DELAYED INACTIVATION OF *CITROBACTER FREUNDII* CEPHALOSPORINASE BY 6β-[3-(2-CHLOROPHENYL)-5-METHYL-4-ISOXAZOLYL]PENICILLIN SULFONE

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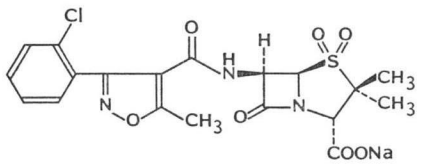
When *Citrobacter freundii* cephalosporinase was incubated with 6β-[3-(2-chlorophenyl)-5-methyl-4-isoxazolyl]penicillin sulfone (cloxacillin sulfone) in phosphate buffer, the enzyme was suddenly inactivated just after the completion of enzymatic degradation of the cloxacillin sulfone. Such delayed inactivation was due to a secondary inhibitor formed from cloxacillin sulfone during the incubation period. The inactivation was delayed due to the protection of the enzyme by cloxacillin sulfone from the attack of the secondary inhibitor. Phosphate anions were essential for the formation of the secondary inhibitor. However, once the secondary inhibitor was formed, the inactivation occurred in the absence of phosphate anions although the degree of the inactivation depended on the length of the preincubation period with phosphate anions. The main species (more than 80%) of the inactivated enzyme was detected as a single protein band with a slightly lower pI value than that of the native enzyme on isoelectric focusing on a plate.

Oxidation of the thiazolidine sulfur of a β-lactamase-stable penicillin is known to convert the antibiotic into a "mechanism-based" inhibitor of β-lactamase[1]. A sulfone may accelerate β-elimination of the 6α proton and cleavage of the bond between C-5 and the sulfur, resulting in a transiently stable 6-aminoacrylate chromophore, which causes enzyme inactivation[1,2]. 6β-[3-(2-Chlorophenyl)-5-methyl-4-isoxazolyl]penicillin (cloxacillin) is a semisynthetic penicillin that is stable for many types of β-lactamases, an exception being OXA-type penicillinases[3]. In our previous study[4], we proved that cloxacillin sulfone (Fig. 1) is, as predicted from the stability of cloxacillin against enzymatic hydrolysis, a strong suicide inhibitor for some β-lactamases. However, cloxacillin sulfone showed no progressive inactivation of *Citrobacter freundii* cephalosporinase, even though cloxacillin is a very poor substrate for the cephalosporinase. On the other hand, we found an unexpected phenomenon concerning the interaction between cloxacillin sulfone and the cephalosporinase, the cephalosporinase was irreversibly inactivated just after completion of the enzymatic degradation of the cloxacillin sulfone by the enzyme. This "delayed inactivation" was most likely not caused by contamination of the cloxacillin sulfone preparation because the inactivation was seen even after repeated purification of the preparation by HPLC using different columns and elution systems. This study was carried out to elucidate the mechanism of the delayed inactivation.

Fig. 1. The chemical structure of cloxacillin sulfone sodium salt.



Experimental

β-Lactam Antibiotics and Related Compounds

Cloxacillin and cephalothin are generous gifts from Meiji Seika Kaisha, Ltd., Tokyo, Japan, and Torii & Co., Ltd., Tokyo, Japan, respectively. Cloxacillin sulfone was synthesized from cloxacillin by two step oxidation *via* the β-oxide of cloxacillin[4]. The cloxacillin sulfone was purified by HPLC on a Nucleosil C_{18} column. ^{1}H NMR of cloxacillin sulfone (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).

Preparation of β-Lactamases

The *C. freundii* and *Proteus vulgaris* cephalosporinases, and type Ib penicillinase, which is identical to TEM-2 penicillinase, were isolated and purified to homogeneity from *C. freundii* GN346, *P. vulgaris* GN76/C-1 and *Escherichia coli* ML1410 (RGN823), respectively, as reported previously[5].

Dilution Assay for Progressive Inactivation

A 5-μM solution of *C. freundii* cephalosporinase in 50 mM phosphate buffer (pH 7.0) was incubated with 3.5 mM cloxacillin sulfone (molar ratio of inhibitor to enzyme (i/e), 700) at 30°C. At different times, 5 μl of the mixture was withdrawn and added to 3 ml of a 100 μM cephalothin solution in the same buffer. The rate of cephalothin hydrolysis was monitored on the basis of the absorption change at 265 nm. As a control, the activity of the enzyme incubated under the same conditions except for the absence of cloxacillin sulfone was also measured. The residual enzyme activity was determined from the slope for cephalothin hydrolysis of the constant rate attained after a short lag.

Gel Filtration Assay for Residual Enzyme Activity

C. freundii cephalosporinase was incubated under the same conditions as for the dilution assay described above. At different times, 200 μl of the reaction mixture was cooled in an ice bath and then filtered through a Sephadex G-25 column (1.0×20 cm) at 5°C. The inactivated enzyme fractions were detected on the basis of the absorption at 280 nm and pooled. Aliquots of the pooled enzyme were diluted in a 100 μM cephalothin solution and the enzyme activity was determined at 30°C. As a control, the enzyme incubated without cloxacillin sulfone was treated in the same way and then its activity was determined.

For assaying of the enzyme reactivation, the enzyme was incubated with cloxacillin sulfone under the same conditions as above for 6 hours. Then the mixture was filtered through a Sephadex column and the inactivated enzyme fractions were pooled. The recovery of the enzyme activity during incubation at 30°C in 50 mM phosphate buffer (pH 7.0) was determined.

Hydrolysis of Cloxacillin Sulfone

Cloxacillin sulfone (3.5 mM) was incubated with 5 μM *C. freundii* cephalosporinase under the same conditions as for the dilution assay. At different times, 50 μl or 20 μl of the reaction mixture was withdrawn and added to 1.2 ml of a 0.15 M sodium tungstate solution to stop the enzymic reaction. The amount of hydrolyzed cloxacillin sulfone was determined by the micro-iodometric method[6].

Removal of Phosphate Anions

Phosphate anions were removed using molybdate-Dowex resin which was prepared from Dowex-1X8 (Cl^- form, 100~200 mesh) followed by the method of TANAKA *et al.*[7].

The residual phosphate concentration was determined by the BARTLETT method[8].

Results

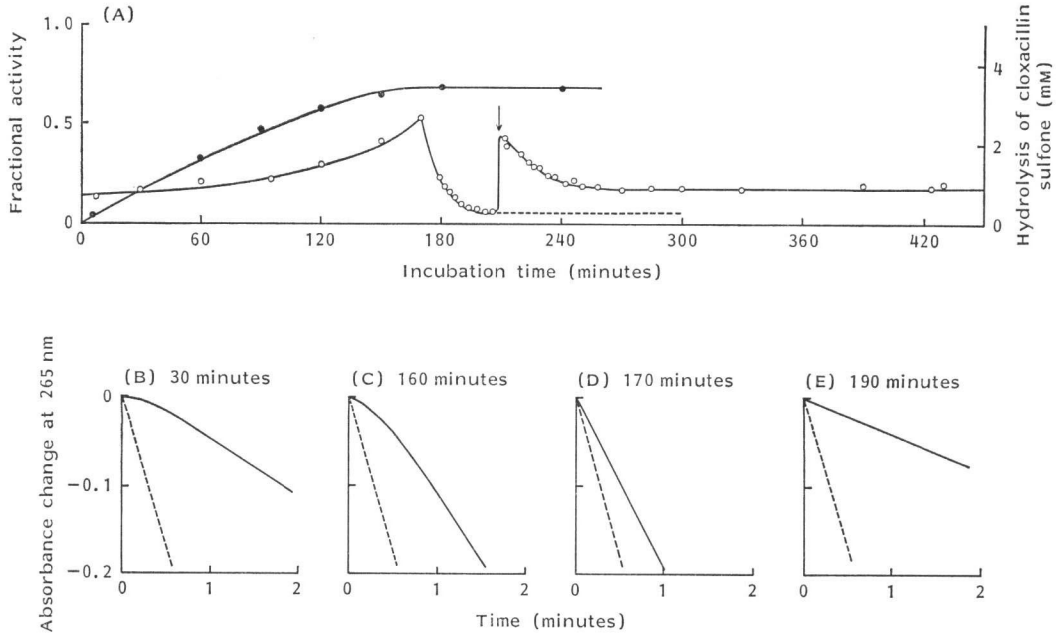
Time Course of the Residual Enzyme Activity

C. freundii cephalosporinase (5 μM) was incubated with cloxacillin sulfone (3.5 mM) in 50 mM sodium phosphate buffer, pH 7.0, at 30°C, and the residual enzyme activity was monitored by the dilution method with cephalothin as the substrate (Fig. 2A). At an early stage of the incubation, the enzyme activity gradually recovered with the enzymatic degradation of cloxacillin sulfone. When

Fig. 2.

(A) Time course of *C. freundii* cephalosporinase activity when 5 μM enzyme was incubated with 3.5 mM cloxacillin sulfone in 50 mM phosphate buffer, pH 7.0. Residual enzyme activity (open circles) was measured by the dilution method described under Materials and Methods. The arrow indicates the time of addition of 100 μl of a 5 μM fresh enzyme solution to 100 μl of the reaction mixture. The broken line indicates the time course of the enzyme activity in the case of no addition of fresh enzyme. Closed circles show the amount of the product of cloxacillin sulfone hydrolysis in the reaction mixture.

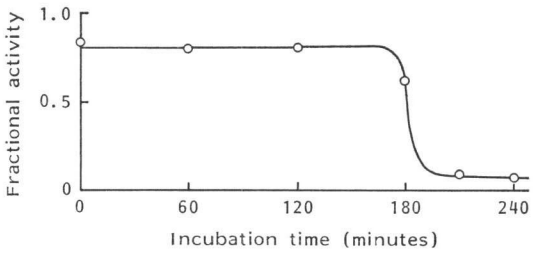
(B)~(E) Traces of the absorption change at 265 nm due to cephalothin hydrolysis, when aliquots of the mixture described above were diluted with a 100 μM cephalothin solution, over the indicated incubation periods. Solid and broken lines show cephalothin hydrolysis by the enzyme incubated with and without cloxacillin sulfone, respectively.



the cloxacillin sulfone had been almost completely hydrolyzed, *i.e.*, after 170 minutes incubation, the recovery of the enzyme activity reached the maximum level, which was about 60% of the native activity. However, just after complete degradation of the cloxacillin sulfone, an unexpected decrease in the enzyme activity was observed (Fig. 2A).

When the residual activity was monitored after gel filtration of the reaction mixture, this phenomenon became more evident (Fig. 3), because the competitive inhibition by free cloxacillin sulfone had been abolished.

Fig. 3. Time course of *C. freundii* cephalosporinase activity, as detected by the gel filtration method, when the enzyme was incubated with cloxacillin sulfone under the same conditions as in Fig. 2.



The lag time for the irreversible decrease in the enzyme activity was proportional to the initial i/e ratio, which was correlated to the time required for the complete degradation of cloxacillin sulfone (data not shown). This inactivation phenomenon with a lag time was called "delayed inactivation".

Fig. 4. Protective effect of cloxacillin sulfone against the cephalosporinase inactivation by the secondary inhibitor.

C. freundii cephalosporinase was incubated with cloxacillin sulfone under the same conditions as in Fig. 2. The enzyme activity was monitored by the dilution method. The arrow indicates the time of addition of 20 μl of 10 mM fresh cloxacillin sulfone to 200 μl of the reaction mixture. Closed and open circles represent the enzyme activity with or without additional fresh cloxacillin sulfone, respectively.

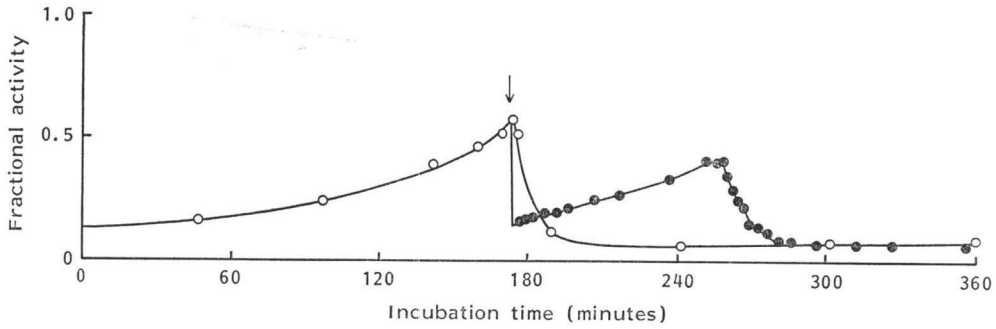
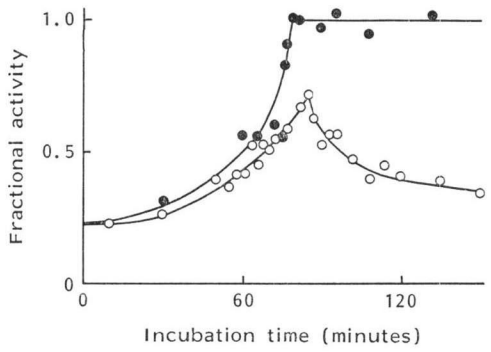


Fig. 5. Time course of the enzyme activity when 5 μM *C. freundii* cephalosporinase was incubated with 2.5 mM cloxacillin sulfone in 50 mM HEPES - NaOH buffer (●) or in 50 mM sodium phosphate buffer (○).

The other conditions and the assay method were the same as in Fig. 2.



When the enzyme was incubated with an initial i/e ratio of greater than 700, the residual enzyme activity after the delayed inactivation was less than 6% of the initial activity. The residual enzyme activity was reciprocally proportional to the initial i/e ratio. It was also confirmed that the residual activity was not increased on prolonged incubation for 24 hours.

Evidence for a Secondary Inhibitor

When fresh enzyme was added to the reaction mixture after the delayed inactivation, the additional enzyme was also inactivated (Fig. 2A), suggesting the presence of a secondary inhibitor in the reaction mixture. The maximum inactivation was seen when the fresh enzyme was added immediately after completion of the delayed inactivation. Then the inactivation activity of the reaction mixture very slowly decreased, with a first order rate constant ($t_{1/2}$, 3.7 hours).

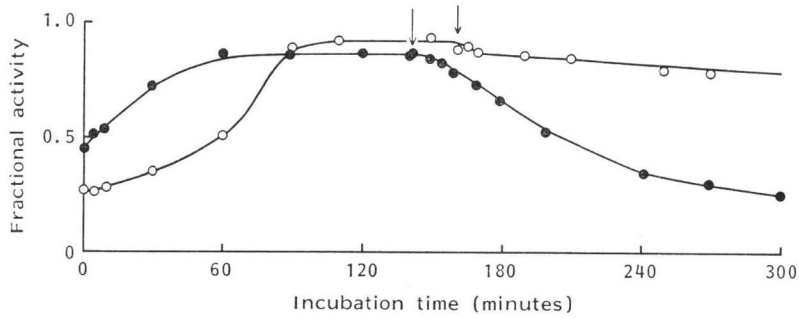
The secondary inhibitor could be separated from the enzyme protein by means of filtration of the reaction mixture through a collodion bag, and the filtrate was confirmed to show progressive inhibition of *C. freundii* cephalosporinase without a lag time. However, the filtrate showed no inhibitory activity against type Ib penicillinase or *P. vulgaris* cephalosporinase (data not shown).

Protection against the Delayed Inactivation

When fresh cloxacillin sulfone (final, 1 mM) was added to a reaction mixture after 170 minutes incubation, the delayed inactivation was itself delayed for about 80 minutes (Fig. 4). During this period, only the competitive inhibition by the added cloxacillin sulfone was observed. This period

Fig. 6. Effect of the addition of sodium phosphate to the reaction mixture of *C. freundii* cephalosporinase and cloxacillin sulfone in HEPES - NaOH buffer.

C. freundii cephalosporinase (5 μM) was incubated in 50 mM HEPES - NaOH buffer, pH 7.0, at 30°C with 2.5 mM fresh cloxacillin sulfone (○) or 2.5 mM cloxacillin sulfone preincubated in distilled water at 30°C for 24 hours (●). Sodium phosphate (final, 50 mM) was added at the time indicated by the arrow. The enzyme activity was monitored by the dilution method.



was proportional to the amount of added cloxacillin sulfone. These observations suggest that cloxacillin sulfone protects the enzyme from the attack of the secondary inhibitor. *C. freundii* cephalosporinase should form a complex with cloxacillin sulfone under such conditions because the enzyme shows high affinity for cloxacillin sulfone (*Km*, 0.28 μM).

This assumption was supported by the following observations; when the reaction mixture was diluted with the cephalothin solution before the complete degradation of the cloxacillin sulfone, a short lag in the attainment of steady state hydrolysis of the cephalothin was observed (Figs. 2B and 2C). On the other hand, this short lag disappeared when the dilution was performed after the complete degradation (Figs. 2D and 2E). This phenomenon suggests that most of the enzyme molecules form a transiently stable complex with cloxacillin sulfone, probably an acyl-enzyme.

Reactivation of the Inactivated Enzyme

When the enzyme, inactivated by the secondary inhibitor, was separated from the low molecular weight compounds in the reaction mixture by gel filtration and then incubated in 50 mM sodium phosphate buffer (pH 7.0) at 30°C, the enzyme activity was very slowly recovered with a first order rate constant ($t_{1/2}$, 2.1 hours). The regeneration rate was significantly accelerated in the presence of 5 mM hydroxylamine ($t_{1/2}$, 0.65 hour), and more than 80% of the initial activity was recovered within 90 minutes.

Isoelectric Point of the Inactivated Enzyme

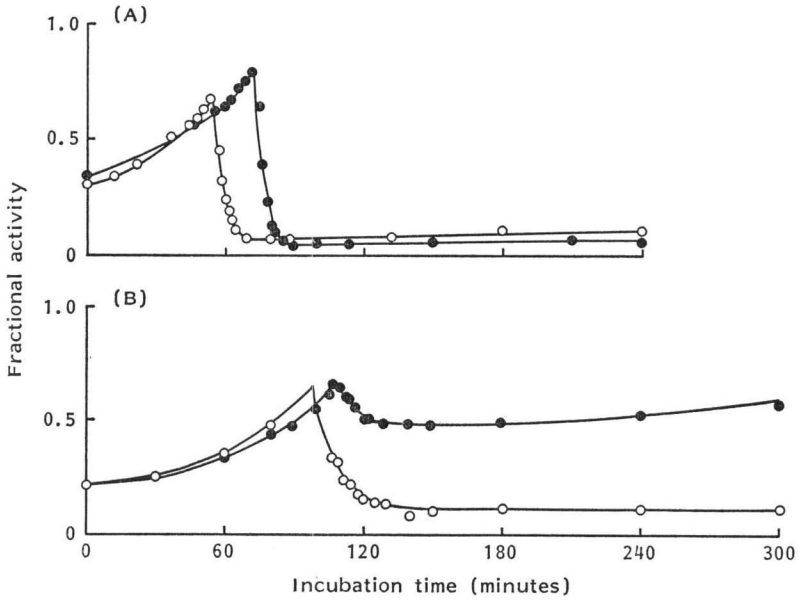
Isoelectric focusing of both the native and inactivated enzymes was performed. The pI value for the native *C. freundii* cephalosporinase was determined to be 9.6. The inactivated enzyme migrated as the main band to a position corresponding to pI 9.25 and was separable from the native enzyme. Two very minor bands at pI 8.8 and 8.0 were found.

Dependence of the Delayed Inactivation on Phosphate Anions

We found that the delayed inactivation did not occur when HEPES - NaOH buffer was used as the reaction medium instead of sodium phosphate buffer (Fig. 5). When *C. freundii* cephalosporinase (5 μM) was incubated with excess cloxacillin sulfone (2.5 mM) in 50 mM HEPES - NaOH buffer (pH

Fig. 7. Delayed inactivation of *C. freundii* cephalosporinase by cloxacillin sulfone preincubated in sodium phosphate buffer.

Cloxacillin sulfone (30 mM) was incubated in 10 mM sodium phosphate, pH 7.0, at 30°C for 15 hours (A) or 1.5 hours (B). Then phosphate anions were removed by molybdate-Dowex resin as described under Materials and Methods. *C. freundii* cephalosporinase (5 μM) was incubated with this phosphate-pretreated cloxacillin sulfone (final, 2.5 mM) in 50 mM HEPES - NaOH buffer (●) or sodium phosphate buffer (○), pH 7.0, at 30°C. The enzyme activity was monitored by the dilution method.



7.0) at 30°C, enzymic hydrolysis of cloxacillin sulfone occurred to the same extent as in the case of sodium phosphate buffer. The enzyme activity was restored to the original level in 80 minutes, and the delayed inactivation was not observed in HEPES - NaOH buffer (Fig. 5).

Addition of sodium phosphate (final, 50 mM; pH 7.0) to the HEPES - NaOH reaction medium after the complete degradation of the cloxacillin sulfone caused only a slight decrease in the enzyme activity (Fig. 6). However, when sodium phosphate was added before the incubation in HEPES - NaOH buffer, the delayed inactivation occurred (data not shown). Therefore, the possibility that the HEPES reagent interfered with the action of the secondary inhibitor could be excluded. It is likely that the secondary inhibitor is produced from cloxacillin sulfone through a catalytic function of phosphate anions, which probably act as a nucleophile. When cloxacillin sulfone was hydrolyzed in 0.1 N NaOH prior to mixing with the enzyme in the phosphate buffer, the secondary inhibitor was not detected in the mixture, suggesting that degradation products of cloxacillin sulfone are not the source of the secondary inhibitor (Fig. 8).

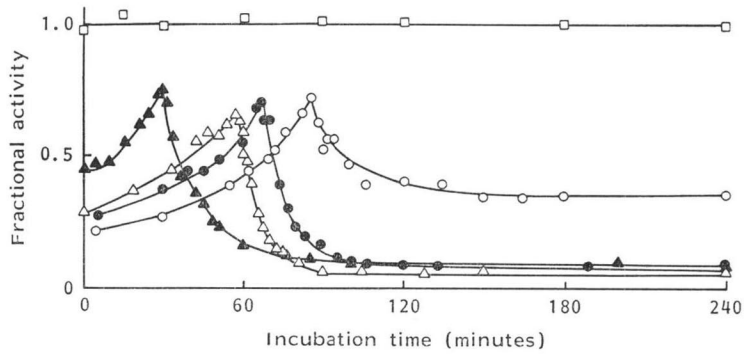
Preincubation of Cloxacillin Sulfone in Sodium Phosphate Buffer

In order to clarify the role of the enzyme in the secondary inhibitor formation, the effect of preincubation in the phosphate buffer prior to the addition of the enzyme was examined. Freshly purified cloxacillin sulfone was incubated in 50 mM sodium phosphate buffer (pH 7.0) at 30°C for 15 hours, and then phosphate anions were removed from the solution by using molybdate-Dowex resin. Then *C. freundii* cephalosporinase (5 μM) was mixed with the phosphate-pretreated cloxacillin sulfone (2.5

Fig. 8. Effect of the preincubation time on the delayed inactivation.

Cloxacillin sulfone (10 mM) was incubated in 50 mM sodium phosphate buffer, pH 7.0, at room temp for 0 hour (○), 6 hours (●), 12 hours (△) or 24 hours (▲) or preincubated in 0.1 N NaOH at 0°C for 10 minutes prior to incubation in sodium phosphate, pH 7.0, for 24 hours (□).

Then *C. freundii* cephalosporinase (5 μM) was incubated with these pretreated cloxacillin sulfone preparation (final, 2.5 mM) in 50 mM sodium phosphate buffer, pH 7.0, at 30°C. The enzyme activity was monitored by the dilution method.



mM) in 50 mM HEPES - NaOH buffer (Fig. 7A). Residual inorganic phosphate in this reaction mixture amounted to less than 3 mM. As a control, the enzyme and the phosphate-pretreated cloxacillin sulfone were also mixed in 50 mM sodium phosphate buffer. The time required for the maximum recovery of the enzyme activity was shorter than that in the case of fresh cloxacillin sulfone, probably due to the partial degradation of the cloxacillin sulfone during the 15 hours preincubation. The delayed inactivation occurred in the absence of phosphate anions when the phosphate-pretreated cloxacillin sulfone was used (Fig. 7A). Furthermore, the degree of the delayed inactivation observed in the HEPES - NaOH buffer was just the same as that in the sodium phosphate buffer.

When the preincubation in the phosphate buffer was performed for 90 minutes, the degree of the delayed inactivation in the HEPES - NaOH buffer was only 25% of that in the phosphate buffer (Fig. 7B). In the case of 5 minutes preincubation, the pretreated cloxacillin sulfone did not cause the delayed inactivation in the HEPES - NaOH buffer (data not shown). Therefore, it may be concluded that the secondary inhibitor is produced through a non-enzymic process in the presence of phosphate anions. The results also indicate that phosphate anions are not concerned in the interaction between the enzyme and the secondary inhibitor.

In contrast to cloxacillin sulfone preincubated in the phosphate buffer, cloxacillin sulfone preincubated in distilled water showed no delayed inactivation of the enzyme in the HEPES - NaOH buffer (Fig. 6), indicating that the secondary inhibitor was not accumulated during the preincubation in distilled water. However, the preparation preincubated in distilled water was clearly different from fresh cloxacillin sulfone as an enzyme inhibitor because, when the preparation was used as an enzyme inhibitor, sodium phosphate added to the reaction mixture after the complete enzymic degradation of the cloxacillin sulfone caused progressive inactivation of the enzyme, whereas in the case of fresh cloxacillin sulfone, sodium phosphate added after the complete degradation of the cloxacillin sulfone had little effect on the enzyme activity (Fig. 6). This may indicate that the secondary inhibitor is formed *via* an intermediate which is accumulated during the preincubation in distilled water, and that the intermediate is stable against enzymic hydrolysis.

Effect of the Preincubation Conditions

The lag period for the delayed inactivation was inversely proportional to the preincubation time in sodium phosphate buffer (Fig. 8). The degree of delayed inactivation was proportional to the length of the preincubation time. These phenomena may reflect the time required for non-enzymic degradation of the cloxacillin sulfone and accumulation of the secondary inhibitor. When the pH of the preincubation medium was varied between 6.0 to 8.0, the degree of the delayed inactivation increased with an increase in pH.

Discussion

The delayed inactivation of the *C. freundii* cephalosporinase by cloxacillin sulfone is an unique phenomenon, which has not been reported previously to the best of our knowledge. It may be concluded that this phenomenon is caused by a secondary inhibitor derived from cloxacillin sulfone on the basis of following experimental results: 1) The activity of the secondary inhibition appeared after the complete hydrolysis of the cloxacillin sulfone by the enzyme, 2) the secondary inhibitor could be separated from the reaction mixture and was found in the fraction containing low-molecular weight materials, and 3) the amount of the secondary inhibitor in the cloxacillin sulfone preparation increased with an increase in the preincubation time in sodium phosphate buffer.

The lag period for the secondary inhibition may be due to the protective effect of the cloxacillin sulfone against the attack of the secondary inhibitor on the enzyme, because cloxacillin sulfone shows high affinity for the *C. freundii* cephalosporinase[4].

The secondary inhibitor most likely was not a contaminant originally existing in the cloxacillin sulfone preparation, since the delayed inactivation still occurred with an extremely purified preparation in sodium phosphate buffer, which was purified by repeated HPLC treatment, and its homogeneity was confirmed on both HPLC and TLC (data not shown).

It was confirmed that phosphate anions are essential for the production of the secondary inhibitor, but the presence of the cephalosporinase is not required for the production. However, once the secondary inhibitor has been formed, the cephalosporinase is inactivated by the secondary inhibitor in the absence of phosphate anions. It should be emphasized that the secondary inhibitor could not be produced through alkaline hydrolysis of cloxacillin sulfone.

When cloxacillin sulfone was incubated in distilled water, the preparation never showed secondary inhibition in the absence of phosphate anions. However, the preparation exhibited inhibitory activity when phosphate anions were added to the reaction mixture, even after the complete enzymatic degradation of the cloxacillin sulfone. These facts indicate that cloxacillin sulfone is transformed to an intermediate which is stable against enzymatic hydrolysis, and then this compound is converted to the secondary inhibitor in the presence of phosphate anions.

The 6-epimer of cloxacillin sulfone can be assumed to be a candidate for the intermediate. Epimerization of penam sulfones in an alkaline solution is possible[9~11], and the solution of cloxacillin sulfone in distilled water used was a mild alkaline solution, because cloxacillin sulfone was used as the sodium salt. This assumption is supported by the fact that an alkaline pH was favorable for accumulation of the secondary inhibitor.

The 6-epimer of cloxacillin sulfone is a poor substrate for *C. freundii* cephalosporinase, but it does not act as an irreversible inhibitor of the enzyme. However, the 6-epimer of cloxacillin sulfone caused the delayed inactivation with a short lag time (several minutes) in the presence of phosphate anions, and the inhibition was higher than that by normal cloxacillin sulfone (our unpublished observation).

The secondary inhibitor was not produced on the degradation of cloxacillin sulfone in a strongly alkaline solution. This excludes the possibility that the secondary inhibitor is derived through post-degradational rearrangement, which was shown to be the case for 6β-bromopenicillanic acid[12] and 6-acetylmethylenepenicillanic acid[13]. KEMAL and KNOWLES[14] suggested that β-elimination during enzymatic or alkaline hydrolysis of penicillanic acid sulfone might result in the formation of an imine

or enamine structure. The secondary inhibitor may have a phosphate-binding imine or enamine structure.

The secondary inhibitor reported is specific to *C. freundii* cephalosporinase among the enzymes investigated. Such a strict specificity is similar to that of aztreonam[15]. Aztreonam is a "tight-binding competitive" inhibitor. On the other hand, suicide inhibitors generally show rather broad specificities[16]. The secondary inhibitor is likely to be the former type of inhibitor on the basis of the following evidence: 1) The inactivated enzyme was detected mainly as a single band on isoelectric focusing, this being similar to what is deserved in the case of inhibition by tight-binding competitive inhibitors[15]. Suicide type inactivation tends to produce multiple species of an inactivated enzyme[17~19]. 2) The enzyme activity was restored completely on the addition of hydroxylamine, which is also a characteristic of tight-binding type inhibitors[15]. However, the chemical structure of the secondary inhibitor is expected to be different from that of aztreonam, because the secondary inhibitor is assumed to contain covalent-bound phosphate and to have no β-lactam ring, on the basis of the results of preliminary experiments now in progress.

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