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# Clavulanate inactivation of *Staphylococcus aureus* β-lactamase

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The interaction of clavulanic acid with β-lactamase from *Staphylococcus aureus* was investigated, particularly with a view to determining whether conformational effects are involved. The inactivation at neutral pH is essentially stoichiometric, leading to an inactive species with an enamine chromophore. Two forms of the enamine were observed, the first-formed having a positive ellipticity with a maximum near 290 nm. This species slowly converted into the stable form of the inactivated enzyme that had a negative ellipticity with a minimum at 275 nm. This change in sign of the ellipticity of the enamine is consistent with the previously proposed *cis–trans* isomerization of the enamine [Cartwright & Coulson (1979) Nature (London) **278**, 360–361). Both the far-u.v. c.d. and the intrinsic viscosity of the inactivated enzyme indicated that negligible change in conformation of the enzyme accompanied inactivation. The rates of inactivation and enamine formation were compared at low temperatures, where the initial rates were slow enough to be monitored. The rate of loss of 95% of the catalytic activity was almost 100-fold faster than the rate of formation of the first-formed enamine species. The remaining 5% activity was lost with a rate comparable with that for formation of the initial enamine. The simplest explanation of these results is that a relatively stable acyl-enzyme intermediate builds up initially and more slowly partitions between turnover (hydrolysis) and enamine formation. The initially formed enamine is in the *cis* conformation but slowly isomerizes to the more stable *trans* form.

## INTRODUCTION

The recently determined [1] high-resolution crystal structure of β-lactamase (EC 3.5.2.6) from *Staphylococcus aureus* reinforces the considerable evidence that this enzyme and other class A β-lactamases are acylated at an active-site serine residue by substrates and by several mechanism-based inhibitors [2]. Acylation of the enzyme by a substrate analogue such as clavulanic acid is accompanied by ring cleavage adjacent to the heteroatom of the five-membered ring, leading to an imine intermediate with the potential both for hydrolysis to products and for formation of a long-lived enzyme-bound enamine [3–5]. The molecular details of clavulanate inactivation are not yet well understood. With the staphylococcal enzyme rapid appearance of near-u.v. absorption, as expected for enamine formation, is accompanied by loss of catalytic activity. A slower change in the u.v. spectrum has been proposed to reflect *cis–trans* isomerization of the enamine to the more stable configuration [4]. In the case of the TEM enzyme from *Escherichia coli* incubation with clavulanate leads to three competing reactions: turnover, transient inactivation and irreversible inactivation [6,7]. The irreversibly inactivated enzyme was reported to exist in three different forms, distinguishable by their isoelectric points [7]. However, permanent inactivation of the staphylococcal enzyme by clavulanic acid has not been found, although inhibition is prolonged [4,8]. Irreversible inactivation of the TEM enzyme by penicillanic acid sulphone has been reported to involve transimination of the imine form of the acyl-enzyme by a lysine group in the active site [5,7].

It is not known whether the protein conformation is affected by interaction with clavulanic acid, although it has been suggested that extensive conformational changes are responsible for irreversible loss of catalytic activity brought about by a penicillin sulphone inhibitor of *Bacillus cereus* β-lactamase I [9]. Since staphylococcal β-lactamase is readily and reversibly perturbed to partly unfolded or expanded states by low concentrations of denaturants (state H and the more compact state I) [10–12] or by low pH (state A) [13], it would not be surprising if substantial conformational changes were to accompany inhibition by clavulanic acid. The present study was undertaken to determine whether conformational changes were associated with clavulanate-induced inactivation, and to clarify whether the observed absorbance changes associated with the enamine arose from *cis–trans* isomerization of the enamine or were due to alternative forms of the enamine (for example, arising from transimination or conformational change), and to ascertain the relationship between loss of catalytic activity and enamine formation.

## EXPERIMENTAL

The extracellular β-lactamase from *Staphylococcus aureus* PC1 was isolated and purified [14] to give a specific activity of 111–145 kat/mol. Clavulanic acid (sodium salt) was a gift from Beecham Pharmaceuticals, Betchworth, Surrey, U.K. Unless otherwise stated, solutions were prepared in Pipes buffer, pH 6.7, which contained 90 mM-NaCl, 1 mM-EDTA and 10 mM-Pipes adjusted with NaOH to pH 6.7 (at 20 °C). Solutions were passed through a 0.45 μm-pore-size filter before use. C.d.

Abbreviations used: pH*, apparent pH of the cryosolvent as measured by a pH-meter with a glass electrode.

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measurements were made with a Jobin Yvon Dichrographe IV or an AVIV model 60DS instrument. β-Lactamase activity in 1 mM-benzylpenicillin was determined from the rate of decrease in $A_{240}$ at 25 °C [15]. For experiments below 0 °C solutions contained 20% (v/v) ethylene glycol, 30% (v/v) methanol, 50 mM-sodium acetate and 25 mM-sodium/potassium phosphate buffer, pH* 6.5. Reactions were monitored with a Cary model 118 or a Hewlett–Packard model HP-8452 spectrophotometer (1 cm-light-path cell). First-order kinetics were analysed by unconstrained non-linear regression [16–18]. For viscosity measurements the enzyme, dialysed against Pipes buffer, pH 6.7, was diluted with the diffusate in which clavulanic acid was dissolved. A glass capillary viscometer, solution capacity 1.5 ml, was used. The temperature was 25±0.01 °C. Unless otherwise stated the molar ratios of clavulanate to enzyme were 2:1.

## RESULTS AND DISCUSSION

### U.v. absorption of clavulanic acid-treated β-lactamase

Molar ratios in excess of 1.5 clavulanate led to greater than 98% loss of enzymic activity. β-Lactamase (69 μM) was incubated at 25 °C at pH 6.7 with 139 μM-clavulanic acid, and samples were diluted 100-fold for measurement of the catalytic activity towards 1 mM-benzylpenicillin. As expected [4,8], there was a rapid and prolonged decrease in catalytic activity: samples taken during a period of 2 min to 4 h posessed no detectable catalytic activity (less than 2% of controls); reversibility of the inhibition after exhaustion of the clavulanate was apparent in that up to 45% of the catalytic activity had returned in samples taken after 21–24 h. This is consistent with the complete re-activation previously noted for the enzyme from *Staphylococcus aureus* at lower pH values and in contrast with the apparent lack of reversibility in the case of the TEM enzyme [8,19]. A control experiment showed that residual free clavulanic acid present in the diluted samples did not interfere with the measurement of catalytic activity by competitive inhibition of benzylpenicillin hydrolysis. In agreement with ref. [4], incubation of the enzyme with clavulanic acid led to rapid formation of a chromophore ($\lambda_{max.}$ 295 nm, $\epsilon = 1.65 \times 10^4$ M$^{-1}$·cm$^{-1}$) that was slowly replaced by a second chromophore ($\lambda_{max.}$ 277 nm, $\epsilon = 2.53 \times 10^4$ M$^{-1}$·cm$^{-1}$) with an isosbestic point at 290 nm.

The species with absorbance maxima at 295 nm and 277 nm have previously been assigned to the enamine in the *cis* and *trans* configurations respectively [4]. The time-dependent changes in absorbance at 295 and 277 nm at selected temperatures are shown in Fig. 1. As can be seen the increase in absorbance at 295 nm, corresponding to enamine formation, was rapid (half-life < 10 s) at 25 °C, but became readily detectable at lower temperatures. The subsequent reaction, corresponding to the putative enamine isomerization, was manifested as a decrease in absorbance at 295 nm and an increase at 277 nm. Some typical rate data are given in Table 1.

### C.d. of clavulanic acid-treated β-lactamase

In similar experiments to the above the reaction was monitored by measurement of the near-u.v. c.d. Mixing enzyme and clavulanate at 25 °C resulted in a very rapid

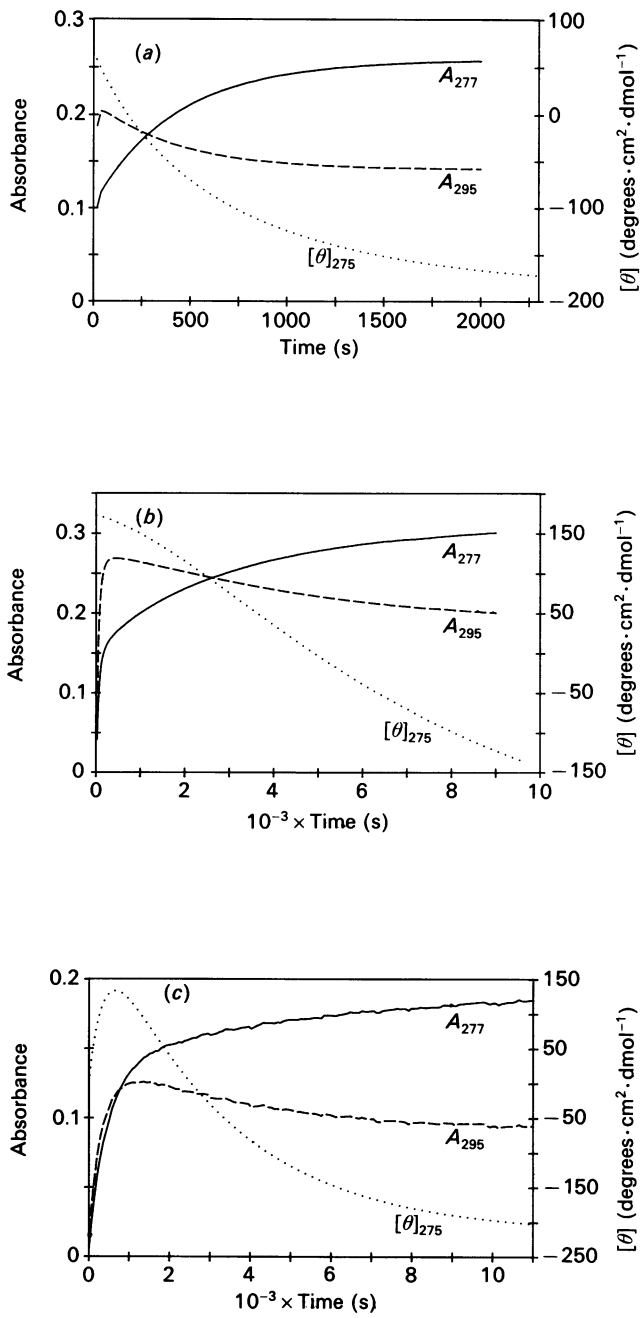



**Fig. 1. Formation and isomerization of the enamine acyl-enzyme**

——, Absorbance at 277 nm; – – – –, absorbance change at 295 nm; · · · · ·, change in ellipticity at 275 nm. Panel (*a*) shows the time-dependent changes at 25 °C. Conditions were pH 6.7, 69 μM-β-lactamase and 140 μM-clavulanic acid for the absorbance changes, and pH 6.7, 32 μM-β-lactamase and 69 μM-clavulanic acid for the change in ellipticity. Panel (*b*) was for 0.0 °C. Conditions were pH 6.7, 14.4 μM-β-lactamase and 53 μM-clavulanic acid for the absorbance experiments and 32 μM-β-lactamase and 69 μM-clavulanic acid for the ellipticity measurements. Panel (*c*) shows the data for −10.0 °C. Conditions were as follows: the solvent was 20% ethylene glycol/30% methanol/50 mM-sodium acetate/25 mM-sodium/potassium phosphate buffer, pH* 6.5; the β-lactamase concentration was 5.4 μM (lower than in the above experiments because of limited solubility in the cryosolvent) and the clavulanic acid concentration was 53.2 μM.

**Table 1. Rate constants for enamine formation and isomerization during the clavulanate inactivation of β-lactamase**

The conditions were Pipes buffer, pH 6.7, for 0 to 25 °C, and 20% ethylene glycol/30% methanol/sodium acetate/phosphate cryosolvent buffer for 0 to −10 °C. At the higher temperature range only the rate of the slower phase is shown.

| Temperature | $k_{obs.}$ ($s^{-1}$) | | | |
|---|---|---|---|---|
| | $A_{295}$ | $A_{277}$ | $\theta_{275}$ | $\theta_{288}$ |
| 25.0 | $6.5\times10^{-3}$ | $6.4\times10^{-3}$ | $6.3\times10^{-3}$ | $6.9\times10^{-3}$ |
| 5.3 | $4.5\times10^{-4}$ | $5.8\times10^{-4}$ | | |
| 0.3 (aqueous) | $1.6\times10^{-2}$ | $1.4\times10^{-2}$ | $1.4\times10^{-2}$ | |
| | $2.3\times10^{-4}$ | $3.3\times10^{-4}$ | $2.2\times10^{-4}$ | $2.3\times10^{-4}$ |
| 0.0 (cryosolvent) | $0.9\times10^{-2}$ | $1.2\times10^{-2}$ | | |
| | $1.6\times10^{-3}$ | $1.6\times10^{-3}$ | | |
| −5.0 | $0.9\times10^{-2}$ | $1.2\times10^{-2}$ | | |
| | $1.2\times10^{-3}$ | $1.0\times10^{-3}$ | | |
| −10.0 | $2.2\times10^{-3}$ | $3.1\times10^{-3}$ | $3.6\times10^{-3}$ | |
| | $2.0\times10^{-4}$ | $2.5\times10^{-4}$ | $3.4\times10^{-4}$ | |

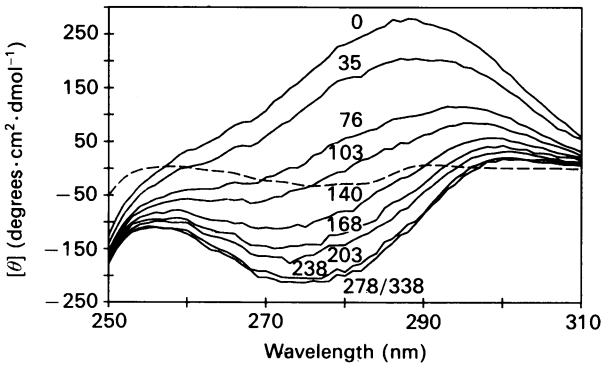



**Fig. 2. Near-u.v. c.d. spectra during the reaction of β-lactamase and clavulanic acid**

Spectra were collected as a function of time throughout the reaction. Selected spectra are shown, the numbers associated with them reflect the time (in minutes) the spectrum was started after initiation of the reaction. The spectra have been corrected by subtraction of the spectrum of the free enzyme and that of clavulanic acid alone. The spectrum shown by the broken line corresponds to that of the free enzyme under the same conditions. The reaction conditions were Pipes buffer, pH 6.7, at 0 °C, the β-lactamase concentration was 32 μM and the clavulanic acid concentration was 69 μM.

increase in the ellipticity, corresponding to the initial rapid increase in absorbance observed at 295 nm, followed by a slower decrease in ellipticity (Fig. 1). The near-u.v. c.d. spectra as a function of time during the reaction are shown in Fig. 2. The spectra at early times, corresponding to the species with absorbance maximum at 295, have positive ellipticities with a maximum around 288 nm. As the reaction proceeds the ellipticity changes to a negative trough with a minimum at 275 nm, corresponding to the species with absorbance maximum

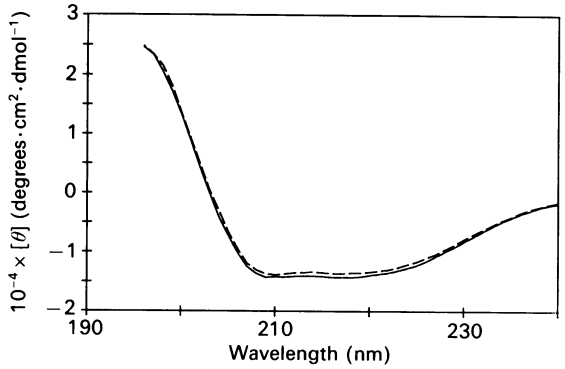



**Fig. 3. Comparison of the far-u.v. c.d. spectra of native β-lactamase and clavulanate-inactivated enzyme**

The experimental conditions were the same as for Fig. 2. ——, Spectrum of native enzyme; – – – –, spectrum of clavulanate-inactivated enzyme.

at 277 nm. As shown in Table 1, the rates for the transients measured by change in ellipticity are in good agreement with the rates measured for the change in absorbance at 295 and 277 nm. At the completion of this reaction the minimum ellipticity at 275 nm was about 5 times more negative than the minimum near-u.v. ellipticity of the native protein (Fig. 2). Because the near-u.v. ellipticity includes contributions from both forms of the enamine as well as from the asymmetry of the environment of tyrosine residues, it is not possible to rule out completely a change in protein conformation during the enamine interconversion. However, a change in protein conformation to produce a state similar to either H [10], A [13] or I [12] would entail an increase of ellipticity approaching 40 deg·cm²·dmol⁻¹ [10], and there was no evidence for such a change with kinetics distinct from that of the main transition.

From analysis of far-u.v. c.d. [20] staphylococcal β-lactamase has been estimated to contain 27% α-helix and 32% β-sheet structure on the basis of the ellipticity at 208 and 222 nm [10]. This gives a total of α+β structure in good agreement with that found by X-ray crystallography [1]. The far-u.v. c.d. spectrum was not significantly affected by incubating 25 μM-enzyme with 51 μM-clavulanic acid for time periods corresponding to completion of the slow increase in absorbance at 277 nm (Fig. 3). These results rule out a significant change in secondary structure in the clavulanic acid-inactivated enzyme. In summary, the results of the c.d. measurements are consistent with a simple interconversion between two forms of the enamine acyl-enzyme, and the inversion in sign of the ellipticity strongly supports the suggested *cis–trans* isomerization of the initially formed enamine derivative [4].

### Reduced specific viscosity of clavulanic acid-treated β-lactamase

The intrinsic viscosity of β-lactamase has been shown to be sensitive to the unfolding of the enzyme to state H [10], although the intrinsic viscosity of the less expanded state I is unknown. The viscosity of the inactivated enzyme was therefore compared with that of a control sample. A solution of enzyme was mixed with clavulanic acid to give final concentrations of 20 mg/ml and 0.7 mM respectively, and the mixture was transferred to the

viscometer. In a control experiment clavulanic acid was absent. After equilibration, the flow time was measured at intervals up to 65 min after mixing. The flow times for solvent in which 0.7 mM-clavulanic acid was present and absent were 84.8 and 84.9 s respectively. No significant dependence of the viscosity on time of reaction was noted. The values of reduced specific viscosity of the enzyme solution containing clavulanic acid and the control sample were $3.08 \pm 0.09$ ml/g (mean $\pm$ S.D. for 22 samples) and $2.93 \pm 0.08$ ml/g (means $\pm$ S.D. for seven samples) respectively. Although this difference in reduced specific viscosity is statistically significant at the 2% confidence level, suggesting a small expansion or increase in asymmetry of the inactivated enzyme, the effect is small compared with the 2.5-fold increase expected if the inactivated enzyme were to unfold to the same extent as the intermediate state H [13,14].

### Kinetics of enamine formation at low temperature

The rates of formation of the enamine derivative (increase in $A_{295}$) and of inactivation were both rapid at room temperature, so that it was not possible to identify the rate-limiting process for loss of catalytic activity. These reactions were therefore investigated at lower temperatures. At temperatures less than 0 °C the rate of formation of enamine becomes sufficiently slow for manual sampling, and the rate of isomerization of the enamine becomes very slow (Fig. 1*c*). For the formation of the enamine, as manifested by the increase in absorbance at 295 nm, there was no significant difference between the rate observed at a temperature close to 0 °C in aqueous solution and in the cryosolvent, suggesting that the rate-determining step in the process is not affected by the cryosolvent (Fig. 4). However, the rate of isomerization increased by 4.5-fold on going from aqueous solution to cryosolvent. We attribute this to the fact that the cryosolvent is a more destabilizing solvent than water for proteins, and thus the cryosolvent preferentially facilitates the isomerization reaction. We have determined that the cryosolvent has no adverse effects on the structural or catalytic properties of the enzyme. Comparison of the rate of loss of catalytic activity with the initial enamine formation (increase in absorbance at 295 nm) at $-10$ °C showed that the catalytic activity was lost in a biphasic process with a rate constant of 0.3 $s^{-1}$ for the faster phase, corresponding to loss of 95% of the catalytic activity (Fig. 5). This was about 100-fold greater than the rate of formation of the enamine ($2.2 \times 10^{-3}$ $s^{-1}$). The remaining catalytic activity was lost with a rate constant of 0.01 $s^{-1}$, about 4.5-fold faster than the rate of enamine formation; the corresponding rate constant for the slower phase was $2.0 \times 10^{-4}$ $s^{-1}$. No evidence of reactivation was noted during the assays for catalytic activity.

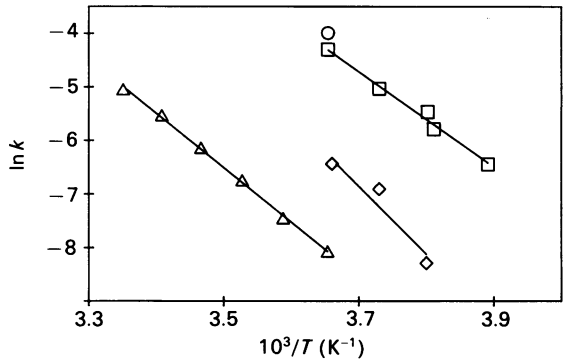



**Fig. 4. Arrhenius plots for the formation and isomerization of the enamine acyl-enzymes**

The formation of the enamine was monitored by the increase in $\Delta A_{295}$, the isomerization by the decrease in $\Delta A_{295}$ (△). Similar rates were obtained from analysis of the absorbance changes at 277 nm (see Table 1). The lines shown were obtained by linear regression. The point shown by the ○ symbol represents the rate for formation of the enamine measured in aqueous solution, and is shown for comparison of the effect of solvent on the reaction rate. The □ symbols represent the rate of formation of the enamine (increase in $A_{295}$) in cryosolvent. The △ symbols represent the rate of isomerization (decrease in $A_{295}$) in aqueous solvent. The ◇ symbols represent the rate of isomerization in cryosolvent. An explanation for the higher rate than that observed in aqueous solution is given in the text. The experimental conditions were 20% ethylene glycol/30% methanol/sodium acetate/phosphate buffer, pH* 6.5, for 0.3 °C and lower temperatures, with 5.4 mM-β-lactamase and 53 μM-clavulanate (□ and ◇), or aqueous buffer, pH 6.7, 14.2 μM-β-lactamase and 54.2 μM-clavulanate (△ and ○) for temperatures above 0 °C.

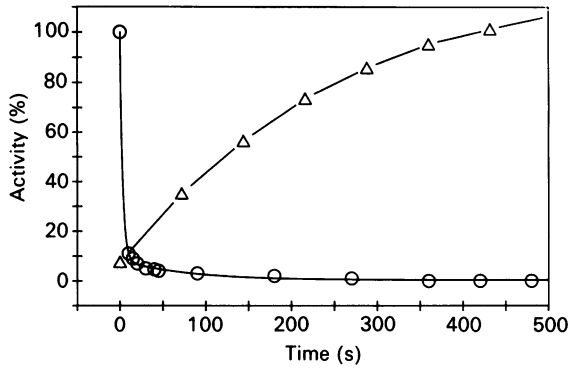
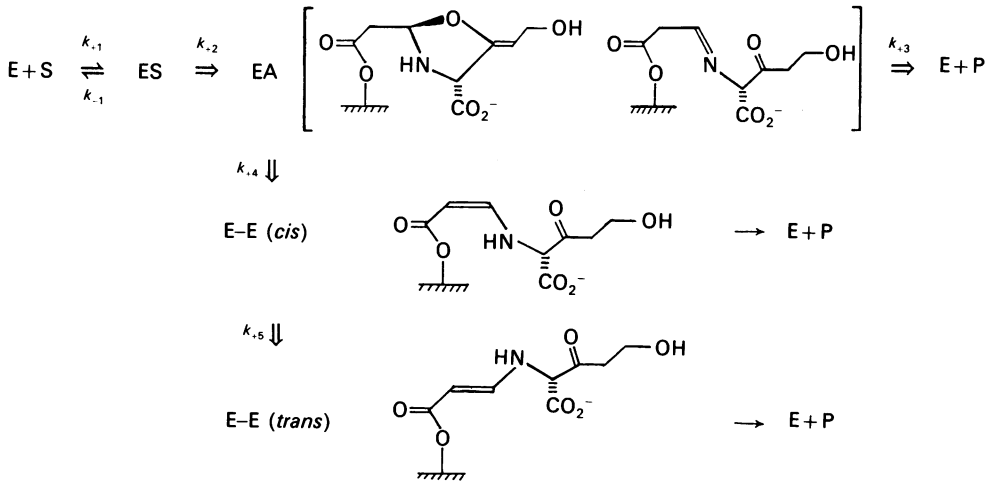



**Fig. 5. Rate of inactivation of β-lactamase by clavulanic acid at −10 °C**

The ○ symbols represent data points obtained from assaying samples with benzylpenicillin. β-Lactamase (5.4 μM) was incubated with 53 μM-clavulanic acid. At each of the times shown as 20 μl portion was transferred to 1.0 mM-benzylpenicillin for measurement of catalytic activity. The line shown is the least-squares best fit to a biphasic exponential model with rates of 0.32 and 0.01 $s^{-1}$ (standard errors 0.02 and 0.003 $s^{-1}$ respectively). The △ symbols represent the rate of formation of the enamine chromophore as measured by the change in $A_{295}$ under the same experimental conditions and are shown for comparison.

### Mechanism of clavulanate inactivation of *S. aureus* β-lactamase

These results may be explained in terms of Scheme 1, where EA represents either the initially-formed acyl-enzyme, or, less likely, the corresponding imine resulting from subsequent scission of the C–O bond, and E–E represents enamine forms of acyl-enzyme. The initial rapid loss of catalytic activity reflects the accumulation of the intermediate EA, the acyl-enzyme. This inter-




**Scheme 1.**

mediate can react either by hydrolysis (deacylation) to give turnover or can form the enamine resulting from cleavage of the C-5–O bond and tautomerization of the resulting imine (manifested as the increase in absorbance or ellipticity at 295 nm). Apparently both these reactions are quite slow, and much slower than formation of the acyl-enzyme (as shown by the loss of catalytic activity). The initially formed enamine is presumably in the *cis* conformation, and this isomerizes in a slower process (manifested as the increase in absorbance at 277 nm or the decrease in ellipticity at 275 nm) into the *trans* conformation of the enamine acyl-enzyme. Interestingly, for the related enzyme from *B. cereus* inactivation by clavulanate leads to a similar increase in ellipticity at 288 nm, but no subsequent decrease (L. M. Ellerby & A. L. Fink, unpublished work). Re-activation of the enamine species presumably occurs via acid-catalysed hydrolysis of the tautomeric imine, which accounts for the more significant re-activation at lower pH [8]. It is also likely that the enamine acyl-enzymes undergo slow deacylation, accounting for a significant proportion of the reactivation at neutral pH. The residual catalytic activity at completion of the first phase (Fig. 5) reflects the steady-state concentration of enzyme not in the form of the acyl-enzyme or enamine. As enamine formation proceeds, this concentration decreases with a rate similar to that for the initial enamine formation. We are unable to distinguish whether the enamine is formed by direct abstraction of the proton on C-6 or is formed via an imine. The enamine form of the acyl-enzyme is expected to be more stable than the normal acyl-enzyme to hydrolysis of the acyl-enzyme bond owing to the conjugation between the ester bond and the double bond of the enamine. By analogy with similar systems we esimate a rate decrease of at least 20-fold. It is also possible that the *cis* and *trans* forms of the enamine differ in their resistance to deacylation as a result of steric effects. We have no evidence for additional imine formation from attack by an active-site lysine residue on the ketone carbonyl group of the enamine form of the acyl-enyme, although we cannot rule out such a reaction [5].

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