CLAVULANIC ACID INHIBITION OF β-LACTAMASE I FROM *BACILLUS CEREUS* 569/H

JON P. DURKIN* and THAMMIAH VISWANATHA**

The Guelph-Waterloo Centre for Graduate Work in Chemistry, Waterloo Campus,
Department of Chemistry, University of Waterloo, Waterloo,
Ontario, N2L 3G1, Canada

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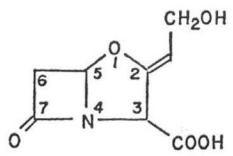
Inactivation of β-lactamase I by clavulanic acid was investigated. Clavulanic acid induced inhibition of the enzyme was found to be progressive with time. Benzylpenicillin provided protection against the adverse effects of the inhibitor initially, however, the enzyme was irreversibly inhibited in a progressive manner even in the presence of substrate. Reaction of β-lactamase I with clavulanic acid, in the presence of ampicillin, led to a very rapid inactivation of the enzyme.

The search for β-lactamase inhibitors has been stimulated by the observed association between bacterial resistance to β-lactam antibiotics and the production of β-lactamases in such resistant organisms. While several inhibitors have been found, they have invariably been derived from the penicillin and cephalosporin family. These inhibitory agents exert their effects in a competitive, reversible manner and are required in relatively high concentration in order to afford a significant degree of protection to β-lactamase sensitive antibiotics. These compounds, therefore, possess minimal value as therapeutically applicable β-lactamase inhibitors. Consequently the recent observations[1] on the ability of clavulanic acid to function as a potent inhibitor of β-lactamases are of considerable significance. Using crude preparations of β-lactamases isolated from a variety of Gram-positive and Gram-negative sources, a direct correlation was found to exist between the extent of clavulanic acid induced β-lactamase inhibition and the susceptibility of the corresponding organisms to β-lactam antibiotics (in the presence of the inhibitor). Inhibition was found to proceed in a progressive manner and the levels of clavulanic acid required to attain 50% inhibition (I_{50}) following a 15-minute interaction period were found to be as low as 0.03 μg/ml. Thus, clavulanic acid by itself proves to be a formidable β-lactamase inhibitor[1].



The structure of clavulanic acid, a naturally occurring β-lactam isolated from *Streptomyces clavuligerus,* has been elucidated[2,3]. While the compound possesses a bicyclic ring system as found in penicillins, its major distinguishing features are: (1) the absence of amino acyl substituent at C_6; (2) replacement of sulfur by oxygen; and (3) presence of a hydroxyethylidine substituent at C_2. Current studies concern the inhibitory effects of clavulanic acid on purified β-lactamase from *Bacillus cereus* 569/H.

* Present address: Cell Biology Laboratory, Salk Institution, P. O. Box 1809, San Diego, California 92112, U.S.A.

** To whom all correspondence should be addressed.

Experimental

Sodium clavulanate was the generous gift of Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey, U.K. It was supplied in the form of the anhydrous freeze-dried sodium salt and stored at −20°C. Fresh samples (1 mg/ml) of the compound were made daily in appropriate buffers.

β-Lactamase I was isolated from a constitutive β-lactamase producing strain of *B. cereus* 569/H, kindly provided by Dr. DAVID SHAPIRO of the Weizmann Institute of Science. Colonies highly active in β-lactamase production were selected[4] and used for the isolation of the enzyme, according to the procedure of MILLER *et al.*[5]. The crude enzyme was further purified by chromatography on DEAE cellulose[6] and Sephadex G–100. Samples were judged as >95% pure based upon SDS-polyacrylamide gel electrophoresis[7]. Purified enzyme samples were stored in either the frozen or lyophilized state at −50°C.

Protein concentration of β-lactamase I was estimated from the molar extinction coefficient ($E^{0.1\%}_{280nm}$, 0.834) established previously[8].

Enzyme activity was measured using the spectrophotometric assay of WALEY[9]. Three-milliliter samples of benzylpenicillin (1 mM) in appropriate buffer were placed in temperature-controlled (30°C) reference and sample compartments of a Cary model 14 spectrophotometer. Measurement of enzymatic activity was initiated by introducing small aliquots (10~50 μl) of enzyme solution to the reference cuvette and monitoring the increase in A_{232} as a function of time.

β-Lactamase stability of clavulanic acid

One hundred microlitres of clavulanic acid (approximately 4.5 mM) at pH 7.0 was treated with 10 μl of a stock solution of β-lactamase to achieve a final concentration of 20 nM with respect to the enzyme and the mixture was incubated at 30°C for 1 hour. A sample of clavulanic acid treated under identical conditions but for the replacement of the enzyme by buffer (10 μl) served as control. Both the control and the β-lactamase treated samples were subjected to thin-layer chromatography (TLC) using a solvent system consisting of *n*-butanol - ethanol - water (4: 1: 5, vol/vol/vol; top phase). The chromatograms were developed using TTC (2,3,5-triphenyl tetrazolium chloride) spray according to published procedure[1]. Clavulanic acid appears as a red spot with an Rf value of 0.44 under these conditions.

The susceptibility of clavulanic acid to degradation by β-lactamase was also monitored using the spectrophotometric method. The procedure employed was identical with the assay for the enzymatic activity described above, except for the replacement of benzylpenicillin by clavulanic acid (1 mM) as substrate.

Irreversible inhibition of β-lactamase I by clavulanic acid

Samples of enzyme solution (18.2 nM) were prepared by transferring 10 μl aliquots of a β-lactamase stock solution to tubes containing 200 μl of phosphate buffer (100 mM), pH 7.0. Following the addition of 10 μl of clavulanic acid solution of appropriate concentration, the samples were incubated at room temperature. The range of concentration of clavulanic acid used in the studies varied from 5- to 16,000-fold molar excess of the reagent over that of the enzyme. Aliquots (50 μl) of each of the samples were transferred to 3 ml of benzylpenicillin (1 mM), pH 7.0 and assayed spectrophotometrically 15 and 30 minutes following addition of clavulanic acid. Enzyme solution, treated in an identical manner, but for the omission of clavulanic acid, served as control. The extent of irreversible inactivation of β-lactamase I by clavulanic acid treatment was estimated by observing the rate of penicillin hydrolysis over extended time periods (~30 minutes).

Competitive inhibition of β-lactamase I by clavulanic acid

Appropriate dilutions of a 15.4 mM benzylpenicillin stock solution were made into 10.0 ml phosphate buffer (100 mM), pH 7.0, to yield substrate concentrations ranging from 20 to 150 μM. In the presence of several fixed concentrations of clavulanic acid, initial rates of penicillin hydrolysis were measured spectrophotometrically (at 232 nm) over the first two minutes following the addition of aliquots (10 μl) of a β-lactamase stock solution (final enzyme concentration, 0.067 nM). Final clavulanic acid concentrations ranged from 0 to 116 μM. The data was plotted as a double reciprocal plot (1/v *vs* 1/S) on a Hewlett-Packard 9866A calculator (equipped with a model 9862A plotter) using the least

squares criteria. *Ki* of the inhibitor was estimated from a secondary plot of slope *vs* inhibitor concentration.

Kinetic experiments on β-lactamase I inhibition by clavulanic acid

(1) Two cuvettes, each containing 3.0 ml of a benzylpenicillin solution (1 mM in 100 mM phosphate buffer, pH 7.0) were placed in the reference and sample compartments of a Cary model 14 spectrophotometer. Aliquots (10 μl) of a clavulanic acid solution were introduced into both cuvettes so as to achieve a concentration of 75 μM with respect to the reagent. After 1 minute, an aliquot (10 μl) of enzyme solution was added to the reference chamber and the rate of benzylpenicillin hydrolysis monitored by following the relative increase in absorbance at 232 nm. The final enzyme concentration in the reference cuvette was 0.6 nM. The above reaction, performed in the absence of clavulanic acid, served as control. In experiments with the sample containing clavulanic acid, a fresh sample (20 μl) of 50 mM benzylpenicillin was introduced (to both the reference and sample solutions) at the end of 20 minutes of initial reaction. The addition of fresh benzylpenicillin restored the concentration of substrate to saturating levels (approximately 1 mM). Such replenishment of benzylpenicillin minimized the probable decrease in the rate of hydrolysis due to substrate depletion and consequently permitted an accurate assessment of the inactivation induced by clavulanic acid.

(2) Following the procedure outlined above, the experiment was repeated using ampicillin as substrate. Penicillin hydrolysis was monitored spectrophotometrically at 236 nm, the optimum wavelength for following ampicillin hydrolysis.

(3) To 3.0 ml of a clavulanic acid solution (75 μM) in phosphate buffer (100 mM), pH 7.0, an aliquot (10 μl) of the enzyme stock solution was added and the cuvette incubated at 30°C. At 1, 15 and 30 minutes, residual enzymatic activity of the sample was assessed by introducing a 10 μl aliquot of a benzylpenicillin stock solution to yield an initial substrate concentration of 1 mM. An identical solution, void of enzyme, served as a reference in the assay.

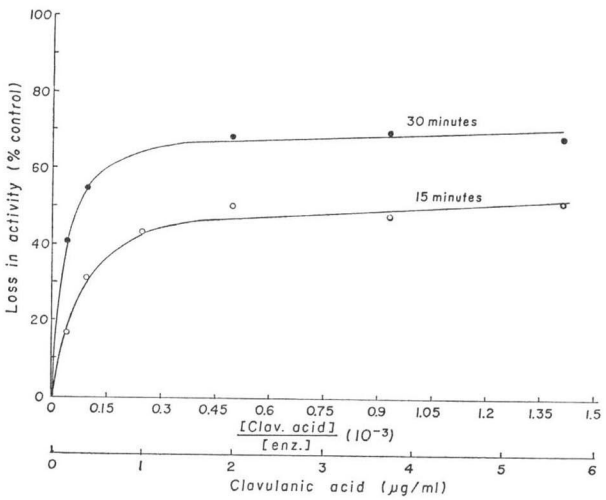
(4) An aliquot (10 μl) of the enzyme stock solution was preincubated at 30°C in 3.0 ml of buffer containing clavulanic acid (75 μM). After 15 minutes, assessment of enzymatic activity was initiated by the addition of an aliquot (100 μl) of ampicillin solution (in buffer) to the cuvette to achieve an initial substrate concentration of 1 mM. Ampicillin hydrolysis was monitored at 236 nm against an identical solution, except for the omission of enzyme.

Fig. 1. Effect of clavulanic acid on β-lactamase I activity

Enzyme solution (18.2 nM) in phosphate buffer (100 mM) at pH 7.0 was treated with varying amounts of clavulanic acid (to achieve the molar excess of the reagent over protein as shown). Subsequent to incubation at room temperature, activity was determined spectrophotometrically at pH 7.0.

Incubation time: 15 and 30 minutes.

Enzyme samples treated under identical conditions, but for the omission of clavulanic acid, served as controls.



Results

No gross destruction of a clavulanic acid could be detected by TLC following 1-hour incubation with β-lactamase. In addition no changes in the spectral properties of clavulanic acid were noted (specifically in the 230 nm region) upon incubation of the compound with the enzyme. Thus, clavulanic acid appears resistant to β-lactamase catalyzed hydrolysis, a finding consistent with that reported earlier[1].

As illustrated in Fig. 1, incubation

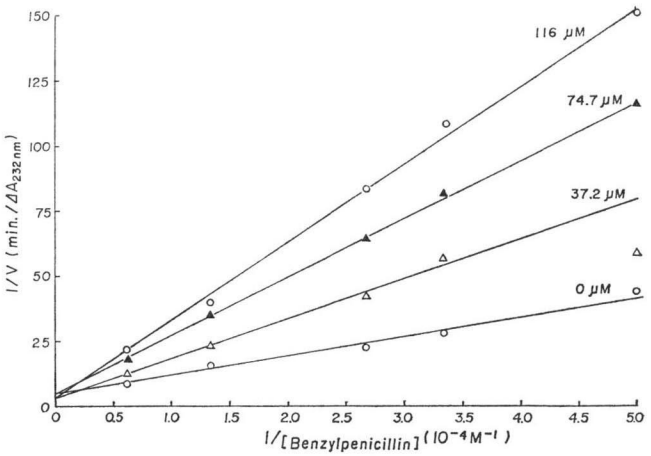
of enzyme with a 50-fold molar excess of clavulanic acid for 15 minutes (at room temperature) resulted in a 14% loss in activity. Approximately 40% inhibition of enzymatic activity was noted when the period of incubation was extended to 30 minutes. Thus, the effects of clavulanic acid appear to be progressive in nature, confirming earlier findings[1]. Although not shown in Fig. 1, incubation of enzyme with a 16,000-fold molar excess of clavulanic acid for 30 minutes resulted in >80% loss in enzymatic activity. It should be noted that <1 μg/ml of clavulanic acid was sufficient to cause >50% inhibition of β-lactamase activity within 30 minutes, under the experimental conditions employed. Thus, incubation with relatively large concentrations of inhibitor for prolonged time periods was required to achieve substantial inactivation of enzyme. In addition, dilution of the inhibitor concentration during the assay for residual enzymatic activity (see Methods) failed to relieve the inhibitory effects of clavulanic acid. Thus, β-lactamase I is seen to undergo inhibition upon extended period of interaction with clavulanic acid.

Fig. 2. Competitive inhibition of β-lactamase I catalysed hydrolysis of benzylpenicillin by clavulanic acid

Double reciprocal plots (1/v *vs* 1/S) of the initial rates of penicillin hydrolysis are shown. Reactions were conducted at varying concentrations of substrate in the presence of several fixed amounts of clavulanic acid.

Initial rates were measured spectrophotometrically using a Cary model 14 spectrophotometer. The concentrations of inhibitor used and R values were: 0 μM, 0.989; 37.2 μM, 0.994; 74.7 μM, 0.999; and 116 μM, 0.997.

Reactions were conducted in phosphate buffer (pH 7.0).



In view of the structural similarity to natural penicillins, clavulanic acid was examined for its ability to function initially as a competitive inhibitor of the enzyme and the results are shown in Fig. 2. The double reciprocal plot is characteristic of the enzyme being inhibited competitively by clavulanic acid. A secondary replot of the data revealed a *Ki* of 34.2 μM (with a curve fit of R^2 equal to 0.999), which represents a binding affinity of the order of that found for natural penicillin substrates[10,11].

Previous *in vivo* studies have shown that the addition of low concentrations of clavulanic acid effectively reduces the minimum inhibitory concentration of ampicillin against β-lactamase producing, penicillin resistant micro-organisms[1]. Thus, two therapeutically valuable penicillins, ampicillin and benzylpenicillin, both class S type substrates[12], were examined for their effects on the rate and extent of clavulanic acid induced β-lactamase inhibition.

Clavulanic acid was added to benzylpenicillin (1 mM) at pH 7.0 and 30°C to achieve a final concentration of 75 μM. Following 1-minute incubation, an aliquot of enzyme was introduced and the rate of benzylpenicillin hydrolysis monitored spectrophotometrically at 232 nm. Enzymatic activity was found to decline gradually (Fig. 3; curve b); the rate of hydrolysis (following 20 minutes of reaction) was approximately 45% of that noted in the control reaction (curve a). This diminution in activity was not due to substrate depletion since addition of fresh benzylpenicillin (final concentration 1 mM

Fig. 3. Effect of clavulanic acid on β-lactamase I catalysed hydrolysis of benzylpenicillin.

In all of the following cases, enzyme and benzylpenicillin concentrations were 0.6 nM and 1 mM respectively. The rates of substrate hydrolysis were measured in phosphate buffer (100 mM), pH 7.0 at 30°C.

Curve a: control (no clavulanic acid)

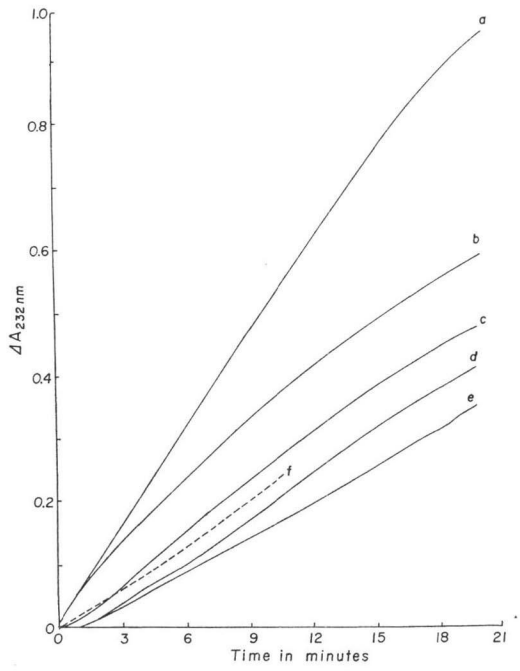
Curve b: enzyme was added at t=0 to a mixture of benzylpenicillin and clavulanic acid (75 μM) preincubated for 1 minute.

Curves c, d, e: enzyme was preincubated with clavulanic acid for 1, 15 and 30 minutes respectively prior to addition of substrates.

Curve f: enzyme was added at t=0 to a mixture of benzylpenicillin and clavulanic acid (75 μM) preincubated for 1 minute. Following 20 minutes of reaction, saturating conditions with respect to substrate were re-established by the introduction of 20 μl of 50 mM benzylpenicillin.

The figure represents the actual rates measured on a Cary model 14 spectrophotometer.

The maximum rate of hydrolysis in curves d and e were 84% and 71%, respectively of that found in curve c.



so as to achieve approximately 14-fold molar excess over that of clavulanic acid) to this solution (after 20 minutes of reaction) failed to produce any enhancement in the rate of substrate hydrolysis (curve f).

In similar experiments, β-lactamase was preincubated with clavulanic acid for 1, 15 and 30 minutes (Fig. 3, curves c, d and e, respectively) prior to the introduction of benzylpenicillin. Upon addition of substrate to the enzyme-clavulanic acid mixture, little or no measurable enzymatic activity could be noted for approximately 1 minute, after which time a gradual increase in activity was observed. Again, the maximum rate of substrate hydrolysis achieved after such treatment was only ~45% of that found for the control reaction.

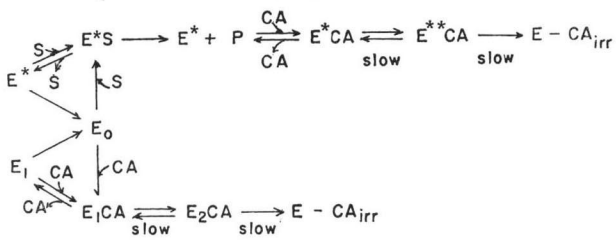
A comparison of the data in curves b with those in c, d and e reveals that benzylpenicillin is capable of affording a substantial degree of protection against clavulanic acid induced inhibition during early stages of reaction.

The above experiments were repeated using ampicillin as substrate. Addition of enzyme to an ampicillin solution preincubated for 1 minute with clavulanic acid resulted in a rapid inactivation, the extent of which far exceeded that observed with benzylpenicillin. As shown in Fig. 4 (curve b), the rate was approximately 15% of that noted in the control (curve a). Replenishment of substrate to saturating levels (1 mM), after 20 minutes of reaction did not significantly alter the degree of enzyme inhibition (Fig. 4, curve c). As was the case with benzylpenicillin (Fig. 3), preincubation of enzyme with clavulanic acid for 15 minutes prior to the introduction of substrate (ampicillin) resulted in a distinct lag before measurable rates of hydrolysis could be observed (curve d).

Discussion

Clavulanic acid was found to inhibit β-lactamase I at low concentrations (<1 μg/ml), yet relatively

Scheme 1.



large excesses of the reagent over extended time periods (Fig. 1) were required to achieve significant levels of enzyme inhibition. These observations indicate the possible occurrence of several unsuccessful bindings of this substrate-like inhibitor prior to the inactivating event. Consequently, clavulanic acid could be expected to function as a competitive inhibitor of the enzyme. Indeed, the results in Fig. 2 demonstrate this to be the case; the apparent affinity of clavulanic acid ($Ki \simeq 35$ μM) for the enzyme is comparable to that found for typical penicillin substrates. The requirement of a specific enzyme conformation(s) sensitive to clavulanic acid may explain the progressive nature of the inactivation. The required conformation may be a natural transient one or, that induced in the presence of the compound, as is the situation known to occur with class A type substrates[12]. Once the necessary conformation is achieved, irreversible inhibition can be envisaged to occur due to interaction between the vinyl moiety of clavulanic acid and some reactive functional group of the enzyme.

The data presented in Fig. 1 shows that with an increasing concentration of inhibitor, the rate limiting step is not its initial binding to the enzyme, but a later event in the inactivation process. However, the apparent Ki (~ 35 μM) of clavulanic acid observed in the presence of benzylpenicillin (Fig. 2) is clearly too large to account directly for the saturation effects seen at very low concentrations of clavulanic acid (~ 1 μg/ml; ~ 4 μM; Fig. 1). In addition, as illustrated in Fig. 3, addition of substrate to clavulanic acid-enzyme mixtures, preincubated up to 30 minutes, resulted in little or no measurable enzymatic activity for about 1 minute (Fig. 3, curves c, d and e), after which time a gradual increase in activity, eventually reaching $\sim 45\%$ of that found for the control, was observed. Benzylpenicillin, on the other hand, was found to offer a substantial degree of protection to the enzyme throughout the initial period of reaction with clavulanic acid (Fig. 3, curve b).

Fig. 4. Effect of clavulanic acid on the rate of β-lactamase I catalysed hydrolysis of ampicillin

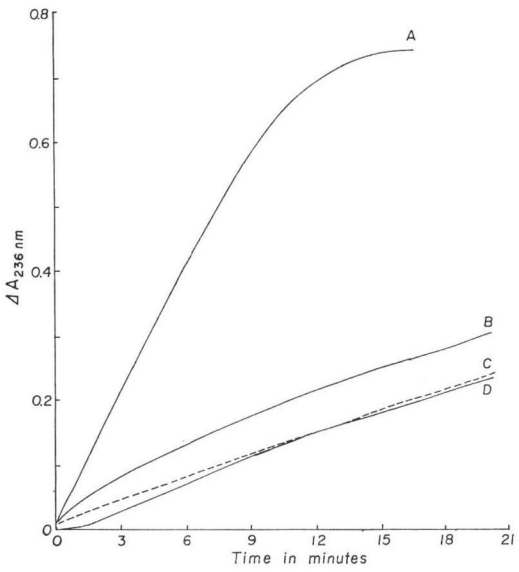
Conditions were identical to that reported in Fig. 3, except that ampicillin served as substrate.

Curve A: control (no clavulanic acid)

Curve B: enzyme was added at t=0 to a mixture of ampicillin and clavulanic acid (75 μM) preincubated for 1 minute.

Curve C: enzyme was added at t=0 to a mixture of ampicillin and clavulanic acid (75 μM) preincubated for 1 minute. After 20 minutes of reaction, saturating conditions with respect to substrate were re-established by the introduction of 20 μl of a 50 mM ampicillin solution.

Curve D: enzyme was preincubated with clavulanic acid (75 μM) for 15 minutes prior to the addition of substrate.



It would appear, therefore, that the process of inactivation of β-lactamase I by clavulanic acid is far from a simple one. While elucidation of the actual mechanism would require a more thorough

investigation, Scheme 1 represents a working model which is consistent with most of the observations made in this study.

In the presence of benzylpenicillin, enzyme in the "resting" state (E_0), is seen to undergo a rapid substrate induced conformational change in forming the MICHAELIS-MENTEN complex (E*S), which rapidly releases product to form, at least transiently, a form of the enzyme (E*) to which clavulanic acid can bind to form the complex E*CA. A slow conversion to an irreversibly inhibited form of the enzyme ($E \sim CA$) is then envisaged to occur. This pathway can account for the enzyme being initially inhibited competitively by clavulanic acid as well as the initial protection afforded to the enzyme (from the effects of clavulanic acid) in the presence of saturating levels of substrate. The $E_0 \rightarrow E^*$ transition is consistent with earlier observations which indicate a relative inertness (of the essential functional groups) of enzyme preparations to classical protein modifying reagents[8]. Thus, the active site of the enzyme may not pre-exist, but is formed in response to the presence of substrate, a concept consistent with an earlier proposal[13].

In the absence of substrate, clavulanic acid, due to its structual similarity to penicillins, may react directly with E_0 (but slower than does benzylpenicillin) to form the reversible complex E_1CA. The relatively low concentration of inhibitor required to achieve "saturating levels" of inhibition in the absence of substrate (Fig. 1) could be accounted for if the E_1CA complex were to dissociate at a slow rate. In addition, preincubation of enzyme with clavulanic acid, in the absence of substrate, allows for a slow conversion to the E_2CA complex, which upon addition of saturating levels of substrate slowly reverts to the active form of the enzyme (E*). Only partial activity would be attained in this manner since a proportion of the enzyme would have already undergone irreversible inhibition. Undoubtedly, other models consistent with the observed results could be conceived and the above scheme is presented only as a working model.

Under the experimental conditions employed, treatment of β-lactamase I with clavulanic acid in the presence of ampicillin (as substrate) led to a more rapid and complete inactivation of the enzyme (Fig. 4) than that noted with benzylpenicillin (Fig. 3). The recorded *Km* values of β-lactamase I for ampicillin and benzylpenicillin are approximately 110 μM and 50 μM respectively[12]. It is not clear whether the different effects of the two penicillins on clavulanic acid induced inhibition of the enzyme are a consequence of the difference in *Km* values of β-lactamase for these compounds. The possibility of ampicillin inducing an enzyme conformation more susceptible to inhibition by clavulanic acid may provide an explanation for the observations.

Subsequent to the preparation of this manuscript, two comprehensive papers from the laboratory of Prof. JEREMY KNOWLES, Harvard University, dealing with the kinetics of clavulanic acid induced inactivation of β-lactamase from *Escherichia coli* RTEM have appeared in the literature[14,15]. In essence, the observations noted with the *E. coli* enzyme are similar to those reported in the current study (dealing with β-lactamase I from *B. cereus* 569/H). Interestingly, both studies arrive at a two step mechanism for the inactivation process.

Acknowledgement

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