Effect of side-chain amide thionation on turnover of β -lactam substrates by β -lactamases

Further evidence on the question of side-chain hydrogen-bonding in catalysis

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Two side-chain-thionated β -lactams, a penicillin and a cephalosporin, have been prepared and found to be not significantly poorer as substrates of typical serine (classes A and C) β -lactamases than are their oxo analogues. This result is interpreted to mean that any hydrogen-bonding site on these enzymes for the β -lactam side-chain amide carbonyl group must be flexible and is more likely to be a passive rather than active or essential feature of the active site. Previously, data from crystal structures and site-directed mutagenesis had suggested that the side chain of Asn-132 of class-A β -lactamases, a component of the conserved SDN loop, forms a hydrogen bond with the side-chain carbonyl of the β -lactam substrate and may provide significant transition-state stabilization during catalysis. The thionocephalosporin was also equally as good as its oxo analogue as a substrate of the class-B β -lactamase II of *Bacillus cereus* and not significantly less effective as an inhibitor of the *Streptomyces* R61 DD-peptidase; a tight hydrogen-bond donor site for the β -lactam side-chain amide is apparently not present in these enzymes either.

INTRODUCTION

There is much interest at present in the structure and function of the active sites of serine β -lactamases. Inspection of amino acid sequences [1,2] and crystal structures [3–5] and functional studies of the native enzymes and of specific mutants [6–10] have indicated the close similarity of the essential amino acid components. Somewhat less clear at present is the role of these components in catalysis. Beyond Ser-70, Lys-73, Lys-234 and Glu-166 (to use the numbering system of Ambler [11]), which have been much discussed and are believed to play important roles in class-A β -lactamase catalysis [1–10], the conserved Ser-130, Asp-131, Asn-132 (SDN) loop [12] stands out. Of this triad, one member, Asn-132, is believed, on the basis of crystal structures [3–5], to interact directly with bound substrate, specifically through hydrogen-bonding from a side-chain amido-NH to the substrate side-chain carbonyl group (1). Asn-132 also appears to be hydrogen-bonded to Asn-104 (in the *Bacillus licheniformis* structure [4]) and to the conserved Lys-73 and thus may well play a significant structural role also. Class-C β -lactamases and other β -lactam-recognizing enzymes, in particular the DD-peptidases, are believed to have a homologous asparagine in a conserved S(Y)XN loop [12]:

The importance to binding or catalysis of a hydrogen bond to a carbonyl group can often be assessed by determination of the effect of changing it to a thiocarbonyl (the ‘S-test’). This transformation has demonstrated, for example, the presence of a catalytic oxyanion hole in serine proteinases [13,14] and serine β -

lactamases [15]. Polgár and co-workers [16] have also used it to show the catalytic importance of a hydrogen bond to the amide carbonyl of the P₂ residue of a papain substrate. In related investigations, Street *et al.* [17] have quantitatively evaluated the importance of specific hydrogen bonds in the binding of sugars to glycogen phosphorylase through employment of deoxy and fluorodeoxy sugars, and, in a complementary fashion, Fersht and co-workers have undertaken site-directed mutagenesis of tyrosyl-tRNA synthetase to remove or add hydrogen-bonding sites [18–20].

In the present paper we use the thionation approach to assess the catalytic significance of the proposed hydrogen bond from the conserved asparagine to the substrate side-chain carbonyl group as depicted in 1. Recently, the results of the complementary approach, site-directed mutation of Asn-132 in a class-A β -lactamase, have been published [21,22]. A comparison of those results and ours yields useful further insight into the role of this residue in β -lactamase catalysis.

EXPERIMENTAL

Materials

The β -lactamases of the RTEM-2 plasmid from *Escherichia coli* W3310, of the PCI plasmid from *Staphylococcus aureus* and of *Enterobacter cloacae* P99 were obtained from the Centre for Applied Microbiology and Research (Porton Down, Wilts., U.K.) and used as supplied. The same source provided a mixture of β -lactamases I and II of *Bacillus cereus* which were separated as described by Davies *et al.* [23]. The soluble D-alanyl-D-alanine transpeptidase/carboxypeptidase (DD-peptidase) of *Streptomyces* R61 was generously provided by Dr. J.-M. Ghuysen and Dr. J.-M. Frère (University of Liège, Liège, Belgium). Benzylpenicillin, 6-aminopenicillanic acid and hippuryl DL-phenyl-lactate were purchased from Sigma Chemical Co. 7-Aminocephalosporanic acid was a gift from Eli Lilly and Co. Dansylpenicillin was available from previous studies in this laboratory [24].

7-Phenylacetamidocephalosporanic acid (Ia) was prepared from the reaction of 7-aminocephalosporanic acid with phenylacetyl chloride (Fluka) in aq. NaHCO₃ solution, essentially as described by Chauvette *et al.* [25]. The sodium salt was purified

by Sephadex G-10 chromatography. The ^1H n.m.r. spectrum of the product met expectations [δ (p.p.m.) ($^2\text{H}_2\text{O}$) 2.06 (3H, s, OAc), 3.32, 3.58 (2H, ABq, J 19 Hz, H-2), 3.63, 3.70 (2H, ABq, J 12 Hz, PhCH_2), 4.66, 4.82 (2H, ABq, J 12 Hz, H-3'), 5.04 (1H, d, J 4 Hz, H-6), 5.58 (1H, J 4 Hz, H-7), 7.3–7.5 (5H, m, ArH)].

Carboxymethyl phenyldithioacetate was prepared from the reaction of benzylmagnesium chloride (1 M solution in diethyl ether; Aldrich Chemical Co.) with carbon disulphide and then chloroacetic acid as described by Kjaer [26]. The product melted at 78 °C (lit. [26] 78 °C) after recrystallization from light petroleum (b.p. 50–110 °C).

7-Phenylthioacetamidoccephalosporanic acid (Ib) was prepared from the reaction of 7-aminocephalosporanic acid with carboxymethyl phenyldithioacetate as follows. 7-Aminocephalosporanic acid (1.0 g, 3.67 mmol), carboxymethyl phenyldithioacetate (1.25 g, 5.53 mmol, 1.5 eq.) and NaHCO_3 (1.54 g, 18.3 mmol, 5 eq.) were stirred together under a nitrogen atmosphere in 20 ml of water at room temperature for 3 h. The mixture was extracted three times with ethyl acetate and then acidified to pH 1. Three ethyl acetate extracts were then taken. The latter extracts, combined, were washed with water and then extracted with water (20 ml) containing 0.62 g of NaHCO_3 (2 eq.). This aqueous solution was stirred under a nitrogen atmosphere with 20 ml of ethyl acetate containing 1 ml of aniline (approx. 4 eq.). This procedure removed all carboxymethyl phenyldithioacetate from the aqueous layer (by reaction with aniline), leaving thioglycolate. After separation of the layers, the aqueous solution was extracted three times with ethyl acetate and then freeze-dried.

The product was purified by chromatography on Sephadex-QAE A-25 ion-exchange resin (eluted with a 0–1 M-NaCl gradient and extracted from acidified fractions) and then, as the sodium salt, on Sephadex G-10 resin (eluted with water). The final product (approx. 100 mg) was obtained as a pale-yellow solid on freeze-drying appropriate fractions from the latter column. Fast-atom-bombardment m.s. (Yale University Instrument Center, New Haven, CT, U.S.A.) yielded strong $M+1$ (H^+) and $M+23$ (Na^+) peaks at m/z 429 and 451 respectively. The u.v.-absorption spectrum [λ_{max} (Mops buffer, pH 7.5) 267 nm, $\epsilon = 13\,500\text{ M}^{-1}\text{ cm}^{-1}$] and ^1H n.m.r. spectrum [δ (p.p.m.) ($^2\text{H}_2\text{O}$) 2.22 (3H, s, OAc), 3.34, 3.62 (2H, ABq, J 18 Hz, H-2), 4.05, 4.15 (2H, ABq, J 14 Hz, PhCH_2), 4.70, 4.87 (2H, ABq, J 12 Hz, H-3'), 5.22 (1H, d, J 4.5 Hz, H-6), 6.03 (1H, d, J 4.5 Hz, H-7), 6.3–6.5 (5H, m, ArH)], were consistent with the required structure (Ib).

6-Phenylthioacetamidopenicillanic acid II could not be obtained in a pure state by the above method. It appeared to be unstable in concentrated solution and could not be purified by the chromatographic methods described above. ^1H n.m.r. spectra of reaction mixtures comprising 6-aminopenicillanic acid, carboxymethyl phenyldithioacetate and NaHCO_3 in the relative amounts used in the preparation of Ib showed the disappearance of the amino compound over 30 min and the appearance of a compound whose spectrum was that expected of the required thionopenicillin [δ (p.p.m.) ($^2\text{H}_2\text{O}$) 1.40, 1.54 (6H, 2s, Me_2), 4.00, 4.13 (2H, ABq, J 14 Hz, PhCH_2), 4.24 (1H, s, H-3), 5.59 (1H, d,

J 3.5 Hz, H-5/6), 5.63 (1H, d, J 3.5 Hz, H-5/6), 7.3–7.5 (5H, m, ArH)]. Also present in solution were carboxymethyl phenyldithioacetate [δ (p.p.m.) ($^2\text{H}_2\text{O}$) 3.88 (2H, s, PhCH_2), 4.88 (2H, s, SCH_2), 7.4–7.5 (5H, m, ArH)] and its hydrolysis products phenylthioacetate (3.15, s) and thioglycolate (4.05, s). A 45 min reaction mixture containing these species was freeze-dried. The dried mixture could be stored unchanged at $-20\text{ }^\circ\text{C}$ for several months.

Analytical and kinetic methods

Concentrations of the various enzymes in solution were determined spectrophotometrically by employing the published absorption coefficients previously listed [27]. Kinetic measurements with the serine β -lactamases were made in 20 mM-Mops buffer, pH 7.5, and with the R61 DD-peptidase in 20 mM-phosphate, pH 7.0, containing 0.02 % gelatin. A buffer composed of 20 mM-Hepes, pH 7.5, and containing 50 μM -zinc sulphate and KCl to 1 M ionic strength was employed for the kinetic studies of *B. cereus* β -lactamase II. The temperature employed in all kinetics experiments was 25 °C. The reactions of compounds Ia and Ib were monitored spectrophotometrically, in most cases at 270 nm ($\Delta\epsilon$ values were $5300\text{ M}^{-1}\text{ cm}^{-1}$ and $3700\text{ M}^{-1}\text{ cm}^{-1}$ respectively), although at higher concentrations it was necessary to use longer wavelengths. Steady-state kinetic parameters were obtained from initial-velocity measurements by the method of Wilkinson [28]. Routine assays of the β -lactamases and the R61 DD-peptidase were performed spectrophotometrically with benzylpenicillin and hippuryl DL-phenyl-lactate [29] respectively as substrates. Pre-steady-state kinetic measurements were made using a Durrum D-110 stopped-flow spectrophotometer. The K_m values of Ia and Ib for reaction with the PCl β -lactamase were too low to determine directly and were obtained as K_i values of Ia and Ib as competitive inhibitors of dansylpenicillin hydrolysis catalysed by this enzyme. The hydrolysis of dansylpenicillin was monitored fluorimetrically, as previously described [24].

RESULTS

The thionocephalosporin Ib is, like the oxo-analogue Ia, a substrate of typical β -lactamases of class A (the TEM-2 β -lactamase, *B. cereus* β -lactamase I and the PCl β -lactamase of

S. aureus), class B (*B. cereus* β -lactamase II) and class C (the β -lactamase of *Enterobacter cloacae* P99). Addition of any one of these enzymes to a solution of Ib leads to β -lactam hydrolysis. This is shown clearly in the ^1H n.m.r. spectrum of the products

$[\delta$ (p.p.m.) ($^2\text{H}_2\text{O}$) 1.90 (3H, s, CH_3CO_2^-), 3.26, 3.71 (2H, ABq, J 15 Hz, H-2), 4.00, 4.19 (2H, ABq, J 12 Hz, PhCH_2), 5.35 (1H, d, J 3 Hz, H-6/7), 5.56 (1H, d, J 3 Hz, H-6/7), 5.63 (1H, s, $=\text{CH}$), 5.68 (1H, s, $=\text{CH}$), 6.3–6.5 (5H, m, ArH)] which can be thereby identified as IIIb and acetate. These arise from the well-established elimination reaction [30–33] of the 3'-leaving group from the initial hydrolysis product IVb. Analogous products, IIIa and acetate, were generated from Ia on its treatment with β -lactamases. These ^1H n.m.r. spectra and structural assignments are well documented [34]. The spectra of the thiono compounds differ from those of the oxo in the significant downfield shifts produced in the phenylacetymethylene hydrogens and H-7 resonances by the thionamide group.

The u.v.-absorption spectrum of Ib, before and after treatment with one of the above β -lactamases, is shown in Fig. 1. The residual absorption (λ_{max} , 272 nm, $\epsilon = 9100 \text{ M}^{-1} \text{ cm}^{-1}$) after β -lactam hydrolysis, and absent after hydrolysis of Ia [34], is of course that of the thionamide group [35].

^1H n.m.r. spectral changes consonant with β -lactam hydrolysis were also produced in samples of II on addition of β -lactamases. The product spectrum [δ (p.p.m.) ($^2\text{H}_2\text{O}$) 1.23, 1.50 (6H, 2s, Me_2), 3.33 (1H, s, H-3), 4.11 (2H, s, PhCH_2), 4.84 (1H, d, J 6.5 Hz, H-5/6), 5.13 (1H, d, J 6.5 Hz, H-5/6), 7.3–7.5 (5H, m, ArH)] is similar to that of benzylpenicilloate [36], but again with distinctive downfield shifts of the resonances of hydrogens

Fig. 1. Absorption spectrum of compound Ib before (—) and after (---) hydrolysis catalysed by a β -lactamase

adjacent to the thionamide group. As described above, the samples of II were not pure but the resonances of other components of the mixture did not change on β -lactamase treatment. The addition of the P99 β -lactamase or, in a separate experiment, the TEM β -lactamase to a solution in an n.m.r. tube containing approximately equal concentrations of benzylpenicillin and II, led to disappearance of both substrates and the appearance of the corresponding penicilloates. The rates of hydrolysis in each case were very similar, with those of II being perhaps slightly greater. Hence $k_{\text{cat.}}/K_m$ values for benzylpenicillin and II must be very similar for both of these enzymes.

The P99 β -lactamase also catalysed methanolysis of Ib. The ^1H n.m.r. spectrum of a mixture containing approx. 5 mM-Ib, 50 mM- NaHCO_3 in $^2\text{H}_2\text{O}$ with 8% (v/v) [$^2\text{H}_4$]methanol (approx. 2 M) to which about 0.1 mg of enzyme had been added showed, after completion of the reaction, the presence of both the hydrolysis product IIIb and the methanolysis product V [δ (p.p.m.) 3.40, 3.71 (2H, ABq, J 15 Hz, H-2), 4.07, 4.14 (2H, ABq, J 15 Hz, PhCH_2), 5.66 (1H, s, $=\text{CH}$), 5.70 (1H, d, J 3.2 Hz, H-6/7), 5.71 (1H, s, $=\text{CH}$), 5.81 (1H, d, J 3.2 Hz, H-6/7), 7.3–7.5 (5H, m, ArH)], with the latter making up about 27% of the total. The class-A enzymes, however, did not catalyse the methanolysis of Ib; this result is of course typical of the reaction of these enzymes with β -lactams in general [29].

Steady-state kinetic parameters pertaining to the hydrolysis of Ia and Ib are given in Table 1. The rate of the P99 β -lactamase-catalysed disappearance of Ib, under close to saturating conditions, increased linearly with methanol concentration (results not shown) which suggested that deacylation of the enzyme is rate-determining during turnover. This is true for this enzyme with cephalosporins in general [37] and for penicillins [29,38], and thus is presumably also true for Ia.

Turnover of cephalothin, a close analogue of Ia, by the PCI β -lactamase is limited by deacylation of a rather inert acylenzyme VIa ($R = \text{thenyl}$) from which the 3'-acetoxy group has been eliminated [39]. The steady-state parameters for hydrolysis of Ia with this enzyme (Table 1) are very similar to those of cephalothin, suggesting that rate-determining deacylation obtained for this substrate also. This conclusion was supported by stopped-flow kinetic measurements. On mixing Ia (50 μM) with the PCI β -

Table 1. Steady-state parameters for the β -lactamase-catalysed hydrolysis of the cephalosporins Ia and Ib

Substrate	Parameter	Enzyme ...	PCI*	TEM*	BCI*	BCII*	P99*
Ia	$k_{\text{cat.}}$ (s^{-1})		0.0039	67 ± 5	—	211 ± 29	710 ± 50
	K_m (μM)		0.14 ± 0.05	690 ± 90	—	1160 ± 240	36 ± 9
	$k_{\text{cat.}}/K_m$ ($\text{s}^{-1} \text{ M}^{-1}$)		2.8×10^4	9.7×10^4	$4.6 \times 10^4 \dagger$	1.8×10^5	2.0×10^7
Ib	$k_{\text{cat.}}$ (s^{-1})		0.34 ± 0.02	2.33 ± 0.15	—	78 ± 9	42 ± 3
	K_m (μM)		5.5 ± 3.0	290 ± 40	—	410 ± 100	9.3 ± 2.7
	$k_{\text{cat.}}/K_m$ ($\text{s}^{-1} \text{ M}^{-1}$)		6.0×10^4	8.1×10^3	$4.7 \times 10^4 \dagger$	1.9×10^5	4.5×10^6
	$(k_{\text{cat.}}/K_m)_{\text{Ib}}/(k_{\text{cat.}}/K_m)_{\text{Ia}}$		2.1	0.084	1.02	1.05	0.23

* PCI, the class-A β -lactamase of the PCI plasmid of *S. aureus*; BCI, the class-A *B. cereus* β -lactamase I; TEM, the class-A β -lactamase of the TEM plasmid of *E. coli*; BCII, *B. cereus* β -lactamase II, a class-B β -lactamase; P99, the class-C β -lactamase of *Ent. cloacae*-P99.

† These values were derived from pseudo-first-order reactions at low (10–20 μM) substrate concentrations.

lactamase (11.6 μM) in the stopped-flow spectrophotometer, two phases of decrease in absorption at 270 nm were observed. The

first, of half-time approximately 0.4 s, preceded a slower phase, the rate of which was dictated by $k_{\text{cat.}}$. Under similar concentration conditions, a fast phase was also observed with Ib, although in this case it was more convoluted with the second phase. Thus it seems likely, from precedent and from these experiments, that $k_{\text{cat.}}$ for turnover of both Ia and Ib by the PC1 β -lactamase relates to the deacylation step.

The R61 DD-peptidase (9.8 μM) reacted with Ia and Ib (100–200 μM) in a pseudo-first-order fashion, as monitored by the decrease in absorption at 270 nm. The derived second-order rate constants, corresponding to $k_{+1}k_{+2}/(k_{-1}+k_{+2})$ of Scheme 1 (see below), were $195 \pm 33 \text{ s}^{-1} \text{ M}^{-1}$ and $107 \pm 11 \text{ s}^{-1} \text{ M}^{-1}$ for Ia and Ib respectively. The enzyme, assayed against hippuryl DL-phenyl-lactate, was inactive after these reactions.

DISCUSSION

Thionamidocephems and penams such as Ib and II seem remarkably poorly documented in the literature [40] beyond a number of scattered patent references and some well-characterized thionopenicillin sulfoxide derivatives [41,42]. Although Ib and II are relatively stable as anions, in dilute (mM) solution and in the solid state, they appeared unstable in concentrated solution, presumably through bimolecular reaction(s). They appear, as demonstrated by the spectroscopic measurements described above and discussed in quantitative detail for Ia and Ib below, to be good β -lactamase substrates. Further, since Ib inhibits the *Streptomyces* R61 DD-peptidase, they most likely also have some antibiotic activity.

A quantitative comparison of Ia and Ib, demonstrating the effect of side-chain thionation of a normal cephalosporin β -lactamase substrate, is available from Table 1. The thiono compound is thus indeed, in general, a good β -lactamase substrate.

The results for the serine (class A and C) β -lactamases and the R61 DD-peptidase can be considered further in terms of Scheme 1, the well-established double-displacement mechanism for these enzymes, where ES' represents the covalent acylenzyme intermediate.

Scheme 1

The major conclusion from the data of Table 1 is that side-chain thionation has relatively little effect on the specificity ($k_{\text{cat.}}/K_m$) of the serine β -lactamases toward a typical cephalosporin substrate. In terms of Scheme 1, $k_{\text{cat.}}/K_m$ is a measure of the free energy of acylation of free enzyme in solution by free substrate (the values of $k_{\text{cat.}}/K_m$ are not large enough, except perhaps in the case of the P99 enzyme and Ia, to reflect rate-determining diffusion together of substrate and enzyme). Thus

stabilization by the enzyme of the transition state for acylation is not greatly changed in thionation. This result is in sharp contrast with the findings of Asbóth *et al.* [16] that analogous substitution in the P₂ peptide bond of a papain substrate destabilized the transition state of papain-catalysed hydrolysis by some three orders of magnitude. It should be noted, however, as indeed did Asbóth *et al.* [16], that a destabilization of the magnitude that they observed is not likely to result simply from the loss of a hydrogen bond from the acylenzyme. The latter change, in itself, should not result in more than a 10-fold (6.3 kJ/mol) change [19]. The additional effect presumably arose from unfavourable steric interactions between the larger sulphur atom (the C=S bond in a thionoamide is some 0.04–0.05 nm longer than the C=O of an amide [35]) and a rather tight hydrogen-bonding site on the enzyme.

The present result can then presumably be interpreted to mean that the positioning of the carbonyl group of the acylamido side chain of a good β -lactamase substrate into a tight hydrogen-bonding site is not essential for effective β -lactamase catalysis. This is not to say that hydrogen-bonding of the carbonyl group, and presumably to Asn-132 or its homologue, does not occur – if it did not then hydrogen bonds would be lost on transfer of the substrate from aqueous solution – but that the hydrogen-bond site must either be loose, in order to also accommodate the thiocarbonyl, or else the thiocarbonyl must bind elsewhere but in a mode not substantially less productive. With either scenario, the conclusion of the first sentence of this paragraph holds. Of the serine β -lactamases, the TEM-2 enzyme appears to have the greatest resistance to the thiono substrate, reflecting perhaps a less flexible side-chain-binding site in this enzyme, or one more constricted around the amide group.

The findings can be compared, on one hand, with those concerning the tight hydrogen-bonding site of the oxyanion hole of serine proteinases [43–45] and β -lactamases [3,15] and the P₂ substituent site of papain, and, on the other, with the more flexible oxyanion hole of papain [14,46,47]. In the latter case, as in the present one, the hydrogen-bond donor is a potentially flexible asparagine side chain rather than a backbone amide. On the other hand, it should be noted that subtilisin, although thought to include an asparagine amido group as a hydrogen-bond donor in its oxyanion hole, only weakly interacts with thiono substrates [14].

Although it is not profitable, in general, to dissect $k_{\text{cat.}}/K_m$ into its components and interpret them separately, one aspect of the more striking changes in $k_{\text{cat.}}$ and K_m seen in Table 1 might be briefly commented on. In particular, the presence and consequences of a good 3'-leaving group in Ia and Ib should be noted. It has been demonstrated for both class-A [39,48] and class-C [37] β -lactamases that, although the initial acylenzyme achieved on reaction with a cephem bearing a 3'-leaving group has the expected structure VII, if the leaving group L is sufficiently good, as is often true for L = acetoxy, an elimination reaction occurs faster than deacylation, leading to acylenzymes of structure VI. In all cases to date, acyl-enzymes of structure VI deacylate much more slowly than those of structure VII. Often in such cases deacylation is rate-determining; typically both $k_{\text{cat.}}$ and K_m are small, the latter reflecting the stability of VI.

For the P99 enzyme, where deacylation is rate-determining at saturation (see the Results section), although the acylenzyme from Ia is most likely VIIa, i.e. this complex undergoes hydrolysis before the elimination reaction can occur [37], that from Ib may be, partly at least, VIb, i.e. the thionation may decrease k_{+3} to an extent that elimination of L (acetoxy) can compete with hydrolysis. Certainly the elimination of acetoxy from IVa in free solution occurs with a rate constant of something greater than 50 s^{-1} [34], although the reaction may be retarded on an enzyme [49,50]. It is possible that a similar partial change in rate-determining step may also be reflected in the data for the TEM β -lactamase. The PCl β -lactamase presents a different situation. It is clear from the above-cited papers that k_{cat} for Ia represents deacylation of VIa. The significantly larger k_{cat} and K_m values obtained for Ib suggest that in this case most turnover proceeds directly through VIIb. Whether this arises predominantly from a more facile hydrolysis of VIIb than VIIa or a slower elimination reaction is not clear, although the latter might seem more likely in view of the interpretation of the data for the P99 and TEM β -lactamases presented above. At any event, these uncertainties do not compromise the interpretation of the k_{cat}/K_m values.

Further evidence for the nature of the hydrogen-binding site is potentially available by removal of the enzymic hydrogen-bond donor. This is in fact the situation likely to be present in the Asn-132-Ala mutant of the class-A β -lactamase of *Streptomyces albus* G prepared by Jacob *et al.* [21,22]. They found this mutant much less active than the wild-type enzyme – k_{cat}/K_m diminished some 300-fold for benzylpenicillin and considerably more so for cephalosporins – and concluded that Asn-132 participated significantly in transition-state stabilization. Although the present work does suggest that the flexibility of the hydrogen-bond donor may vary from enzyme to enzyme, the substantially smaller effects obtained from substrate thionation suggest that a major effect of the Asn-132-Ala mutation may be structural. Although the gross thermal stability of the mutant was reported to be unaffected, it is rather difficult to see that removal of the amido side chain of Asn-132, if it is in fact hydrogen-bonded to the essential and invariant active-site residue Lys-73 and in some cases at least to another residue also [4], would not lead to some structural change even if it were replaced by water molecules. Indeed, Jacob *et al.* report that the mutation also caused a 3000-fold decrease in k_{cat}/K_m for 6-aminopenicillanic acid, which does not contain an amido side chain.

The conclusions above with respect to the serine β -lactamases appear to apply also to the β -lactam-binding site of the *Streptomyces* R61 DD-peptidase during its acylation and inhibition by β -lactams. This enzyme is also believed to possess the S(Y)XN triad, YSN in this case, at the active site [12], but again thionation of the β -lactam had essentially no effect on the rate of acylation. Although it is possible that the peptide substrate and β -lactam side-chain-binding sites may not overlap [27,51], it is clear at least that tight hydrogen-bonding to the carbonyl of the β -lactam side chain is not essential to effect inactivation.

Finally, it should be noted that similar conclusions must also apply to the class-B enzyme, *B. cereus* β -lactamase II. This enzyme is, of course, a metallo- β -lactamase and completely unrelated to the serine enzymes. No high-resolution crystal structure is yet available [52] and no analogue of Asn-132 has thus been proposed. The complete lack of effect of side-chain thionation (Table 1) also gives no indication of side-chain amide binding to the active-site zinc ion. On the other hand, however, an investigation of the interaction of this enzyme with a β -lactam-thionated cephalosporin did suggest direct interaction between the β -lactam carbonyl group and the zinc ion [53].

The general conclusion is that serine β -lactamases and DD-peptidases probably possess a soft, flexible hydrogen-bonding

site, available, when needed, to accommodate the amide side chains of β -lactams, a passive rather than active feature of the active site. This flexibility may ensure a low substrate side-chain specificity in β -lactamases, an important feature of their biological role. In the present work, the importance of complementary evidence, from modification of the substrate on one hand and of the enzyme on the other, is clearly seen.

Preliminary syntheses related to this project were carried out by Nilantha Sirisoma. Financial support for this research was provided by the National Institutes of Health.

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Received 3 January 1992/25 February 1992; accepted 27 February 1992