Interaction of clavulanate with the β -lactamases of *Streptomyces albus* G and *Actinomadura* R39

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The reactions of β -lactamases of *Actinomadura* R39 and *Streptomyces albus* G with clavulanate proceed along branched pathways. Both enzymes perform the hydrolysis of this β -lactam with rather high efficiencies ($k_{\text{cat.}} = 18 \text{ s}^{-1}$ and 52 s^{-1} respectively). If large clavulanate/enzyme ratios are used, complete inactivation of the enzymes is observed. At lower ratios, inactivation is only partial. Irreversible inactivation occurs after 400 and 20000 turnovers for the *A.* R39 and *S.* *albus* G enzymes respectively. With the *A.* R39 β -lactamase, a transiently inhibited complex is also formed that remains undetectable with the *S.* *albus* G β -lactamase. Kinetic models are presented and studied for the interaction between clavulanate and both enzymes. A tentative general reaction scheme is also discussed.

Clavulanic acid, a novel β -lactam, was isolated from cultures of *Streptomyces clavuligerus* (Howarth *et al.*, 1976). It was soon found to inhibit or inactivate a large number of β -lactamases, from both Gram-positive and Gram-negative bacteria (Brown *et al.*, 1976; Reading & Cole, 1977; Neu & Fu, 1978; Pechere *et al.*, 1980; Van Klingeren & Van Wijngaarden, 1981). The interaction between this novel β -lactam and the *Escherichia coli* R TEM-2 β -lactamase has been carefully studied by Charnas *et al.* (1978), Fisher *et al.* (1978), Charnas & Knowles (1981) and Reading & Farmer (1981). The results obtained by those authors could only be explained on the basis of a complex branched reaction pathway. A similar reaction scheme appeared to be valid for the β -lactamases of *Klebsiella pneumoniae* E70 and *Proteus mirabilis* C889 (Reading & Farmer, 1981). In contrast, it seemed that the interaction between clavulanic acid and the β -lactamases from *Staphylococcus aureus* (Reading & Hepburn, 1979; Cartwright & Coulson, 1979) and *Enterobacter cloacae* P99 (Reading & Farmer, 1981), other *Escherichia coli* β -lactamases (Labia & Peduzzi, 1978) and the *Streptomyces cacaoi* β -lactamase (Ogawara & Mantoku, 1981) proceeded via a simple linear pathway involving little or no turnover of the β -lactam. Whatever the pathway, there are good reasons to believe that at least some of these interactions involve the formation of an acyl-enzyme with a serine residue of the protein. In a preliminary study (Kelly *et al.*, 1981), the inter-

action between the *Actinomadura* R39 β -lactamase and clavulanate was demonstrated to follow a branched pathway, similar to that observed by Fisher *et al.* (1978), whereas the inactivation of the *Streptomyces albus* G β -lactamase appeared to be a simple linear pathway. No chemical studies have been performed with the minute amounts of enzymes so far purified, but indirect evidence indicates that these enzymes are probably serine enzymes. The present paper describes further studies of these interactions, including the measurement of several kinetic constants involved in the hypothetical reaction pathways.

Materials and methods

The enzymes were those purified previously (Duez *et al.*, 1981, 1982). Clavulanic acid and ^{14}C -labelled clavulanic acid (uniformly labelled, $1.1 \text{ mCi} \cdot \text{mmol}^{-1}$) were gifts from Beecham Pharmaceuticals, Research Division, Betchworth, Surrey, U.K.

The enzyme activity was routinely determined by using the chromogenic substrate nitrocefin (a gift from Glaxo Research Laboratories, Greenford, Middx., U.K.). Hydrolysis of the β -lactam amide bond of this substrate induces the appearance of a chromophoric group with $\lambda_{\text{max.}} = 482 \text{ nm}$ (O'Callaghan *et al.*, 1972). Cephalothin was a gift from Dr. D. Boyd (Eli Lilly and Co., Indianapolis, IN, U.S.A.).

Inactivation experiments

They were performed at 24°C in 50 mM-sodium phosphate, pH 7.0, containing, in the case of the *S. albus* G enzyme, 10% (v/v) ethylene glycol and 10% (v/v) glycerol. The enzyme sample (5–10 µl) was directly added to a solution containing clavulanate and 50 µM-nitrocefins. In the absence of inactivator, the increase of A_{482} remained linear throughout the experiment. In the presence of clavulanate, the rate of hydrolysis of nitrocefins progressively decreased and the pseudo-first-order rate constant for the inactivation was computed from plots of $\ln(v_0/v_t)$ against t , where v_0 and v_t were the rates of increase of A_{482} at zero time and at time t respectively. These experiments were performed with a Beckman DU-8 spectrophotometer. Absorbance measurements were recorded every minute in the 'kinetic' mode, or, if the inactivation was too fast, continuously in the 'time-drive' mode. Under these latter conditions, the mixing dead-time did not exceed 7–8 s.

Stopped-flow experiments were performed with a u.v./visible Aminco-Morrow DW2 apparatus (optical pathlength 10 mm). One of the syringes delivered about 150 µl of enzyme solution and the other exactly the same volume of clavulanate solution.

U.v. spectra of the unstable products were obtained by using a Hewlett-Packard 8450 A u.v.-visible recording spectrophotometer which allowed rapid recording of spectra (1 s/scan). High-voltage paper electrophoresis at pH 6.5 was performed as described by Duez *et al.* (1981).

Results

Kinetics of the interaction between clavulanate and the *S. albus* G β -lactamase

Proposed model. Previous results (Kelly *et al.*, 1981) had been obtained at very high (≥ 50000) [inactivator]/[enzyme] ratios. When this ratio was decreased below 20000, only a partial inactivation of the enzyme was observed. However, no spontaneous recovery of the enzymic activity occurred, even after further prolonged incubation (24 h). These data were in agreement with a simple branched pathway (model 1a, a model for the interaction between clavulanate and the β -lactamase of *S. albus* G):

Model 1(a)

In this model, EC would be the Henri Michaelis complex or a tetrahedral intermediate and EC* the acyl-enzyme.

This type of mechanism has been extensively analysed by Waley (1980) and Tatsumi *et al.* (1981). In the present work, however, we tried to choose conditions where one branch of the pathway had negligible effects on the other. Thus if on the one hand the appearance of P can be monitored after very short times of contact, EC[†] is negligible, and the usual parameters k_{cat} , $[(k_{+2}k_{+3})/(k_{+2} + k_{+3})]$ and K_m $[(k_{+3}K)/(k_{+2} + k_{+3})]$ can be determined. If, on the other hand, the progressive inactivation of the enzyme is monitored at high C_0/E_0 ratios (where C_0 and E_0 are the initial concentrations of clavulanate and enzyme respectively) so that [C] can be considered as constant throughout the process, a pseudo-first-order rate constant:

$$k_i = \frac{(k_i)_{\text{lim}}[C]}{[C] + K_m} \quad (1)$$

can be determined, where $(k_i)_{\text{lim}}$ [i.e. $(k_{+2}K_{+4})/(k_{+2} + k_{+3})$] characterizes the velocity of the inactivation process at saturating concentrations of the inactivator. In the presence of nitrocefins, an apparent rate constant $(k_i)_{\text{nit}}$ is measured. Assuming a competitive interaction:

$$(k_i)_{\text{nit}} = \frac{(k_i)_{\text{lim}}[C]}{[C] + K_{m_1} \left(1 + \frac{[S]}{K_{m_2}} \right)} \quad (2)$$

where K_{m_1} and K_{m_2} are the K_m values for clavulanate and nitrocefins respectively and [S] is the concentration of nitrocefins. The graph of $[C]/[(k_i)_{\text{nit}}]$ against [C] yields a line of slope $1/(k_i)_{\text{lim}}$ and of intercept:

$$\frac{K_{m_1} \left(1 + \frac{[S]}{K_{m_2}} \right)}{(k_i)_{\text{lim}}}$$

It is easy to see that:

$$\frac{k_{+3}}{k_{+4}} = \frac{k_{\text{cat}}}{(k_i)_{\text{lim}}} \quad (3)$$

When the C_0/E_0 ratio is such that incomplete inactivation is observed, the concentration of inactivated enzyme [EC[†]] can be computed from the stable, residual activity and:

$$\frac{[C_0]}{[E_0]} = \frac{k_{+3}}{k_{+4}} \frac{[\text{EC}^{\dagger}]}{[E_0]} \quad (4)$$

Determination of K_m and $(k_i)_{\text{lim}}$ by complete-inactivation experiments. The enzyme (2 ng in 5 µl) was added to 300 µl of 50 µM-nitrocefins containing clavulanate at concentrations ranging from 20 to 190 µM (so that the final enzyme concentration was

Fig. 1. Determination of K_m , $(k_i)_{lim}$ and k_{cat} for the interaction of the *S. albus G* enzyme with clavulanate (a) Plot of $[C]/(k_i)_{nit.}$ against $[C]$ obtained from complete-inactivation experiments. (b) Plot of $[C]/v$ against $[C]$ for the hydrolysis of clavulanate. The ΔA values are those obtained from the linear part of recordings similar to that presented in Fig. 2. For conditions, see the text.

0.2 nm). A_{482} was determined every 60 s and the apparent rate constant $(k_i)_{nit.}$ was calculated as explained in the Materials and methods section. Under the same experimental conditions, K_m was 250 μM (M. Lenzi & J. M. Frère, unpublished work). The plot of $[C]/[(k_i)_{nit.}]$ against $[C]$ yielded values of 22 μM and $2.4 \times 10^{-4} s^{-1}$ for K_m and $(k_i)_{lim}$ respectively (Fig. 1a).

An approximation of the value of K_m could also be obtained by measuring the velocity of nitrocefin hydrolysis during the first few minutes of the reaction, assuming that it represented a true initial velocity and that the interaction was competitive. A value of 28 μM was found from the slope of a Dixon graph.

Determination of the $k_{cat}/(k_i)_{lim}$ ratio by partial inactivation. The enzyme, at a final concentration of 89 nM, was incubated in the presence of 0.72, 1.08 and 1.34 mM-clavulanate. After 20 min, the activity

stabilized at 35, 58 and 71% of the initial value respectively and no further decrease of activity was observed after 24 h. By using eqn. (4), the k_{+3}/k_{+4} (i.e. k_{cat}/k_{lim}) ratio values were 22 900, 20 700 and 21 000, i.e. $21 600 \pm 1200$. Thus a value of $52 s^{-1}$ could be attributed to k_{cat} .

Determination of K_m by the initial rate of clavulanate turnover. During the partial-inactivation experiments reported above, a rapid increase of A_{280} was observed, followed by a slower decrease and stabilization at a value corresponding to the absorbance of the enzyme itself. The maximum absorbance was too high to be attributed to the transient complex EC^* involving the enzyme, and it was therefore assumed that this A_{280} was due to an unstable product, similar to that observed by Kemal & Knowles (1981) with penicillanic acid sulphone. Model 1(a) was accordingly corrected (Model 1b):

The initial rate of the A_{280} increase was monitored with the stopped-flow apparatus. (Note that the value really measured was $A_{280} - A_{310}$.) The final enzyme concentration was 0.044 μM and that of clavulanate varied from 20 to 75 μM . The C_0/E_0 ratio never exceeded 2500 and consequently the enzyme inactivation during the first 5 s was negligible. Fig. 2 shows the two observed phases of the variation of $A_{280} - A_{310}$. The initial rate (v) was estimated from the linear increase in absorbance during the first 5–10 s. However, since the absorption coefficient of the unstable product was not known, the velocities were expressed as $\Delta A \cdot min^{-1}$. From a graph of $[C]/v$ against $[C]$ (Fig. 1b), a K_m value of 44 μM and a V_{max} value $\Delta A = 0.268/min$ could be computed. The K_m value was in fair agreement with that determined in the inactivation experiments (Table 1). Since the k_{cat} value had been obtained by a different method, the $\epsilon_{280} - \epsilon_{310}$ value ($1950 M^{-1} \cdot cm^{-1}$) for the unstable product P could be computed. The data presented in Fig. 2 also permitted estimation of the first-order rate constant, k' , for the disappearance of the A_{280} : a value of $1.37 (\pm 0.13) \times 10^{-2} s^{-1}$ was found (four determinations).

Kinetics of the interaction between clavulanate and the *Actinomadura R39* β -lactamase

Proposed model. To make a choice between the two different schemes proposed previously (Kelly *et al.*, 1981), the following experiment was carried out. The enzyme (12.5 nM) was incubated with 8.9 μM -clavulanate and, after increasing times of

Fig. 2. Recording of $A_{280}-A_{310}$ (optical pathlength 10 mm) after rapid mixing of equal volumes of 88 nM-*S. albus* G β -lactamase and 100 μ M-clavulanate

After 35 s (see the arrow), the rate of recording was decreased by a factor of 10 to permit observation of the absorbance decrease.

contact (5–60 min), 10 μ l samples were removed and added to 300 μ l of 0.25 μ M-cephalothin. The hydrolysis of cephalothin was monitored at 30°C by continuously measuring A_{260} . The rate of cephalothin hydrolysis increased with time and did not reach a constant value before at least 50 min. This increase in velocity corresponded to the spontaneous degradation of EC^{**} into active enzyme and an unidentified product (P' in Model 2 below). The constant velocity observed after 50 min was used to compute the proportion of enzyme which had accumulated in the form of the irreversible complex EC^i at the time of the sampling. On the basis of these data, the graph $\ln[1-(EC^i/E_0)]$ against time (Fig. 3) was biphasic, indicating that EC^{**} could not be an intermediate in the formation of EC^i (Model 2, a model for the interaction between clavulanate and the β -lactamase of *Actinomadura* R39):

Model 2

The formation of EC^i appeared to be rapid during the first 5–6 min of contact ($k_{a1} = 3 \times 10^{-3} \text{ s}^{-1}$) and then to slow down by a factor of 15 ($k_{a2} = 2 \times 10^{-4} \text{ s}^{-1}$).

The k_{+6} value and the relationships between the values of the other constants is obtained by performing experiments under conditions where the effect of only one or two branches of the interaction can be individually analysed.

(1) The value of k_{+6} is obtained either from Fig. 3 ($k_{+6} = k_{a2}$) or from the rate of recovery of enzyme activity when incomplete inactivation has occurred.

(2) The constants k_{cat} and K_m are determined from initial-velocity measurements of formation of P under conditions where formation of both EC^{**} and EC^i is negligible.

(3) The value of $(k_i)_{lim}$ and K_m can be determined by measuring the velocity of enzyme inactivation for large C_0/E_0 ratio values and using periods of incubation such that the spontaneous decay of EC^{**} is negligible. Eqns. (1), (2) and (3) also apply to model 2, k_{+4} being replaced by $k_{+4} + k_{+5}$.

(4) The ratio k_{+4}/k_{+5} can be obtained either from Fig. 3 (see below) or from the ratio (recoverable activity)/(unrecoverable activity) after partial inactivation. Finally, the ratio C_0/E_0 just necessary to obtain complete inactivation yields the ratio $(k_{+3} + k_{+4} + k_{+5})/k_{+4}$.

The value of k_{+6} . A value of $0.2 \times 10^{-3} \text{ s}^{-1}$ was computed from Fig. 3 and a value of $0.3 \times 10^{-3} \text{ s}^{-1}$ was obtained on the basis of enzyme-activity recovery after partial inactivation.

The value of k_{+5}/k_{+4} . Extrapolation of the late linear portion of the graph (Fig. 3) to $t = 0$ yielded $[\ln(v_t/v_0)]_{ex} = -1.1$. This value indicated that 33% of the enzyme was transiently involved in complex EC^{**} before it was definitively immobilized in complex EC^i . A k_{+5}/k_{+4} value of 0.5 was obtained. However, measurements of the ratio (recoverable activity)/(unrecoverable activity) after partial inactivation yielded a higher, although not very reproducible, value of 1.2 ± 0.5 (five experiments). All these results indicated that the k_{+4} and k_{+5} values were very similar and it was assumed that $k_{+4} \simeq k_{+5}$.

Determination of the approximate value of $k_{cat}/(k_i)_{lim}$. The C_0/E_0 ratio just necessary to induce complete, irreversible inactivation was measured. A constant concentration of clavulanate (10 μ M) was

incubated with the enzyme at concentrations ranging from 13 to 40 nM (C_0/E_0 from 770 to 250). A complete, irreversible inactivation was observed at a minimal C_0/E_0 ratio of about 400. It followed that $(k_{+3} + k_{+4} + k_{+5})/k_{+4} = 400$, and since $k_{+4} \simeq k_{+5}$, both k_{+4} and k_{+5} were negligible when compared with k_{+3} , so that $k_{+3}/k_{+4} = 400$. Moreover, $k_{cat}/(k_i)_{lim} = k_{+3}/(k_{+4} + k_{+5})$, and since $k_{+4} \simeq k_{+5}$, $k_{cat}/(k_i)_{lim} \simeq 200$.

Table 1. Values of the constants (24°C)

	<i>Streptomyces albus</i> G β -lactamase	<i>Actinomadura</i> R39 β -lactamase	Method
1. $(k_i)_{lim}$	$2.4 \times 10^{-3} s^{-1}$	$0.09 s^{-1}$	Inactivation at high C_0/E_0 ratio
2. K_m	22 μM	153 μM	Inactivation at high C_0/E_0 ratio
	28 μM	97 μM	Competition with nitrocefin
	44 μM	164 μM	Initial rate of clavulanate turnover
3. $k_{cat.}$	$21\,600 \pm 1200$	200	Partial inactivation (<i>S. albus</i> G)
	$(= k_{+3}/k_{+4})$	$(= k_{+3}/k_{+4} + k_{+5})$	Number of turnovers before inactivation (R39)
4. $k_{cat.}$	$50 s^{-1}$	$18 s^{-1}$	Calculated from lines 1 and 3 above
5. k_{+4}/k_{+5}	k_{+5} step not detectable	≈ 1	Fig. 3 and recoverable activity after partial inactivation
6. k_{+6}	Not detectable	$(0.2-0.3) \times 10^{-3} s^{-1}$	Fig. 3 and rate of recovery after partial inactivation
7. k'	$1.37 (\pm 0.13) \times 10^{-2} s^{-1}$	$1.34 (\pm 0.3) \times 10^{-2} s^{-1}$	Rate of decrease of A_{280} in clavulanate-turnover experiments
8. k_{+2}/K	$(1-2.3) \times 10^6 M^{-1} \cdot s^{-1}$	$(1.2-1.8) \times 10^5 M^{-1} \cdot s^{-1}$	From lines 2 and 4 above
9. $\varepsilon_{280}-\varepsilon_{310}$ of P	$1950 M^{-1} \cdot cm^{-1}$	$2060 M^{-1} \cdot cm^{-1}$	Initial rate of clavulanate turnover and from line 4 above

Determination of K_m and $(k_i)_{lim}$ by complete-inactivation experiments. The enzyme samples (8–24 ng, i.e. 1.7–5 nM final concns.) were added to 300 μl of 56 μM -nitrocefin solutions containing concentrations of clavulanate ranging from 20 to 230 μM . The ratio C_0/E_0 was always larger than 10000. The hydrolysis of nitrocefin was continuously monitored during 2 min (see Fig. 4). As described above, the values of $(k_i)_{nit.}$ were determined and the graph of $[C]/(k_i)_{nit.}$ against $[C]$ yielded values of $0.09 s^{-1}$ and 153 μM for $(k_i)_{lim}$ and K_m respectively. The K_m value for nitrocefin was that measured previously (85 μM ; Duez *et al.*, 1982). The velocities of nitrocefin hydrolysis during the first 15 s of reaction were regarded as true initial velocities. Assuming a competitive interaction, a value of 97 μM was found for the K_m of clavulanate.

Determination of K_m by the initial rate of clavulanate turnover. As with the *S. albus* G enzyme, a transient increase of A_{280} was observed at C_0/E_0 ratios lower than 400. By using a 0.22 μM enzyme concentration in stopped-flow experiments, the initial rate was estimated from the linear increase of A_{280} during the first 5 s (clavulanate concentrations ranged from 25 to 150 μM). The values of K_m (169 μM) and V ($\Delta A = 0.495 min^{-1}$) were computed from the graph of $[C]/v$ against $[C]$.

Since $k_{cat.} \approx 200 (k_i)_{lim}$, one could compute $k_{cat.} \approx 18 s^{-1}$, and from both this value and that of $V_{max.}$ ($\Delta A min^{-1}$) as determined above, a $\varepsilon_{280}-\varepsilon_{310}$ value of $2060 M^{-1} \cdot cm^{-1}$ was attributed to the unstable product, P.

Finally, measurements of the rate of decrease of $A_{280}-A_{310}$ after longer contact times yielded a value of $1.34 (\pm 0.3) \times 10^{-2} s^{-1}$ for the first-order rate constant (k') characteristic of the spontaneous decay of P.

Fig. 3. Plot of $\ln(v_t/v_0)$ against time during inactivation of the *A. R39* β -lactamase by clavulanate

v_0 and v_t are the velocities of cephalothin hydrolysis. Enzyme (12.5 nM) was incubated with 8.5 μM -clavulanate. A sample was immediately removed and the velocity of cephalothin hydrolysis determined (v_0). Other samples were removed after increasing times of contact and added to the cephalothin solution. For these samples, the velocity of cephalothin hydrolysis (v_t) was that observed after reaching a maximum constant rate (50 min or more); v_t/v_0 was thus proportional to $(E_0 - EC^t)/E_0$.

Attempts to identify products P and P*

The good agreement between the values of k' and of $\varepsilon_{280}-\varepsilon_{310}$ computed for product P obtained with

Fig. 4. Time course of nitrocefin hydrolysis in the absence (a) and in the presence of $20\mu\text{M}$ (b) or $40\mu\text{M}$ (c) clavulanate

The broken portion of the curves represents the mixing dead time. For other conditions, see text.

Fig. 5. U.v. spectra of the unstable products formed by the *S. albus* G (continuous line) and *A. R39* (broken line) enzymes

For conditions, see the text. In each case the spectrum recorded after 20 min was subtracted from the most intense spectrum obtained after 25 s.

both enzymes suggested that the same unstable compound was formed by the action of both enzymes. A complete u.v. spectrum of this unstable compound was obtained with a rapid-scan recording spectrophotometer. To 1 ml of $150\mu\text{M}$ -clavulanate, $4\mu\text{g}$ of *S. albus* G or $5\mu\text{g}$ of R39 enzyme were added.

Spectra (1 s) were recorded every 5 s during 1 min and, eventually, after 20 min. This last spectrum was subtracted from the most intense spectrum in order to eliminate the slight contribution of the enzymes to the spectra. At a concentration of $150\mu\text{M}$, clavulanate itself and the final product of degradation of P had no significant absorbance between 240 and 350 nm. Fig. 5 shows the spectra obtained for the unstable product formed by both enzymes. The spectra were similar and had a maximum at 265 nm. From these spectra and the ϵ_{280} – ϵ_{310} values determined above, ϵ_{264} values of $4400\text{M}^{-1}\cdot\text{cm}^{-1}$ and $4800\text{M}^{-1}\cdot\text{cm}^{-1}$ were found for the product formed by the *S. albus* G and *A. R39* β -lactamases respectively.

Clavulanate ($2\mu\text{l}$ of a 10 mM solution) was mixed with $0.5\mu\text{g}$ of *S. albus* G β -lactamase in $200\mu\text{l}$ of 50 mM-sodium phosphate buffer, pH 7. When the A_{265} value reached a maximum (about 0.10), $10\mu\text{l}$ of a 1 M solution of NaOH was added. The A_{265} value immediately increased to 0.5 and remained stable. The spectrum exhibited a maximum at 259 nm. This spectrum and those obtained by adding NaOH after P had totally decayed into P* or by adding NaOH to clavulanate alone were superimposable. In all cases, the effects of varying the pH on the spectrum were fully reversible and indicated an apparent pK of 11. Further attempts to characterize P* were carried out with [^{14}C]clavulanate. To $300\mu\text{l}$ solutions of 1 mM-clavulanate ($0.3\mu\text{Ci}/\mu\text{mol}$), the *S. albus* G β -lactamase was added at concentrations ranging from 0.2 to $0.004\mu\text{M}$. After 15 min, the residual enzyme activity was determined (on 1–50 μl samples, depending on the concentration of enzyme used), and in parallel with this, all the samples (50 μl) were submitted to a 30-min high-voltage paper electrophoresis at pH 6.5 and 60 V/cm. Under these conditions, the mobility of intact clavulanate was $18\text{cm}\cdot\text{h}^{-1}$. When no residual enzyme activity was observed ($C_0/E_0 > 22000$), only residual clavulanate was detected. In the other cases, the clavulanate had been turned over by the enzyme and had completely disappeared but, nevertheless, no significant radioactivity peak could be detected on the electrophoretograms.

Discussion

The interactions between clavulanate and both *S. albus* G and *A. R39* β -lactamases follow branched pathways. Both enzymes effectively catalyse the hydrolysis of clavulanate, with k_{cat} values of 50 and 18s^{-1} for the *S. albus* G and *A. R39* β -lactamases respectively. The K_m value is similar to, and slightly lower than, those observed with good substrates in the case of the *A. R39* (Duez *et al.*, 1982) and *S. albus* G β -lactamases (Duez *et al.*, 1981) respectively. The number of turnovers before inactivation

is 20000 for the *S. albus* G enzyme and 400 for the *A. R39* enzyme.

The observation that an unstable u.v.-absorbing product was formed upon turnover of clavulanate by both enzymes is central to the present study. This product has properties consistent with an enamine structure such as compound (I), although it is diffi-

cult to ascertain the structure of the right-hand portion of the molecule (Scheme 1). Kemal & Knowles (1981) described the properties of compound (II) obtained from penicillanic sulphone by hydrolysis at high pH; it exhibits an absorption maximum at 267 nm ($\epsilon = 20000$) and has a very short half-life of about 2–4 s near pH 7.0 and at 30°C. In comparison, the compound observed in the course of the present work exhibits an absorption maximum at 264 nm ($\epsilon \approx 4000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and has a half-life of 50 s at pH 7.0 and 24°C. This difference in the ϵ values may arise from the fact that the value reported by Kemal & Knowles (1981) was measured at pH 12. Moreover, both P and compound (II) decompose to yield (a) product(s) having similar pH-dependent spectral characteristics, i.e. λ_{max} at 258 nm at pH > 12 and loss of absorption at a lower pH with a pK of about 11. This degradation product has not been identified. It is probably volatile or paraffin (kerosene)-soluble, since it was lost before or during the high-voltage electrophoresis. Upon reaction between [^{14}C]clavulanate and the *E. coli* R TEM-2 β -lactamase, Reading & Farmer (1981)

(I)

(II)

Scheme 1. Possible pathways for the interaction between clavulanate and the *A. R39* β -lactamase

EC^I (1) may be several complexes. One of them is re-activated by hydroxylamine and another might be a Michael adduct of a side chain of the enzyme on the nitrogen atom of the clavulanate moiety (Kelly *et al.*, 1981; Charnas & Knowles, 1981).

also observed the formation of low-molecular-weight fragments. If the u.v.-absorbing enamine compound is not the primary product of the enzymic turnover (see Scheme 1), the transformation of the primary product into the postulated enamine must be very rapid, since no delay in the increase of A_{280} is observed. The exact structure and fate of the various inactive EC^{**} and EC^I complexes are more difficult to discuss. In Model 2, EC^{**} is assumed to decay directly, yielding free enzyme and an unidentified product, P' . However, Charnas & Knowles (1981) and Brenner *et al.* (1981) have proposed an alternative and attractive hypothesis: the k_{+5} step would be the tautomerization of the acyl-enzyme to a more stable enzyme-bound enamine and the k_{-5} step the slow reversal of this reaction. In Scheme 1, both this possibility and that of a direct hydrolysis of the transiently inhibited enzyme (k_{+6} step) are represented. Scheme 1 starts with the tetrahedral intermediate, which is assumed to be in rapid equilibrium with the Michaelis complex, itself in rapid equilibrium with the free enzyme and free clavulanate. It should be stressed that this Scheme represents a possible explanation that is in good agreement with the experimental observations. Strictly speaking, Scheme 1 is only valid for the *A. R39* enzyme, since no transiently inhibited complex, EC^{**} , has been detected with the *S. albus* G enzyme. That does not necessarily mean that, for the *S. albus* G β -lactamase, $k_{+5} = 0$. For example, if k_{+5} is substantially smaller than k_{+4} , a small amount of EC^{**} may escape detection. If, in addition, k_{-5} or k_{+6} is also small, both formation and degradation of EC^{**} may remain below the limits of experimental error. Finally, if k_{+5} and k_{+6} (or k_{-5}) are large, a steady state can be rapidly reached in the branch containing the transiently inhibited enzyme. In this respect, one should mention that the enzyme itself may play an active role in the steps characterized by k_{+5} and/or k_{+6} (or k_{-5}). The importance of the enzyme in the various rearrangements is emphasized by the 40-fold decrease in $(k_1)_{lim}$ observed with the *S. albus* G enzyme when compared with the *A. R39* enzyme. According to the results of Charnas & Knowles (1981), it is probable that this or these step(s) also involve(s) rearrangements of the clavulanate moiety.

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References

- Brenner, D. G., Knowles, J. R. & Rihs, G. (1981) *Biochemistry* **20**, 3680–3687
- Brown, A. G., Butterworth, D., Cole, M., Hanscomb, G., Hood, J. D., Reading, C. & Robinson, G. N. (1976) *J. Antibiot.* **29**, 668–669
- Cartwright, S. J. & Coulson, A. F. W. (1979) *Nature (London)* **278**, 360–361
- Charnas, R. L. & Knowles, J. R. (1981) *Biochemistry* **20**, 3214–3219
- Charnas, R. L., Fisher, J. & Knowles, J. R. (1978) *Biochemistry* **17**, 2185–2189
- Duez, C., Frère, J. M., Klein, D., Noël, M., Ghuysen, J. M., Delcambe, L. & Dierickx, L. (1981) *Biochem. J.* **193**, 75–82
- Duez, C., Frère, J. M., Ghuysen, J. M., Van Beeumen, J., Delcambe, L. & Dierickx, L. (1982) *Biochim. Biophys. Acta* **700**, 24–32
- Fisher, J., Charnas, R. L. & Knowles, J. R. (1978) *Biochemistry* **17**, 2180–2184
- Howarth, T., Brown, A. & King, T. (1976) *J. Chem. Soc. Chem. Commun.* 266–267
- Kelly, J., Frère, J. M., Duez, C. & Ghuysen, J. M. (1981) *Biochem. J.* **199**, 137–143
- Kemal, C. & Knowles, J. R. (1981) *Biochemistry* **20**, 3688–3695
- Labia, R. & Peduzzi, J. (1978) *Biochim. Biophys. Acta* **526**, 572–579
- Neu, H. C. & Fu, K. P. (1978) *Antimicrob. Agents Chemother.* **14**, 650–655
- O'Callaghan, C., Morris, A., Kirby, S. A. & Shingler, A. H. (1972) *Antimicrob. Agents Chemother.* **1**, 283–288
- Ogawara, H. & Mantoku, A. (1981) *J. Antibiot.* **34**, 1341–1346
- Pechere, J. C., Letarte, R. & Guay, R. (1980) *Curr. Chemother. Infect. Dis.* **1**, 323–326 (*Proc. Int. Cong. Chemother. 11th*)
- Reading, C. & Cole, M. (1977) *Antimicrob. Agents Chemother.* **11**, 852–857
- Reading, C. & Hepburn, P. (1979) *Biochem. J.* **179**, 67–76
- Reading, C. & Farmer, T. (1981) *Biochem. J.* **199**, 779–787
- Tatsumi, S., Yago, N. & Hosoe, M. (1981) *Biochim. Biophys. Acta* **662**, 226–235
- Van Klingeren, B. & Van Wijngaarden, L. J. (1981) *J. Antimicrob. Chemother.* **8**, 79–80
- Waley, S. G. (1980) *Biochem. J.* **185**, 771–773