Mechanism of inactivation of TEM-1 β -lactamase by 6-acetylmethylenepenicillanic acid

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The interaction between 6-acetylmethylenepenicillanic acid (compound Ro 15-1903; AMPA) and TEM-1 β -lactamase was investigated in order to elucidate the mechanism of action of AMPA. Formation of the enzyme–inhibitor complex (EA*) was accompanied by a shift of the absorbance maximum from 292 nm to 303 nm and an increase in the absorption. Regeneration of activity was very slow and incomplete, reaching about one-third of the initial activity after 48 h at 37°C. This behaviour indicated a branched pathway of the decay of the inactivated enzyme. Kinetic and isoelectric-focusing experiments proved this assumption. The first-order constant of regeneration of active enzyme was 6×10^{-6} – $10 \times 10^{-6} \text{ s}^{-1}$, whereas the rate constant leading to inactive enzyme (EA') was 10×10^{-6} – $15 \times 10^{-6} \text{ s}^{-1}$ at pH 7.0. Both constants became larger at higher pH. Inactive enzyme (EA') consisted of two major species, with pI 5.36 (EA₁') and 5.30 (EA₂'). The former increased at the beginning of incubation but decreased after prolonged incubation. From consideration of these results and previous data [Arisawa & Then (1983) *Biochem. J.* **209**, 609–615], a likely mechanism of inactivation of TEM-1 β -lactamase by AMPA is discussed.

6-Acetylmethylenepenicillanic acid (compound Ro 15-1903) is a potent and broad-spectrum β -lactamase inhibitor (Then *et al.*, 1982). It inactivates TEM-1 β -lactamase with a large second order constant ($0.61 \mu\text{M}^{-1} \cdot \text{s}^{-1}$) and produces two forms of inactivated enzyme discernible by isoelectric focusing (designated as EA with pI 5.25 and EA* with pI 5.15) in the early stage of inactivation. Only EA* is found after 30 min incubation at 37°C. One-to-one inactivation is also observed by measuring the stoichiometry (Arisawa & Then, 1983).

For elucidation of the chemical reactions between AMPA and the enzyme, a spectroscopic study of the inactivated enzyme was undertaken. Regeneration of catalytic activity was also examined at various pH values to characterize the nature of the chemical bond between the enzyme and the AMPA-derived moiety. The results of these studies are described in the present paper, and a likely mechanism of inactivation of TEM-1 β -lactamase that accommodates the data is discussed.

Abbreviation used: AMPA, sodium 6-acetylmethylenepenicillinate.

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Materials and methods

Materials

AMPA and its analogues were synthesized by S. A. in our laboratory (for details see Hoffmann–La Roche, 1981). Purified *Escherichia coli* TEM-1 β -lactamase and all other materials used were the same as described in a previous paper (Arisawa & Then, 1983). Varian A 60 (Palo Alto, CA, U.S.A.) and MS9 AEI (Manchester Instruments, Manchester, U.K.) were used for n.m.r. and mass spectrometry respectively.

Enzyme assay and isoelectric focusing

The enzyme assay and isoelectric focusing were performed as outlined in a previous paper (Arisawa & Then, 1983). Sodium phosphate buffer, pH 7.0 (0.1 M), was used throughout the experiments unless otherwise stated.

U.v. spectrometry

Difference spectra were obtained by the use of quartz double-layer cuvettes (light-path $l = 4.375 \text{ mm} \times 2$). Absorbances calculated for $l = 10 \text{ mm}$ are shown in Fig. 1. Since the absorbance of the β -lactamase at $8 \mu\text{M}$, which was the highest concentration used for scanning of the chromophore of the inactivated enzyme, was 1.25 at

230 nm, difference spectra above 230 nm could be reliably measured by the spectrophotometer Uvicon 810 (Kontron, Zürich, Switzerland). Compounds (Ia) and (Ib) (see the Results and discussion section) were dissolved in 0.1 M-sodium phosphate buffer, pH 7.0. Changes in the absorbance at 232 and 303 nm were also measured in the double-layer cuvette.

Pivaloyloxymethyl ester compounds were dissolved in ethanol at 1 mM, and spectra of those compounds were scanned after dilution into 0.1 M-sodium phosphate buffer, pH 7.0, to give 50 μ M.

Regeneration of native enzyme

β -Lactamase (12.6 to 2.6 μ M) was inactivated by mixing with AMPA solutions to give AMPA/enzyme molar concentration ratios in the range 1–5:1.

After 30 min incubation at 37°C, the mixture was cooled in an ice/water bath and extensively dialysed at 4°C against 10 mM-sodium phosphate buffer, pH 7.0, through cellulose tubing. Storage of the inactivated and dialysed enzyme at –20°C for up to a month did not influence its properties.

Regeneration of catalytic activity was studied by incubating the mixture of inactivated complex with 15% (v/v) glycerol at 37°C. At appropriate time intervals 5 μ l amounts of the mixture were transferred into 1 ml of 2 mM-benzylpenicillin (37°C), and the hydrolysis rate was measured spectrophotometrically.

The effect of hydroxylamine on regeneration was tested by the addition of 40 mM-hydroxylamine hydrochloride dissolved in 100 mM-sodium phosphate buffer, pH 7.0, to an equal volume of inactivated enzyme solution. For regeneration at various pH values, 100 μ l portions of inactivated enzyme were each mixed with 60 μ l of 50% (v/v) glycerol and 40 μ l of 0.5 M-sodium/potassium phosphate buffer, pH 5.0, 7.0 and 8.0. The final pH of the mixture was checked directly by using a micro-electrode (Ingold Co., Zürich, Switzerland).

Preparation of compound (Ia)

A 1 ml volume of 1 M-sodium methoxide in methanol was added drop by drop to 10 ml of methanol solution containing 1 mmol of AMPA. After 5 min at room temperature, the reaction mixture was evaporated. The residue was dissolved with about 20 ml of ice-cold 5 mM-citric acid, pH 3, and extracted with ethyl acetate. The organic phase was washed with water, dried over MgSO_4 , filtered and evaporated to give a foamy material. N.m.r. spectrum: δ (p.p.m.) ($[\text{}^2\text{H}]$ chloroform) 6.26 (1H, d, $J \sim 1$ Hz, $\text{CH}_3\text{--C=CH}$), 4.48 (1H, s, $\text{CH--CO}_2\text{H}$), 3.76 (3H, s, $\text{CO}_2\text{--CH}_3$), 2.18 (3H, d, $J \sim 1$ Hz, $\text{CH}_3\text{--C=CH}$), 1.76 (3H, s) and 1.66 (3H, s). Mass spectrum: m/e 269 (the molecular ion): additional

fragments at m/e 222, 238 and 192 (high intensity). U.v. spectrum: λ_{max} at 298 nm (ϵ 6000 $\text{M}^{-1}\cdot\text{cm}^{-1}$).

Isolation of compound (Ib) from the interaction of AMPA with the enzyme

Dialysed inactivated protein (prepared as above at an AMPA/enzyme concentration ratio of 5:1) was incubated for 48 h at 37°C at pH 7.0. The solution was acidified and treated as described above for compound (Ia) to extract released compound (Ib). The product has a high-resolution mass spectrum very similar to that of compound (Ia), with characteristic fragments at m/e 222 and 192.

Results and discussion

Relationship between structure of the C-6 side chain and inhibitory activity

Table 1 summarized the inhibitory activities of AMPA derivatives. Since acid forms of those compounds had been found to be slightly unstable (S. Adam, unpublished work), pivaloyloxymethyl ester forms were examined. Table 1 indicates that a ketone group in the C-6 side chain is essential for the compound to exhibit inhibitory activity against TEM-1 β -lactamase. This suggests that the ketone group should play a key role in the interaction with TEM-1 β -lactamase.

Generation of a new chromophore

Spectral changes caused by the addition of AMPA were studied by difference spectroscopy. AMPA had a u.v. spectrum with λ_{max} at 233 nm (ϵ 12600 $\text{M}^{-1}\cdot\text{cm}^{-1}$) and 292 nm (ϵ 2200 $\text{M}^{-1}\cdot\text{cm}^{-1}$). When TEM-1 β -lactamase and AMPA were mixed at 37°C, a new spectrum with two peaks at about 303 and 240 nm was obtained, as shown in Fig. 1. Absorbance at 303 nm increased 3-fold, reaching ϵ 5500 $\pm$ 900 $\text{M}^{-1}\cdot\text{cm}^{-1}$ (mean $\pm$ s.d. for four independent experiments). This peak shifted to about 296 nm without apparent change of ϵ when guanidinium chloride (2.6 M) was added, suggesting some influence of protein structure. Absorbance at 240 nm decreased to about half the value of that of AMPA. Generation of the peak at 303 nm was so fast that the rate of appearance could not be measured over the range of concentrations that could conveniently be studied. This fits the large second-order inactivation constant that has been determined (Arisawa & Then, 1983). Of particular interest was the change of absorbance at 232 nm. This was not as fast as the increase of absorbance at 303 nm. It took about 3 min to reach a stable value, the reaction obviously not obeying the same kinetics.

Spectra of model compounds

From the chemical point of view there are three active sites in AMPA: the ketone group and the

Table 1. Structure-activity relationship for AMPA and related compounds as β -lactamase inhibitors

All compounds except AMPA were dissolved in ethanol to give a 1 mM solution and diluted with 0.1 M-sodium phosphate buffer, pH 7.0, to appropriate concentrations. To 900 μ l of 0.1 M-sodium phosphate buffer, pH 7.0, containing 1.6 pmol of TEM-1 β -lactamase was added 10 μ l of inhibitor solution. After 10 min at 37°C, 100 μ l of 10 mM-benzylpenicillin was added, and the remaining activity of the enzyme was measured by spectrophotometry as described in the Materials and methods section. The concentration of inhibitor required to inhibit TEM-1 β -lactamase by 50% (IC_{50}) was determined graphically.

Compound	R-	R'-	IC_{50} (μ M)
AMPA	$\begin{array}{c} \text{O} \\ \\ \text{CH}_3-\text{C}-\text{O} \end{array}$	Na^+	0.0014
piv-AMPA	$\begin{array}{c} \text{O} \\ \\ \text{CH}_3-\text{C}-\text{O} \\ \\ \text{O} \\ \\ \text{H}_2\text{N}-\text{C}- \end{array}$	piv-*	0.072
(II)	$\begin{array}{c} \text{O} \\ \\ \text{H}_2\text{N}-\text{C}- \end{array}$	piv-	>50
(III)	$\begin{array}{c} \text{O} \\ \\ \text{C}_2\text{H}_5-\text{O}-\text{C}- \\ \\ \text{OH} \end{array}$	piv-	26
(IV)	$\begin{array}{c} \text{CH}_3-\text{CH}- \\ \\ \text{N}-\text{OH} \\ \end{array}$	piv-	>50
(V)	$\begin{array}{c} \text{CH}_3-\text{C}- \\ \\ \text{N}-\text{O}-\text{CH}_3 \\ \end{array}$	piv-	0.73
(VI)	$\begin{array}{c} \text{CH}_3-\text{C}- \\ \\ \text{O} \end{array}$	piv-	>50

* piv- = $-\text{CH}_2-\text{O}-\text{C}(\text{CH}_3)_2$.

methylene bond in the C-6 side chain, and the β -lactam ring. Addition reaction at the C-6 methylene bond, known as the Michael reaction, would produce non-chromophoric compounds. Attempts to isolate an AMPA derivative with just the β -lactam ring opened failed. Reaction of AMPA with equimolar hydroxylamine in 0.1 M-sodium phosphate buffer, pH 7.0, produced its oxime (yield >80%), confirming the reactivity of the ketone group. The ester form of AMPA oxime was employed for the spectroscopic study, for preparation reasons (Fig. 2). An excess amount of hydroxylamine, which

Fig. 1. Absorption spectra of AMPA and the new chromophore, and effects of guanidinium chloride. AMPA (10 μ M) in 0.1 M-sodium phosphate buffer, pH 7.0, and the enzyme solution (16 μ M) in 0.01 M-sodium phosphate buffer, pH 7.0, were each poured into one compartment of a quartz double-layer cuvette (0.8 ml each). The reference cuvette contained 0.1 M-sodium phosphate buffer instead of AMPA solution. Scans before and after mixing of solutions in each cuvette gave the spectra of AMPA (spectrum A) and the new chromophore (spectrum B) respectively. Guanidinium chloride (199.5 mg) was added to each mixture (sample or reference cuvette, respectively) at a final concentration of 2.6 M (spectrum C). The cuvettes containing the enzyme and 0.1 M-sodium phosphate buffer, pH 7.0, in each compartment were used for the measurement of the baseline (spectrum D).

caused opening of the β -lactam ring, changed peak absorption from $\lambda_{\max}$ 273 nm (ϵ 11 000 $\text{M}^{-1} \cdot \text{cm}^{-1}$) to $\lambda_{\max}$ 293 nm (ϵ 5000 $\text{M}^{-1} \cdot \text{cm}^{-1}$). This spectrum was found to resemble that of the new chromophore generated in the reaction with TEM-1 β -lactamase. With regard to the absorbance change at 232 nm, it is not clear whether the change could be attributed to a structural change of the ligand or to a conformational change of the enzyme.

Fig. 2. Spectra of AMPA derivatives in comparison with that of new chromophore

Spectrum A, pivaloyloxymethyl ester of AMPA (50 μ M); spectrum B, oxime of pivaloyloxymethyl ester of AMPA (50 μ M); spectrum C, oxime of pivaloyloxymethyl ester of AMPA with opened β -lactam ring, obtained by the reaction of the oxime (50 μ M) with hydroxylamine (20 mM) in 0.1 M-sodium phosphate buffer, pH 7.0; spectrum D, new chromophore generated in the inhibition of TEM-1 β -lactamase with AMPA (spectrum C in Fig. 1 was transferred with expanded absorbance).

Regeneration of catalytic activity from inactivated enzyme

In order to characterize the nature of the chemical bond between the chromophore and protein of the enzyme-inhibitor complex, regeneration of active enzyme and the effects of some factors on it were studied.

When inactivated enzyme was incubated at 37°C, slow regeneration of catalytic activity was detected. Fig. 3 shows the regeneration as a function of time at three different pH values. Since native TEM-1 β -lactamase has partially lost its activity in 10 mM-sodium phosphate buffer, pH 7.0, after 48 h at 37°C, glycerol (15%, v/v) was added to stabilize the enzyme. Glycerol stabilized the active enzyme at all pH values tested, without affecting regeneration rate and final recovery (see below). Re-activated enzyme

Fig. 3. Regeneration of active β -lactamase at different pH values

Inactivated and dialysed TEM-1 β -lactamase was incubated at 37°C at different pH values. At times indicated, 5 μ l samples were withdrawn and mixed into 1 ml of 2 mM-benzylpenicillin for measurement of the activity. Activities are expressed as percentages of the original activity of enzyme present before inactivation. Experiments were done at: ●, pH 5.6; ▲, pH 7.0; ○, pH 7.8. The continuous curves are calculated in accordance with eqn. (1) by using rate constants for group 1 given in Table 2. The effect of hydroxylamine (20 mM) was also tested at pH 7.0 (△).

was partially recovered, and about one-third of the original activity was obtained at pH 7.0 after 48 h, but less at other pH values. On the other hand previous results (Arisawa & Then, 1983) showed that only one species of inactivated enzyme form (EA^*) was found by isoelectric focusing under the conditions employed here (30 min inactivation at 37°C). These observations indicated that not all the inactivated enzyme molecules could recover catalytic activity, and that some might be converted into another inactive form. This result, together with the fact (see below) that inactivated forms other than EA^* were detected by isoelectric focusing during re-activation, demands an assumption of a pathway from EA^* to the tertiary inactive forms. A branched pathway can thus be drawn as shown in Scheme 1, k_{+3} and k_{+4} being taken as the rate constants in the re-activation and conversion processes respectively. The process may therefore be formulated according to Scheme 1, and the following equation can be derived to express the concentration of enzyme [E] at time t :

$$[E] = \frac{k_{+3}}{k_{+3} + k_{+4}} \cdot [EA^*] \cdot (1 - e^{-(k_{+3} + k_{+4})t}) + C \quad (1)$$

where $[EA^*]$ is the concentration of EA^* , and C is a residual concentration of active enzyme present in the inactivated enzyme solution before regeneration,

Scheme 1

E represents free enzyme, A represents AMPA, EA represents the primary form of inactivated enzyme, EA* represents the secondary form of inactivated enzyme, P represents degradation product(s) of AMPA and EA' represents tertiary forms of inactivated enzymes.

Table 2. Determination of k_{+3} and k_{+4} in the inactivation of β -lactamase by AMPA
 k_3 and k_4 were calculated as follows: $k_3/(k_3 + k_4)$ could be obtained as

$$\frac{[E]_1^2}{(2[E]_1 - [E]_2)[EA^*]}$$

if the concentrations of active enzyme [E] at time t_1 and $2 \times t_1$ were given as $[E]_1 + C$ and $[E]_2 + C$ respectively, which were calculated by the equation

$$[E]_0 \, V/V_0$$

where $[E]_0$ is the original concentration of enzyme used for inactivation to generate EA*, V is the enzyme activity after regeneration, V_0 is the enzyme activity measured with $[E]_0$, C is the residual concentration of active enzyme present in the inactivated enzyme solution before regeneration and $[EA^*] = [E]_0 - C$. k_3 and k_4 were obtained by substituting into eqn. (1) values for $[E]$, $k_3/(k_3 + k_4)$, $[EA^*]$, t and C . Three independent preparations of inactivated enzyme were made and subjected to regeneration experiments with or without glycerol. In the latter case reaction was followed only for 24 h. For group 1 $[EA^*] = 1.27 \mu\text{M}$, +15% (v/v) glycerol, for group 2 $[EA^*] = 6.3 \mu\text{M}$, +15% (v/v) glycerol, and for group 3 $[EA^*] = 4.9 \mu\text{M}$, glycerol absent.

Exptl. group	pH	$10^6 k_{+3} \text{ (s}^{-1}\text{)}$	$10^6 k_{+4} \text{ (s}^{-1}\text{)}$	Final recovery (%)	Residual activity found before regeneration (%)
				$\left(\frac{k_{+3}}{k_{+3} + k_{+4}} \times 100\right)$	$\left(\frac{C}{[E]_0} \times 100\right)$
1	5.6	2.7	5.8	32	1.2
	7.0	$6.4 \pm 0.3 \text{ (3)*}$	$12 \pm 2.5 \text{ (3)*}$	$35 \pm 4 \text{ (3)*}$	$1.8 \pm 0.2 \text{ (3)*}$
	7.8	16	56	22	1.7
2	5.6	5.9	8.0	42	3.8
	7.0	12	13	48	3.9
	7.8	17	29	37	3.8
3	7.0	6.7	14	33	2.4

* Mean value $\pm$ s.d. (no. of replicates).

being of the order of a few per cent. k_{+3} and k_{+4} were calculated by using the data of Fig. 3, which are summarized in Table 2 together with the results of additional experiments. Although the difference in values obtained from two different groups of experiments was at the most a factor of 2, reproducibility within the same group, where the same preparation of inactivated enzyme was used, was satisfactory. The parameters in the same group can be compared directly. The higher the pH value, the higher the k_{+3} value. The increase of k_{+3} was steeper from pH 7.0 to pH 7.8 than from pH 5.6 to pH 7.0. Groups 1 and 2 both showed similar dependency of k_{+3} on the pH value. k_{+4} was also

affected by the pH; it was larger at higher pH values. Consequently, the final recovery of catalytic activity was highest at pH 7.0.

Neither glycerol (15%, v/v) nor hydroxylamine (20 mM) (Fig. 3) affected the rate constants or extent of final recovery. No difference was observed during re-activation of enzyme inactivated at an $[A]/[E]$ ratio of 1:1 or 11:1 (results not shown). The decay constant of EA*, a sum of k_{+3} and k_{+4} , was very low, about $2 \times 10^{-5} \text{ s}^{-1}$ at pH 7.0.

Isoelectric focusing of inactivated enzyme

To provide evidence in support of eqn. (1),

Fig. 4. Densitometer scans of the different forms of β -lactamase after polyacrylamide-gel isoelectric focusing (a) Mixtures of native enzyme ($5.0\mu\text{M}$) and AMPA ($5.0\mu\text{M}$) (upper) or $25.0\mu\text{M}$ (lower) were incubated in 0.1 M -sodium phosphate buffer, pH 7.0, for 18.5 h at 37°C . A 40 ml sample of the mixture was applied on to the polyacrylamide gel (LKB Ampholine PAGplate) and focused. (b) Inactivated enzyme ($6.3\mu\text{M}$, experimental group 2 in Table 2), free from excess of AMPA (see the Materials and methods section), was analysed by isoelectric focusing after regeneration of active enzyme for 20.5 h (upper) and 44 h (lower) in 0.1 M -sodium phosphate buffer, pH 7.0, at 37°C . Densitometry was performed after staining for protein.

percentages of existing E and EA^* after prolonged incubation were determined by isoelectric focusing. As shown in Fig. 4(a), when native enzyme and AMPA were incubated at an $[\text{A}]/[\text{E}]$ ratio of 5:1, three bands were detected after 18.5 h incubation, but no catalytic activity was found. On the other hand, the sample mixed at an $[\text{A}]/[\text{E}]$ ratio of 1:1 showed four protein bands in the same incubation conditions. One additional band focused at pH 5.4, the same pI as that of native enzyme, and amounted to 34% of all protein, which corresponded to 27% of the catalytic activity of a control with enzyme alone. Therefore the other three bands could effectively be regarded as inactive. The difference in the number of protein bands between the two samples is explained by the fact that regenerated active enzyme is inactivated again by excess of AMPA in the experiment at an $[\text{A}]/[\text{E}]$ ratio of 5:1 but not at an $[\text{A}]/[\text{E}]$ ratio of 1:1.

Fig. 4(b) illustrates the patterns of enzyme forms at pH 7.0 after 20.5 and 44 h incubation at 37°C .

After 20.5 h regeneration the four main bands observed contained 99% of all protein, whereas they accounted for 93% after 44 h incubation. The fractional amount of each band and activity expressed as a percentage of control were compared with those values calculated in accordance with eqn. (1) by using the data in Table 2. Table 3 shows that percentages of regenerated activity coincided with those of native enzyme detected and calculated theoretically by using eqn. (1). Similar close agreement was obtained between the percentages of existing EA^* observed and those calculated. These data validated Scheme 1 as well as eqn. (1). For unknown reasons the samples incubated at pH 7.8 did not give a clear isoelectric-focusing pattern on the gel in the pH range 4–6.5 (though probably because of disturbance of the pH gradient by the high pH value).

Isoelectric focusing also indicated that the inactivated enzyme comprised two main species, with pI values of 5.36 and 5.30. The protein with pI 5.36 decreased with incubation time and was not found in the sample incubated at pH 5.6. It may be concluded that the inactive complex with pI 5.36 is an intermediate form.

Products formed from AMPA

Since acylation of a serine residue at the active site of β -lactamase seems to be the general scheme of hydrolysis of β -lactam compounds (Knott-Hunziker *et al.*, 1982), the reaction of AMPA with certain nucleophiles could provide a good model for the chemical reactions of AMPA during the inactivation of TEM-1 β -lactamase. The methods used by Orlek *et al.* (1979) and Reading & Hepburn (1979) with 6 β -bromopenicillanic acid and clavulanic acid respectively were employed to study the reactions of AMPA with sodium methoxide as nucleophilic reagent. Only one compound, (Ia), was formed. The structure of compound (Ia) was identified by spectroanalysis and confirmed by X-ray-diffraction analysis of its benzhydryl ester. Compound (Ia) is the monomethyl ester of compound (Ib), which in turn could be isolated after prolonged incubation of the inactivated enzyme at neutral pH, and which was identified by its mass spectrum.

Likely mechanism of inactivation by AMPA

The following experimental observations have to be taken into consideration when discussing possible reaction mechanisms. (1) AMPA-inactivated enzyme had an absorption maximum at about 303 nm. This is shifted to 296 nm by 2.6 M-guanidinium chloride. (2) The spectrum resembled that of an oxime derivative of AMPA pivaloyloxymethyl ester whose β -lactam ring was opened by the

Table 3. Comparison of the activity regenerated with the amount of native β -lactamase detected after isoelectric focusing or calculated in accordance with eqn. (1)

Inactivated enzyme (100 μ l), 40 μ l of 0.5M-potassium phosphate buffer, pH5.0, 7.0 or 8.0, and 60 μ l of sterile 50% (v/v) glycerol were mixed and incubated at 37°C. At the indicated times 50 μ l samples were withdrawn and kept at -20°C. Isoelectric focusing was performed by applying 20 μ l of the sample on to a LKB Ampholine PAGplate, pH range 4-6.5. Quantification was done densitometrically after staining with Coomassie Brilliant Blue R250. Activities were measured with the use of 1ml of 2mM-benzylpenicillin at 37°C. Experimental groups correspond to those described in Table 2. The calculations were performed in accordance with eqn. (1), with k_{+3} , k_{+4} and C values as indicated in Table 2. Calculated values (in parentheses) are expressed as percentages of the original amount of enzyme present before inactivation.

pH	Exptl. group	Incubation time (h)	Activity regenerated (%)	E (pI5.4)	EA* (pI5.15)	EA' = EA' ₁ + EA' ₂ + minor forms (pI5.36) (pI5.3)			
5.6	1	48	25	28	27	46	=	0	+ 35 + 11
				(27)	(23)	(50)			
	2	44	34	38	7	55	=	0	+ 46 + 9
				(38)	(9)	(53)			
7.0	1	24	28	36	25	39	=	5	+ 26 + 8
				(31)	(25)	(44)			
	2	20.5	43	41	13	46	=	16	+ 29 + 1
				(40)	(14)	(46)			
	2	44	45	45	3	52	=	5	+ 40 + 7
				(46)	(1.5)	(52.5)			

treatment with hydroxylamine. (3) EA* was considerably stable, decaying with an apparent first-order constant of about $2 \times 10^{-5} \text{ s}^{-1}$. The constant became greater under basic conditions. (4) EA* obviously decayed in two directions, producing further inactivated forms (mainly EA'₁ and EA'₂) and native enzyme. (5) Compound (Ia) was produced in a model reaction of AMPA with sodium methoxide in methanol; the corresponding diacid compound (Ib) was formed as a decomposition product of inactivated enzyme. Moreover, additional results obtained in the present study and in previous experiments (Arisawa & Then, 1983) must be considered. (6) AMPA seems most likely to be an active-site-directed inhibitor. (7) Four major enzyme forms besides the native one were detected by isoelectric focusing, corresponding to EA, EA*, EA'₁ and EA'₂ in Scheme 1.

On taking these experimental data into consideration, a most likely mechanism is that illustrated in Fig. 5. A rather stable inactivated enzyme, EA*, could be formed via EA (Arisawa & Then, 1983), although the detailed mechanism of this step remains unclear. EA* could be stabilized by the imine-eneamine tautomerism. The absorbance at 303 nm is consistent with the presence of a β -aminoacrylyl ester. From the assumed structure of EA*, a branched pathway in the decay of EA* is possible. During prolonged incubation either slow hydrolysis of the acyl bond or rearrangement to EA'₂ could occur. Release of compound (Ib) to produce active enzyme could follow the hydrolysis.

Since the amino group of the thiazolidine is a good nucleophile, it could displace the Schiff base, resulting in the formation of EA'₂. In acid medium (pH 5.6) the protonated amino group of the thiazolidine would become less potent for the attack of the Schiff base. On the other hand, a lone pair of the amino group could displace the Schiff base more easily under basic conditions (pH 7.8). The acyl bond is known to be more unstable in basic solution. The stability of EA'₂ may be explicable on account of pyrrole structure of the rearranged AMPA. The conjugated ester behaves as a vinylogous urethane and is resistant to hydrolysis (Orlek *et al.*, 1980). The structure of EA'₁ cannot be proposed from the present study, although it is possible to speculate that EA'₁ is an intermediate between EA* and EA'₂.

The suggested structures may qualitatively explain the relative pI values of each enzyme form. EA* could have one more negative charge than EA'₂, which in turn could have one more negative charge than the intact form (E). As a result, the order of acidity among the three would be EA* > EA'₂ > E, which is consistent with the observed pI values.

In summary, AMPA belongs to a new class of potent β -lactamase inhibitors. The incorporation of an $\alpha\beta$ -unsaturated keto group at C-6 makes the compound a poor β -lactamase substrate and a potent β -lactamase inhibitor. In comparison with other inhibitors, the inactivation constant of AMPA is much larger (Arisawa & Then, 1983). Most compounds of this class are effective inhibitors of a variety of β -lactamases (Then *et al.*, 1982).

Fig. 5. Likely mechanism of inhibition of TEM-1 β -lactamase by AMPA and the chemical reaction of AMPA with sodium methoxide.

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