CONVERSION OF CLOXACILLIN INTO A PROGRESSIVE INHIBITOR OF β-LACTAMASES BY SULFONATION AND ITS ACTIVITY AGAINST VARIOUS TYPES OF THESE ENZYMES

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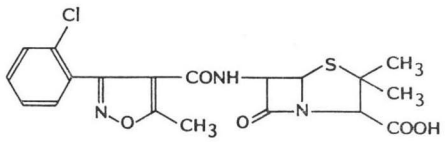
On the assumption of the theory that the sulfone of a penam derivative should effectively act as a suicide substrate against β-lactamases to which the parent compound is a poor substrate, the action of cloxacillin sulfone on four different types of β-lactamases was studied. As we expected, cloxacillin sulfone showed strong mechanism-based irreversible inactivation against type Ib penicillinase and *Proteus vulgaris* cephalosporinase whereas it showed no progressive inactivation against cloxacillin-hydrolyzing type II penicillinase. However, an unexpected result was that cloxacillin sulfone could not inactivate *Citrobacter freundii* cephalosporinase which itself could hardly hydrolyze the parent cloxacillin. The number of hydrolytic events which occurred before inactivation of type Ib penicillinase, and *P. vulgaris* cephalosporinase, by the inactivator was 190 and 13, respectively. These values indicate that cloxacillin sulfone is far more effective as a suicide substrate against the two types of β-lactamases than penam sulfones so far reported. The inactivation proceeded *via* the formation of an irreversible enzyme-inhibitor complex which could be detected by isoelectric focusing.

A major cause of bacterial resistance to β-lactam antibiotics is the ability to produce β-lactamases[1]. Recently, a new approach to the β-lactamase problem has been taken by utilizing the β-lactamase inactivators with characteristics of "suicide" or "mechanism-based" irreversible inactivation[2~14]. More recently, other types of inactivators based on "tight-binding competitive" inactivation have also been reported[15,16]. The mechanism of suicide inactivation has been investigated mainly using plasmid-coded penicillinases. However, since the chromosomally-mediated cephalosporinases play an important role in the resistance of Gram-negative bacteria to cephalosporins and penicillins[1], it is necessary to elucidate the inactivation mechanisms of such cephalosporinases by the novel inactivators.

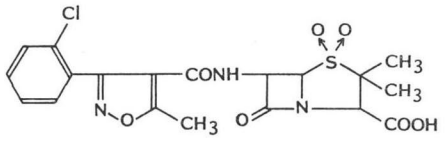
FISHER *et al.*[2,17] assumed that suicide inactivation of TEM penicillinase by penam sulfones may be caused by β-elimination of the 6α-proton resulting in cleavage of the bond between C-5 and the heteroatom of the chromophore of the acyl-enzyme intermediate. The resulting β-aminoacrylate chromophore may be stable. Such a β-elimination process must compete with the normal hydrolysis of the acyl-enzyme. The same authors showed that only penicillin sulfones derived from poor substrates for TEM penicillinase were efficient inactivators of the penicillinase[2]. It is interesting to know whether such a relationship between the inactivation activity of sulfones and the stability of the parent compounds to β-lactamase hydrolysis is also observed in the cases of cephalosporinases and penicillinases other than the TEM type.

Cloxacillin is one of the isoxazolyl penicillins, stable to many β-lactamases except the OXA-type penicillinase[18]. In this study, we synthesized cloxacillin sulfone and examined the relationship between

Fig. 1. Chemical structure of cloxacillin and cloxacillin sulfone.



Cloxacillin



Cloxacillin sulfone

its ability to inactivate the four types of β-lactamases including two cephalosporinases, and the stability of cloxacillin to those enzymes.

Materials and Methods

Preparation of β-Lactamases

Type II penicillinase[18] is an OXA-type penicillinase with high ability to hydrolyze cloxacillin. Type Ib penicillinase[19] corresponds to the TEM-2 type in the MATTHEW's classification[20], which has a relatively broad substrate specificity but its cloxacillin-hydrolyzing ability is lower than that of type II penicillinase. *Proteus vulgaris* and *Citrobacter freundii* cephalosporinase are species specific cephalosporinases mediated by chromosomal gene(s)[1]. The former has a broader substrate specificity than the latter, that is, the former can hydrolyze penicillins in some degree other than cephalosporins whereas the substrate specificity of the latter is limited to cephalosporins[1]. Cloxacillin is a poor substrate for these two cephalosporinases, especially for *C. freundii* cephalosporinase.

Type II and type Ib penicillinases and *P. vulgaris* and *C. freundii* cephalosporinases were prepared from *Escherichia coli* ML1410 (RGN238) and ML1410 (RGN823), *P. vulgaris* GN76/C-1 and *C. freundii* GN346, respectively as described in the previous paper[16]. The enzymes were purified to homogeneity as judged by SDS-polyacrylamide gel (PAG) electrophoresis.

β-Lactam Antibiotics

Cloxacillin and benzylpenicillin were kindly provided by Meiji Seika Kaisha, Ltd., Tokyo, Japan. Cephalothin was a gift from Torii & Co., Tokyo, Japan. Cloxacillin sulfone was synthesized from cloxacillin as follows;

Ten gram (21.86 mmol) of cloxacillin in 50 mM phosphate buffer (pH 6.0) was oxidized to the β-oxide of cloxacillin by the dropwise addition of 4.676 g (21.86 mmol) of $NaIO_4$ in the same phosphate buffer. The solution was then stirred for 15 minutes at room temperature. The resulting β-oxide was purified by Diaion HP-50 column chromatography. The β-oxide fraction was pooled, concentrated and dried under vacuum. One gram (2.12 mmol) of cloxacillin β-oxide in the phosphate buffer was further oxidized at ice bath temperature by dropwise addition of 0.36 g of $KMnO_4$ in the same buffer. During the reaction, the pH of the mixture was maintained between 6.2 ~ 6.5 by addition of 10% phosphoric acid. After 30 minutes, excess $KMnO_4$ was destroyed by addition of sodium bisulfite. The reaction mixture was twice filtered through Hyflo super cell, and the cloxacillin sulfone then extracted from the filtrate into 100 ml ethyl acetate. The resulting cloxacillin sulfone was concentrated and dried under vacuum.

The purity of the cloxacillin sulfone was about 81% as judged by thin-layer chromatography and HPLC using Nucleosil C_{18} column eluted with a one-to-one mixture of methanol and 0.1 M sodium acetate buffer (pH 6.0). The IR spectrum of the product showed a characteristic absorption peak at 1790 cm^{-1}, which suggested the presence of an intact β-lactam ring structure. ^1H NMR (D_2O, TMS=0.0) δ 1.40 (s, 3H), 1.51 (s, 3H), 2.69 (s, 3H), 4.27 (s, 1H), 5.18 (d, 1H, J=4.3 Hz), 6.09 (d, 1H, J=4.3 Hz), 7.75 (m, 4H).

Determination of *Ki* Value

Benzylpenicillin (200, 80, 50, 40, 30, 20 or 15 μM) for penicillinases, and cephalothin (100, 60, 50, 40, 30, 25, 20, 15 or 10 μM) for cephalosporinases, was mixed with cloxacillin (0, 10, 15 μM) or cloxacillin sulfone (0, 15, 30 μM) in 50 mM phosphate buffer (pH 7.0) and prewarmed to 30°C. Fifty microliters of the enzyme (approx 0.1 μM) was added to the mixture. Hydrolysis of benzylpenicillin and cephalothin was measured by the change in absorbance at 232 nm and 265 nm, respectively. Under these conditions, the absorption change due to hydrolysis of the inhibitors was negligible. The *Ki* value was cal-

Table 1. Kinetic properties of cloxacillin against four β-lactamases.

Enzyme	*Km* or *Ki* (μM)	Rate constants (second^{-1})		k_{cat}/Km (M^{-1} second^{-1})
		k_{cat}	k^{a}_{react}	
Type II penicillinase	15 (*Km*)	28.3	—	1.9×10^{6}
Type Ib penicillinase	15 (*Ki*)	12.2	—	0.8×10^{6}
P. vulgaris cephalosporinase	0.27 (*Ki*)	1.6×10^{-1}	1.1×10^{-2}	0.6×10^{6}
C. freundii cephalosporinase	0.027 (*Ki*)	5.3×10^{-4}	8.3×10^{-4}	2.0×10^{4}

[a] The value of k_{react} was determined as a first-order rate constant of the reactivation of the enzyme in Fig. 2.

Table 2. Kinetic properties of cloxacillin sulfone against four β-lactamases.

Enzyme	*Km*[a], *Ki* or Km_{inact}[b] (μM)	Rate constants (second^{-1})		k_{cat}/Km (M^{-1}second^{-1})
		k_{cat}	k_{inact}	
Type II penicillinase	900 (*Km*)	96.7	—	1.1×10^{5}
Type Ib penicillinase	850 (Km_{inact})	4.17	1.7×10^{-2}	4.9×10^{3}
P. vulgaris cephalosporinase	700 (Km_{inact})	5.2×10^{-2}	9.5×10^{-3}	7.4×10
C. freundii cephalosporinase	0.28 (*Ki*)	8.3×10^{-2}	—	3.0×10^{5}

[a] *Km* and k_{cat} values were calculated from the double reciprocal plot of the initial rate of cloxacillin sulfone hydrolysis by β-lactamases.

[b] Km_{inact} and k_{inact} values were calculated from the double reciprocal plot of the initial rate of inactivation of the enzyme activity by cloxacillin sulfone.

culated from a Lineweaver-Burk plot of initial hydrolysis rate against substrate concentration at several inhibitor concentrations.

Dilution Assay of Progressive Inactivation

A 1 μM solution of β-lactamase in 50 mM phosphate buffer (pH 7.0) was incubated with the indicated concentrations of cloxacillin or cloxacillin sulfone at 30°C. At time intervals, 5 μl of the mixture was withdrawn and added to 3 ml of either 200 μM of benzylpenicillin for penicillinases or 100 μM of cephalothin for cephalosporinases unless otherwise stated. Enzyme activity was determined from the initial rate of substrate hydrolysis as measured spectrophotometrically. As a control, the activity of the enzyme incubated without inhibitor was measured in the same way. The values of Km_{inact} and k_{inact} for progressive inactivation were calculated from the double reciprocal plot of the initial rate of inactivation against inhibitor concentration as reported in the previous paper[21].

Hydrolysis of Cloxacillin and Cloxacillin Sulfone

Two hundred μl of indicated concentrations of cloxacillin or cloxacillin sulfone was added to 2 ml of 1 μM β-lactamase in 50 mM phosphate buffer (pH 7.0) and the mixture was then incubated at 30°C. At time intervals, aliquots were withdrawn and added to 1.2 ml of 0.15 M sodium tungstate to terminate the enzyme catalysis. The amount of hydrolyzed β-lactam was measured by microiodometric assay[22].

Recovery of β-Lactamase Activity after Gel Filtration

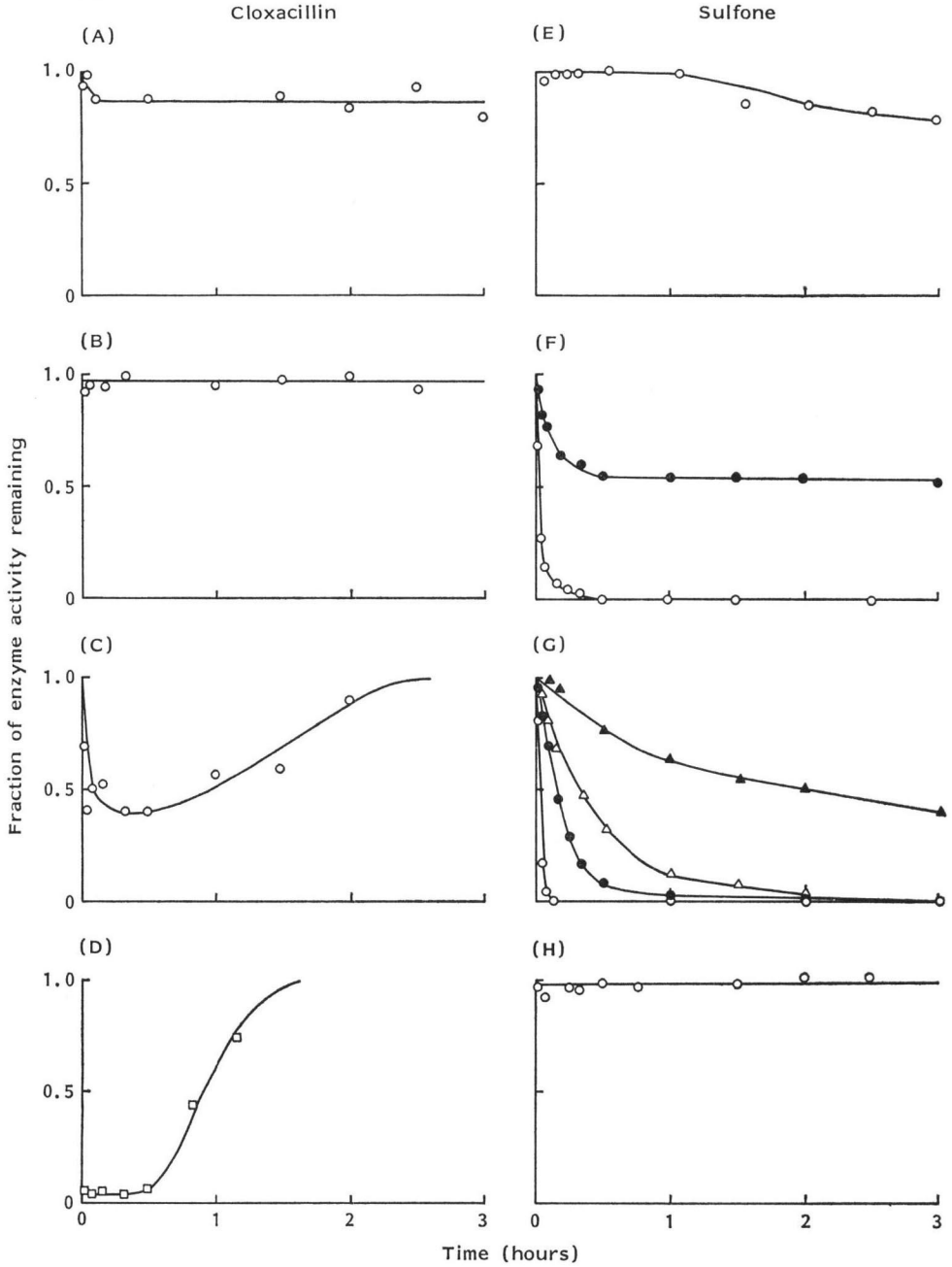
One hundred μl of 10 μM β-lactamase was added to 100 μl of 10 mM cloxacillin sulfone in 50 mM phosphate buffer (pH 7.0) and the mixture was then incubated at 30°C for 30 minutes. Excess cloxacillin sulfone was then removed by gel filtration through a Sephadex G-25 column. Inactivated enzyme fractions were pooled and incubated at 30°C. At time intervals, an aliquot of the sample was withdrawn and the enzyme activity was measured spectrophotometrically using 200 μM benzylpenicillin or 100 μM of cephalothin as substrate. As a control, enzyme was incubated without inhibitor followed by gel filtration.

Isoelectric Focusing

Acrylamide and *N,N'*-methylene-bis(acrylamide) were purchased from Kodak; carrier Ampholytes were purchased from LKB. Slab gel isoelectric focusing was run on an ATTO model SJ1071 apparatus.

Fig. 2. Progressive inactivation pattern of four β-lactamases by cloxacillin and cloxacillin sulfone.

Type II (A, E) and type Ib (B, F) penicillinases and *P. vulgaris* (C, G) and *C. freundii* (D, H) cephalosporinases were incubated with cloxacillin (left column) and cloxacillin sulfone (right column). Enzyme activity remaining was measured by the dilution method described in Materials and Methods. Enzyme concentration was 0.91 μM and initial ratio of inhibitor to enzyme (i/e) was 800 (○), 80 (●), 40 (△), 8 (▲) and 2 (□).



Forty μl of 20 μM type Ib penicillinase or *P. vulgaris* cephalosporinase was incubated with 400 μl of excess inhibitor (cloxacillin or cloxacillin sulfone) at 30°C for 1 hour. The mixture was then cooled in an ice bath. Thirty μl of the mixture was applied to Ampholyte (pH 3.5~9.5) PAG plate and run for 3 hours.

Results

Kinetics of the Inhibition of the Four β-Lactamases by Cloxacillin and Cloxacillin Sulfone

Competitive inhibition of the β-lactamases by cloxacillin and the hydrolysis of cloxacillin by these β-lactamases was measured. As shown in Table 1, the cephalosporinases have high affinity for cloxacillin, while the penicillinases show moderate affinity. On the other hand, the rate of cloxacillin hydrolysis (k_{cat}) by the cephalosporinases is very much lower than that seen with the penicillinases. Judging from the value of k_{cat}/Km (or k_{cat}/Ki), cloxacillin is a slightly poorer substrate for type Ib penicillinase and *P. vulgaris* cephalosporinase than for type II penicillinase for which cloxacillin is a moderately good substrate, while cloxacillin is a very poor substrate for *C. freundii* cephalosporinase.

Oxidation of the thiazolidine sulfur to the sulfone caused a drastic decrease in the affinity of cloxacillin for all the β-lactamases tested (Table 2). On the other hand, the variation in k_{cat} value differs with the different β-lactamases. It should be noted that the k_{cat} value for *C. freundii* cephalosporinase was markedly increased by the oxidation.

The Progressive Inactivation of the Four β-Lactamases by Cloxacillin and Cloxacillin Sulfone

Fig. 2 shows the time dependence of the residual activity of the four β-lactamases during incubation with cloxacillin or cloxacillin sulfone. The residual activity was monitored by the dilution method described in Materials and Methods. In this assay method, simple competitive inhibition by free inhibitor was excluded, and the loss of enzyme activity represents only the progressive part of the inactivation[21].

Cloxacillin was not a progressive inactivator for the penicillinases (Fig. 2A, B). However, cloxacillin did show progressive inactivation of the cephalosporinases although the enzyme fully recovered its activity after the consumption of the inhibitor (Fig. 2C, D). When *C. freundii* cephalosporinase was treated with cloxacillin at an initial enzyme/inhibitor (i/e) ratio higher than about 10, any recovery of the enzyme activity, at least within several hours, could not be observed because hydrolysis of cloxacillin by the enzyme was very slow (data not shown). Such a reversible inactivation of *C. freundii* cephalosporinase by cloxacillin could be explained in terms of high stability of the acyl-enzyme intermediate on the normal pathway of enzymatic hydrolysis of cloxacillin from the following two facts; 1) the hydrolysis of cloxacillin by *C. freundii* cephalosporinase did not show an initial burst, which is characteristic for a branched inactivation mechanism[21], and 2) the k_{cat} value was roughly equal to the k_{react} value for *C. freundii* cephalosporinase (Table 1). On the other hand, in the case of *P. vulgaris* cephalosporinase, the reversible inactivation might be due to the reversible branching from the normal enzymic pathway as indicated from the following two facts, 1) an initial burst of cloxacillin hydrolysis up to 12-fold for the enzyme concentration was observed (data not shown) and 2) the k_{cat} value was very much higher than k_{react} value for *P. vulgaris* cephalosporinase (Table 2).

Cloxacillin sulfone showed the characteristics of a typical progressive inactivator against two of the β-lactamases, namely, type Ib penicillinase and *P. vulgaris* cephalosporinase (Fig. 2F, G), whereas the sulfone did not show any progressive inactivation against type II penicillinase and *C. freundii* cephalosporinase (Fig. 2F, H). It may be of interest to note here that the β-oxide of thiazolidine sulfur of cloxacillin did not show such a progressive inactivation against any of these four β-lactamases (data not shown).

Irreversible Inactivation of Type Ib Penicillinase and *P. vulgaris* Cephalosporinase by Cloxacillin Sulfone

When type Ib penicillinase was incubated with cloxacillin sulfone at less than two hundred molar excess to the enzyme, the residual enzyme activity reached a constant level, depending on the initial i/e ratio, and the reactivation could not be observed even after prolonged incubation (Fig. 2F). The lack of reactivation may be a characteristic for the enzyme inactivation by cloxacillin sulfone, because the inactivation of the same enzyme by other suicide substrates is known to be partially reversible[9,21]. In the case of *P. vulgaris* cephalosporinase, the residual activity appeared not to reach a constant level even after 3-hour incubation with the inactivator (Fig. 2H), but this was due to the slow rate of enzyme inactivation. When the initial concentration of both the enzyme and cloxacillin sulfone was sufficiently high, the residual activity of *P. vulgaris* cephalosporinase also reached a constant level, depending on the initial i/e ratio (Fig. 3A). As shown in Fig. 3B, there was no biphasic nature of the semilogarithmic plot of the inactivation against incubation time, indicating the absence of a transiently stable complex which competes with irreversible complex formation from acyl-enzyme intermediate[9].

The residual enzyme activity after prolonged incubation was inversely proportional to the initial i/e ratio (Fig. 4). By extrapolation of the plot to the x-intercept, the minimum i/e ratio required for complete inactivation of the enzyme could be obtained. These values for type Ib penicillinase and *P. vulgaris* cephalosporinase were 250 and 13, respectively.

The time-course of the cloxacillin sulfone hydrolysis by type Ib penicillinase or *P. vulgaris* cephalosporinase, at an initial i/e ratio of about 80, was followed together with the time-course of the loss of the enzyme activity (Fig. 5). Under these experimental conditions, *P. vulgaris* cephalosporinase was completely inactivated before complete consumption of cloxacillin sulfone (Fig. 5B), whereas type Ib penicillinase completely hydrolyzed cloxacillin sulfone before one half of the initial activity was lost (Fig. 5A). After the consumption of cloxacillin sulfone, the enzyme activity of type Ib penicillinase kept constant. In both cases, the molar amount of enzymatically hydrolyzed cloxacillin sulfone was larger than that of the enzymes inactivated during the incubation period, suggesting that the inactivation was based on the branched mechanism (suicide inactivation). The number of hydrolytic events of cloxacillin sulfone

Fig. 3.

A) Progressive inactivation of *P. vulgaris* cephalosporinase by cloxacillin sulfone.
Residual enzyme activity was monitored by the dilution method. Enzyme concentration was 5 μM. Initial i/e ratio was 20 (○), 10 (●), 7 (△), 4 (▲) and 2 (□).

B) Semilogarithmic plot of progressive inactivation in (A). Initial i/e ratio was 10 (●) and 7 (○).

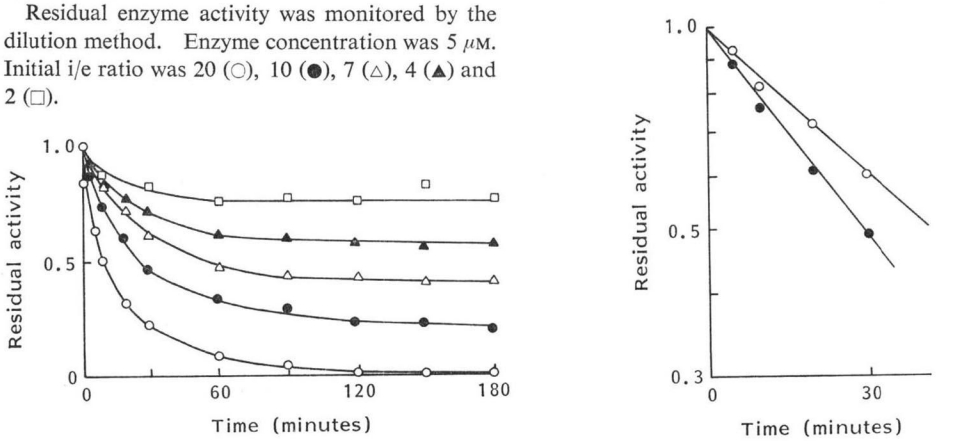


Fig. 4. Fractional activity remaining after incubation of type Ib penicillinase (A) and *P. vulgaris* cephalosporinase (B) with cloxacillin sulfone for 120 minutes at 30°C was plotted against initial i/e ratio. Enzyme concentration was 5 μM.

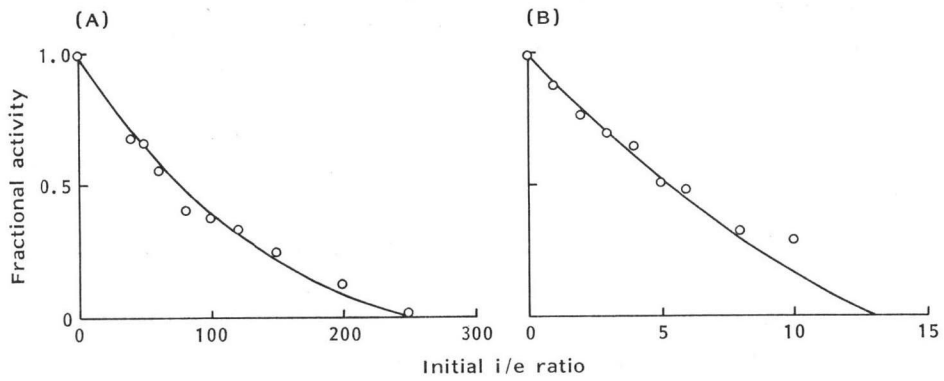
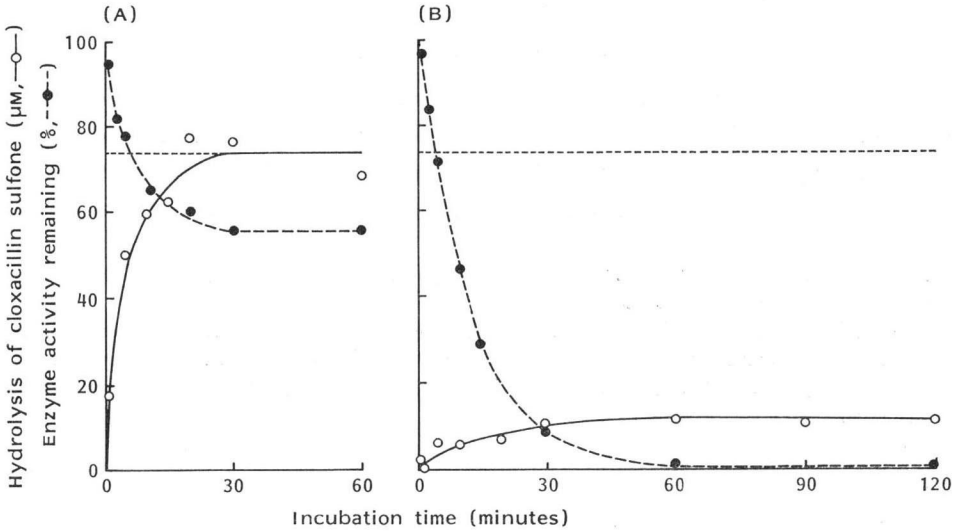


Fig. 5. Hydrolysis of cloxacillin sulfone by type Ib penicillinase (A) and *P. vulgaris* cephalosporinase (B). Open circles with solid line represent the amount of hydrolyzed product detected by microiodometric method as described in Materials and Methods. Closed circles with broken line represent the enzyme activity remaining under the same condition of the assay of hydrolysis. Enzyme concentration was 0.91 μM. Initial concentration of cloxacillin sulfone was 74 μM.

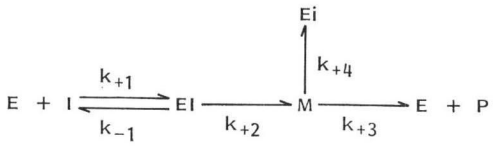


before inactivation of one enzyme molecule was calculated to be about 13 for *P. vulgaris* cephalosprinase and 190 for type Ib penicillinase. These values are in good agreement with the values of minimum i/e ratio required for complete inactivation of the enzymes described above.

When type Ib penicillinase and *P. vulgaris* cephalosporinase were completely inactivated by excess cloxacillin sulfone and then the free cloxacillin sulfone was removed by gel filtration, the recovery of the enzyme activity was hardly observed even after several hours incubation at 30°C (data not shown). Although only 6% (type Ib penicillinase) and 8% (*P. vulgaris* cephalosporinase) of the initial enzyme activity was recovered after overnight incubation at 30°C, we could not confirm whether the initial enzyme can recover its activity completely by further prolonged incubation though pseudo-irreversible inactiva-

Fig. 6. The outline scheme of the inactivation mechanism of type Ib penicillinase and *P. vulgaris* cephalosporinase by cloxacillin sulfone.

E, free enzyme; I, cloxacillin sulfone; EI, Michaelis complex; M, acyl-enzyme intermediate; Ei, irreversively inactivated enzyme; P, enzymatically degradation product.



tion is a common characteristic for so-called suicide inactivation of β-lactamases[21]. The activity of inactivated enzyme could not be restored after several hours incubation with 12.5 mM NH_2OH at pH 7.0.

Steady State Analysis of the Inactivation Mechanism of *P. vulgaris* Cephalosporinase by Cloxacillin Sulfone

We further investigated the inactivation kinetics of *P. vulgaris* cephalosporinase by cloxacillin sulfone as a representative case of suicide inactivation. According to the WALEY's steady state analysis of suicide inactivation as shown in Fig. 6[23], a progressive decrease in the residual enzyme activity can be expressed as follows:

$$-d\ln a/dt = Ai/(B+i) \quad \text{(i)}$$

where a and i represent the residual enzyme activity and the inactivator concentration, respectively. A and B could be written using rate constants as follows:

$$A = k_{+2}k_{+4}/(k_{+2}+k_{+3}+k_{+4}) \quad \text{(ii)}$$

$$B = ((k_{-1}+k_{+2})/k_{+1})(k_{+3}+k_{+4})/(k_{+2}+k_{+3}+k_{+4}) \quad \text{(iii)}$$

In Fig. 7A, $\ln(a/e_0)$ in the case of *P. vulgaris* cephalosporinase inactivation was plotted against the incubation time. The inactivation represented by Eq (i) is not strictly first-order, but the deviation from linearity was not apparent if i_0 is greater than $k_{+3}k_{+4}e_0$, in other words, initial i/e ratio is greater than the number of hydrolytic events before inactivation of enzyme molecule. The double reciprocal plot of

Fig. 7. Progress curve analysis of *P. vulgaris* cephalosporinase inactivation by cloxacillin sulfone using steady state kinetics of WALEY[23].

a, Active enzyme remaining; e_0, total enzyme; i, initial concentration of cloxacillin sulfone. Enzyme concentration was 0.91 μM. (A) The plot of $\ln(a/e_0)$ *versus* incubation time. (B) Replot of the slope of the plot in (A) *versus* 1/i. (C) Similar plot in (B) in the case of high initial concentration of cloxacillin sulfone.

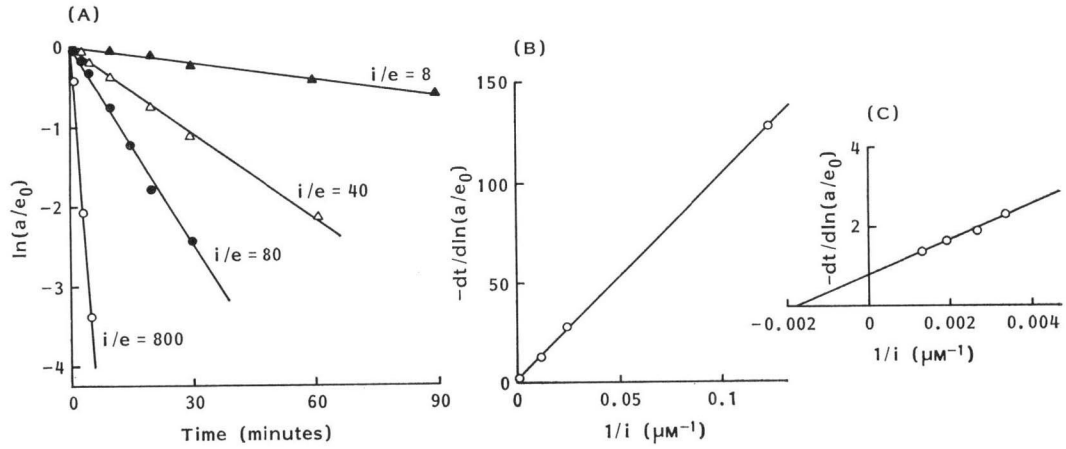
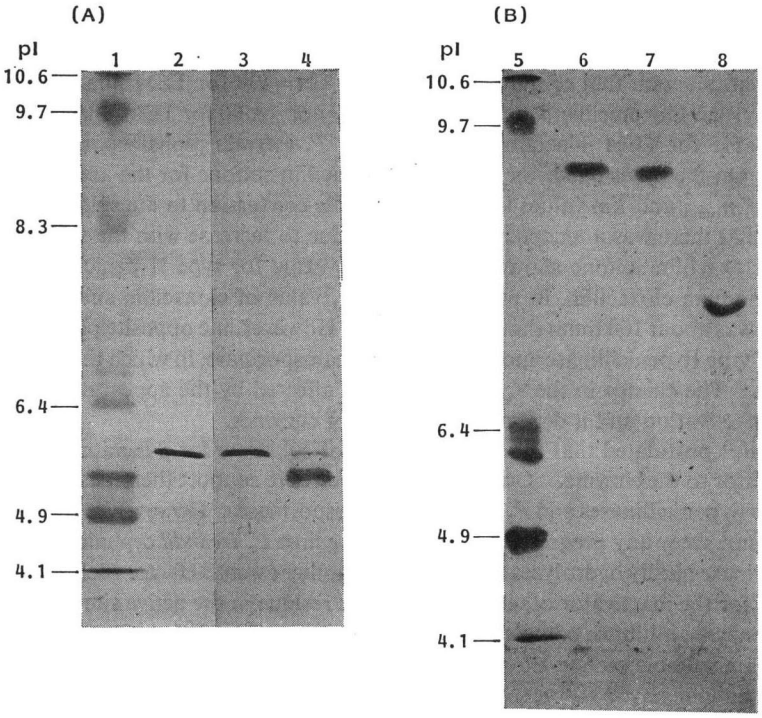


Fig. 8. Polyacrylamide isoelectric focusing of type Ib penicillinase (A) and *P. vulgaris* cephalosporinase (B) after the inactivation.

Lane 1 and 5, pI marker proteins purchased from Oriental Yeast Co., Tokyo, Japan; lane 2 and 6, native enzyme; lane 3 and 7, enzyme inactivated by excess cloxacillin; lane 4 and 8, enzyme inactivated by excess cloxacillin sulfone.



$dIn(a/e_0)/dt$ obtained from the slope of the plot in Fig. 8A against i_0 shows a linear relationship (Fig. 7B). The linearity is more explicit when initial cloxacillin sulfone is raised (Fig. 7C), The values of A and B could be calculated from y and x-intercepts to be 1.25 min^{-1} and 541 μM, respectively. These analyses indicate that the scheme shown in Fig, 6 is in good agreement with the inactivation mechanism of *P. vulgaris* cephalosporinase by cloxacillin sulfone.

Isolation of the Inactivated Enzyme Proteins by Isoelectric Focusing

The pI values of the native form of type Ib penicillinase and *P. vulgaris* cephalosporinase were 5.8 and 9.2, respectively (Fig. 8). When *P. vulgaris* cephalosporinase was inactivated by 100 molar excess of cloxacillin sulfone and applied on the isoelectric focusing, only one species of the inactivated enzyme was detected at a position of lower pI value (7.6) than the native enzyme. However, the pI value of the cephalosporinase was not affected by the incubation with cloxacillin. When type Ib penicillinase was incubated with cloxacillin sulfone, one main species (pI=5.45), two satelite bands (pI=5.5 and 5.4) around the main species, and a very minor species (pI=5.7) in addition to trace amounts of intact species at pI=5.8 (Fig. 7A) were detected. It was also confirmed that the pI value of type Ib penicillinase is not affected by incubation with cloxacillin.

Discussion

Cloxacillin sulfone as an inactivator of β-lactamase was first reported by BRENNER *et al.* as preliminary work in a short review[24]. The work described here is the first report of the detailed characteri-

stics of cloxacillin sulfone as an inactivator of various types of β-lactamases. Cloxacillin sulfone showed high ability to cause progressive inactivation of type Ib penicillinase and *P. vulgaris* cephalosporinase to which cloxacillin itself could not act as a suicide inactivator. The efficiency of cloxacillin sulfone as a suicide inactivator against type Ib penicillinase and *P. vulgaris* cephalosporinase is expressed as the minimum i/e ratio required for complete inactivation of the enzyme activity (turnover number; TN), which is 250 for type Ib penicillinase and 13 for *P. vulgaris* cephalosporinase, respectively. Those values are far lower than those of sulbactam (TN=5,000 for TEM β-lactamase[21]). The potency of cloxacillin sulfone is comparable with that of quinacillin sulfone (TN=400 for TEM β-lactamase[2]), 6-(trifluoromethane sulfonyl) amidopenicillanic acid sulfone (TN=approx 60 for TEM β-lactamase[25]) and clavulanic acid (TN=115 for TEM β-lactamase[9] and 70 for *P. vulgaris* cephalosporinase (our unpublished observation)). On the other hand, the affinity of cloxacillin sulfone for the active site of β-lactamases judged by *Ki*, Km_{inact} and *Km* values is decreased by the conversion to the sulfone (Table 2). FISHER *et al.* reported that there was a tendency for the k_{cat} value to increase with the sulfonation[2]. We also observed that cloxacillin sulfone showed a higher k_{cat} value for type II penicillinase and *C. freundii* cephalosporinase than cloxacillin, in particular the k_{cat} value of cloxacillin sulfone for *C. freundii* cephalosporinase was about 160 times that of cloxacillin. However, the opposite phenomenon was observed in the case of type Ib penicillinase and *P. vulgaris* cephalosporinase, in which the k_{cat} values were somewhat decreased. The change in the k_{cat} value may be affected by the appearance of the branching to cause enzyme inactivation and it depends on the type of enzymes.

FISHER *et al.*[2] postulated that the sulfone of a poor substrate for β-lactamases tends to act as an effective inactivator to the enzyme. Our results described here support their idea at least with respect to type II and type Ib penicillinases and *P. vulgaris* cephalosporinases. However, it is unclear why cloxacillin sulfone can not show any progressive inactivation against *C. freundii* cephalosporinase even though the cephalosporinase hardly hydrolyzes cloxacillin. Another essential factor such as a second covalently binding residue for the inactivator other than the serine residue in the active site may be responsible for an irreversible enzyme-inhibitor complex formation as suggested by BRENNER and KNOWLES[5]. It is assumed that such a suitable second site is absent from the surroundings of the active site of *C. freundii* cephalosporinase.

It is characteristic of the inactivation mechanism by cloxacillin sulfone that there was no transiently inactivated form of the enzyme, which is general for suicide inactivation[9,25,26]. Asparenomycin may be an example of showing similar inhibitory mechanism[27], but the antibiotic has a carbapenem nucleus, so β-elimination should not contribute to the inactivation function. β-Elimination of 6α-proton and the bond between C-5 and sulfur is considered to be responsible for the transiently inactivated form[2,5]. Such a transiently inactivated form is made competitive to irreversible enzyme-inhibitor complex formation[2]. In consideration of the good correlation between turn over number (TN) and k_{cat} of cloxacillin sulfone to the β-lactamases except *C. freundii* cephalosporinase, the β-elimination is thought to play an important role in suicide inactivation by cloxacillin sulfone. The β-elimination may inevitably cause simultaneous covalent binding of the enamine chromophore to an additional amino acid residue other than the serine residue. The recovery of the cloxacillin sulfone-inactivated enzymes could not be enhanced by the presence of hydroxylamine. This fact also strongly suggests the second bond formation.

The multiplicity of the inactivated form of type Ib penicillinase as shown by isoelectric focusing suggests a complex rearrangement or conformational change after β-elimination reaction in this enzyme similar to the case of the inactivation of this enzyme by clavulanic acid[5] or 6-acetylmethylene penicillanic acid[26].

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