On the substrate specificity of bacterial DD-peptidases: evidence from two series of peptidoglycan-mimetic peptides

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The reactions between bacterial DD-peptidases and β-lactam antibiotics have been studied for many years. Less well understood are the interactions between these enzymes and their natural substrates, presumably the peptide moieties of peptidoglycan. In general, remarkably little activity has previously been demonstrated *in vitro* against potential peptide substrates, although in many cases the peptides employed were non-specific and not homologous with the relevant peptidoglycan. In this paper, the specificity of a panel of DD-peptidases against elements of species-specific D-alanyl-D-alanine peptides has been assessed. In two cases, those of soluble, low-molecular-mass DD-peptidases, high activity against the relevant peptides has been demonstrated. In these cases, the high specificity is towards the free N-terminus of the peptidoglycan fragment. With a number of other enzymes, particularly high-molecular-mass DD-peptidases, little or no activity against these peptides was observed. In separate experiments, the reactivity of the enzymes against the central, largely invariant, peptide stem was examined. None of the enzymes surveyed showed high activity against this structural element although weak specificity in the expected direction towards the one structural variable (D-γGln versus D-γGlu) was observed. The current state of understanding of the activity of these enzymes *in vitro* is discussed.

Key words: bacterial DD-peptidase, enzyme specificity, peptide substrate, peptidoglycan, penicillin-binding protein, steady-state kinetics.

INTRODUCTION

The DD-peptidases, transpeptidases or penicillin-binding proteins (PBPs) play an essential role in peptidoglycan biosynthesis in bacteria. These enzymes catalyse the terminal stage of the bio-synthetic pathway, comprising the final disposition of the oligo-peptide moiety of peptidoglycan monomer units [1]. They have been classified by Ghuysen [2] on the basis of amino acid sequence and presumed three-dimensional homology. The higher-molecular-mass group is believed to catalyse the trans-peptidation reaction that cross-links the glycan strands, while the lower-molecular-mass members appear to catalyse DD-carboxy-peptidase and endopeptidase reactions that sculpt details of the final peptidoglycan cell-wall structure [3]. Most of these enzymes are membrane-bound on the outer leaflet of the cell membrane.

Inhibition of the DD-peptidases is the basis of the antibiotic activity of β-lactams [4]. This situation is complicated, however, because each bacterial species typically has more than one representative of both high- and low-molecular-mass DD-peptidases and there is apparently some possible degree of overlap in function between them [3]. Therefore, specific and complete inhibition of a particular DD-peptidase does not necessarily lead to cessation of bacterial growth.

Much research has been devoted to the detection, isolation, purification and characterization of these enzymes. In certain instances, peptidoglycan synthase activity has been detected in the pure preparations [5]. The conditions required for such activity, even at the low level generally observed, namely lipid-linked substrates in detergent/aqueous organic solvent media, do not, however, lend themselves to high-resolution kinetics and structural studies. Water-soluble forms of many DD-peptidases have therefore been prepared by deletion of small membrane-binding segments from the holoenzyme. These solubilized constructs generally have some enzymic activity (see below), are inhibited by β-lactams, and are of considerable value for structural studies. Generally absent, however, is any significant amount of DD-peptidase activity against small soluble peptides. In particular, the higher-molecular-mass group, analogues of *Escherichia coli* PBP1a and 1b, PBP2 and PBP3, do not show any peptidase activity under these conditions (see, for example, the careful studies of *E. coli* PBP1b [6] and *Streptococcus pneumoniae* PBP2x [7,8]). They do show modest activity against small non-specific (thio)depsipeptides [9]. There has been little systematic evaluation, however, of the activity of these solubilized enzymes against peptides that are direct analogues of the peptidoglycan relevant to the specific enzyme in question. Positive results of such studies would be of obvious use in functional and structural studies and in the design of novel inhibitors.

Recently [10], we (J. W. A. and R. F. P.) synthesized peptide 1 (Figure 1), which includes the amine acceptor terminus of *Streptomyces* sp. (Figure 1, **2**). This peptide is an excellent substrate of the DD-peptidase of *Streptomyces* R61 (a low-molecular-mass, class B enzyme [2]). The steady-state kinetic parameters were $k_{cat} = 69$ s^{-1}, $K_m = 7.9$ μM and $k_{cat}/K_m = 8.7 \times 10^6$ s$^{-1} \cdot$ M^{-1}. The specificity of this molecule as a substrate resides primarily in the low K_m value, which, since acylation is rate-determining [10], corresponds to the dissociation of the initial non-covalent complex. Crystal structures of the R61 DD-peptidase in the presence of this peptide reveal a specific binding site for the N-terminus [11].

Abbreviation used: PBP, penicillin-binding protein.
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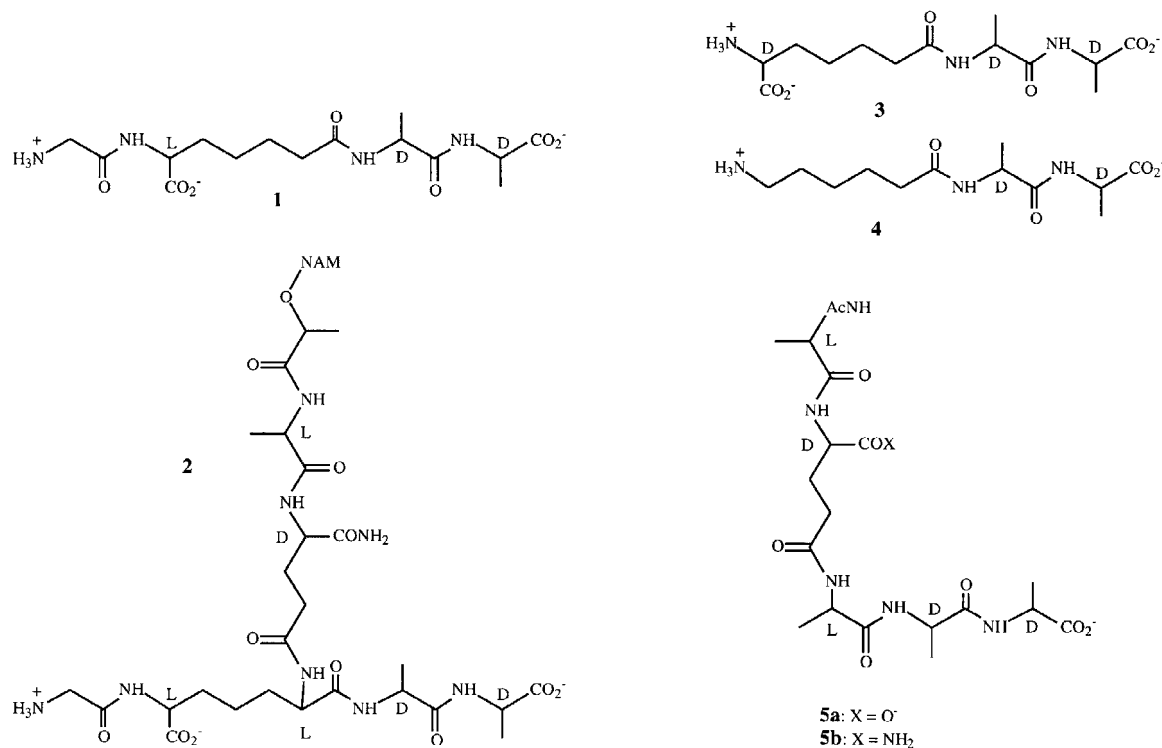
Figure 1   DD-Peptidase substrates

Abbreviation: NAM, *N*-acetylmuramyl.

We have now prepared the analogous peptides **3** and **4** (Figure 1), which correspond to *E. coli* and *Streptococcus pneumoniae* peptidoglycans, respectively, and describe here their reactivity with various DD-peptidases in order to assess the degree of generality of the findings with structure **1**. We have also examined the reactivity of these enzymes with the stem peptide analogues **5** (Figure 1) to detect any strong affinity for this segment of the peptidoglycan. The results show that representative low-molecular-mass class A and high-molecular-mass class B DD-peptidases do not display significant affinity for the sections of their respective peptidoglycan represented by the peptides **3–5**. On the other hand, it appears that representative low-molecular-mass class C enzymes (analogues of *E. coli* PBP4) do show specificity for the N-terminus of **3**, but not for the stem peptide analogue **5**. The general implications of these findings are discussed.

EXPERIMENTAL

Materials

The following DD-peptidases were prepared as described previously: *Streptomyces* R61 DD-peptidase [12], *Streptomyces* K15 DD-peptidase [13], *Actinomadura* R39 DD-peptidase [14] and *E. coli* PBP5 [15]. *Neisseria gonorrhoeae* PBP3 and PBP4 were prepared in the laboratory of R. A. N. by the same procedure employed for *N. gonorrhoeae* PBP2 [16]. *E. coli* PBP2 was generously supplied by Dr H. Adachi (University of Tokyo, Tokyo, Japan) [17], *Streptococcus pneumoniae* PBP2x by Dr O. Dideberg (Institut de Biologie Structurale Jean-Pierre Ebel, Grenoble, France) [18] and *S. pneumoniae* PBP2b by Dr W. Keck (Morphochem, Basel, Switzerland). The peptide substrates **5a** and **5b** were purchased from Multiple Peptide Systems; mass-spectral and HPLC analysis showed them to be > 97 % pure. Peptide **1** was available from a previous study [10]. Diacetyl-L-lysyl-D-alanyl-D-alanine (**6**) was purchased from Sigma.

D-α-Aminopimelyl-ε-D-alanyl-D-alanine (3)

D-α-Aminopimelic acid was prepared by the method of Wade et al. [19] as modified by Bommarius et al. [20]. The amino group was then protected by reaction with benzyl chloroformate [21] and then the α-carboxyl group by cyclization with formaldehyde [22]. Condensation of the resulting *R*-3-benzyloxycarbonyl-5-oxo-4-oxazolidine valeric acid with D-alanyl-D-alanine methyl ester was achieved by dicyclohexyl carbodi-imide coupling [23]. Deprotection of the condensation product was achieved by alkaline hydrolysis followed by catalytic hydrogenation over 20 % Pd(OH)$_2$/C. The product, a colourless solid, was purified as the zwitterion by Sephadex G chromatography in water and characterized as follows: ^{1}H-NMR (^{2}H$_2$O) δ 1.33 (3H, d, $J = 9$ Hz, CH$_3$), 1.38 (3H, d, $J = 9$ Hz, CH$_3$), 1.41 (2H, m, γ-CH$_2$), 1.65 (2H, quint, $J = 6$ Hz, β-CH$_2$), 1.87 (2H, m, δ-CH$_2$), 2.32 (2H, t, $J = 6$ Hz, ε-CH$_2$), (1H, t, $J = 6$ Hz, C<u>H</u>-CH$_2$), 4.22 (1H, q, $J = 9$ Hz, C<u>H</u>-CH$_3$), 4.30 (1H, q, $J = 9$ Hz, C<u>H</u>-CH$_3$); electrospray mass spectrum ($+$) 318.2.

ε-Aminohexanoyl-D-alanyl-D-alanine (4)

N-Benzyloxycarbonyl-ε-aminohexanoic acid was coupled to D-alanyl-D-alanine methyl ester by the method of Blaakmeer et al. [23]. The condensation product was deprotected by catalytic hydrogenation over Pd/C followed by alkaline hydrolysis. The final product, a colourless solid, was purified as the zwitterion by Sephadex G-10 chromatography in water and characterized as follows: ^{1}H-NMR (^{2}H$_2$O) δ 1.33 (3H, d, $J = 9$ Hz, CH$_3$), 1.38 (3H, d, $J = 9$ Hz, CH$_3$), 1.35 (2H, m, γ-CH$_2$), 1.65 (4H, m, β-CH$_2$,

δ-CH$_2$), 2.32 (2H, t, $J = 8$ Hz, α-CH$_2$), 2.99 (2H, t, $J = 8$ Hz, ε-CH$_2$), 4.13 (1H, q, $J = 9$ Hz, CH), 4.30 (1H, q, $J = 9$ Hz, CH); electrospray mass spectrum (+) 274.2.

Enzyme kinetics

In general, the reactivity of the peptides **3–5** with the various enzymes was determined spectrophotometrically, where the loss of peptide bond was monitored at wavelengths between 210 and 230 nm. The analytical wavelengths for hydrolyses of the various peptides were: **1**, 222 nm ($\Delta\varepsilon = 64$ cm$^{-1} \cdot$ M^{-1}); **3**, 214 nm ($\Delta\varepsilon = 380$ cm$^{-1} \cdot$ M^{-1}) or 220 nm ($\Delta\varepsilon = 260$ cm$^{-1} \cdot$ M^{-1}); **4**, 220 nm ($\Delta\varepsilon = 290$ cm$^{-1} \cdot$ M^{-1}); **5a**, 230 nm ($\Delta\varepsilon = 110$ cm$^{-1} \cdot$ M^{-1}); **5b**, 230 nm ($\Delta\varepsilon = 130$ cm$^{-1} \cdot$ M^{-1}). The reaction conditions chosen were, in general, from all literature precedent, likely to induce enzyme activity. The *Streptomyces* R61 DD-peptidase reactions were carried out in 20 mM Mops buffer, pH 7.5, at 25 °C in order to compare the results directly with those obtained previously [10]. The reactions of all other enzymes were studied at 37 °C. The buffers employed were 25 mM Tris/0.4 M NaCl, pH 7.2 (*Streptomyces* K15 DD-peptidase), 0.1 M Tris/1.25 mM MgCl$_2$, pH 7.7 (*Actinomadura* R39 DD-peptidase), 50 mM sodium phosphate/0.5 M NaCl, pH 7.0 (*E. coli* PBP2), 0.1 M sodium pyrophosphate/10 % glycerol, pH 8.5 (*E. coli* PBP5, *N. gonorrhoeae* PBP3 and PBP4), 10 mM sodium phosphate/0.2 M NaCl, pH 7.0 (*S. pneumoniae* PBP2x) and 20 mM Tris/0.115 M NaCl, pH 7.0 (*S. pneumoniae* PBP2b). In general, initial rates of hydrolysis were measured, and these were used subsequently to obtain steady-state parameters by the method of Wilkinson [24]. Enzyme concentrations were, typically, 0.5–2 μM. In cases where substrate concentrations approaching K_m were not achieved, values of k_{cat}/K_m were obtained by non-linear least-square exponential fits to the total progress curves. The highest substrate concentrations employed were usually 1 mM. Total progress curves were analysed to determine whether the above condition obtained by means of the Dynafit program [25].

In a number of cases (see below), no indication of enzyme activity against a particular substrate was obtained from the spectrophotometric assay. In several such instances, the negative result was checked by ^{1}H-NMR. For this experiment, samples of substrate in buffer were freeze-dried and reconstituted by addition of ^{2}H$_2$O. Enzyme solutions in ^{2}H$_2$O were prepared in a similar manner. After addition of a suitable aliquot of one of the latter solutions to the former, ^{1}H-NMR spectra were recorded at appropriate time intervals. The activity of the enzyme solutions was checked against a known (depsi)peptide substrate.

In several cases also, the binding (K_m) of the peptide substrate to a particular enzyme was determined independently from a competition experiment. This was done by employing either a chromogenic substrate, *m*-[(*N*-phenylacetyl)-D-alanyl]-oxybenzoic acid [10] or phenylacetylglycyl-D-thiolactic acid [26], or a chromogenic or fluorophoric β-lactam, 6-β-bromo-penicillanic acid [26] or dansylpenicillin [10].

RESULTS

The DD-peptidases, in general, catalyse the hydrolysis and aminolysis of cell-wall peptides that terminate in D-Ala-D-Ala, such that the terminal D-Ala is cleaved from the chain (Scheme 1). In this work, the hydrolysis reaction was generally monitored, although with some of the enzymes, e.g. the K15 DD-peptidase, the aminolysis pathway seems kinetically preferred even at low (specific) amine concentrations [27]. Since the same acyl-enzyme is thought to occur in aminolysis as in hydrolysis, substrate
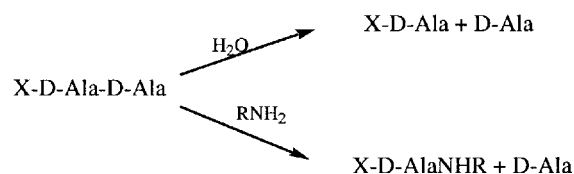
Scheme 1   Carboxypeptidase and transpeptidase activity of DD-peptidases

X-D-Ala-D-Ala represents the peptidoglycan acyl donor and RNH$_2$ is the acyl acceptor in the transpeptidation reaction.

specificity should be expressed as well in studies of the latter reaction as in the former. ^{1}H-NMR experiments indicated that the reactions observed in the present studies involved hydrolysis of the terminal D-Ala.

The results obtained for the hydrolysis of **1**, **3** and **4** by the panel of available enzymes is given in Table 1. Some values for the non-specific peptide substrate *N*,*N'*-diacetyl-L-lysyl-D-alanyl-D-alanine (**6**) are included for comparison. A number of points of interest can be found here. As described previously [10], the *Streptomyces* R61 DD-peptidase, a class B low-molecular-mass DD-peptidase, has high specificity for **1**, which has the *Streptomyces* peptidoglycan motif at its free N-terminus. This enzyme also catalyses the hydrolysis of **3** and **6**, although at considerably lower rates. An immediate subsequent observation from Table 1, however, is that the *Streptomyces* K15 DD-peptidase, which one would expect also to have affinity for **1**, does not appear to react at all with this peptide. An NMR experiment confirmed this result: no D-alanine was detected over a 24 h period in a reaction mixture consisting of 1 mM peptide **1** and 5 μM enzyme, in both the absence and presence of 10 mM glycylglycine: the latter amine was found to be an effective acyl acceptor where **6** was employed as a substrate [27]. The K15 DD-peptidase did, however, react with β-lactams, specifically with dansylpenicillin, where the second-order rate constant of acylation was determined fluorimetrically to be 1.3 s$^{-1} \cdot$ M^{-1}. The rate of this acylation was reduced by 50 % by 1 mM peptide **1**, suggesting a dissociation constant of **1** from the enzyme of approx. 1 mM, and thus no indication of specific interaction. Thus despite the common cell-wall structure of their parent organisms, the R61 and K15 enzymes are very different in their response to **1**. An important difference between the enzymes, however, is that the K15 enzyme is a class A low-molecular-mass DD-peptidase and thus has significantly different overall and active-site structure.

A disappointing result was that the *E. coli* enzymes PBP2, a class B high-molecular-mass DD-peptidase, and PBP5, a class A low-molecular-mass DD-peptidase, did not react with the peptide **3** at all. The former result was confirmed by an NMR experiment. Further, **3** (1 mM) did not decrease the rate of turnover of phenylacetylglycyl-D-thiolactate (4 mM) by this enzyme or the rate of its acylation by 6-β-bromopenicillanic acid (86 μM). Ishino and Matsuhashi [28] showed that turnover of substrates by PBPs can be promoted by a medium designed to be more 'membrane-like', e.g. one including 0.08 % reduced Triton X-100, 11 % glycerol and 14 % methanol; however, no turnover of **3** by PBP2 in this medium was observed. The peptide **3** therefore has little or no affinity for solubilized *E. coli* PBP2. The situation with PBP5 is even more puzzling. The absence of turnover of **3** by this enzyme was confirmed by NMR in the usual way; the amine acceptor glycine [29] had no effect on this result, nor did addition of the potential ligands NAGNAM-L-Ala-D-γGln and NAM-L-Ala-D-γGln (where NAG is *N*-acetylglucosamine and NAM is *N*-acetylmuramic acid), both of which might conceivably assist in inducing an active conformation. The rate of hydrolysis of

Table 1   Steady-state parameters for hydrolysis of peptides 1, 3, 4 and 6 by DD-peptidases

A dash denotes experiment not performed; n indicates that no reaction was observed spectrophotmetrically or in the NMR experiment either.

Enzyme	Peptidoglycan structure	Peptide...	k_{cat}/K_m (s^{-1}·M^{-1}) 1	3	4	6
Streptomyces R61	*Streptomyces* (**2**)		8.7×10^6* $k_{cat} = 69$ s^{-1} $K_m = 7.9$ μM	710 $k_{cat} = 1.3 \pm 0.2$ s^{-1} $K_m = 1.8 \pm 0.3$ mM	–	3.5×10^3† $k_{cat} = 34.5$ s^{-1} $K_m = 9.8$ mM
Streptomyces K15	*Streptomyces* (**2**)		n‡	–	–	10§
Actinomadura R39	Gram-negative		$(4.0 \pm 0.2) \times 10^3$ $k_{cat} \geqslant 4$ s^{-1} $K_m \geqslant 1$ mM	$(5.7 \pm 1.8) \times 10^6$¶ $k_{cat} = 7.4 \pm 0.9$ s^{-1}¶ $K_m = 1.3 \pm 0.5$ μM¶ $(3.4 \pm 1.0) \times 10^6$**	$(3.1 \pm 0.1) \times 10^3$ $k_{cat} \geqslant 3.1$ s^{-1} $K_m \geqslant 1$ mM	6.3×10^4†† $k_{cat} = 18.5$ s^{-1} $K_m = 0.28$ mM
E. coli PBP5	Gram-negative		460 ± 30 $k_{cat} \geqslant 0.46$ s^{-1} $K_m \geqslant 1$ mM	n‡‡	590 ± 120 $k_{cat} \geqslant 0.59$ s^{-1} $K_m \geqslant 1$ mM	220§§
E. coli PBP2	Gram-negative		–	n	–	–
N. gonorrhoeae PBP3	Gram-negative		–	$(1.0 \pm 0.1) \times 10^4$ $k_{cat} \geqslant 10$ s^{-1} $K_m \geqslant 1$ mM	–	2.9×10^4¶¶ $k_{cat} \geqslant 550$ s^{-1} $K_m \geqslant 10$ mM
N. gonorrhoeae PBP4	Gram-negative		–	$(1.8 \pm 0.1) \times 10^3$ $k_{cat} \geqslant 1$ s^{-1} $K_m \geqslant 0.5$ mM	–	60 $k_{cat} \geqslant 1.8$ s^{-1}¶¶ $K_m \geqslant 30$ mM¶¶
S. pneumoniae PBP2x	*Streptococcus*		–	–	n	–
S. pneumoniae PBP2b	*Streptococcus*		–	–	n	–

* Data from [9].
† Data from [41].
‡ Addition of glycylglycine (10 mM) [28] had no effect on this result.
§ In the absence of an acyl acceptor; in the presence of 2 mM glycylglycine, $k_{cat} = 0.31$ s^{-1}, $K_m = 6.2$ mM and $k_{cat}/K_m = 50$ s^{-1}·M^{-1} [42].
¶ Direct spectrophotometric determination.
** Competition with m-[(N-phenylacetyl)alanyl]oxybenzoic acid.
†† Data from [43].
‡‡ Addition of glycine (1 mM) [31] or NAGNAM-L-Ala-D-γGln (1 mM; where NAG is N-acetylglucosamine and NAM is N-acetylmuramic acid) had no effect on this result.
§§ Data from [29].
¶¶ Data from the laboratory of R. A. N.

phenylacetylglycyl-D-thiolactate (4 mM) was not reduced by **3** (1 mM). On the other hand, however, PBP5 did appear to catalyse the hydrolysis, albeit at low rates, of the (presumed) non-specific peptides **1**, **4** and **6**. This result is very surprising.

The more interesting result with peptide **3** was its reactivity with the *Actinomadura* R39 DD-peptidase. This enzyme derives from an organism with the same free N-terminus of the peptidoglycan monomer as Gram-negative bacteria such as *E. coli* [30]. As is evident from the data of Table 1, peptide **3** is an excellent substrate of this enzyme, with a k_{cat}/K_m value of approx. 6×10^6 s^{-1}· M^{-1} and with tight binding ($K_m = 1.3$ μM). Data specific to this reaction are shown in Figures 2 and 3. In each, the tight binding of **3** is demonstrated by the tight curvature of the trace as the substrate is consumed; in Figure 2, comparable data with the non-specific peptide **6** are also presented; in the latter case, shallow curvature denoting weak binding is evident. An NMR experiment confirmed the rapid and stoichiometric formation of D-alanine from **3**. In a manner reminiscent of the R61 DD-peptidase, the R39 enzyme also catalysed hydrolysis of non-cognate peptides **1** and **4** and the non-specific **6**, but with greatly reduced specificity. As is evident from comparison of the results from **3** with those from **6**, the specificity of the former compound derives from K_m, presumably the non-covalent binding, rather than k_{cat}; this is also true for **1** with the R61 DD-peptidase.

PBP3 of *N. gonorrhoeae*, like the *Actinomadura* R39 enzyme [31], is a class C low-molecular-mass DD-peptidase, sharing homology with *E. coli* PBP4. As Table 1 shows, the *N. gonorrhoeae* enzyme, as might be hoped of an enzyme from a
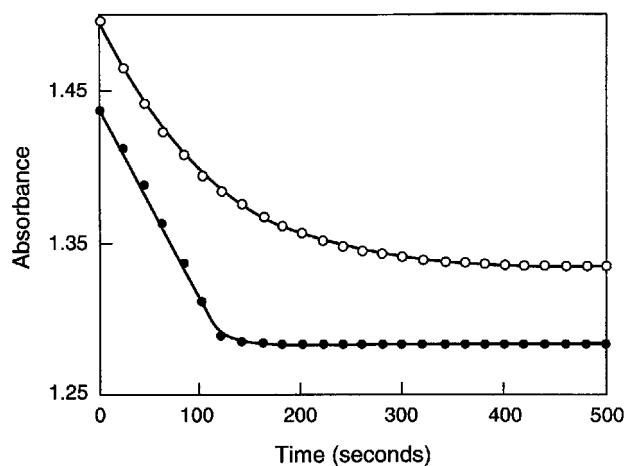
Figure 2   Absorption changes at 220 nm on hydrolysis of peptides 3 (●) and 6 (○) catalysed by *Streptomyces* R39 DD-peptidase

Each peptide was tested at 0.4 mM, and the enzyme concentration was 0.69 μM. The solid lines are derived from the relevant parameters in Table 1.

Gram-negative organism, does catalyse the hydrolysis of peptide **3**. The degree of specificity for **3**, however, is much less than that demonstrated by the *Actinomadura* R39 enzyme. The value of k_{cat}/K_m is 1.1×10^4 s^{-1}· M^{-1}, which is smaller than that for the non-specific peptide **6**. It is also of interest that such specificity

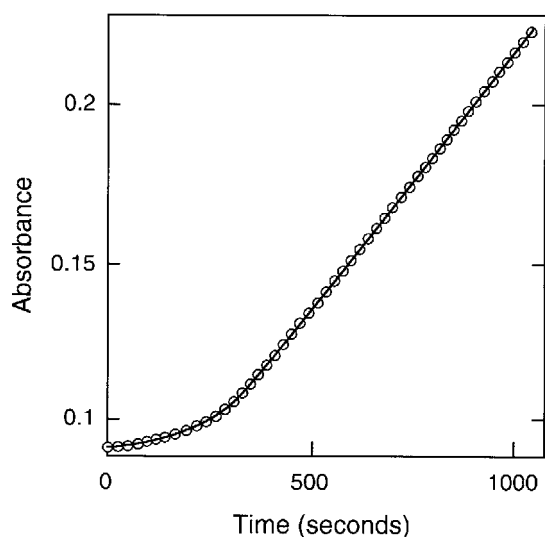**Figure 3   Absorption changes at 300 nm on the hydrolysis of *m*-[(*N*-phenylacetyl)-D-alanyl]oxybenzoate (0.81 mM) in the presence of peptide 3 (0.1 mM) and *Streptomyces* R39 DD-peptidase (69 nM)**

The solid line is derived from the relevant parameters in Table 1.

Table 2   Steady-state parameters for hydrolysis of peptides 5a and 5b by DD-peptidases

n indicates no reaction.

Enzyme and source	Peptidoglycan structure	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	
		5a (γGlu)	5b (γGln)
Streptomyces R61	γGln	180 ± 20	270 ± 15
Streptomyces K15	γGln	n	n
Actinomadura R39	γGln	860 ± 240	$(2.9 \pm 0.7) \times 10^3$
E. coli PBP5	γGlu	150 ± 40	110 ± 10
E. coli PBP2	γGlu	n	n
S. pneumoniae PBP2x	γGlu	n	n
N. gonorrhoeae PBP3	γGlu	$(3.5 \pm 0.2) \times 10^3$	$(1.1 \pm 0.1) \times 10^3$
N. gonorrhoeae PBP4	γGlu	n	n

DISCUSSION

A striking feature of the literature on bacterial DD-peptidases is the absence of evidence for good peptide substrates under *in vitro* conditions with purified enzymes [34]. It is, however, not clear just what values of the steady-state parameters might be optimal or required for the *in vivo* role of these enzymes. Values of K_m of $\geqslant 1$ mM that are commonly found for small peptides such as *N*,*N*′-diacetyl-L-lysyl-D-alanyl-D-alanine (**6**) are not impressive, although, *in vivo*, the substrates could be restrained significantly by the cell membrane, other membrane proteins, and/or the peptidoglycan itself. Thus the unfavorable entropy of substrate binding could be greatly diminished when compared with that in free solution, i.e. the K_m values required for *in vivo* function could appear to be rather high when measured in solution. On the other hand, reported k_{cat} values often appear too small to achieve observed bacterial growth rates.

One parameter that has not been considered systematically is the match of DD-peptidase with the peptidoglycan structure of the parent organism. The stem pentapeptide itself does vary between species, and is often supplemented with additional amino acids, particularly in Gram-positive bacteria. One would anticipate substantial DD-peptidase specificity towards the peptide moiety of the peptidoglycan, characteristic of its species of origin. The results of experiments designed to test this expectation against a panel of representative DD-peptidases are given in Tables 1 and 2. As described above in detail, the general finding is that most of the enzymes show little specificity for their cognate peptide, either in binding or in catalysis. The exceptions to this are a class B and a class C low-molecular-mass DD-peptidase, those of *Streptomyces* R61 and *Actinomadura* R39 respectively, where specific peptide turnover at rates approaching the likely diffusion limit have been observed (Table 1). These rates reflect specificity for the free N-terminus of the peptidoglycan peptide. The enzymes presumably have an immediately available specific binding site for the N-terminus. This has been observed in the case of the R61 enzyme [11], but a crystal structure for the R39 DD-peptidase is not yet available. Despite the specific interactions of these two enzymes with the N-termini of peptides **1** and **3**, respectively, they have little affinity for the general stem peptide analogues **5a** and **5b** (Table 2). It is clear therefore that the specificity of these enzymes towards the acyl donor resides in the N-terminus.

The remaining enzymes of the panel studied have little reactivity in solution for the peptides resembling their particular peptidoglycan. This is true of both class A low-molecular-mass DD-peptidases and of both class B high-molecular-mass enzymes. It might also be noted in passing here that solubilized class A

as is present seems to reside in k_{cat} rather K_m; the K_m value, greater than 1 mM, does not indicate strong affinity of **3** for the enzyme. Low specificity for **3** is also shown by PBP4a from *B. subtilis* (where the peptidoglycan structure is the same as that of *E. coli*), another class C low-molecular-mass DD-peptidase [32]; $k_{cat} = 23$ s^{-1}, $K_m = 2.1$ mM and $k_{cat}/K_m = 1.1 \times 10^4$ $s^{-1} \cdot M^{-1}$ (C. Duez and J.-M. Frère, unpublished work). Class C low-molecular-mass DD-peptidases apparently differ in their specificity for **3**. Another *N. gonorrhoeae* DD-peptidase, PBP4, a homologue of *E. coli* PBP7, also turned over **3**, with specificity that was greater than for **6**, but not high on an absolute scale.

Peptide **4** was designed as a peptidoglycan analogue of *S. pneumoniae*. It was not, however, detectably a substrate of either PBP2b or PBP2x from this organism, either in purely aqueous medium or under the conditions of Ishino and Matsuhashi [28]. No inhibition of β-lactam binding to either enzyme was observed. These high-molecular-mass class B enzymes therefore follow the pattern of inactivity established by *E. coli* PBP2. The crystal structure of a solubilized construct of PBP2x has been obtained [33]. The active site, in general appearance, seems intact, although subtle issues of overall shape have not yet been addressed. The structure of an acyl-adduct with a β-lactam is also available [33].

In Table 2, the results of experiments to test for the specificity of DD-peptidases for the basic stem peptide structure, as represented by the peptides **5a** and **5b** (Figure 1), are shown. There are three major conclusions from these data. First, there is little sign of particular specificity for the stem peptide from any of the tested enzymes. The K_m values in all cases were greater than 1 mM and the k_{cat}/K_m values did not exceed 5000 $s^{-1} \cdot M^{-1}$. Second, the enzymes showing activity were the same ones that showed activity against peptides **1**, **3**, **4** and **6**. In particular, the higher-molecular-mass enzymes, *E. coli* PBP2 and *S. pneumoniae* PBP2x, showed no activity against either type of substrate. Finally, the relative specificity of the active enzymes towards **5a** versus **5b** directly reflects the presence of γGlu versus γGln at the second amino acid position. This difference in specificity however is very small and cannot represent a major source of substrate specificity in these enzymes.

high-molecular-mass enzymes have not yet demonstrated significant, or in most cases any, activity against small peptides in aqueous solution [6]. Low activity towards small (thio)-depsipeptides has been demonstrated by these enzymes [6,9,26,34], although peptidoglycan-mimicking analogues of this type have not yet been prepared. In striking contrast with the situation with peptides, it is found that these enzymes appear to react with β-lactams as rapidly *in vitro* (solubilized) as *in vivo* (membrane-bound) [35]. The active sites of the solubilized enzymes may therefore be constricted into a more β-lactamase-like conformation that reacts with β-lactams, but not with peptides. It may be that a conformational adaptation pathway [36,37] between the N-terminal binding site and the reaction centre has been blocked or that the general shape of the active site is changed. It is thought that the general shape of the active site of the free enzyme is a significant issue in the difference between a β-lactamase and a DD-peptidase, the former site optimal for interaction with a bicyclic β-lactam which is tetrahedral at the nitrogen atom of the scissile bond, and the latter for the planar amide group of a peptide substrate [38]. Some activity against depsipeptides could be retained by the solubilized DD-peptidases because of the higher chemical reactivity of these substrates and their greater ease of distortion of the scissile bond into a conformation resembling a bicyclic β-lactam [39]. In general, the DD-peptidase activity of these enzymes parallels their (thio)depsipeptidase activity.

At this time, one can only speculate about how the active conformation of the DD-peptidase is maintained *in vivo* and thus on how it could be attained *in vitro*. It seems likely that *in vivo*, many of these enzymes exist in multienzyme complexes [1,5] embedded in the membrane, and it seems not unlikely that reactivity would be dictated by this environment. It is probably not a coincidence therefore that the enzymes where impressive peptidase activity has been detected, the *Streptomyces* R61 and *Actinomadura* R39 DD-peptidases, are water-soluble monomeric proteins; the role of these particular enzymes *in vivo* is, however, unknown. It has been speculated that the role of the R61 enzyme is defensive, namely the enzyme is used to mop up β-lactams threatening the organism, a first-generation β-lactamase [40].

Although it is not clear at present how to induce a reactive conformation in these enzymes, this is an important goal for the rational design of alternative antibiotics to β-lactams, and the application of the emerging crystal structures for this purpose. However, the indication from the present results that substrate specificity in that reactive conformation is towards the N-termini of peptidoglycans may ultimately be useful in this regard.

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