Relative specificities of a series of β-lactam-recognizing enzymes towards the side-chains of penicillins and of acyclic thioldepsipeptides

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In an attempt to understand more of the subtle differences between bacterial β-lactamases and DD-peptidases, comparisons have been made between the specificities of these enzymes towards the phenylacetyl side chain, generally thought to be favoured by β-lactamases, and the *NN'*-diacetyl-L-lysyl side chain, widely employed in low-molecular-mass substrates of DD-peptidases. These comparisons were carried out with both a penicillin and an acyclic thioldepsipeptide reaction nucleus and employing a range of both β-lactamases and DD-peptidases. Rather contrary to general expectations, a general preference for reaction of both groups of enzymes with penicillins rather than thioldepsipeptides was observed and for the phenylacetyl rather than the *NN'*-diacetyl-L-lysyl side chain. Quantitative comparisons suggested that the side chains of penicillins may be bound in relatively similar sites in all of the enzymes whereas the side chains of thioldepsipeptides are more heterogeneously bound, both with respect to each other and to the comparable side chains of penicillins.

INTRODUCTION

The antibiotic activity of β-lactams against bacteria is largely determined by the interactions of these molecules with two groups of enzymes. On one hand, the D-alanyl-D-alanine transpeptidase/carboxypeptidases or DD-peptidases that catalyse the final step in the biosynthesis of the bacterial cell wall are inhibited by β-lactams. This inhibition leads to the cessation of bacterial growth and in some circumstances, through complex intracellular mechanisms, to cell death (Tomasz, 1979). On the other hand, the β-lactamases catalyse the hydrolysis of β-lactams, which brings about the destruction of their antibiotic activity and thus bacterial resistance to these antibiotics. An effective β-lactam antibiotic must therefore have high affinity for the former enzymes and as little as possible, preferably, for the latter. Since different species and strains of bacteria contain a variety of each of these enzymes, with differing β-lactam specificities, a single β-lactam is unlikely to be effective in all clinical situations (Sykes et al., 1987; Frére et al., 1988; Neu, 1992).

There is now considerable evidence for the suggestion by Tipper and Strominger (1965) that these two groups of enzymes have much in common through a shared ancestry. It is now generally believed that the β-lactamases are evolutionary descendants of the DD-peptidases. Crystal structures of these proteins show significant similarity in overall architecture and also in the nature and distribution of active site functional groups (Kelly et al., 1986, 1989; Herzberg and Moult, 1987; Moews et al., 1990; Oefner et al., 1990; Ghuysen, 1991; Strynadka et al., 1992; Lobkovsky et al., 1993). Striking similarities in the active-site catalytic chemistry have also been identified and analysed (Pratt and Govardhan, 1984; Murphy and Pratt, 1991). Nevertheless, there are clear functional differences between the two groups of enzymes which directly relate to their biological function but the structural basis of which is not yet well understood: DD-peptidases cannot efficiently catalyse β-lactam hydrolysis and β-lactamases cannot catalyse the hydrolysis of acyclic peptides.

There have been many broad surveys of the specificity of both groups of enzymes against their respective substrates, acyclic peptides in the case of DD-peptidases (Ghuysen et al., 1979) and β-lactams of β-lactamases (Galleni and Frére, 1988; Galleni et al., 1988; Matagne et al., 1990), and of the specificity of DD-peptidases against β-lactam inhibitors (Ghuysen et al., 1979; Frére et al., 1992; Neu, 1992). Much has been learned from such studies, but in order to pinpoint the apparently subtle essential differences between the active sites of the two groups, a close analysis of their response to a single important perturbation can also be valuable. In this paper we assess the relative specificity of a variety of these enzymes towards two substrate side chains, phenylacetyl on one hand, favoured by most β-lactamases, and on the other, *NN'*-diacetyl-L-lysyl, which is thought, on the basis of investigations of low-molecular-mass members of the group, to be preferred by DD-peptidases (Ghuysen et al., 1979). Furthermore, we compare the response to this side-chain perturbation between two molecular systems, the penicillins (Figure 1, structure 1) on one hand and the thioldepsipeptides (Figure 1, structure 2) on the other. Acyclic depsipeptides and their thiolester analogues are substrates common to both β-lactamases and DD-peptidases (Pratt and Govardhan, 1984; Govardhan and Pratt, 1987; Adam et al., 1990). The results are analysed in terms of the modes of binding of these molecules to the various enzymes.

The enzymes employed in this study were two typical class-A β-lactamases, those of *Staphylococcus aureus* PC1 and of the TEM plasmid, a class-B β-lactamase (*Bacillus cereus* β-lactamase II), a class-C β-lactamase (that of *Enterobacter cloacae* P99), the water-soluble low-molecular-mass DD-peptidase of *Streptomyces* R61, a water-soluble, active construct of a low-molecular-mass, membrane-bound DD-peptidase, *E. coli* pbp5, and water-soluble active constructs of two high-molecular-mass membrane-bound

Abbreviation used: pbp, penicillin-binding protein.

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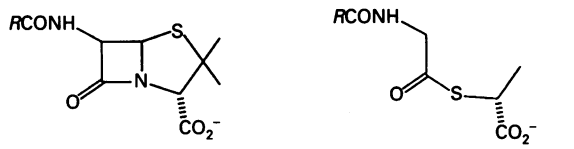

1a, $R = PhCH_2$-
1b, R = *N,N'*-diacetyl-L-lysyl-

2a, $R = PhCH_2$-
2b, R = *N,N'*-diacetyl-L-lysyl-

Figure 1 Structure of (1) penicillins and (2) thioldepsipeptides

DD-peptidases, pbp2 of *E. coli* and pbp2b of *Streptococcus pneumoniae*.

EXPERIMENTAL

Materials

The β-lactamases were purchased from the Centre for Applied Microbiology and Research (Porton Down, Wilts., U.K.), and used as received. The penicillin-binding protein (pbp)5 of *Escherichia coli* (Van der Linden et al., 1992) and pbp2b of *Streptococcus pneumoniae* (W. Keck, unpublished work) were prepared in the laboratory of Dr. W. Keck and pbp2 of *E. coli* in that of Dr. H. Adachi (Adachi et al., 1987). The DD-peptidase of *Streptomyces* R61 was a generous gift from Dr. J.-M. Ghuysen and Dr. J.-M. Frére (University of Liege, Belgium). Benzylpenicillin (Figure 1, structure 1a) was purchased from Sigma Chemical Co. Cephalothin was a gift from Eli Lilly and Co.

Phenylacetylglycyl-D-thiol-lactic acid (Figure 1, structure 2a)

D-Thiolactic acid, a clear, pale yellow oil, b.p. 67–70 °C/1–1.2 Torr (1 Torr = 133.322 Pa) [^{1}H-n.m.r. spectrum: (400 MHz, C^2HCl_3) δ (p.p.m.): 1.55 (d, J 7 Hz, 3H, CH_3), 2.22 (d, J 8 Hz, 1H, SH), 3.55 (quint., J 7 Hz, 1H, CH); a spectrum after the addition of the chiral shift reagent (L)-α-methylbenzylamine demonstrated the presence of a single enantiomer], was prepared from L-2-chloropropanoic acid (Ruud-Christensen et al., 1984) by the method of Strijtveen and Kellogg (1986). Carbonyldiimidazole (4.9 g, 30.3 mmol) was added with stirring to a solution of phenylacetylglycine (Govardhan and Pratt, 1987) (5.0 g, 25.9 mmol) in tetrahydrofuran (125 ml, freshly distilled from lithium aluminium hydride) at 0 °C. After a reaction time of 1 h, D-thiolactic acid (2.5 ml, 28.3 mmol) was added and the solution stirred for 4 days at 4 °C. After removal of the solvent by evaporation under vacuum, the residue was dissolved in ethyl acetate and washed successively with water, 10% aq. citric acid and water. The ethyl acetate solution (dried over $MgSO_4$) was then evaporated to dryness under vacuum. The resulting product was recrystallized from a benzene/chloroform (60/40, v/v) mixture yielding colourless crystals (2.8 g, 35%) of m.p. 109–110.5 °C and $[\alpha]_D^{25} = +43.0°$ (13.2 mM ethanol). The ^{1}H-n.m.r. spectrum [(400 MHz, C^2HCl_3) δ (p.p.m.): 1.53 (d, J 7.1 Hz, 3H, CH_3), 3.68 (2H, s, $PhCH_2$), 4.16 (dd, J 6.1, 17.7 Hz, $NHCH_2CO$), 4.20 (q, J 7.1 Hz, 1H, SCH), 4.23 (dd, J 6.1, 17.7 Hz, $NHCH_2CO$), 6.02 (br t, 1H, NH), 7.23–7.42 (5H, m, ArH)] and elemental analysis (Calc. for $C_{13}H_{15}NO_4S$: C, 55.50; H, 5.37; N, 4.98; S, 11.40. Found: C, 55.38; H, 5.25; N, 4.94; S, 11.22) confirmed the product as having structure 2a (Figure 1).

Potassium *NN'*-diacetyl-L-lysyl-6-β-aminopenicillanate (Figure 1, structure 1b)

p-Nitrobenzyl 6β-aminopenicillanate (Brain et al., 1975) was condensed with *NN'*-diacetyl-L-lysine (Greenstein and Winitz, 1984) as described by Bentley and Stachulski (1983) who prepared the DL-lysyl derivative. The product acid was purified by ion-exchange chromatography (QAE-Sephadex A-25, gradient elution by 0–1 M triethylammonium bicarbonate) and the resultant triethylammonium salt converted into the potassium analogue, an off-white solid, by its elution in water through a Dowex 50X 4-400 (K^+ form) cation-exchange column. The ^{1}H-n.m.r. spectrum of the product {(400 MHz, ^2H_2O) δ (p.p.m.): 1.20–1.90 [m, 6H, $(CH_2)_3$], 1.54 and 1.65 (s, 3H, CH_3C), 2.00 and 2.07 (s, 3H, CH_3CO), 3.20 (t, J 6 Hz, 2H, CH_2NH), 4.30 (t, J 7 Hz, 1H, CH_2CH), 4.26 (s, 1H, 3-H of penicillin), 5.42 (br s, 1H, 6-H of penicillin), 5.58 (br s, 1H, 5-H of penicillin)} was very similar to that reported by Bentley and Stachulski (1983) for the DL-lysyl analogue.

Potassium *NN'*-diacetyl-L-lysyl-glycylthiol-lactate (Figure 1, structure 2b)

This compound was prepared by the condensation of *NN'*-di-t-Boc-L-lysylglycine (5.0 g, 12.4 mmol, Bachem, Bioscience Inc.) with D-thiolactic acid (1.25 ml, 14.2 mmol) in the presence of carbonyldi-imidazole (2.5 g, 15.3 mmol), as described above for Structure 2a. The crude product (50% yield) was taken up into the minimum volume of hexane/ethyl acetate/acetic acid (53:45:2, by vol.) and flash-chromatographed on a silica gel column in the same solvent. Evaporation of appropriate column fractions yielded *NN'*-di-t-Boc-L-lysylglycyl-D-thiol-lactic acid as a colourless solid (0.9 g, 15% yield).

This material (0.5 g, 1.0 mmol) was dissolved in trifluoroacetic acid (10 ml) and stirred at room temperature for 2 h. After evaporation of the trifluoroacetic acid from the reaction mixture, the residue was held under oil-pump vacuum for 1.5 h and then dissolved in 11 ml of a dioxan/water mixture (1/1, v/v). To this solution, stirred and cooled at 0 °C, triethylamine (0.7 ml) was added, followed by acetic anhydride (0.3 ml). After a reaction time of 2 h, the solvents were removed by evaporation under reduced pressure. The residue was taken up into water (10 ml), extracted with diethyl ether (4 × 10 ml), and the resulting aqueous solution freeze-dried. The product acid was purified by passage through a Dowex 50X4 cation-exchange column (H^+ form) and converted into the colourless potassium salt (having structure 2b, 0.78 g) by a column of the same resin in the K^+ form. The latter was characterized by its ^{1}H-n.m.r. spectrum: (400 MHz, ^2H_2O) δ (p.p.m.): 1.45 (d, J 7 Hz, 3H, $CHCH_3$), 1.4–2.0 [m, 6H, $(CH_2)_3$], 2.00 and 2.08 (s, 3H, $COCH_3$), 3.2 (t, J 7 Hz, 2H, CH_2NH), 4.02 (q, J 7 Hz, 1H, $CHCH_3$), 4.15 (ABq, J 18, 2H, $NHCH_2CO$), 4.32 (m, 1H, CHNH).

Analytical and kinetic methods

Absorption spectra and spectrophotometric reaction rates were measured by means of Perkin–Elmer Lambda 4B and Hewlett–Packard 8452A spectrophotometers. The β-lactamase and R61 DD-peptidase concentrations were determined spectrophotometrically using published absorption coefficients, as in previous experiments (Murphy and Pratt, 1991). The concentrations of pbp solutions were taken as supplied.

All kinetic experiments were carried out at 25 °C in the following buffers: 0.1 M phosphate, pH 7.5 (TEM, PC1 β-lactamase); 200 mM succinate/500 mM NaCl/100 μM $ZnSO_4$, pH 6.0 (*B. cereus* β-lactamase II); 20 mM Mops, pH 7.5 (P99 β-lactamase); 20 mM phosphate, pH 7.0 (R61 DD-peptidase); 50 mM phosphate/500 mM NaCl, pH 7.0 (pbp2); 20 mM Mops, pH 7.0 (pbp2B); 10 mM Tris, pH 8.0 (pbp5).

Steady-state kinetic parameters for enzyme-catalysed

hydrolyses of compounds with structures **1** and **2** were determined from spectrophotometrically determined initial rate measurements by the method of Wilkinson (1961), except as noted below. The hydrolysis of the penicillins **1a** and **1b** were monitored at 232 nm and 230 nm respectively [$\Delta\epsilon$ values of 940 $M^{-1}\cdot cm^{-1}$ (Waley, 1974) and 545 $M^{-1}\cdot cm^{-1}$ respectively, were employed]. The hydrolysis of **1b** in the presence of *B. cereus* β-lactamase II was monitored at 240 nm ($\Delta\epsilon = 321\ M^{-1}\cdot cm^{-1}$). The hydrolysis of the thioldepsipeptides **2** were studied in the presence of 4,4′-dipyridyldisulphide (approx. 1 mM) which reacted rapidly and stoichiometrically with the thiol-lactate product, producing characteristic absorption at 350 nm, $\Delta\epsilon = 2.74\times10^3\ M^{-1}\cdot cm^{-1}$ (Brocklehurst and Little, 1973) whose increase with time was measured to provide initial rates. Corrections for the relatively slow background hydrolysis of **2** were made by subtracting initial rates in the absence of enzyme from those in its presence.

The K_m values of **1a** with the PC1 β-lactamase and **1b** with the P99 β-lactamase were too small to determine directly because of the small absorption coefficient changes. These K_m values were therefore obtained as K_i values from experiments where these compounds were used as competitive inhibitors of the more chromophoric substrates, furylacryloylpenicillin (Calbiochem) in the case of the PC1 β-lactamase ($\Delta\epsilon$ 3050 $M^{-1}\cdot cm^{-1}$ at 330 nm, $K_m = 7.8\ \mu M$) and cephalothin in the case of P99 enzyme ($\Delta\epsilon$ 2980 $M^{-1}\cdot cm^{-1}$ at 270 nm, $K_m = 18.0\ \mu M$). The $k_{cat.}$ values for **1a** and **1b** with these β-lactamases were determined by direct measurements at high substrate concentrations ($> 10\ K_m$).

Second-order rate constants for reaction of the penicillins **1a** and **1b** with DD-peptidases were also determined by competition with a more chromophoric inhibitor or substrate: cephalothin in the case of the R61 DD-peptidase ($k_2/K_s = 166\ s^{-1}\cdot M^{-1}$), 6-β-bromopenicillanic acid in the case of pbp2 and pbp2B ($\Delta\epsilon$ 9333 $M^{-1}\cdot cm^{-1}$ and 6425 $M^{-1}\cdot cm^{-1}$, both at 325 nm, for pbp2 and pbp2B respectively, and $k_2/K_s = 24\ s^{-1}\cdot M^{-1}$ and $12\ s^{-1}\cdot M^{-1}$), and **2a**/4,4′-dipyridyldisulphide in the case of pbp5. The rate parameters could be determined from non-linear least squares fitting to the experimental data in the cases were pseudo-first-order conditions were achievable (**1a** and **1b** with pbp5) and by numerical simulation with Simplex optimization (Anderson and Pratt, 1983) otherwise.

RESULTS AND DISCUSSION

Most of the enzymes of this study are serine hydrolyses, i.e. they employ a serine hydroxyl group as the principal nucleophile of the active site and catalyse hydrolysis of their substrates by the double-displacement mechanism of Scheme 1 (Waley, 1992; Frére et al., 1992).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot S \xrightarrow{k_2} E-S \xrightarrow[H_2O]{k_3} E + P_2 \qquad \textbf{Scheme 1}$$
$$(+P_1)$$
$$(K_s = k_{-1}/k_1)$$

In this reaction scheme, E·S represents the non-covalent complex of enzyme and substrate, and E–S the acyl-enzyme intermediate where the active-site serine hydroxyl group is acylated by the substrate. P_1 represents the leaving group of an acyclic substrate which is released from the enzyme on formation of the acyl-enzyme and P_2 is the product carboxylate formed on hydrolysis of the acyl-enzyme. The same scheme, with $k_3 \ll k_2$, applies to the inhibitory interaction of β-lactams with DD-peptidases. Henceforward, S will therefore also denote β-lactam inhibitors of DD-peptidases.

The one exception to the above mechanistic generalization is the class-B β-lactamase II of *Bacillus cereus* which is a metalloenzyme and presumably employs a carboxypeptidase A-type mechanism of catalysis (Waley, 1992). In accord with this presumption, no acyl-enzyme intermediates have been detected in the turnover of substrates by this enzyme (Bicknell and Waley, 1985).

We have determined, in the ways described in the Experimental section, steady-state parameters for turnover of **1** and **2** by the β-lactamases, and of **2** by the DD-peptidases (Table 1). In particular, second-order rate constants for reaction of free E and S to form the ES complex directly beyond the first irreversible step of catalysis have been determined. These rate constants of course correspond to the steady-state kinetic parameter $k_{cat.}/K_m$. This should correspond to the parameter k_2/K_s in Scheme 1, provided that the initial binding, described by the equilibrium constant K_s, is fast with respect to acylation (k_2), i.e. that the substrate is not 'sticky'. This may not be true in some instances. In such cases, where $k_2 > k_{-1}$ and the first step effectively becomes the first irreversible one, $k_{cat.}/K_m$ will more closely approximate to k_1, the rate constant for association of E and S to form the productive non-covalent Michaelis complex E·S. This may represent a purely diffusion-controlled process, which should be recognizable by the magnitude of k_1 (i.e. of $k_{cat.}/K_m$) (Eigen, 1964; Hiromi, 1979) and presumably also by its small side-chain dependence, or, if slower, it may represent the rearrangement of an encounter complex, formed by diffusive contact, into a second non-covalent complex where S is specifically bound to the active site. In the latter case the nature of the side chain would be important.

The reaction of benzylpenicillin with class-A and -C β-lactamases represents a situation where the values of $k_{cat.}/K_m$ (Table 1) appear to approach levels where diffusional control seems likely. Indeed it has been shown from studies of the effect of solvent viscosity on $k_{cat.}/K_m$ that the rate of diffusion of E and S together partly determines $k_{cat.}/K_m$ values for the interaction of benzylpenicillin with the typical class-A β-lactamases of *B. cereus* (β-lactamase I), *Staphylococcus aureus* PC1 and the TEM plasmid (Hardy and Kirsch, 1984; Christensen et al., 1990). On the other hand, however, the values of k_2 generally do not exceed k_{-1} and thus the value of (side-chain responsive) k_2 remains a significant contributor to $k_{cat.}/K_m$. It is also not certain that substrate recognition does not play some role in the viscosity-dependent contribution to $k_{cat.}/K_m$ (Berg, 1985). The values of k_1 for benzylpenicillin and the three class-A enzymes referred to above are reported to be significantly different from each other (Christensen et al., 1990), suggesting that some specific interaction with the enzyme is involved in the reaction steps associated with this rate constant. The discussion of the present results, below, assumes significant influence of enzyme–substrate recognition in all the values of $k_{cat.}/K_m$ in Table 1. Little reference is made to individual $k_{cat.}$ and K_m values since, in many cases, their identification with specific reaction steps has not been made, and because, for specificity comparisons, $k_{cat.}/K_m$ is the most relevant kinetic parameter.

The first generalization presented by Table 1 is that the enzymes, as a whole, prefer to react with the penicillins **1a** and **1b** than with the respective thioldepsipeptides **2a** and **2b**. This is probably not simply a matter of chemical reactivity since second-order rate constants for reaction of **1a** and **2a**, for example, with hydroxide ion are 0.10 (Pratt and Govardhan, 1984) and $0.90\ s^{-1}\cdot M^{-1}$ respectively. This preference for penicillins by the β-lactamases has been previously interpreted in terms of the

Table 1 Kinetic parameters for the interaction of compounds 1 and 2 with β-lactam-recognizing enzymes

The units of K_m, $k_{cat.}$ and $k_{cat.}/K_m$ are mM, s^{-1} and $s^{-1} \cdot M^{-1}$ respectively. Abbrevations: n.o., no steady-state turnover observed; constants relate to inhibition by acylation; n.d., insufficient reactivity to determine these constants.

		Pencillin			Thioldepsipeptide		
Enzyme	Kinetic parameter	**1a**	**1b**	k^P/k^L	**2a**	**2b**	k^P/k^L
TEM*	K_m	0.020‡	0.074	–	17.5	> 5	–
	$k_{cat.}$	2000‡	405	–	9.43	> 0.1	–
	k_{cat}/K_m	1×10^8‡	5.5×10^6	18	538	24.3	22
PC1*	K_m	1.9×10^{-3}	0.064	–	0.295	19.6	–
	$k_{cat.}$	64.1	49.5	–	0.10	0.052	–
	$k_{cat.}/K_m$	3.4×10^7	7.8×10^5	44	342	2.62	130
BCII*	K_m	4.6	7.68	–	3.38	4.58	–
	$k_{cat.}$	490	416	–	0.49	0.14	–
	$k_{cat.}/K_m$	1.1×10^5	5.4×10^4	2.0	146	31	4.7
P99*	K_m	0.89×10^{-3}	1.1×10^{-3}	–	3.34	6.65	–
	$k_{cat.}$	54.0	0.861	–	22.3	5.08	–
	$k_{cat.}/K_m$	6.07×10^7	7.83×10^5	77	6670	763	8.7
R61†	K_m	n.o.	n.o.	–	0.565	1.54	–
	$k_{cat.}$	n.o.	n.o.	–	3.28	5.93	–
	$k_{cat.}/K_m$	1070	91.7	12	5800	3860	1.5
pbp2b†	K_m	n.o.	n.o.	–	n.d.	5.18	–
	$k_{cat.}$	n.o.	n.o.	–	n.d.	0.19	–
	$k_{cat.}/K_m$	2020	82.4	25	< 0.9	37.4	< 0.03
pbp2†	K_m	n.o.	n.o.	–	n.d.	n.d.	–
	$k_{cat.}$	n.o.	n.o.	–	n.d.	n.d.	–
	$k_{cat.}/K_m$	397	12.5	32	< 1.4	< 0.73	–
pbp5†	K_m	n.o.	n.o.	–	2.83	n.d.	–
	$k_{cat.}$	n.o.	n.o.	–	2.34	n.d.	–
	$k_{cat.}/K_m$	3.0×10^4	4.5×10^2	67	827	< 1.1	> 800

* β-Lactamases: TEM, the class-A β-lactamase of the TEM plasmid of *E. coli*; PC1, the class-A β-lactamase of the PC1 plasmid of *S. aureus;* BCII, *B. cereus* β-lactamase II, a class-B β-lactamase; P99, the class-C β-lactamase of *Enterobacter cloacae* P99.

† DD-Peptidases: R61, the DD-peptidase of *Streptomyces* R61; pbp2b, pbp2 and pbp5, penicillin-binding proteins of *S. pneumoniae, E. coli* and *E. coli* respectively.

‡ From Govardhan and Pratt (1987).

greater loss of conformational entropy in binding acyclic substrates and also in the energy required to distort the acyclic substrate about the scissile bond into a penicillin-like shape (Govardhan and Pratt, 1987; Murphy and Pratt, 1991). The latter of these explanations would not apply to DD-peptidases, and the former alone is probably insufficient to encompass the highest selectivities observed (e.g. for pbp2b, $k_{cat.}/K_m$ for **1a** is $\geqslant 2000$ times that for **2a**). Thus there is the indication, analysed below, of greater complexity in the binding of these substrates. A further indication of this conclusion is the one clear exception to the preference for β-lactams, observed with both the phenylacetyl and lysyl side chains, provided by the R61 DD-peptidase.

A second general impression produced by the data of Table 1 is that the enzymes surveyed, both β-lactamases and DD-peptidases, prefer the phenylacetyl to the *N*,*N*′-diacetyl-L-lysyl side chain, both in penicillins and in the acyclic substrates. For the β-lactamases at least, where the data are more complete, this preference is seen in both a smaller $k_{cat.}$ and a larger K_m and thus a smaller $k_{cat.}/K_m$ for the latter side chain. Even the DD-peptidases, which might have been expected to favour the lysyl side chain, prefer, with one exception, (i.e. pbp2b with the acyclic substrates), phenylacetyl. This result is in qualitative agreement with the observations of Bentley and Stachulski (1983) and, more recently, of Hanessian et al. (1993), who found that penicillins with acyclic peptidoglycan-like peptide side chains were, in general, inferior antibiotics to those with aromatic side chains. The absence of a change in this specificity in the acyclic substrates is something of a surprise.

The data can be analysed somewhat more quantitatively by means of the ratio of $k_{cat.}/K_m$ for the phenylacetyl side chain to that of the lysyl side chain (k^P/k^L) in both **1** and **2**. The values of this ratio are also presented in Table 1.

The simplest model for interpretation of these numbers assumes that the scissile bond in all substrates is oriented in exactly the same way with respect to the catalytic machinery of a given enzyme. In the serine enzymes, one might imagine that the scissile bonds of all substrates, in the acylation transition state, the energy of which with respect to the ground state is given by $k_{cat.}/K_m$ (i.e. k_2/K_s), would be fixed in position by their juxtaposition to the serine nucleophile, the oxyanion hole, and the hydrogen-bond donor and acceptor to the amido part of the side chain, all of which are thought to be common elements of these enzyme active sites (Figure 2) (Ghuysen, 1991). If this were so, one would then expect, for a given enzyme, that k^P/k^L would be very similar for **1** and **2** if the side chains occupied the same region in space, i.e. the same binding sites, for those two classes of substrate. Inspection of Table 1 shows that this is not convincingly so, except for the TEM β-lactamase, where the result could be through coincidence.

In general then the phenylacetyl and lysyl side chains are not placed with respect to one another in enzyme-bound penicillins as they are in the thioldepsipeptides. This situation presumably arises through differences in overall substrate disposition induced by the different leaving groups and by the presence of the ring structure in **1**. The leaving groups differ not only in steric structure but also chemically. The β-lactam nitrogen requires protonation

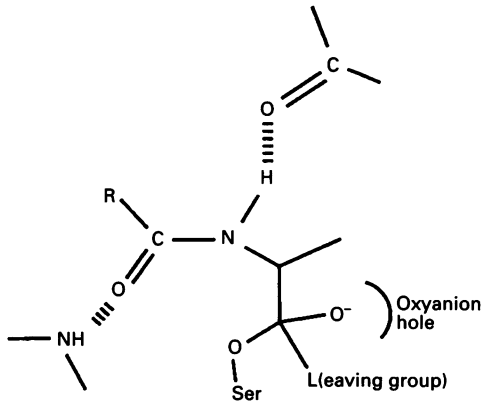

Figure 2 Interactions of a substrate with the active site

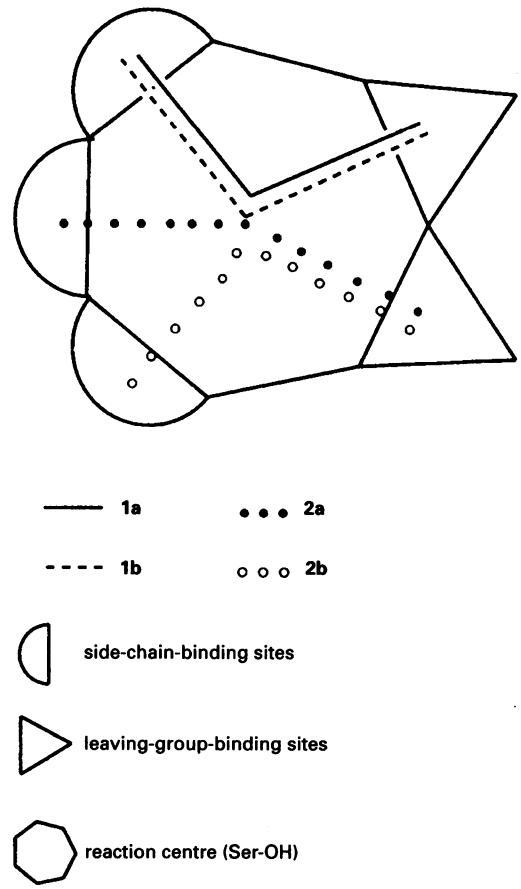

Figure 3 Disposition of β-lactams and thioldepsipeptides in the active site

prior to or concerted with its departure (Page, 1987), while the thiolate sulphur probably has no such requirement. [Nucleophilic attack of thiols, as distinct from alcohols, on carbonyl groups is not general base catalysed (direct attack of the thiolate anion is observed) and conversely, as anticipated from microscopic reversibility arguments, elimination of thiolates is not general acid catalysed (Lienhard and Jencks, 1966; Barnett and Jencks, 1967, 1969); this reflects the high nucleophilicity of the thiolate anion and the weak basicity of neutral sulphides (Jensen and Jencks, 1979).] The positioning of the β-lactam nitrogen adjacent to a general acid catalyst would therefore not be required of the thiolester sulphur atom. This greater freedom of the leaving group could be coupled to the side-chain disposition via the degree of freedom provided by rotation about the CH_2–CO bond of the depsipeptide, corresponding to C-6–C-7 of the penicillin where the rotation is not possible because of the β-lactam ring.

The most striking regularity of Table 1 is the rather similar value of k^P/k^L for the penicillins for all enzymes, with an average value of 35 and S.D. of 26 (39 ± 25 if *B. cereus* β-lactamase II is excluded). This similarity applies to the serine β-lactamases, the DD-peptidases and to the class-B metallo-β-lactamase (although, in the latter case, the value of the ratio is at the extreme low end of the range and might well be taken as significantly different from those of the serine enzymes). It can be interpreted to mean that the relative nature of the phenylacetyl- and lysyl-binding sites remains essentially the same for these penicillins irrespective of the enzyme. The simplest and most likely situation whereby this would occur is if the two side-chain binding sites overlapped and the area of overlap dominated the effect of side-chain binding on the operative transition state. A hydrophobic binding site appropriate for the aryl side chain would also accommodate, although less optimally, the aliphatic lysyl side chain. It seems likely that β-lactamases, including those of class B, would have an aryl-group-specific binding site since, as enzymes of β-lactam-resistance, they should evolve to most effectively inactivate the most prevalent/potent β-lactam antibiotics. There is currently no explanation, however, of why DD-peptidases should have and maintain such a site. Presumably its presence must somehow be essential to the catalysis of peptidoglycan synthesis.

The ratio k^P/k^L for the thioldepsipeptides varies much more than that for penicillins. This, by the converse of the above argument, can be interpreted to mean that the phenylacetyl and lysyl side chains are more likely to occupy different, and more enzyme specific, sites when present in thioldepsipeptides than in penicillins. It might seem plausible that the phenylacetyl side chain would occupy the same site as in penicillins, but that the lysyl side chain would be preferentially situated elsewhere, perhaps in a more DD-peptidase-specific site better suited to the transpeptidase reaction. If this were true, however, one would presumably predict that the ratio of $k_{cat.}/K_m$ for phenylacetylpenicillins versus that for phenylacetylthioldepsipeptides would be very similar for all enzymes. This is not true, certainly not to the extent that k^P/k^L for penicillins is similar. Nor is it close to true for lysyl side chains. Thus the disposition of side chains of the thioldepsipeptides must be more heterogeneous than those of β-lactams, reflecting perhaps the DD-peptidase specificity. Vestiges, at least, of these sites may, however, remain with β-lactamases, as has been previously proposed (Pazhanisamy and Pratt, 1989).

Previous work in this area has led, from the different side-chain specificities of β-lactam inhibitors and acyclic substrates, to the idea of different binding sites for these moieties on DD-peptidases (Ghuysen et al, 1979; Lamotte-Brasseur et al., 1984; Lamotte et al., 1991; Neuhaus and Georgopapadakou, 1991) but the four-way comparison, over a range of enzymes including both β-lactamases and DD-peptidases, is novel to this work.

The simplest explanation of these results therefore is that of Figure 3, showing the disposition of substrates **1** and **2** in the $k_{cat.}/K_m$ transition states. The structural nature of these sites is largely unknown at present but may emerge on further analysis of present and future high-resolution crystal structures of these enzymes (Herzberg and Moult, 1987; Moews et al., 1990; Oefner et al., 1990; Lamotte-Brasseur et al., 1991; Strynadka et al.,

1992; Lobkovsky et al., 1993). The productive binding of the thioldepsipeptides is substantively different from that of penicillins, for reasons described above that may relate specifically to thiol esters. These differences in modes of binding may of course, as always, reflect differences in protein structure (via an induced fit process) rather than, or as well as, substrate orientation. To what extent the above picture holds for the natural peptide substrates of DD-peptidases cannot yet be answered.

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