	> 1000	> 1000	≥ 900

[a] See equation 1 and text.