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Review

Substrate specificity of bacterial DD-peptidases (penicillin-binding proteins)

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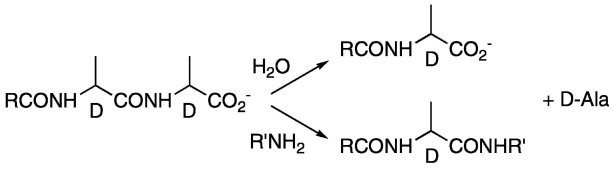
Abstract. The DD-peptidase enzymes (penicillin-binding proteins) catalyze the final transpeptidation reaction of bacterial cell wall (peptidoglycan) biosynthesis. Although there is now much structural information available about these enzymes, studies of their activity as enzymes lag. It is now established that representatives of two low-molecular-mass classes of DD-peptidases recognize elements of peptidoglycan structure and rapidly react with substrates and inhibitors incorporating these elements. No members of other DD-peptidase classes, including the high-molecular-mass enzymes, essential for bacterial growth, appear to interact strongly with any particular elements of peptidoglycan structure. Rational design of inhibitors for these enzymes is therefore challenging.

Keywords. DD-peptidase, penicillin-binding protein, peptidoglycan, substrate specificity, β-lactam.

The bacterial cell wall has intrigued and stimulated scientists both before and after its unambiguous definition by electron microscopists in the middle of the 20th century, as described, for example, by Salton [1]. Its presence in bacteria and its absence in mammalian cells established its metabolism as a focus of antibiotics research; indeed, chemical studies of the bacterial cell wall have been intertwined with those of β-lactam antibiotics for the past 50 years. In 1957, Park and Strominger [2] suggested explicitly that penicillin inhibited some element of the metabolic pathway of cell wall biosynthesis, and in 1964, Martin [3] proposed that peptidoglycan cross-linking was blocked. Then, in 1965, Wise and Park [4] and, independently, Tipper and Strominger [5] suggested that the penicillin target was a D-alanyl-D-alanine transpeptidase. Soon thereafter, the inhibition by β-lactams of the transpeptidation reaction, which led to cell wall cross-linking, was demonstrated [6].

The composition and structure of bacterial cell walls as a peptidoglycan polymer was established between 1950 and 1970 [7]. The 1970–1990 time period was dominated by the isolation and characterization of the enzymes that were inhibited by β-lactams (penicillin-binding proteins, PBPs) and which did indeed turn out to be D-alanyl-D-alanine peptidases (DD-peptidases, transpeptidases) involved in cell wall (peptidoglycan) biosynthesis [8, 9]. At the same time, the kinetics and mechanism of peptide turnover by these enzymes and their inhibition by β-lactams were investigated [9–11]. It became clear that the reaction catalyzed is nucleophilic displacement of the terminal D-alanine of the peptidoglycan stem peptide (described in more detail below) by water or by an amine moiety of an adjacent peptidoglycan strand, effecting cross-linking (Scheme 1). More recently, since 1990, studies of these enzymes have been illuminated and extended by the availability of crystal structures [12, 13]. Although much is now known about the structures of bacterial

DD-peptidases and their general reactivity, there remain puzzles concerning their substrate specificity and quantitative aspects of their catalysis. This review will focus on these latter issues and also sum up current thoughts on the mechanism(s) of catalysis employed by these enzymes.



Scheme 1.

Table 1. Classification of the bacterial DD-peptidases on the basis of amino acid sequence and function.

DD-peptidase class	Function *in vivo*
High molecular mass A (HMMA)	transglycosylase, transpeptidase (bifunctional)
High molecular mass B (HMMB)	transpeptidase
Low molecular mass A (LMMA)	carboxypeptidase
Low molecular mass B (LMMB)	carboxypeptidase
Low molecular mass C (LMMC)	carboxypeptidase/endopeptidase

Adapted from reference 17.

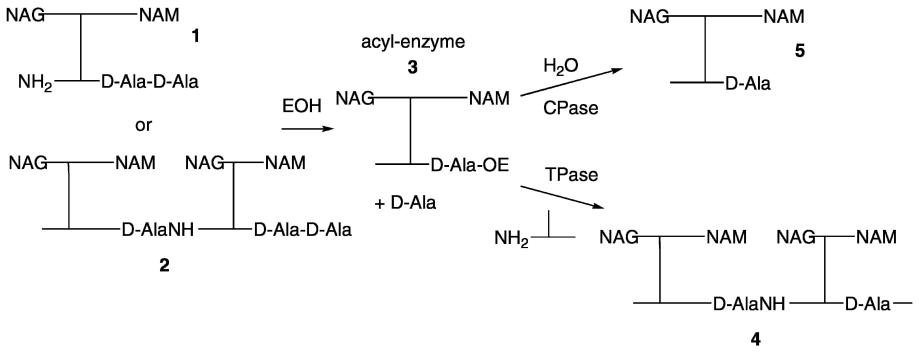
Classification of DD-peptidases

The DD-peptidases comprise a protein superfamily that may derive from an ancient serine hydrolase. Current members of the superfamily have widely different amino acid sequences but retain common active site functional groups, most notably the SXXK motif, and a common protein fold; further details of structure/function are discussed below. On the basis of amino acid sequence homologies, evolutionary trees have been constructed which subdivide the DD-peptidases into groups of related proteins. These groups, in general, correlate with function [14–18]. Sub-classes have also been proposed, which also may have functional significance [17]. Although there have been some attempts to regroup these enzymes, mainly for convenience [13, 16, 17], this review will retain the original scheme of Ghuysen [15] since it does also classify the enzymes with respect to their *in vivo* function, to the extent that it is now known (Table 1). The reactions catalyzed by all of these enzymes are believed to be the same (Scheme 1) but they differ in place and time: different PBPs are localized in different positions of the cell and operate optimally at different stages of the cell cycle, regulated to produce normal cell morphology and cell division stages [19, 20]. The β-lactamases, enzymes that catalyze hydrolysis of β-lactams and thereby lead to β-lactam resistance in bacteria, are also members of this superfamily [15, 18]. They are not, however, effective peptidases [21, 22] and will not be further considered in this review.

Functional form *in vivo*

Although, following classical reductionist methodology, most detailed kinetics and structural studies of these enzymes have been carried out with water-soluble proteins in aqueous solutions, there is good reason to believe that, *in vivo*, many of them operate in a more condensed and heterogeneous medium. First, it is well established that the HMMA and HMMB enzymes are bound to the cell membrane by an N-terminal transmembrane peptide anchor [15]. There is evidence that the membrane anchor and the adjacent periplasmic peptide of *Escherichia coli* PBP3, for example, play an important role in the proper orientation and positioning of the enzyme for optimal activity [23]. There also may be, in some cases, such as the HMM enzymes, secondary membrane association sites [24, 25]. Many of the LMM enzymes are also membrane associated. LMMA *E. coli* PBP5 [26] and the closely similar *Streptococcus pneumoniae* PBP3 [27] appear to be attached to the outer leaflet of the cell membrane by means of a C-terminal amphiphilic helix. Lacking such a helix [28], another LMMA enzyme, that of *Streptomyces* K15, is weakly associated with the membrane, probably via a hydrophobic surface patch [29]. The much-studied LMMB DD-peptidase of *Streptomyces* R61 is apparently a completely solubilized enzyme with little similarity to the membrane-bound DD-peptidase of this organism [9]. A typical LMMC enzyme, such as *E. coli* PBP4, is weakly attached to the cell membrane, probably by electrostatic interactions [30, 31].

In addition to membrane association, it is likely that, *in vivo*, many DD-peptidases are also involved in protein-protein interactions within oligoprotein complexes. The evidence for this assertion has been cogently summarized recently by Vollmer [32, 33]. For example, interactions between *E. coli* PBP1A and PBP3 have been demonstrated [32]. It is also likely that HMMA proteins are present *in vivo* and optimally functional as dimers [34–36]. Other proteins, in complexes with the DD-peptidases, may also be involved, monofunctional transglycosylases for example [37]. It is also well known that HMMB enzymes



Scheme 2.

require at least one additional protein for significant transpeptidase activity; *E. coli* PBP2 requires the rod A protein, for example [38, 39], and PBP3, the FtsW protein [40, 41]. The likely existence of these protein complexes suggests, as proposed by Höltje [42], that peptidoglycan biosynthesis may be catalyzed *in vivo* by multiprotein complexes. Although such a complex in functional form has not yet been isolated, it is interesting to note that Alaedini and Day [43] have cross-linked *in vivo* an aggregate of PBP1A, PBP3, PBP4 and PBP5 and another of PBP 1B, PBP2, PBP4 and PBP5 from *Haemophilus influenzae*. Various PBP aggregates have also been detected in *Caulobacter crescens* by immunoprecipitation [44]. Localization and co-localization of a variety of these proteins in cells have been observed by fluorescence microscopy [45].

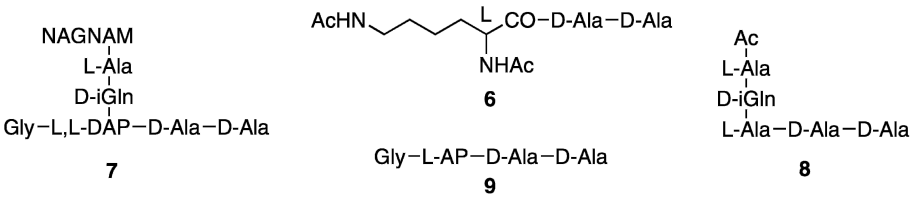
There is, therefore, quite a good reason to suspect that the reactivity of the bacterial DD-peptidases *in vivo* may be quantitatively and perhaps qualitatively different from that observed from solubilized enzymes *in vitro*.

Enzyme activity and substrate specificity

As indicated above, the demonstrated general activity of the DD-peptidases is shown in Scheme 1 and Table 1, and now in more detail in Scheme 2 (note that in Scheme 2 and the structures **15** and **17**, the solid lines often represent various different linear elements of structure of the stem peptide rather than single chemical bonds). *In vivo*, the stem peptide monomer **1** and/or already cross-linked analogues **2**, react with a specific enzyme EOH to yield the acyl-enzyme **3** which then undergoes either aminolysis (the transpeptidase, TPase, reaction) to yield the cross-linked (from **1**, or further cross-linked, from **2**) peptidoglycan **4**, or hydrolysis (the carboxypeptidase, CPase, reaction) to limit cross-linking by formation of **5**.

Some idea of the rates at which these reactions must occur can be estimated from the growth rates of bacteria under optimal conditions. For example, it is estimated that *E. coli* cells contain 3×10^6 monomers in the cell wall [46], some 50% of which are cross-linked [47]. It is also estimated that there is a 50% turnover of peptidoglycan per cell generation [48, 49]. Thus, the number of cross-linked monomers created per cell generation would be circa 2.25×10^6. If the cell doubling time were 30 min, the lower limit of cross-link formation rate would be around 10^3 cross-links per second. Finally, assuming that all cross-linking is catalyzed by HMMA and HMMB enzymes, and that *E. coli* contain 350 ± 30 of the former and 250 ± 30 of the latter [50], the average turnover rate of these enzymes must be about two cross-links/enzyme per second. If the enzymes were performing under half-saturated conditions, the average kcat required would be $4\ s^{-1}$. A similar turnover rate would be required by the LMMA enzymes. Greater rates may be required in Gram-positive bacteria where more peptidoglycan is present but comparable numbers of DD-peptidases are found, in *Staphylococcus aureus*, for example [51]. It is also possible that the enzymes may, *in vivo*, be capable of processing specific substrates even more rapidly than these numbers suggest if, as is quite likely, cell generation times are limited by other factors.

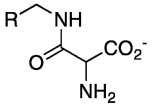
The best-studied DD-peptidase as an enzyme is an LMMB representative from *Streptomyces* R61. This water-soluble enzyme provided Ghuysen, Frère and their group in Belgium with a case study DD-peptidase for over 30 years, interest in it only waning when it became clear that the LMMB enzymes were not typical DD-peptidases and not essential for bacterial survival [15]. Nonetheless, it still stands as the best-studied DD-peptidase from the point of view of substrate specificity and mechanism. Early investigations showed that it catalyzed hydrolysis and aminolysis by specific amine acceptors of peptides such as N,N-diacetyl-L-lysyl-D-alanyl-D-alanine, **6** [10] and, more recently, of small (thiol) depsipeptides [52, 53]. These studies established the double displacement mechanism of Scheme 2. More recently, also, the substrate specificity of the R61 DD-peptidase



with respect to peptidoglycan structure was more closely investigated. The stem peptide of *Streptomyces* sp. has the structure **7** [54] (AP denotes 6-aminopimelic acid and DAP 2,6-diaminopimelic acid). Although the peptide **8**, was a poor substrate for hydrolysis by the enzyme, (kcat/Km = 270 s^{-1} M^{-1}, inferior even to the generic peptide **6**, where kcat = 34.5 s^{-1}, Km = 9.8 mM, kcat/Km = 3.52 × 10^3 s^{-1} M^{-1} [55]), the peptide **9** appeared to be a very specific substrate (kcat = 69 s^{-1}, Km = 7.9 μM, kcat/Km = 8.7 × 10^6 s^{-1} M^{-1}) [56]. Crystal structures of complexes of the enzyme with **9** or its hydrolysis product, glycyl-L-aminopimelyl-D-alanine, showed a specific binding site for the glycyl-L-aminopimelyl terminus [57]. The structure of an analogue of the hydrolysis transition state of **9** displayed the same binding site [58]. Structural variants of **9**, where the methylene chain was longer or shorter, or where the terminal amine or carboxylate was removed, were orders of magnitude less effective as substrates [59]. These results strongly suggest that the substrate of the R61 DD-peptidase *in vivo* contains the stem peptide N terminus and that this moiety represents the dominant substrate recognition element.

The R61 DD-peptidase also catalyzes aminolysis of the acyl-enzyme **3** by specific small amines, notably D-amino acids and glycyl-L-amino acid dipeptides [53, 60–63]; it therefore shows a dual acyl acceptor specificity. The need for the latter is readily understood in terms of one possible *in vivo* role for and the evolutionary history of this enzyme. D-amino acid acyl acceptors are analogues of the D-alanine leaving group of natural substrates and glycyl-L-amino acids can be rationalized as short analogues of the glycyl-aminopimelyl moiety of a putative acceptor. Molecular modeling led to suggestions for the orientation of these acceptors at two separate sites [62] but direct experimental evidence has not yet been obtained. Indirect evidence in the form of the bifunctional acceptors **10**, proposed to occupy both sites simultaneously, was, however obtained; these compounds were found to be very effective acceptors [63].

Of considerable interest from these aminolysis studies was the fact that extended amines, such as **9** itself, that would, in principle, better mimic a 'real' transpeptidase acceptor, were inactive as acceptors when **9** was the acyl donor. This may reflect the low population of



10a: R = Me

10b: R = Ph

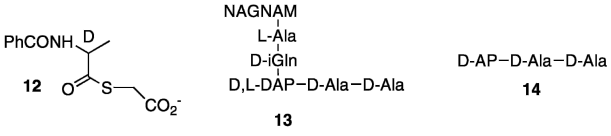
Gly–N-Ac-L-Lys–D-Ala–D-Ala
11

these extended acceptors in an active conformation or the fact that the R61 DD-peptidase is not a transpeptidase *in vivo*, but rather a pure carboxypeptidase or even a β-lactam trap [64]. The low Km of the enzyme for **9** would certainly permit it to efficiently hydrolyze stem peptides at low concentration in solution or at low density on the cell surface. Inspection of the wild-type crystal structure [65] and of models with acceptors [62] suggests that larger acceptors and thus any *in vivo* transpeptidase reaction may be precluded by a flap over the active site, comprising particularly the type I turn between the β2d and β2e strands [65]. It should be noted, however, that early studies of this enzyme indicated that it catalyzed oligomerization of the substrate analogue **11** [66].

Although there is, therefore, limited direct evidence for a functional extended acceptor binding site on this enzyme, there are some indications of binding sites beyond the immediate acyl donor and acceptor sites. There is kinetics evidence, for example, that additional acyl donor substrate binding can affect partitioning of the acyl enzyme intermediate between hydrolysis and aminolysis [62, 67], and α-hydroxyacids and glycolyl-L-amino acids are mixed inhibitors of turnover of a peptidoglycan-mimetic substrate [68]. These non-productive sites may represent the vestiges of a complete transpeptidase acceptor binding site, the latter lost in the evolution of a carboxypeptidase.

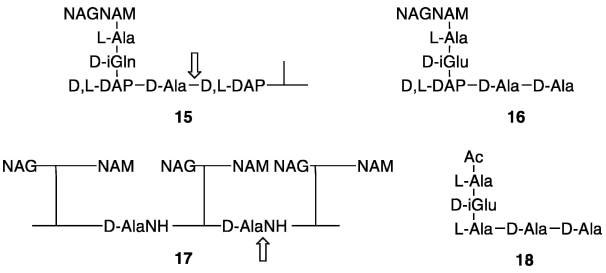
There are clear indications that the LMMC enzymes also recognize and efficiently turn over peptidoglycan-mimetic peptides. Several of these enzymes have been studied, most particularly the soluble DD-peptidase of *Actinomadura* R39. This enzyme catalyzes the hydrolysis and aminolysis of generic small peptides such as **6** [10] and thiolesters such as S2d, **12** [52, 69], with kcat/Km values typically 10^3–10^4 s^{-1} M^{-1}. The stem peptide of *Actinomadura* R39 has the structure **13**, and, in a fashion analogous to that observed for the R61 DD-peptidase, the peptide **8** is a poor substrate (kcat ≥ 2.9 s^{-1}, Km ≥ 1 mM, kcat/Km

$= 2.9 \times 10^3\ s^{-1}\ M^{-1}$) and **14** is an excellent substrate (kcat $= 7.4\ s^{-1}$, Km $= 1.3\ \mu M$, kcat/Km $= 5.7 \times 10^6\ s^{-1}\ M^{-1}$) [55]. A crystal structure of **14** bound to the enzyme reveals a specific D-aminopimelyl binding site [E. Sauvage, A. J. Powell, J. Heilemann, H. R. Josephine, P. Charlier, C. Davies and R. F. Pratt, unpublished data]. As with the R61 DD-peptidase, early studies suggested that this enzyme was capable of catalyzing the transpeptidase reaction with small-stem peptide analogues [70].



Another LMMC enzyme, PBP4 of *E. coli*, has a very similar structure to that of the R39 DD-peptidase [71]. The former enzyme appears to have endopeptidase activity *in vivo* [72] and may be involved in the processing of mature cross-linked peptidoglycan. If the R39 DD-peptidase has a similar role *in vivo*, the above results indicate that its specific substrate may be the stem peptide dimer **15** (the cleavage site is indicated by the arrow – note, by the way, that this is also a DD-carboxypeptidase reaction). PBP4a of *Bacillus subtilis* is another LMMC enzyme. It also shows pronounced reactivity towards peptidoglycan-mimetic substrates [73, 74] although the optimal substrate has not yet been prepared. A crystal structure of a complex with **14** suggests the presence of a side chain binding pocket similar to that of the R39 DD-peptidase [Sauvage et al., unpublished data]; such a pocket is presumably also present in *E. coli* PBP4 [71]. The specific role of PBP4a *in vivo* is not clear at this time, but it does appear to localize at the lateral wall during vegetative growth [75]. PBP3 of *Neisseria gonorrhoeae*, another enzyme of this clan is also active against small peptides [76]. Substrate inhibition of this enzyme by certain peptides was observed, indicating the presence of an extended substrate-binding site. Despite its origin from a Gram-negative bacterium whose stem peptide is **16**, PBP3 is not more reactive with **14** than with **6** [55]. It does have demonstrated endopeptidase activity, so its substrate *in vivo* may be a trimer, **17**, or a higher polymer, rather than the dimer **15** [77].

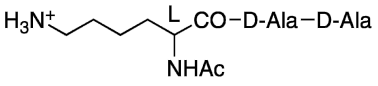
In contrast to the LMMB and LMMC enzymes described above, LMWA enzymes demonstrate generally low activity against small peptides and depsipeptides and no enhanced activity against peptidoglycan-mimetic analogues. The best-studied examples of these enzymes are PBP5 of *E. coli*, the DD-peptidase



of *Streptomyces* K15, and PBP3 of *S. pneumoniae*. Early studies of the membrane-attached *E. coli* PBP5 revealed low activity, both hydrolytic and aminolytic, against small peptides such as **6** [9]. Similar results were obtained with other orthologues, e.g. *S. aureus* PBP4. More recent investigations of the *E. coli* enzyme have employed a water-solubilized version where the membrane-interacting C-terminal peptide has been removed. This enzyme catalyzes hydrolysis of **6** and other small peptides at comparable rates to those of the membrane-bound version [77]. Little evidence of substrate specificity has been observed and significantly enhanced rates were not observed with **14**, **18** or more elaborate peptidoglycan-mimetic peptides [55, 78]. Another ortholog, *N. gonorrhoeae* PBP4, exhibited similar low and non-specific activity against small peptides [55, 79].

The *Streptomyces* K15 DD-peptidase is a membrane-associated, although not by means of a C-terminal peptide, LMMA enzyme [28, 29]. It slowly catalyzes the hydrolysis and aminolysis of **6** and generic depsipeptides such as **12** [80, 81]. Complex kinetics in some of these reactions indicate the presence of an extended binding site. The K15 DD-peptidase does not, however, catalyze these reactions of the peptidoglycan-mimetics **8** and **9** at any appreciable rate [55]. *S. pneumoniae* PBP3, an orthologue of *E. coli* PBP5 [27], and similarly attached to the bacterial membrane through a C-terminal peptide, catalyzes hydrolysis of **6** and **12** considerably more effectively than the latter enzyme: **6**, kcat $= 110\ s^{-1}$, Km $= 19$ mM, kcat/Km $= 5689\ s^{-1}\ M^{-1}$; **12**, kcat/Km $= 5.05 \times 10^4\ s^{-1}\ M^{-1}$ [27]. It is striking, however, that this enzyme does not appreciably catalyze the hydrolysis of the peptidoglycan-mimetics **8**, **19** and **20**. The greater activity of this enzyme against **6** may indicate that its substrate *in vivo* is more likely a dimer analogous to **15**.

The LMMA DD-peptidases therefore, unlike the LMMB and LMMC enzymes, do not, in general, show high reactivity or specificity towards model substrates, even peptidoglycan-mimetics. This situation is even more apparent in the HMM enzymes described below. Possible reasons for it are also discussed below.



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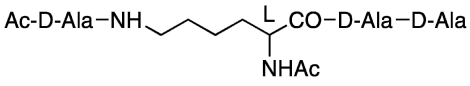

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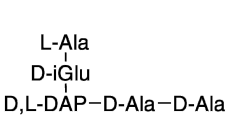
Studies of the membrane-anchored HMM DD-peptidases were complicated for many years by the question of which of them were bifunctional, catalyzing both the transglycosylase reaction – synthesis of the murein polysaccharide – as well as the transpeptidase. This controversy was finally settled in 1992 when van Heijenoort et al. [82] showed that although the HMMA enzyme *E. coli* PBP1b catalyzed transglycosylation, the HMMB enzyme *E. coli* PBP3 did not. Earlier experiments had suggested that both PBP1b and PBP3 were bifunctional [83, 84]. From the vantage point of today, it is clear that HMMA DD-peptidases catalyze both transglycosylation and transpeptidation reactions in murein biosynthesis whereas HMMB enzymes catalyze only the latter reaction [16]. The latter enzymes, therefore, like the LMM enzymes, most likely act on preformed peptidoglycan or combine with monofunctional glycosyltransferases in the synthesis of peptidoglycan [37, 42, 85].

The mechanism of the transglycosylation reaction, catalyzed by HMMA DD-peptidases, particularly *E. coli* PBP1a and PBP1b, and its relationship to the transpeptidation reaction, has been recently intensively investigated. These studies will be further accelerated by recent crystal structures of the transglycosylase active site [86, 87]. Lipid-linked peptidoglycan serves as the glycosyl donor and lipid II as the glycosyl acceptor in the transglycosylase reaction [36, 88–90]. Inactivation of the transpeptidase site of PBP1a and PBP1b by reaction with a β-lactam [91, 92] or by mutation [88] does not significantly impede polysaccharide formation. On the other hand, elimination of the transglycosylase activity of PBP1a and PBP1b, by moenomycin binding or by mutation, produced proteins unable to catalyze transpeptidation [88, 92]. These results suggest, but do not prove, that HMMA DD-peptidases are only active when transglycosylation is in progress.

It should be noted that the experiments with *E. coli* PBP1a and PBP1b described above were performed in homogeneous media containing detergent. Under these conditions, the measured transglycosylase rates were many times lower than those apparently required for cell growth (see above). For example, Terrak et al. [92] determined a kcat value of 0.07 s^{-1} for *E. coli* PBP1b with lipid II as substrate; lower rates were obtained by Bertsche et al. [36] and slightly higher (0.11 s^{-1}) by Schwartz et al. [93]. Earlier measurements, including those made with the enzymes retained in membrane fragments, also achieved rates much too low to support growth [9, 82]. Thus, although the results reported above represent very important advances in the study of the HMWA enzymes, it appears that conditions closely approximating those *in vivo* have not yet been achieved.

A number of other reports describe the behavior of purified HMMA enzymes in homogeneous solution towards a variety of substrate analogues. In general, under the particular conditions employed, none of these enzymes were found to catalyze the hydrolysis or aminolysis of small peptides such as **6** [15]. *E. coli* PBP1b, for example, does not catalyze either of these reactions with either **6** or with the peptidoglycan-mimetic peptides **21** and **22** [92]. *S. pneumoniae* PBP1b had no effect on the peptides **8** and **18** [94]. On the other hand, certain of these enzymes exhibited weak hydrolytic activity against non-specific thiodepsipeptides such as **12** [92, 95–97].



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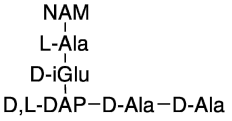

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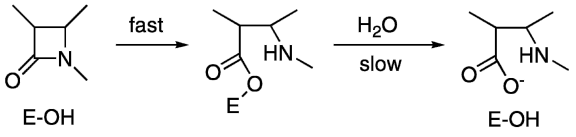
The situation with HMMB DD-peptidases is similar but different. There is not the issue of intrinsic transglycosylase activity [16, 82], but it seems likely that the HMMB enzymes can facilitate *ab initio* peptidoglycan synthesis in combination with either an HMMA enzyme or a monofunctional transglycosylase [42, 98–100]. As described above, there is good evidence that these enzymes, *in vivo*, form part of multiprotein complexes in which their activity could be modulated. As with the HMMA enzymes, there is no evidence that purified HMMB DD-peptidases catalyze acyl transfer reactions of small peptides *in vitro* [15]. For example, *E. coli* PBP2 does not react with **14** or **18**, nor PBP3 with **13** [101], nor *S. pneumoniae* PBP2b or PBP2a with **6**, **8** or **19** [55, 102], nor *S. aureus* PBP2a with **6** [103]. Some do, however, weakly (kcat/Km $< 10^4$ s^{-1} M^{-1}), with no evidence of specific binding, hydrolyse and aminolyse certain thiodepsipeptides [92, 101, 104, 105].

To sum up, it seems that although LMMB and LMMC DD-peptidases catalyze hydrolysis and aminolysis of

small peptides and demonstrate enhanced activity against specific peptidoglycan-mimetic peptides, the LMMA and HMM classes exhibit little or no activity against these substrates and, to date, show no sign of particular peptidoglycan affinity. It is possible that, in some cases at least, the general reactivity of the active site and the substrate side chain specificity may be, or appear to be, separable issues. The former may depend on the conformation of the protein and active site, which may vary with external conditions.

Inhibition by β-lactams

Much of the interest in bacterial DD-peptidases has traditionally centered around their role as the targets of β-lactam antibiotics. β-Lactams inhibit these enzymes by acylation of their active site serine hydroxyl group [106, 107] in a reaction (Scheme 3) analogous to that with substrates (Scheme 2).

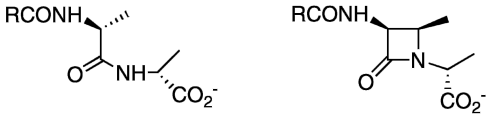


Scheme 3.

Unlike the situation with substrates, the deacylation step is slow, leading to the accumulation of an inert acyl-enzyme and effective inhibition of DD-peptidase activity. This inhibition leads to disruption of bacterial cell wall biosynthesis followed by cessation of growth and, in many cases, cell death. β-Lactams are particularly effective inhibitors of the DD-peptidase reaction because they are substrate analogues [5] and a blend of transition state analogue inhibitor and mechanism-based inhibitor [21, 108]. The former two attributes promote fast acylation of the active site while the third is responsible for the slow deacylation (see below).

One source of bacterial resistance to β-lactams arises from structural changes in the DD-peptidases themselves. Point mutations may lead to resistance [109], interspecies homologous recombination may lead to mosaic proteins where up to 100 single sites are modified [110], and in some cases, entire new proteins have been recruited e.g. PBP2a in *S. aureus* [111] and PBP5 in *Enterococcus faecium* [112]. It is likely that, for a given enzyme, mutational hotspots can be identified producing resistance [113]. Nonetheless, it is likely that an effective β-lactam can be developed for any specific resistant enzyme, if necessary, e.g. for PBP2a of *S. aureus* [114].

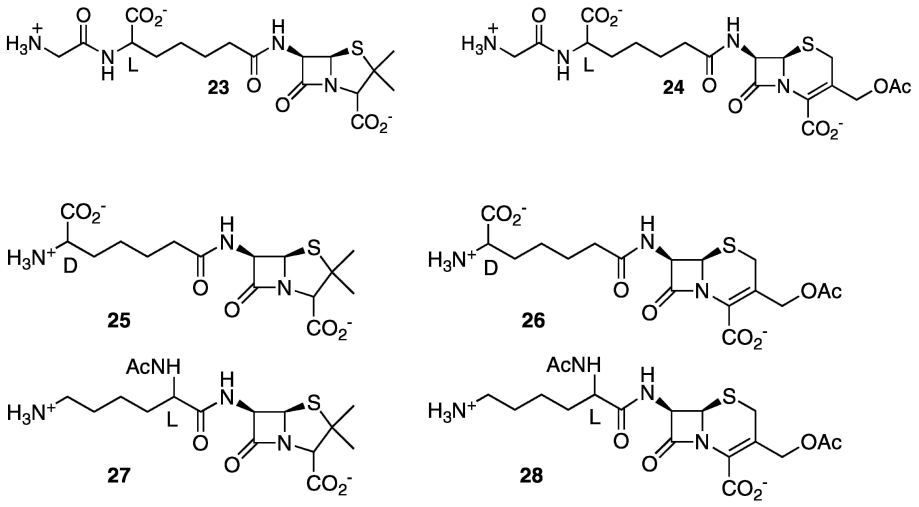
The analogy between the structures of D-alanyl-D-alanine peptides and β-lactams (Scheme 4) [5] would suggest that very effective β-lactam antibiotics might be achieved by synthesis of lactams with peptidoglycan-mimetic side chains (R; Scheme 4). This idea was suggested many years ago but did not appear to be seriously followed up [5, 94].



Scheme 4.

A number of peptide side chains were, at various times, incorporated into β-lactams, but these were not designed to be specific to a particular bacterium nor tested with cognate enzymes [115, 116]. A variety of early specificity studies with non-peptidoglycan-mimetic side chains suggested that the binding site of a particular side-chain on a particular enzyme differed, depending on whether a β-lactam or an acyclic substrate (peptide or depsipeptide) was involved [10, 104, 117–119]. It is certainly understandable that, if the side chain binding were non-specific, then overall binding may be dominated by the reaction nucleus (D-alanyl D-alanine in a peptide, for example, and the β-lactam ring in a β-lactam) and thus, given the different geometry of these nuclei [117, 118, 120], that the side chains may bind differently and thereby induce differential reactivity, particularly between peptides and β-lactams [121]. If, on the other hand, a tight specific side-chain-binding site were present, then binding may be controlled by the side chain, which could then dictate the observed rates.

The latter situation, described above, certainly seems to apply for the *Streptomyces* R61 DD-peptidase. As noted in the previous section, peptide **9**, incorporating a central element of the *Streptomyces* stem peptide **7**, is an excellent substrate of this enzyme ($k_{cat}/K_m = 8.7 \times 10^6\ s^{-1}\ M^{-1}$). Incorporation of the same side chain into β-lactams, producing the penicillin **23** and the cephalosporin **24**, yielded two excellent inhibitors of the R61 DD-peptidase (**23**, $k_i = 1.5 \times 10^7\ s^{-1}\ M^{-1}$; **24**, $k_i = 5.6 \times 10^5\ s^{-1}\ M^{-1}$) [122]. It is clear from the data in the cited reference that the presence of the peptidoglycan-mimetic side chain greatly enhanced the reactivity of both a D-alanyl-D-alanine peptide and a β-lactam with this enzyme. This suggests that a common binding site for the specific side chain influences the reactivity of both reaction nuclei in a similar way. Crystal structures support this conclusion (see below) [57, 123]. The specific side chain may have less effect, however, on substrate deacylation rates.

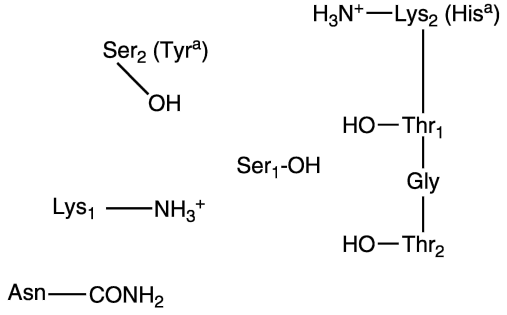


The same conclusion was reached for the LMMC *Actinomadura* R39 DD-peptidase. Peptide **14**, including the essential element of the *Actinomadura* stem peptide, **13**, is an excellent substrate of this enzyme [55]. Similarly, the β-lactams **25** and **26** are excellent inhibitors [94]. The presence of a specific D-aminopimelyl-binding site is confirmed by crystal structures [Sauvage et al., unpublished data].

On the other hand, in direct contrast, as noted above, peptide **14** is a very poor substrate of the LMMA *E. coli* PBP5 [55]. So too are **25** and **26** relatively poor inhibitors for this enzyme with k_i values of $2.2 \times 10^3\ s^{-1}\ M^{-1}$ and $4.7 \times 10^3\ s^{-1}\ M^{-1}$, respectively, both comparable to that of benzylpenicillin, $k_i = 8.0 \times 10^3\ s^{-1}\ M^{-1}$ [94]. One would predict that no specific D-aminopimelyl-binding site exists at the active site of this enzyme. This conclusion seems confirmed by crystal structures [Sauvage et al., unpublished data]. Also in agreement with the peptide results, HMMA *S. pneumonia* PBP1b and *S. pneumoniae* PBP2x do not react with **27** and **28** more rapidly than they do with benzylpenicillin and cephalothin, respectively, and *E. coli* PBP2 reacts sluggishly, if at all, with **25** [94]. No sign of specific interaction, therefore, is seen with these enzymes either.

Protein structure

Much progress has been made in the determination of DD-peptidase structures in recent years, to the extent that at least one structure is now available for each of the major classes of these enzymes [12, 13]. The common fold of the SXXK superfamily of β-lactam-recognizing enzymes is now well established. The HMM enzymes contain an additional module, the tranglycosylase domain in HMMA enzymes, and one of still unknown but not catalytic function in the HMMB group.



Scheme 5. Ser_1 is the active site nucleophile; Ser_2/Tyr and the two lysine residues are believed to be involved in proton transfer/transition state stabilization. Thr_1 and Thr_2 (the latter seems conserved in HMM enzymes) are probably involved in binding the substrate (or β-lactam) carboxylate group (see text). [a]LMMB DD-peptidases.

The conserved catalytic residues of the active site are well-established, a nucleophilic serine of the SXXK motif and three residues involved in proton transfer/transition state stabilization: serine/tyrosine, lysine and lysine/histidine (Scheme 5). Only LMMB enzymes have the tyrosine and histidine combination; the default set is serine and lysine. The active site residues, and general organization of the active site are very similar to those in β-lactamases, which, of course, are evolutionary descendants of the DD-peptidases [5, 21]. LMMB DD-peptidases have the active site composition of class C β-lactamases, while the other classes resemble class A β-lactamases. Differences in the spatial distribution of active site residues between DD-peptidases and β-lactamases are subtle, but the β-lactamases differ in having a more effective β-lactam deacylation catalyst or catalytic

system than is present in the DD-peptidases – Glu 166 in the class A β-lactamases, Tyr 150/Lys 65 (Lys_1) in class C, and the carboxylated lysine (Lys_1) in class D [124].

Although the general polypeptide fold of the DD-peptidases is, as noted above, very similar [13], reflecting their common heritage [5, 17], and the catalytic active site residues are also similar, there seems to be a distinct difference with respect to the overall shape of the active site in the HMM and in the LMM enzymes. In the latter group, the active site residues lie at the bottom of a rather broad bowl, whereas, in the former, the catalytic residues are found on the bottom of strikingly deep and narrow clefts (Fig. 1). This may reflect a greater substrate specificity in the latter enzymes – the requirement for more extended substrates which interact with more of the protein for significant activity – and the concomitant poor reactivity with small substrate analogues and inhibitors based on them (see above and below). The HMM active sites, essential for bacterial survival, may thus be more strongly protected against small substrate analogue inhibitors.

To date, the analysis of crystal structures of unliganded DD-peptidases have not directly led to detailed information concerning substrate specificity and mechanism. Although it has been suggested, for example, that the distance between O_γ of the nucleophilic active site serine (Ser_1) and N_ζ of the SXXK lysine (Lys_1) may be a measure of reactivity [125], a survey of all the presently available structures shows this to be not generally true; this distance in the reactive *Streptomyces* R61 DD-peptidase, for example, is 4.13 Å, while that for the unreactive *S. aureus* PBP2a is 2.89 Å, the latter reflecting hydrogen bonding and apparent readiness for general base catalysis.

It seems, in general, that these structures should be cautiously interpreted, particularly those of HMM enzymes, since there are indications of conformational flexibility in solution and conformational sampling in crystals. Macheboeuf et al. obtained a crystal structure of the solubilized transpeptidase domain of *S. pneumoniae* PBP1b [126]. It showed a narrow active site cleft, which did not appear directly accessible to substrates or β-lactams. Presumably because of this, direct reaction of β-lactams with the crystalline enzyme did not occur. After exposure of the crystals to a small depsipeptide substrate, however, the crystals did react with β-lactams [126] and lactivicin [127]. The structure then displayed an 'open' conformation where the active site cleft had widened, sufficiently to allow access of the inhibitors. Macheboeuf et al. [127] suggested that the binding of substrates, both in solution and *in vivo,* might require this conformational change, which may then be externally controlled to regulate enzyme activity. Subsequently, however, Strynadka and coworkers described another crystal form of the unliganded protein in the 'open' conformation [128]. It is thus not clear just what the resting and active conformations of PBP1b might be under optimally functional conditions. The crystal structure of β-lactam-resistant PBP2a of *S. aureus* also contains a distorted active site that β-lactams probably cannot react with directly [129]; this distortion is lost on acylation of the enzyme by a β-lactam. There is also evidence from circular dichroism studies in solution that this enzyme changes

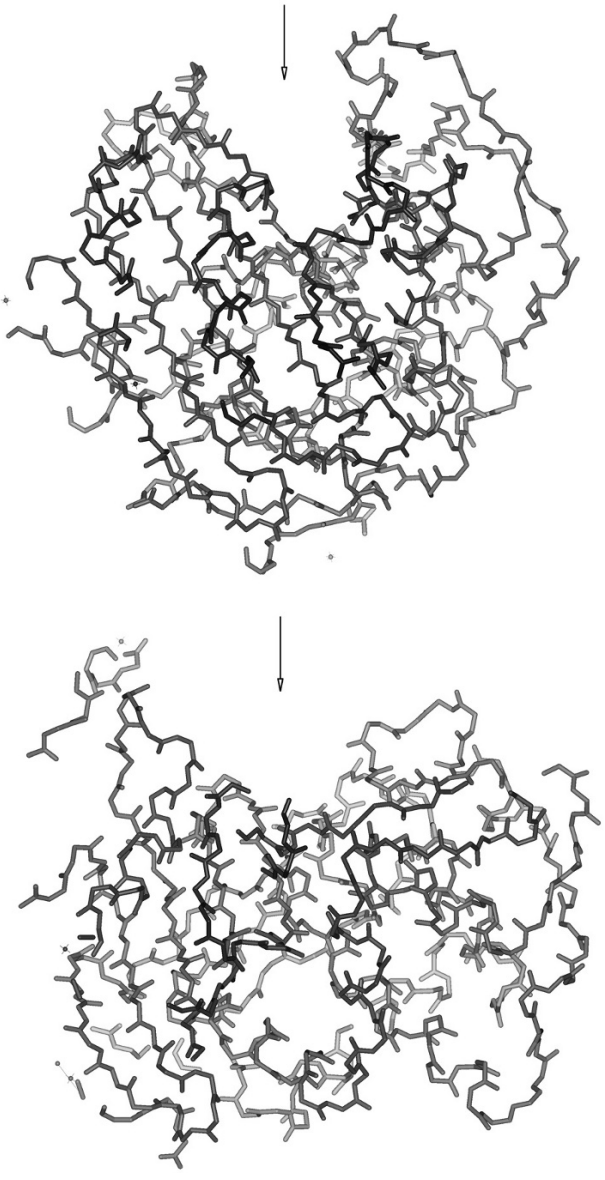


Figure 1. Active site clefts of a typical HMM DD-peptidase (*S. pneumoniae* PBP 2x [140], upper panel) and a typical LMM enzyme (*S. pneumoniae* PBP 3 [27], lower panel). In each case, a 20-Å sphere centered at the active site serine residue is shown.

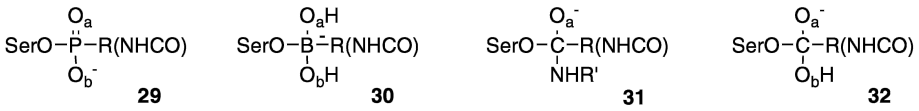
conformation on acylation by β-lactams [130, 131]. Infra-red studies of *S. pneumoniae* PBP2x indicate conformational change on acylation by β-lactams [132]. In view of the above, it was not unreasonable to conclude that the distorted active site structure observed in crystals of PBP2a is responsible for its slow acylation by β-lactams and the β-lactam resistance of bacteria expressing this protein [129], but in view of the situation with *S. pneumoniae* PBP1b, described above, and the apparent inability of the *S. aureus* enzyme to catalyze any peptide or depsipeptide hydrolysis *in vitro* [103], one cannot be completely sure that the distortions observed in the crystal structure are the direct and only cause of the low reactivity of this enzyme in solution. A more detailed combination of structural and kinetics studies may be needed to answer this question. The same uncertainty arises with other β-lactam-resistant DD-peptidases where, at present, the source of the resistance has been directly attributed to observations made from the crystal structures [133–135].

An important question, at present, for any given DD-peptidase, is that of the structure of the active conformation of that protein *in vivo* and how similar to this is the structure of solubilized enzymes in homogeneous solution and in the crystalline form. From the discussion above, it seems likely that the reactive LMMB and LMMC enzymes are in optimally active form, both in solution and in crystals, but this may not be true for many LMMA and HMM enzymes. Even *in vivo*, when not actually engaged in catalyzing a reaction, these enzymes may not be in a fully active conformation. As noted above, Macheboeuf et al. [127] suggested this possibility to explain the requirement of a poor substrate to induce a conformation of *S. pneumoniae* PBP1b reactive toward β-lactams. To directly interrogate the conformation of DD-peptidases *in vivo*, we determined the MICs of **25** and **26** as antibiotics against *E. coli*, and of **27** and **28** against *S. pneumoniae.* In neither case were the peptidoglycan-mimetic β-lactams better antibiotics than generic β-lactams [136]. These experiments led to the conclusion that, even *in vivo*, the DD-peptidases that represent β-lactam killing sites (the HMM enzymes), are not, either statically or dynamically, in conformations that recognize elements of peptidoglycan structure directly adjacent to the reaction center. It may be that quite extended structures, well beyond **25** and **26**, even with saccharides attached [78], are required to induce active conformations of these enzymes. Such a conclusion would have important messages for inhibitor design (see below).

Finally, it should be noted that the crystal structures of DD-peptidases with β-lactams do appear to largely explain the inability of these enzymes to hydrolyze acyl-enzymes derived from their reaction with these molecules, i.e. the molecular basis of the antibiotic activity of β-lactams. The general reason appears to be the steric hindrance to hydrolysis provided by the still present leaving group, which limits access of a hydrolytic water molecule and its attendant general base catalyst to the acyl-enzyme. This issue has been defined in terms of a combination of dihedral angles [21]; more recent structures continue to support that definition. Still not understood, however, is just why it is not possible for the acylated DD-peptidases to relax these dihedral angles, as allowed by β-lactamase active sites, to allow water access. The reason must be strongly coupled to acylation of the active site by peptides but the molecular details are not known.

Mechanism of action

Thoughts on the mechanism of catalysis by DD-peptidases of the carboxypeptidation and transpeptidation reactions and of the reaction of those enzymes with β-lactams have naturally derived from those on β-lactamases because of the structural similarity of the active sites and the much more intensive studies of mechanism in the latter enzymes [21, 137]. Both β-lactamases and DD-peptidases catalyze hydrolysis and aminolysis of acyclic depsipeptides [52, 138], an overlap that allows direct comparisons of structure/activity relationships and mechanism. Most enzymes of both groups contain the SXXK, SXN and KT(S)G amino acid sequence boxes that contain most of the functional active site residues (Scheme 5). There is agreement that β-lactamase and DD-peptidase mechanisms share the nucleophilic Ser_1, the attack by which on the substrate leads to an acyl-enzyme intermediate. Acylation of the enzyme in both cases and deacylation of β-lactamases is generally facilitated by an oxyanion hole composed of backbone NH moieties contributed by Ser_1 and X of the KT(S)GX β strand, and by an amido side chain of the substrate hydrogen-bonded to the side chain NH of Asn from the SXN motif and to the backbone carbonyl of X in KT(S)GX. Finally, the terminal carboxylate of the substrate is generally hydrogen-bonded to elements of the KT(S)GX box and, in individual cases, to side chains of more distant, not strictly conserved, residues. Notably, most crystal structures of covalent complexes of β-lactams with DD-peptidases (*S. pneumoniae* PBP1a [113], PBP1b [126] and PBP2x [139], *S. aureus* PBP2a [129], *Streptomyces* R61 DD-peptidase [140], *Actinomadura* R39 DD-peptidase [141], and *E. coli* PBP4 [71]) show the β-lactam carboxylate interacting with the side chain hydroxyl



group of X in KT(S)GX, where X in all of these enzymes is Thr or Ser. It seems likely that peptide substrate carboxylates would be similarly accommodated [why else is Thr (Ser) at this position?] although it has been observed, in the only crystal structure of an intact substrate bound to a DD-peptidase, admittedly the atypical LMMB R61 DD-peptidase, that the terminal carboxylate of a peptide substrate is held between Thr 299 and Arg 285 rather than Thr 301 [57]. This binding pattern seen in the R61 enzyme with a specific peptide seems to apply to penicillin inhibitors also [123], although cephalosporin carboxylates appear to prefer hydrogen-bonding to the two Thr side chains [140]. Specific binding of substrate side chain amide and the carboxylate, as described above, and the penultimate D-alanine methyl group [21], presumably settles the substrate into the active site and induces productive geometry for catalysis.

Most discussion of DD-peptidase mechanisms, as is also true of β-lactamases, revolves around the catalysts of proton transfer. During formation of the acyl-enzyme intermediate, a proton must be removed from the active site serine hydroxyl group and one must be added to the amine leaving group. A similar proton movement must be facilitated in deacylation. The identity of the general acid and base catalysts involved in these proton transfers is the question. The situation appears simpler with DD-peptidases since the number of candidates is smaller than in β-lactamases, of class A at least. The possibilities in DD-peptidases seem to revolve around Lys_1, Lys_2 and Ser_2 of Scheme 5. Evidence on their participation in catalysis is described below.

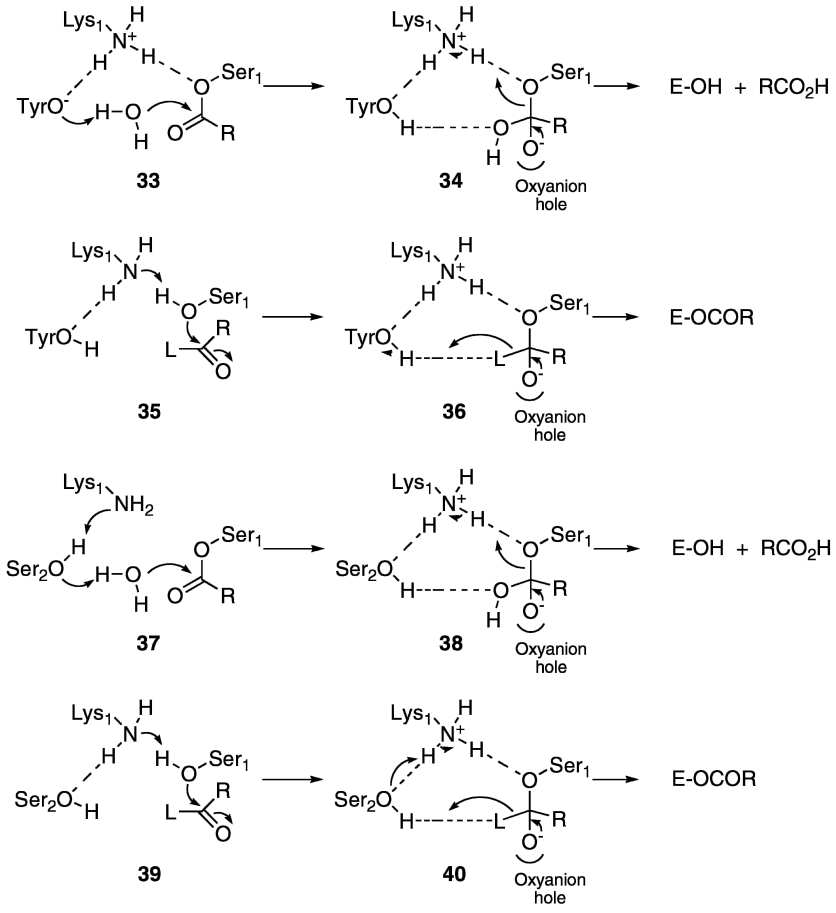
In general, the crystal structures of enzymes with specific ligands (substrate, substrate analogs, transition state analogues) bound should give a better indication of the distribution of functional groups during catalysis, and hence of mechanism, than unliganded structures. There are many structures of PBPs in covalent complexes with β-lactams, but these complexes are catalytically incompetent and thus could well be misleading on issues of mechanism. More useful would be crystal structures with transition state analogues bound. These, in principle, if the analogues are good ones, with the latter always the question, would reveal the disposition of functional groups during catalysis, including proton transfer [142]. At present, two such structures are available (more would be welcome!), one a phosphonate complex **29** of the R61 DD-peptidase [58] and the other a boronate complex **30** of *E. coli* PBP5 [143]. Both represent tetrahedral anionic structures that can be assumed to closely resemble the tetrahedral intermediates of acylation **31** and deacylation **32** formed during substrate turnover. In each case, one oxygen, PO_a^-, or $B(O_aH)^-$, resides in the oxyanion hole, and the side chain amide is hydrogen-bonded to the enzyme as described above.

It should also be noted, however, that neither of these analogues contains a leaving group and thus presumably most likely resemble deacylation transition states, **32**, as analyzed below. It is possible that the leaving group (D-alanine) carboxylate might also participate in catalysis of the acylation step, as has been suggested for β-lactamases [144, 145] (although not generally accepted in that case) and considered for DD-peptidases [146].

The structure of the phosphonate complex with the R61 DD-peptidase, representing LMMB enzymes, shows Tyr 159 O_ζ within hydrogen-bonding distance of O_b (**29**) and Lys 65 N_ζ similarly close to Ser 62 O_γ. This has been interpreted as a model of the transition state for formation of the tetrahedral intermediate in deacylation, where Tyr 159 O^- would play the role of general base catalyst of water attack and Lys 65 NH_3^+ would serve to stabilize Tyr 159 O^- and assist the reaction electrostatically (**33**).

In the breakdown of the tetrahedral intermediate, Lys 67 NH_3^+ would then presumably serve as a general acid for protonation of the leaving group, Ser 62 O_γ (**34**). If the reaction mechanism were symmetrical with respect to acylation and deacylation, Lys 65 would then be the general base catalyzing nucleophilic attack of Ser 62 on the substrate (**35**) and Tyr 159 the general acid for loss of the leaving group from the tetrahedral intermediate (**36**). It is possible however, given the positions of these residues, that they might exchange roles or, in a deletion mutant, either one could, less effectively perhaps, carry out both roles.

The boronate complex of *E. coli* PBP5 shows that the only catalytic residue directly interacting with the ligand is Lys 47 (Lys_1), where its N_ζ is within hydrogen-bonding distance of Ser 44 (Ser_1) O_γ. O_b is hydrogen-bonded to two water molecules, one of which (Wat 182) is also hydrogen-bonded to Ser 110 O_γ, which, in turn is apparently hydrogen-bonded to Lys 213 N_ζ (Lys_2). This may be best interpreted as an analogue of the transition state for breakdown of the tetrahedral intermediate in deacylation (**38**). The transition state



for formation of the tetrahedral intermediate is less clear. One possibility is that the proton transfer does occur through an extended relay and Wat 182. Alternatively, as Nicola et al. [143] point out, Ser 110, perhaps still accompanied by Lys 213, may move to displace Wat 182 with its own hydroxyl group [indeed, a short molecular dynamics run, based on the boronate complex structure, suggested that this movement of Ser 110 is facile; R. F. Pratt, unpublished data]. That movement would allow the more direct relay shown in **37**, where Lys 47 is the (indirect) general base. It should be noted, however, that the boronate inhibitor used to form the complex did not contain the side chain of a specific substrate; it is possible that a better analogue would yield a different picture. If the mechanism of hydrolysis were also symmetrical, then the sequence of events in acylation would be that indicated in **39** and **40**, where the general base initiating Ser 44 attack on the substrate is again Lys 47.

These mechanisms represent the simplest possibilities based on the most relevant structures. In each case, more complex possibilities exist, involving longer chains of proton transfers. Transition state structures **33–40** may, in principle, be found in all other classes of DD-peptidases and, in fact, similar mechanisms have been suggested for several of them [13, 27, 74, 79, 125, 143, 147–150].

An issue that immediately arises when mechanisms such as those proposed above are put forward is that of pH rate profiles and the pKa values of the catalytic residues. Questions on this issue have pervaded the literature on β-lactamase mechanisms for many years and are still not completely answered to universal satisfaction. pH rate profiles for several DD-peptidases have been determined, for poor and non-specific substrates in most cases. A number, for example those of the S*treptomyces* R61 DD-peptidase (LMMB) [151], *N. gonorrhoeae* PBP3 (LMMC) [76], *N. gonorrhoeae* PBP4 (LMMA) [79], *Actinomadura* R39 DD-peptidase (LMMC) [S. A. Adediran and R. F. Pratt, unpublished data], and *S. pneumoniae* PBP2x (HMMB) [148], display typical bell-shaped curves for kcat/Km with pKas of 5–7 and 7.5–10. As usually interpreted, these results suggest a general base catalyst of pKa 5–7. The only candidate among the catalytic residues described above would be a very low pKa lysine (or, alternatively, a low pKa Tyr 159 in the R61 DD-peptidase). As demonstrated by the β-lactamase precedent [152, 153], it is possible to more

directly measure these pKa values, although not easily. To date, with the exception noted immediately below, no direct measurements have been made with a DD-peptidase and therefore a low pKa lysine remains unconfirmed.

It has been reported that the pKa values of *E. coli* PBP5 are higher than those mentioned above: 8.2 and 11.1 [77], or 9.1 and 10.8 [150], with maximal activity around pH 10. Although these pKas are closer to those expected of lysine, the enzyme has very little activity at neutral pH. The pKa of 9.1 as lysine is supported by measurements on an 8-thialysine mutant of Lys 67 [150]. Complicating factors here, however, are the non-specific nature of the substrate used and the possibility that PBP5 resides in solution largely in an inactive form [Sauvage et al., unpublished data]; the pKa values of the latter would then be measured. Interestingly, a lower pH shoulder (pKa 6.1) is seen in the data of Zhang et al. [150], which might represent the real active enzyme. Computational approaches to DD-peptidase mechanisms have also been reported [145, 146, 154, 155].

In summary

The bacterial DD-peptidases are still an enticing target for antibacterial agents although they are strongly defended. The design of new antibiotics directed at these targets will require a better understanding than is currently available of their substrate specificity and catalysis *in vivo*.

The structure/activity relationships for LMMB and LMMC enzymes are now quite well understood. Both groups have well-defined peptidoglycan-binding sites. It is clear that new specific inhibitors of these enzymes could readily be designed. Since these enzymes are not essential for bacterial growth, in the short term at least, such inhibitors are unlikely, however, to become antibiotics. The situation with respect to the essential HMM DD-peptidases and less essential LMMA groups is much less advanced. There are issues of reactivity and substrate specificity with these enzymes that are not resolved. It seems likely that many of these enzymes are in unreactive conformations when isolated as soluble constructs and when crystallized. Inhibitor design based on such structures is thus problematic unless closely accompanied by careful kinetics studies. It also seems that these essential enzymes do not tightly interact with monomeric stem peptide analogues and thus small peptidoglycan-mimetic inhibitors are unlikely to be effective.

At present, the only direct approaches to rational inhibitor design targeted at these enzymes would appear to be, on the one hand, the classical one of directing transition state analogues and mechanism-based inhibitors at the reaction center, and, on the other, to use structure-based design on specific enzymes. The latter might well, of course, only produce narrow-spectrum antibiotics but this might be advantageous in particular cases, e.g. *S. aureus*; they would, however, probably be readily susceptible to resistance by mutation. Random screening for inhibitors of specific enzymes, which has been attempted [156–158], appears to be only modestly productive [159], and would, more likely than not, run into the same problem. Competitive measurements against specific enzymes *in vivo* [136] would seem at present to be the least problematic means of assessment of new inhibitors of the HMM DD-peptidases. Closer studies of bacterial DD-peptidases exhibiting *in vivo*-like activity and/or under *in vivo*-like conditions are needed for real progress.

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Note added in proof: A recent publication [Macheboeuf, P., Lemaire, D., Dos Santos Martins, A., Dideberg, O., Jamin, M. and Dessen, A. (2008) Trapping of an acyl-enzyme intermediate in a penicillin-binding protein (PBP)-catalyzed reaction, J. Mol. Biol. 376, 405-413] describes the crystal structure of an acyl-enzyme derived from reaction of *S. pneumoniae* PBP1b with a poor thiodepsipeptide substrate. It is of particular interest because of the unusual hydrogen bonding of the amido side chain, which may explain why these non-specific substrates turn over so slowly.