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ß-LACTAM-RECOGNIZING ENZYMES EXHIBIT DIFFERENT STRUCTURAL SPECIFICITY IN ACYCLIC AMIDE AND ESTER SUBSTRATES: A STARTING POINT IN ß-LACTAMASE EVOLUTION?

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Abstract: Changes in the structure of acyclic substrates, $RNHCHR'COXCH(CH_3)CO_2^-$, from R = N,N'-diacetyl-L-lysyl to phenylacetyl and R' = methyl to hydrogen had little effect on the turnover of esters (X=O) and thiolesters (X=S) by a DD-peptidase and a structurally similar ß-lactamase, but did in the case of amides (X=NH) and the DD-peptidase. Amides interact differently with these enzymes than do esters.

Bacteria remain an ever-present and still-emerging threat to human welfare.[1] The efficacy of even the most generally useful antibacterial drugs, the ß-lactams, is continually challenged by the genetic plasticity of bacteria. [2,3] Hence detailed studies of the nature of the resistance to these antibiotics are important and continue. An important focus of this research involves the interaction of ß-lactams with two groups of bacterial enzymes, the transpeptidases or DD-peptidases and the ß-lactamases. The former group catalyzes the final step in bacterial cell wall biosynthesis and is essential for normal bacterial growth. The inhibition of this group of enzymes by ß-lactams is the source of the antibiotic action of these molecules. Bacteria resist such inhibition in many ways, including the production of the ß-lactamases which catalyze the hydrolytic destruction of ß-lactams. The effectiveness of a particular ß-lactam antibiotic is strongly determined by the extent of its interactions with these two groups of enzymes.

In recent years, molecular studies of both of these groups of enzymes have proceeded apace. It is now clear that the hypothesis of Tipper and Strominger,[4] based on the structural similarity between their substrates, D-ala-D-ala terminating peptides in the case of DD-peptidases and ß-lactams in that of ß-lactamases, that ß-lactamases are evolutionary descendants of DD-peptidase progenitors, is very likely correct. X-ray crystal studies have demonstrated the clear three-dimensional structural similarities between representatives of these enzymes[5,6] and these, together with the analysis of amino-acid sequence homologies and chemical studies,[7] have pinpointed the functional components of the active sites. These functional groups also turn out to be largely common to the two groups of enzymes. Thus of perhaps more interest now is the question of how the two groups differ, rather than that of whether they are similar or share common ancestry. It is the differences, of course, that should more usefully inform drug design.

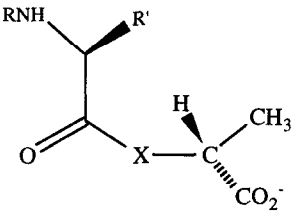
The significant functional differences between the two classes of enzymes which essentially define their respective biological roles and for which the structural basis is still unclear are as follows:

1. DD-peptidases catalyze acyl-transfer reactions of amides (peptides); ß-lactamases cannot.

2. ß-Lactamases efficiently catalyze the hydrolysis of the acyl-enzymes generated on their reaction with ß-lactam antibiotics; DD-peptidases cannot.

The molecular basis for these differences may be provided by a comparison of high resolution crystal structures of these enzymes, but none such of a DD-peptidase is yet available. A plausible rationale for the latter of these differences, for class A ß-lactamases at least, revolves around the proposed specific ability of the ß-lactamase to provide a specifically placed ("occluded") water molecule for deacylation.[8,9] The former, which more directly relates to this paper, is less well understood, although it may be partly explained by experiments in this laboratory which demonstrated the preference of the ß-lactamase active site, in contrast to the DD-peptidase active site, for a "bent" or penicillin-shaped substrate rather than a flat or peptide-shaped one.[10]

Functional comparisons between the ß-lactamases and DD-peptidases have been expedited in recent years by the discovery of common ester and thiolester substrates,[8,11,12] which are close structural analogs of the DD-peptidase amide substrates 3. There are clear indications in the literature that the nature of R and R' is important to the ability of 3 to act as DD-peptidase substrates.[13,14] It was not clear however to what extent this specificity extended to the ester substrates 1 and 2, and through them to ß-lactamases; this paper is addressed to that point.



1 X = O
2 X = S
3 X = NH

a, R = phenylacetyl, R' = H
b, R = N,N'-diacetyl-L-lysyl, R' = H
c, R = phenylacetyl, R' -CH_3
d, R = N,N'-diacetyl-L-lysyl, R' = CH_3

The best studied DD-peptidase from the point of view of substrate specificity and, indeed, in promise of a soon-to-be-realized high resolution crystal structure, is that of *Streptomyces* R61. This enzyme has long been the object of much research by Ghuysen, Frére and coworkers at the University of Liege, Belgium. It appears to strongly prefer R = N,N'-diacetyl-L-lysyl in its peptide substrates to phenylacetyl (the latter favored in ß-lactams by ß-lactamases), and R' = CH_3 rather than R' = H.[13,14] The extension, if any, of these preferences into the ester substrates and into a ß-lactamase was therefore examined. The ß-lactamase structurally closest to this peptidase would seem to be from class C;[6,7] that of *Enterobacter cloacae* P99 was chosen. The substrates employed were 1a-d, 2a, 2b and 3a-d.

RESULTS AND DISCUSSION

The steady state kinetic parameters for turnover of the depsipeptides 1a - 1d and the thiol depsipeptides 2a and 2b by the *Ent. cloacae* P99 ß-lactamase and the DD-peptidase of *Streptomyces* R61 obtained in this work[15] are presented in Table 1. Also shown in the Table are data for the comparable peptides 3a - 3d, largely taken from the literature. The ß-lactamase does not catalyze peptide hydrolysis, as previously noted[8] and reproduced here.

Table 1 Steady State Kinetic Parameters for Enzyme-Catalyzed Hydrolysis of Compounds 1 - 3.

	Enzyme					
	P99 ß-lactamase			R61 DD-peptidase		
Substrate	$k_{cat}^{a}(s^{-1})$	K_m(mM)	$k_{cat}/K_m(s^{-1}M^{-1})$	$k_{cat}^{a}(s^{-1})$	K_m(mM)	$k_{cat}/K_m(s^{-1}M^{-1})$
1a	27.3	8.1	3390	4.3	11.8	366
1b	17.8	36.1	493	10.4	9.8	1060
1c	29.0	23.4	1240	24.6	6.8	3630
1d	63.0	26.9	2340	65.9	50.6	1300
2a	22[b]	3.3[b]	6670[b]	6.1	0.63	9660
2b	5.1	6.7	763	11.9	2.0	5850
3a		--[c]			--[c]	
3b		--[c]		0.019	15	1.3[d]
3c		--[c]		2.0	7	286[e]
3d		--[c]		55	10	5500[f]
				55	7	7890[d]

[a] Average uncertainties in k_{cat} and K_m were (8.4±6)%. [b]Data from Xu et al.[17]. [c]No turnover observed; k_{cat}/K_m values significantly lower than 1 $sec^{-1}M^{-1}$. [d]Data from Georgopapadakou et al.[14] Reaction conditions: pH 7.5, 10 mM phosphate buffer, 30°C. [e]Data from Ghuysen et al.[13]. [f]Data from Kelly et al.[5]

Catalysis of peptide hydrolysis, diagnostic of the DD-peptidase active site appears, from these data, to be characterized by a distinct preference for the N,N'-diacetyl-L-lysyl rather than phenylacetyl side chain (k_{cat}/K_m for 3d is some 20 times larger than that for 3c), and for D-alanine rather than glycine in the acyl moiety of the cleaved amide bond (R' = CH_3 rather than H; k_{cat}/K_m for 3d is some 5000 times larger than that for 3b). These preferences were of course remarked upon in the source literature.[5,13,14]

In striking contrast to the side chain specificities observed with the amides, we find here little sign of strong preference for either of these structural characteristics with either the depsipeptide or thiol-depsipeptide substrates, in either the ß-lactamase or the DD-peptidase, and in either the k_{cat} or the k_{cat}/K_m kinetic parameter. These specificities apparently relate therefore only to the turnover of peptides (by the R61 DD-peptidase).

The most straightforward interpretation of this result would presumably be that significant differences exist between the ways in which the DD-peptidase interacts with amide and depsipeptide (ester) substrates. Such an interpretation would be of some importance because of the extensive recent application of depsipeptides as model substrates for both the ß-lactamases[8,11,19] and DD-peptidases.[12,20,21] The similarity in

response to the esters by the ß-lactamase and the DD-peptidase however suggests that these substrates might be handled in a similar fashion by these two structurally related classes of enzymes.

Previous thought on amide structural specificity involved induced fit interpretations where the correct alignment of catalytic functionality was induced only on the binding of appropriate side chains, particularly, N,N'-diacetyl-L-lysyl.[13] Subsequent to the introduction of acyclic depsipeptide substrates,[11] the chemical nature of the substrate became more a center of attention. An exploration of acyclic substrates[8,10] led to the hypothesis that DD-peptidase and ß-lactamase active sites differ in that planar (at the scissile bond) amides could not productively bind at the ß-lactamase active site which, through evolutionary processes, had become optimized for distorted amides such as is found in penicillins. Penicillins could productively interact with (and inhibit) DD-peptidases however since these enzymes must be able to rearrange to accommodate a distorted amide -- the tetrahedral intermediate and associated transition states of enzyme acylation must involve a pyramidalized nitrogen atom.[22] Thus DD-peptidases must be able to productively accommodate two conformations of bound substrate. ß-Lactamases are required to, and must, since amides are not substrates, accommodate only one.

The depsipeptides could be incorporated into this scenario with the proposition that their ability to act as ß-lactamase substrates resulted from their greater ease of distortion through rotation about the ester C-O bond into a penicillin-like conformation, relative to that of amides about the analogous C-N.[8] The current results can also be accommodated. If the depsipeptides are able to bind to a ß-lactamase active site through facile distortion, they may also to a DD-peptidase active site, just as a penicillin readily binds to both. Induction of the ß-lactam and depsipeptide-reactive protein conformation may then not require a specific side chain. We have recently provided evidence[17] that the side chains of ß-lactams and depsipeptides are not bound to these enzymes in exactly the same way, i.e. no common mode of induced fit can be present.

More detailed quantitative interpretation of the data of Table 1 is complicated by the fact that the meaning of the kinetic constants for many of these substrates is not well-established. Minimally it can be assumed that reaction Scheme 1 applies to all the substrates S, where ES and E-S represent the non-covalent Michaelis complex and the acyl-enzyme, respectively, and P_1 and P_2 are the leaving group and carboxylic acid hydrolysis products, respectively.

Scheme 1

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E\text{-}S + P_1 \xrightarrow{k_3} E + P_2$$

A complementary, or possibly alternative, interpretation of the results to that given above, specifically in terms of Scheme 1, would be that the side chain and D-alanyl-specific effects seen with the peptide substrates are not seen in the depsipeptide data since the step or steps that are rate-determining to (both!) k_{cat} and k_{cat}/K_m in the former substrates and not so in the latter. The step which is chemically likely to be rate-determining to turnover of an amide and less likely to be so for an ester is of course the acylation step with rate constant k_2. There is evidence in fact with the R61 DD-peptidase, based on experiments designed to determine the amount of accumulating acyl-enzyme, that $k_{cat} = k_2$ for 3d and $k_{cat} = k_3$ for 1d.[23] Deacylation also seems rate-determining at saturation for a variety of thioldepsipeptides with this enzyme.[20] The situation

with respect to the P99 ß-lactamase is less clear at present. With an m-carboxyphenyl depsipeptide, k_{cat} was thought to be k_2 and k_{cat}/K_m to be k_1, the value of the latter largely determined by a conformational rearrangement. [8,19] The similarity of k_{cat} values for rather different leaving groups (compare the values obtained in this work for 2a with those of Govardhan and Pratt[8]) leaves the chemical nature of the step rate-determining to k_2 for this enzyme still somewhat problematic. The meaning of k_{cat}/K_m for the DD-peptidase is also unclear. It is thus not impossible at the present state of knowledge, although perhaps unlikely, that the parameter k_2, containing the substrate structural specificities seen in the peptide substrates, does not appear in either k_{cat} or k_{cat}/K_m for the depsipeptides. This explanation would however still beg the question of why the P99 ß-lactamase does not catalyze peptide hydrolysis. Further experiments are needed to decide this point absolutely.

One further point that seems unequivocal enough to warrant comment is the rather similar values of k_{cat} and of k_{cat}/K_m for turnover by both enzymes of lactates and thiollactates with the same side chain, viz, 1a cf. 2a and 1b cf. 2b. Chemically, esters and thiolesters of otherwise identical structure will be attacked by nucleophiles at comparable rates, i.e. rates of formation of tetrahedral intermediates should be comparable, but will differ in that unassisted loss of the leaving group, i.e. breakdown of the tetrahedral intermediate will be considerably faster (perhaps 10^2 fold) in thiolesters.[24-27] A difference even approaching this magnitude is observable only in the k_{cat}/K_m values for the phenylacetyl substrates (1a and 2a) of the R61 DD-peptidase. If acid catalysis of leaving group departure were available, and this should certainly be necessary for the peptide substrates of the DD-peptidase, one would expect a comparable or larger difference in favor of the ester because of the much lower proton basicity of sulfur than oxygen.[27] Since neither of these differences is visible in the kinetic parameters of Table 1, it is likely that leaving group departure is not rate-determining under any conditions of substrate structure and concentration; different combinations of acid catalysis and rate-determining steps could of course also, fortuitously, produce the observed results.

Although more needs to be done to interpret these data in terms of individual reaction steps, it seems likely that there is a real difference in the way ester and amide substrates are handled by these enzymes. It is not clear why a DD-peptidase should handle an ester differently from an amide, but it is possible that the former reaction mode correlates with that of a ß-lactam. This anomalous reactivity of the active site apparently cannot be easily lost without loss of DD-peptidase function, but may have been turned to advantage as the starting point of ß-lactamase evolution.[4,10] Further pursuit of these lines of enquiry should promote understanding of the essential functional differences between a DD-peptidase and a ß-lactamase.

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The initial rates of hydrolysis of the thioldepsipeptides were determined spectrophotometrically at 350 nm with the aid of the chromophoric thiol reagent, 4,4'-dipyridyldisulfide, as previously described.[17] The hydrolysis of the depsipeptide was monitored by an acid-case indicator method.[16] The indicator employed in this work was phenol red at a concentration of 37 μM; proton release on ester hydrolysis was monitored at 550 nm. The response of the indicator in the relevant buffers was calibrated by the addition of aliquots of 0.10 M hydrochloric acid. The P99 ß-lactamase kinetics studies were conducted at 25°C in 20 mM MOPS buffer at pH 7.5 and those of the R61 DD-peptidase at 37°C in 20 mM phosphate buffer at pH 7.0. Steady-state rate parameters were obtained from the initial rates by the method of Wilkinson.[18]

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