Inactivation of the RTEM-1 cysteine β -lactamase by iodoacetate

The nature of active-site functional groups and comparisons with the native enzyme

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The pH-rate profile for inactivation of the RTEM-1 cysteine β -lactamase by iodoacetate supports previous evidence [Knap & Pratt (1989) *Proteins Struct. Funct. Genet.* 6, 316–323] for the activation of the active-site thiol group by adjacent functional groups. The enhanced reactivity of iodoacetate, with respect to that of iodoacetamide, suggests the influence of a positive charge in the active site. The reactivity of iodoacetate is not affected by dissociation of an active-site functional group of pK_a 6.7, which increases the reactivity of neutral reagents, probably because of a compensation phenomenon; it is, however, lost on dissociation of an acid of pK_a 8.1. It is concluded that the active cysteine β -lactamase has four functional groups at the active site, one nucleophilic thiolate of Cys-70, one neutral acid (most probably the carboxy group of Glu-166, from the crystal structures) and two cationic residues (most probably Lys-73 and Lys-234). A comparison of these results with the pH-dependence of reactivity of the native RTEM-2 β -lactamase suggests that the active form of the latter enzyme is also monocationic, although the nucleophile (Ser-70) is likely to be neutral in this case and the carboxylic acid dissociated. A mechanism of class A β -lactamase catalysis is discussed where the Glu-166 carboxylate acts as a general base/acid catalyst and Lys-73 is principally required for electrostatic stabilization of the anionic tetrahedral intermediate.

INTRODUCTION

For many years the mechanism of action of β -lactamases, and indeed the nature of the functional groups at the active sites of these enzymes, were a great mystery. The classical functional-group reagents, for example, were unable to shed much light on the subject [1]. Finally, a series of mechanism-based inhibitors led to the identification of Ser-70 (in the numbering system of Ambler [2]) as the primary active-site nucleophile [3]. The auxiliary functionality of the active site was subsequently only revealed, or at least indicated, by recent crystal structures [4–6]. These suggest that two conserved lysine residues (Lys-73 and Lys-234) and perhaps a conserved glutamic acid residue (Glu-166) may be involved in catalysis by class A β -lactamases. Site-specific mutagenesis experiments, where Lys-73 [7] and Glu-166 [8] have been replaced, support the importance of these residues. There remains the task of determining the chemical properties of these groups and thereby defining their role in catalysis.

We have recently approached the question of the chemical properties of the functional groups of the class A β -lactamase active site through employment of the RTEM-1 cysteine β -lactamase. This enzyme was created by site-specific mutagenesis (Ser-70 $\rightarrow$ Cys) of the native RTEM-1 β -lactamase [9], and does employ its active-site thiol group as the primary nucleophile in catalysis [10]. It is a useful target for investigation because the active-site thiol group is not only a much better intrinsic probe of the active site than the serine hydroxy group of the native enzyme, but also a more versatile point of attachment of extrinsic probes. In previous work with this enzyme [11] we have demonstrated that the active-site thiol group is an active nucleophile towards neutral thiol-specific reagents at low pH, indicating a low pK_a (≤ 4.0) for this thiol group. We have suggested that this is due to specific stabilization of the thiolate anion by two hydrogen-bond donors, at least one of them positively charged. This mono-dissociated form of the enzyme is the active cysteine β -lactamase. One of the hydrogen-bond donors dissociates with

a pK_a of 6.7, increasing the nucleophilicity of the thiolate towards neutral reagents, but abolishing β -lactamase activity.

In the present paper, the reaction of the cysteine β -lactamase with an anionic alkylating agent, iodoacetate, is described. This reagent is sensitive to the dissociation of a further functional group. We also examined the reactivity of the native RTEM-1 β -lactamase as a function of pH, and propose correlations between its behaviour and that of the cysteine enzyme. The paper concludes with a brief discussion of the mechanism of action of class A β -lactamases.

EXPERIMENTAL

Materials

The HB101 strain of *Escherichia coli* K12 containing the pOTBL plasmid was generously provided by E. I. duPont de Nemours and Co. Cells were grown and the cysteine RTEM-1 β -lactamase was extracted, purified and stored as previously described [9,12]. The wild-type RTEM-2 β -lactamase was purchased from the P.H.L.S. Centre for Applied Microbiology and Research, Porton Down, Wilts., U.K., and used as received. Iodoacetic acid (Sigma Chemical Co.) was recrystallized from carbon tetrachloride before use. Benzylpenicillin and methane-sulphonyl fluoride were purchased from Sigma Chemical Co. Nitrocefin was obtained as a gift from B.B.L. MicroBiology Systems.

Enzyme concentrations and activities against benzylpenicillin were routinely determined spectrophotometrically as previously described [10,11]. The buffers used for the pH-rate profiles were 0.5 M-sodium acetate (pH 4–5.5), 0.1 M-sodium phosphate (pH 6–8) and 0.05 M-Bicine/HCl (pH 8–9.5). All buffers contained 9% (v/v) glycerol (for protein stabilization), 1 mM-EDTA, and had an ionic strength of 1.0, adjusted with NaCl. Buffers were sparged with N_2 for 20–25 min before use. All experiments were performed at 25 °C, except where specified otherwise.

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pH-rate profile for inactivation of the cysteine RTEM-1 β -lactamase by iodoacetate

This was determined from measurements of the decrease with time in activity of the β -lactamase against nitrocefin in buffers containing iodoacetate (5.0 or 10.0 mM), exactly as was done with iodoacetamide [11]. The enzyme concentration was 4 μ M. Pseudo-first-order rate constants were obtained by semi-logarithmic plots and converted into second-order rate constants through division by the iodoacetate concentration; the inactivation was first-order in iodoacetate to 10 mM. The concomitant disappearance of the active-site thiol group was monitored through its reaction with 4,4'-dipyridyl disulphide, also as described previously [11].

pH-rate profile for the RTEM-2 β -lactamase-catalysed hydrolysis of benzylpenicillin

Steady-state parameters were determined at each pH by the method of Wilkinson [13] from spectrophotometric (232 nm) initial-velocity measurements.

pH profile of boric acid inhibition of the RTEM-2 β -lactamase

The initial rates of hydrolysis of benzylpenicillin (at concentrations about twice K_m) at each pH were determined spectrophotometrically in the absence and in the presence of appropriate concentrations of boric acid (1.2–120 mM). Competitive inhibition constants were determined by the method of Waley [14]. The pK_a of boric acid was taken to be 9.34; this was the pH of a 5.8 mM solution of $Na_2B_4O_7$ at an ionic strength of 1.0.

pH-rate profiles for inactivation of the RTEM-2 β -lactamase by methanesulphonyl fluoride

These were determined at 15 °C, 25 °C, 35 °C and 45 °C essentially by the method used for iodoacetate. The concentration of enzyme was approx. 3 μ M and that of methanesulphonyl fluoride was 71.5 mM; the latter reagent was added as a portion of the neat liquid. All manipulations of methanesulphonyl fluoride were performed in an efficient fume-hood. The pH values of buffers were carefully determined at the above-mentioned temperatures with an electrode standardized at those temperatures.

The pH profiles were fitted to theoretical equations (see the Results section) by a non-linear least-squares program [15]. The

Fig. 1. pH-rate profile for inactivation of the RTEM-1 cysteine β -lactamase by iodoacetate

The points are experimental and the lines were calculated as described in the text (Scheme 1 and eqn. 1).

Scheme 1.

significance of additional parameters was evaluated by the likelihood ratio test [16].

RESULTS

The RTEM-1 cysteine β -lactamase was inactivated by iodoacetate, as it had been by other thiol-reactive reagents [11,12]. As would be expected, the loss of β -lactamase activity was accompanied by loss, presumably through alkylation, of the active-site thiol group. A pH-rate profile for the iodoacetate reaction is shown in Fig. 1. This can be accommodated by the simple model shown in Scheme 1, where one form of enzyme, EH, present at low pH, which is inactivated by iodoacetate, I, is converted into another form E, which is little or not affected by iodoacetate, by

Fig. 2. pH profiles for catalysis by and inhibition of the RTEM-2 β -lactamase

The points are experimental and the lines were calculated as described in the text and with the use of the parameter values of Table 1. (a) $k_{\text{cat.}}/K_m$ for steady-state hydrolysis of benzylpenicillin (Scheme 2 and eqn. 2). (b) Competitive inhibition by borate (Scheme 3 and eqn. 3). (c) Inactivation by methanesulphonyl fluoride (Scheme 4 and eqn. 4).

Scheme 2.

dissociation of an enzymic acid. The continuous curve of Fig. 1, fitting these data, is derived from eqn. (1):

$$k_i = \frac{k_i^{\text{EH}}[\text{H}^+] + k_i^{\text{E}}K_a}{K_a + [\text{H}^+]} \quad (1)$$

with $\text{p}K_a = 8.13 \pm 0.27$, $k_i^{\text{EH}} = 0.35 \pm 0.02 \text{ s}^{-1} \cdot \text{M}^{-1}$ and $k_i^{\text{E}} = 0.058 \pm 0.045 \text{ s}^{-1} \cdot \text{M}^{-1}$.

For comparison with the current and previous [11] results with the cysteine β -lactamase, the pH-dependence of β -lactamase activity and chemical modification of the native RTEM-2 β -lactamase (no significant differences in β -lactamase activity between the RTEM-1 and RTEM-2 β -lactamases have been detected [17]) was also determined. A pH profile for β -lactamase activity ($k_{\text{cat.}}/K_m$) is shown in Fig. 2(a). These data were fitted to Scheme 2 and the derived eqn. (2):

$$k_{\text{cat.}}/K_m = \frac{(k_{\text{cat.}}/K_m)^{\text{EH}_2}[\text{H}^+]^2 + (k_{\text{cat.}}/K_m)^{\text{EH}}K_{a_1}[\text{H}^+]}{K_{a_1}K_{a_2} + K_{a_1}[\text{H}^+] + [\text{H}^+]^2} \quad (2)$$

The parameters giving rise to the best fit (the continuous line in Fig. 2a) are presented in Table 1. The best fit to the data with $(k_{\text{cat.}}/K_m)^{\text{EH}_2} = 0$, i.e. if EH were the only active species, was statistically poorer than that given in Fig. 2(a), although the $\text{p}K_a$ values were not greatly different ($\text{p}K_{a_1} = 5.62 \pm 0.3$ and $\text{p}K_{a_2} = 7.79 \pm 0.3$).

Borate and boronates are known to be competitive inhibitors of β -lactamases [18–20]. They are thought to form transition-state analogue structures by covalent attachment to the active-site serine hydroxy group, as with serine proteinases [21]. The pH-dependence of the reciprocal of the inhibition constant (i.e. of the binding constant) of borate as an inhibitor of the native RTEM-2 β -lactamase is shown in Fig. 2(b). These data were fitted to Scheme 3 and the derived eqn. (3):

$$\frac{1}{K_i} = \frac{(K_{a_1}/K_a)[\text{H}^+] + (1/K_3)[\text{H}^+]^2}{(K_{a_1}K_{a_2} + K_{a_1}[\text{H}^+] + [\text{H}^+]^2)(1 + K_a/[\text{H}^+])} \quad (3)$$

where K_i is the observed inhibition constant at any pH, and K_a is the dissociation constant of boric acid $[\text{B}(\text{OH})_3 + \text{H}_2\text{O} \xrightleftharpoons{K_a} \text{B}(\text{OH})_4^- + \text{H}^+]$. In order to fit these data it must be assumed (as

Scheme 3.

with serine proteinases [22–25]) that the serine hydroxy-group proton released on formation of the borate complex is taken up by another functional group whose $\text{p}K_a$ must increase on formation of the borate adduct. If the proton were released into solution, the order with respect to $[\text{H}^+]$ in each term of the numerator of eqn. (3) would be decreased by one, and the equation no longer fits the data. Crompton *et al.* [20] have shown that a proton is not released on reaction of specific boronates with *Bacillus cereus* β -lactamase I. The fit of the data of Fig. 2(b) to eqn. (3) yields values for the parameters given in Table 1. A statistically poorer fit was obtained if it was assumed that $1/K_3 = 0$. The reality of the low-pH reactivity, with both borate and benzylpenicillin, is uncertain. We consider that more data would be needed to be confident of this. It is not seen in the reaction of the enzyme with methanesulphonyl fluoride (see below), and is not further referred to in the present paper.

The native RTEM-2 β -lactamase was also inhibited by methanesulphonyl fluoride. A plot of the second-order rate constants for this reaction is shown in Fig. 2(c). The data, particularly at higher pH, were not precise, and its quality could not be improved. It was fitted to Scheme 4 and eqn. (4):

$$k_i = \frac{k_i^{\text{EH}}K_{a_1}[\text{H}^+] + k_i^{\text{E}}K_{a_1}K_{a_2}}{K_{a_1}K_{a_2} + K_{a_1}[\text{H}^+] + [\text{H}^+]^2} \quad (4)$$

with the assumption that $\text{p}K_{a_2} = 7.5$ (the value obtained from the benzylpenicillin and borate data). This procedure yielded the line shown in Fig. 2(c) and the parameters in Table 1. Variation in the assumed value of $\text{p}K_{a_2}$ from 7.0 to 8.0 did not shift $\text{p}K_{a_1}$ outside of the uncertainty limits given. The addition of an $[\text{OH}^-][\text{I}]$ term to the reaction of E did not improve the fit in a

Scheme 4.

Table 1. Kinetic and equilibrium parameters from fitting the data of Fig. 2

Reagent	Parameter	EH ₂	EH	pK _{a1}	pK _{a2}
Benzylpenicillin	$k_{\text{cat.}}/K_m^*$ (s ⁻¹ ·M ⁻¹)	$(1.9 \pm 0.6) \times 10^7$	$(1.1 \pm 0.2) \times 10^8$	6.18 ± 0.25	7.59 ± 0.17
Borate	$1/K_i^\dagger$ (mM ⁻¹)	43 ± 26	4.6 ± 1.0	5.78 ± 0.30	7.45 ± 0.25
Methanesulphonyl fluoride	$k_i^\ddagger$ (s ⁻¹ ·M ⁻¹)	0.024 ± 0.04	0.012 ± 0.03	5.43 ± 0.30	7.5§

* Fig. 2(a), Scheme 2 and eqn. (2).

† Fig. 2(b), Scheme 3 and eqn. (3).

‡ Fig. 2(c), Scheme 4 and eqn. (4).

§ Fixed (see the text).

statistically significant way. Much poorer fits were obtained on setting $k_1^E = 0$.

Kinetic data were also obtained for methanesulphonyl fluoride inhibition at 15 °C, 35 °C and 45 °C, and the value for pK_a derived as above. The ΔH° value for dissociation of EH_2 to EH could then be obtained from a linear plot of pK_a versus $1/T$ (not shown). The value of ΔH° thus derived varied somewhat depending on the value assumed for pK_a . If pK_a was assumed to be 7.0, 7.5 and 8.0 at each temperature (although the assumption of 7.0 gave the best fits except at 15 °C), the corresponding ΔH° values were 13.8 ± 20.9 kJ/mol (3.3 ± 5.0 kcal/mol), 20.5 ± 20.9 kJ/mol (4.9 ± 5.0 kcal/mol) and 26.4 ± 16.7 kJ/mol (6.3 ± 4.0 kcal/mol). If it was assumed that pK_a was 7.5 at 25 °C and ΔH° for pK_a was 50.2 kJ/mol (12.0 kcal/mol) [as might be the case if a lysine dissociation were involved (see the Discussion section)], ΔH° for pK_a was 8.4 ± 20.9 kJ/mol (2.0 ± 5.0 kcal/mol). The latter procedure in fact gave the best fit to the data overall.

DISCUSSION

The pH-rate profile for the inactivation of the RTEM-1 cysteine β -lactamase by iodoacetate is very striking, particularly when it is compared with the pH-rate profiles obtained with neutral reagents including iodoacetamide [11], the interpretation of which is summarized in Scheme 5. It should be noted with respect to the structural assignments in Scheme 5 and elsewhere in this paper that the β -lactamase crystal structures currently available do not include the RTEM enzyme. It seems likely, however, and it is assumed in this paper, that the structure of the RTEM enzyme, particularly with respect to active-site functionality, is very similar to published structures of other class A β -lactamases [4–6].

On one hand, the iodoacetate results do support the presence of thiol-group dissociation at low pH ($pK_a \leq 4$), which is required to explain the plateau in reactivity in the pH 4–6 region of the profile. The reactivity of the enzyme with iodoacetate in this region, where it exists as structure (I), the active β -lactamase, is very similar to that with iodoacetamide ($k_1 = 0.4 \text{ s}^{-1} \cdot \text{M}^{-1}$ [11]). Since in model reactions with low- M_r thiolates [26] the reactivity of iodoacetate is about an order of magnitude less than that of iodoacetamide, the results with the cysteine β -lactamase suggest an enhanced iodoacetate reactivity with respect to iodoacetamide. This is suggestive of the situation with papain and several other cysteine proteinases, where the reactivity of haloacetates is enhanced by the presence of an adjacent positively charged residue, the histidinium ion of the active-site ion-pair [27–29]. The existence of a suitably placed positive charge (lysine ammonium ion) at the β -lactamase active site, perhaps as shown in Scheme 5, seems reasonable in view of the presence of two conserved lysine residues (Lys-73 and Lys-234) in the vicinity [4–6]. The extent of enhancement of the rate of inactivation by

iodoacetate with cysteine β -lactamase is much less than that with papain, suggesting that the positive charge cannot in the former enzyme achieve the optimal position or environment found in papain. It should be noted, however, that the extent of enhancement of iodoacetate reactivity is a sensitive function of the cysteine enzyme involved, and in some cases, e.g. thiolsubtilisin [26] and a bean proteinase [30], does not seem to occur at all, although a thiolate–imidazolium ion-pair is thought to be present at the active site.

On the other hand, however, the pH-rate profile for inactivation by iodoacetate in Fig. 1 is decidedly different from those of neutral reagents ([11] and Scheme 5). The dissociation with pK_a 6.7, which markedly increases the nucleophilicity of the cysteine β -lactamase towards such diverse reagents as iodoacetamide, methyl methanethiolsulphonate and 4,4'-dipyridyl disulphide, and leads to loss of β -lactamase activity [11], is not seen in Fig. 1. Although, in principle, there may be an artifactual explanation of this [31], such an explanation does not seem likely in view of the self-consistent results generated by the neutral reagents mentioned above. It should be noted that under second-order conditions, i.e. when the reaction between free enzyme and free modifying reagent is followed, the nature of a chemical modifying reagent, e.g. whether it is charged or not, should not change the apparent pK_a of an enzyme functional group, but should change only the absolute and relative reactivity of the two enzyme forms. Thus it seems likely that the absence of a change in reactivity of the enzyme with iodoacetate at pH 6.7 must result from a compensation phenomenon. The dissociation of HX, which, since it removes a stabilizing hydrogen bond from the thiolate and which thus should increase the reactivity of the thiolate [32], as seen with the neutral reagents [11], must be accompanied by a change that decreases the reactivity, such that the net effect is zero. Although a conformational change may be the reason for this, it is not necessary to invoke such an effect. The requisite compensation could be achieved electrostatically within the framework of Scheme 5. If HX were positively charged, the increase in reactivity on loss of the hydrogen bond to the thiolate might well be offset by the loss of the positive charge on X. Conversely, if HX were neutral, the same compensation could be achieved through generation of an electrostatically unfavourable (to iodoacetate) negative charge on X. The distinction between these alternatives is discussed further below.

Finally, Fig. 1 indicates that the dissociation of an acidic functional group with pK_a 8.1 leads to a significant decrease in the reactivity of the cysteine β -lactamase towards iodoacetate. The reactivity towards neutral reagents, however, is not affected by this dissociation [11]. This dissociation is therefore unlikely to be that of Lys-73, in the position shown in Scheme 5, since it should then give rise to a further increase in the reactivity of the enzyme towards the neutral reagents. The pK_a of Lys-73 was

Scheme 5.

Scheme 6.

previously assigned to be greater than 9 on this basis [11]. The effect of the adjacent S^- would also argue for a high pK_a for Lys-73. It seems likely, then, that it is the dissociation of another functional group (with pK_a 8.1) that gives rise to the decrease in reactivity towards iodoacetate. This is most probably the conserved Lys-234. Interaction of iodoacetate with the ammonium ion of this residue in the protonated form would then give rise to the enhanced reactivity towards iodoacetate discussed above. It would thereby follow that HX must be the carboxy group of Glu-166, given that this is the only other functional group present (see the Introduction). The complete dissociation sequence of the cysteine β -lactamase active site would therefore be that of Scheme 6.

The most reasonable alternative to Scheme 6, where Lys-234 and Glu-166 exchange places, seems less likely. The pK_a values 6.7 and 8.1 seem better assigned to the former version. Lys-234, which is thought [4,6] to interact with the important carboxylate group of the substrate, should be at some distance from the active-site nucleophile, as in Scheme 6, rather than hydrogen-bonded to it. The crystal structures do not show Lys-234 within hydrogen-bonding distance of the Ser-70 hydroxy group [4-6].

Scheme 6 can then be compared with the results obtained with the native RTEM-2 β -lactamase, where, from the β -lactamase activity and borate inhibition data, two dissociations with pK_a values about 6.0 and 7.5 define the active β -lactamase. The dissociation sequence of the native enzyme should differ from that of the cysteine enzyme, in that the active-site nucleophile, the Ser-70 hydroxy group, will dissociate at a much higher pH than did the thiol group. It will be assumed, as appears to be the case with serine proteinases, that the pK_a of the Ser-70 hydroxy group is greater than 9.0. Thus it seems likely that Scheme 7

would best accommodate the data for the native enzyme, where the pK_a values of 6.0 and 7.5 are assigned to Glu-166 and Lys-234 respectively, both decreased with respect to the corresponding pK_a values of the cysteine enzyme because of the absence of the negative charge that would be on the sulphur atom in the latter enzyme.

The active form of both enzymes is the EH_3 form, containing three functional groups in the acidic form and one in the basic form. The manner in which these might combine to catalyse β -lactam hydrolysis is discussed below. EH_3 is also the species that forms the most stable borate complex. As noted in the Results section, a proton is not released in borate binding but must be taken up by the enzyme, and perhaps by the carboxylate group of Glu-166, to yield structure (II). EH_2 , corresponding to E^{OH} of Scheme 3, forms a much less stable borate complex, which must reflect the importance of electrostatic stabilization of structure (II) by the Lys-234 ammonium ion.

That pK_a of the native enzyme corresponds to the dissociation of Lys-234 rather than to a component of the 'catalytic triad' (Ser-70, Glu-166 and Lys-73) is supported by the methanesulphonyl fluoride results. The reactivity of methanesulphonyl fluoride with the RTEM-2 β -lactamase appears on dissociation of an acidic functional group of pK_a close to that of pK_{a1} in

Scheme 7.

Abbreviation: MSF, methanesulphonyl fluoride.

Scheme 7, and which may reflect the same first dissociation of Scheme 7. The pK_a value from Fig. 2(c) appears lower than those from Figs 2(a) and 2(b), but in view of the quality of the former data the conclusion that different dissociations are involved cannot be made with confidence. The methanesulphonyl fluoride reactivity differs from the β -lactamase activity and borate complex stability in that it remains high throughout the higher pH range. Although the data at high pH are not precise, it is clear that Scheme 7 cannot be fitted to them without assignment of considerable reactivity to EH_2 . This suggests that the dissociation with pK_a does not greatly affect the methanesulphonyl fluoride reaction, i.e. that the catalytic apparatus of the enzyme is still significantly intact in EH_2 , although it is incomplete as far as specific substrates are concerned; the dissociation of Lys-234, which is thought to bind to the carboxylate group of specific substrates [4,6] and to stabilize the negatively charged borate complex (see above), would therefore accommodate these observations.

With respect to the mechanism of action of class A β -lactamases, an uncertainty that must be further addressed is whether HX of Scheme 5 is really the carboxylic acid group of Glu-166. The rationalization presented above and given in Scheme 7 is reasonable, but not by any means tight. Uncertainty on this point is fuelled by the available crystal structures [4–6], and particularly that of the *Bacillus licheniformis* enzyme [6], which place Glu-166 further away from Ser-70 than represented in Scheme 7. Glu-166 is, however, on a flexible (Ω) loop, and its movement to a place more closely adjacent to Ser-70 does not seem impossible. Alternatively, Glu-166 could be separated from Ser-70 by a hydrogen-bonded bridge, such as provided by a water molecule. The pK_a assigned to Glu-166 in the native enzyme, 6.0, certainly suggests a carboxylic acid group whose dissociation is retarded, such as by a hydrophobic environment, or by specific hydrogen-bonding (e.g. to the side-chain carbonyl group of the conserved, in class A β -lactamases, Asn-170), i.e. not a carboxylic acid group on a flexible loop in free solution. It should be also borne in mind that the dissociation constants of Scheme 7 should pertain to the free enzyme, and on binding specific substrates the disposition of some functional groups may change: class A β -lactamases have long given evidence of conformational adaptation to substrates and this phenomenon, as Moews *et al.* have also suggested [6], may relate directly to the flexible loop containing Glu-166. It is noteworthy that in a related class A β -lactamase, that of *Staphylococcus aureus* PC1, pK_a must be considerably lower than 6.0 [33]. This may reflect the difference in the specific environment, but may also signal a different dominant conformation of the free enzyme: the PC1 enzyme may prefer an 'open' Ω -loop conformation in solution and the RTEM-2 enzyme a 'closed' conformation.

A final point should be noted with respect to the identity of HX. The temperature-dependence of pK_a , derived from the methanesulphonyl fluoride data, yielded a ΔH° value of less than 42 kJ/mol (10 kcal/mol) and probably closer to 8 kJ/mol (2 kcal/mol). This is more strongly supportive of a carboxylic acid dissociation than a lysine ammonium dissociation [34]. The observed value could, of course, be specifically influenced by the local environment, and changes in the local environment on dissociation, but ΔH° values for ammonium ions do not generally occur below 42 kJ/mol (10 kcal/mol) or for carboxylic acids above 21 kJ/mol (5 kcal/mol).

In summary, the experiments of this and the previous paper [11] provide evidence that active RTEM β -lactamases, EH_3 (Schemes 6 and 7), require three functional groups in the acidic form and one in basic form. The data are in accord with one of these being anionic, one neutral and two cationic. In the native enzyme the active-site nucleophile, the Ser-70 hydroxy group is

the neutral group, whereas in the cysteine enzyme the active-site nucleophile is probably the anionic Cys-70 thiolate group. The available crystal structures show that there are indeed three functional groups in the vicinity of the primary nucleophile that meet the charge and acid strength requirements of the other functional groups detected, namely Lys-73, Lys-234 and Glu-166. The present work provides evidence that these functional groups are in fact adjacent to the active-site nucleophile when the enzyme is in solution. Of these functional groups, it is likely that two are at a distance to be able directly, or indirectly via a hydrogen-bonded bridge, to hydrogen-bond to the primary nucleophile. One of these must be positively charged and is likely, from the crystal structures, to be Lys-73. The other may be the carboxylate group of Glu-166 (or the carboxylic acid group in the cysteine enzyme). The remaining lysine ammonium ion, which is further from the active-site nucleophile in the crystal structures, is probably involved in binding to the carboxylate group of specific substrates (and interacts with the carboxylate group of iodoacetate, leading to enhanced reaction rates with the cysteine enzyme).

Finally, what can be said about the mechanism of class A β -lactamase catalysis in terms of these structural assignments of catalytic residues? These β -lactamases, it seems, may have a 'catalytic triad', like serine proteinases, but consisting of serine, glutamic acid and lysine residues (with no linear sequence necessarily implied) rather than the Ser-His-Asp of the latter enzymes. It seems likely in the native enzyme that the basic functional group present, the carboxylate group of Glu-166, would act as a general base catalyst of nucleophilic attack by the serine hydroxy group on the β -lactam carbonyl group of the substrate. This has also been proposed by Waley, Page and co-workers [35] on the basis of other evidence [8,35–37] for the presence of a carboxylate group at the class A β -lactamase active site. Nucleophilic attack is apparently also aided, as in serine proteinases, by the presence of an oxy-anion hole [4,38].

The novel feature of the β -lactamase active site, distinguishing it from serine proteinases, is the presence of a lysine ammonium ion (Lys-73). Herzberg & Moulton have proposed a rather novel mechanism wherein this ion catalyses proton transfer electrostatically [4]. Although the positive charge would certainly depress the pK_a of the hydroxy group of Ser-70 [11], we think it more likely that the main electrostatic role of this ion is to provide stabilization of the anionic tetrahedral intermediate, beyond that provided by the oxy-anion hole and the α_2 -helix macrodipole [4,6]. It is also possible that the positive charge of Arg-244, noted by Knox and co-workers [6] as closely adjacent to the active site, might also assist with this stabilization. It has been recently proposed, and evidence provided for the proposition, that the histidinium ion of serine proteinases (achieved by removal of the serine hydroxy-group proton on formation of the tetrahedral intermediate) is important for electrostatic stabilization of the anionic tetrahedral intermediate [39–41]. Conversely, the histidinium ion is stabilized by the tetrahedral anion, as demonstrated in anionic transition-state analogue inhibitor complexes [25,42–44]. We propose that since an enzyme, such as a β -lactamase, employing a carboxylate group as a general base, does not directly generate a stabilizing cation on forming the tetrahedral intermediate, another auxiliary functional group is needed to act in this way. The ability of a phosphonate monoester monoanion to inhibit a β -lactamase is also suggestive of an electrostatic role for Lys-73 [45]. It is possible that the Lys-73 ammonium ion also serves to help organize the other functional groups (serine hydroxy group, glutamic acid carboxylic acid group) of the active site in a fashion comparable with that proposed for Asp-102 in trypsin [43].

Collapse of the tetrahedral intermediate with β -lactam-ring-

opening may require general acid catalysis [46], and, if so, this could be provided either by the carboxylic acid group or by the Lys-73 ammonium ion. Employment of the probably more acidic former group would restore the original charge and proton distribution, leaving the enzyme well placed to catalyse deacylation by way of a symmetrical mechanism, where, as with the serine proteinases, water replaces the serine hydroxy group. Direct interaction of either the carboxy group or the ammonium group with the bound substrate is not, however, supported by model building of β -lactams on to the extant crystal structures of the *S. aureus* [4] and *B. licheniformis* [6] β -lactamases. Some conformational change of the protein would appear to be required in either case, although movement of the Glu-166 carboxy group would seem easier, in the manner described above.

Conformational changes may also be needed at the acyl-enzyme stage, so that the amine leaving group can be replaced by a (possibly occluded [4,47]) water molecule. These might well be triggered by conformational relaxation across the C-5–C-6 bond of a penicillin substrate to relieve steric interactions between the β -face substituents on the erstwhile β -lactam ring. Such conformational relaxation has been observed in the inert complexes of the *Streptomyces* R61 DD-peptidase with a variety of β -lactams [48,49].

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