Kinetic Studies on Inactivation of *Citrobacter freundii* Cephalosporinase by Sulbactam

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The inactivation kinetics for inhibition by sulbactam (CP45,899) of *Citrobacter freundii* GN346 cephalosporinase were studied in detail and compared with those of type Ib penicillinase or TEM-2 β-lactamase mediated by R plasmid RGN823. The rate constant for progressive inactivation of the cephalosporinase was significantly larger than that measured with the penicillinase. The number of sulbactam molecules required to cause complete inactivation of one cephalosporinase molecule (turnover number) was 80. The turnover number for the penicillinase was 5,200. The powerful inhibition by sulbactam of this cephalosporinase is similar to clavulanic acid inhibition of the penicillinase (turnover number, 115; reported by others). The affinity of sulbactam for the cephalosporinase, expressed as $K_i$, was 500 μM; this value was much higher than that for the penicillinase, which was estimated to be 0.5 μM. These results indicated that sulbactam is an effective progressive inactivator but a poor competitive inhibitor for the cephalosporinase. Our study also revealed that the cephalosporinase and sulbactam formed a long-lived inhibitor-enzyme complex which we termed the pseudo-irreversible complex. The half-life of the complex was 550 min at pH 7.0 and 30°C.

Several β-lactamase inhibitors have been discovered recently (3, 7, 12). These inhibitors are expected to be powerful tools for overcoming the resistance of pathogenic bacteria to β-lactam antibiotics, because resistance is mainly due to the production of β-lactamase. A suicide or mechanism-based model for the inactivation of R plasmid or *Staphylococcus aureus* penicillinase by clavulanic acid or sulbactam (penicillanic acid sulfone or CP45,899) was proposed by Brenner and Knowles (1), Fisher et al. (4, 5), Kemal and Knowles (8), Labia et al. (9), Labia and Peduzzi (10), and Reading and Hepburn (14). According to this model, the inactivation process involves the formation of two different types of enzyme-inhibitor complex in addition to the Michaelis complex, namely, a transiently inhibited complex which decomposes to free enzyme and an irreversibly inactivated complex.

The cephalosporinases produced by gram-negative bacteria have become increasingly important because clinical isolates producing these enzymes show high resistance to both cephalosporins and penicillins (21). However, little is known about the kinetic mechanism of cephalosporinase inactivation by these inhibitors. Fu and Neu (6) reported that sulbactam is a poor competitive inhibitor for the cephalosporinases produced by *Citrobacter, Enterobacter*, and *Proteus* spp. Most recently, Reading and Farmer (13) demonstrated that clavulanic acid is a poor inhibitor of a crude cephalosporinase preparation from *Enterobacter cloacae* P99. Bush et al. have reported that the monobactam derivative SQ26,917 forms a very stable but completely reversible complex with the cephalosporinase of *E. cloacae* P99 (2).

Recently, we proposed a set of bacterial strains for estimating the stability of β-lactam antibiotics to β-lactamases; these strains produce nine distinct types of β-lactamases (21). We prepared three representative β-lactamases from these strains and performed comparative kinetic studies with various β-lactamase inhibitors such as cefoxitin, sulbactam, clavulanic acid, and imipenem (N-formimidoyl thienamycin) (20). This wide survey revealed that sulbactam is a very effective progressive inhibitor for the cephalosporinase from *Proteus morganii*. The present paper describes more detailed kinetic studies on the inactivation by sulbactam of *Citrobacter freundii* cephalosporinase in comparison with type Ib (19) penicillinase, which corresponds to TEM-2 β-lactamase.

**MATERIALS AND METHODS**

**Bacterial strains.** *C. freundii* GN346 is a clinical isolate that is highly resistant to cephalosporins and penicillins; this isolate produces a species-specific, typical cephalosporinase semiconstitutively (17). *Escherichia coli* ML1410 RGN823 harbors the R plas-
mid RGN823, which mediates the constitutive synthesis of type Ib or TEM-2 penicillinase (19).

**β-Lactam antibiotics and related compounds.** β-Lactam antibiotics and related compounds were kindly provided by the following pharmaceutical companies: benzylpenicillin and 6-amino penicillinic acid, Meiji Seika Co., Tokyo, Japan; cephalothin, Torii Pharmaceutical Co., Tokyo, Japan. Subactam was synthesized from 6-amino penicillinic acid in our laboratory.

**Enzyme preparation.** β-Lactamases from *E. coli* ML1410 RGN823 and *C. freundii* GN346 were purified to homogeneity by adsorption and elution on a Sephadex ion-exchange column and gel filtration on a Sephadex G-75 column. Purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The specific activity of purified cephalosporinase was 130 U/mg of protein when cephalothin was used as a substrate. The activity of the purified penicillinase was 857 U/mg of protein when benzylpenicillin was used as a substrate. One unit of β-lactamase was defined as the amount of enzyme which hydrolyzed 1 μmol of substrate per min at pH 7.0 and 30°C.

**Determination of *K*~i~ value for inhibition of β-lactamase by subactam.** Cephalothin (10, 15, 20, 30, 40, or 50 μM) was preincubated with subactam (0, 100, or 300 μM) in 3 ml of 50 mM sodium-potassium phosphate buffer (pH 7.0) at 30°C for 1 min. Fifty microliters of cephalosporinase was added to initiate the reaction. Cephalothin hydrolysis was measured as the change in absorbance at 265 nm in a temperature-controlled spectrophotometer (model 24; Beckman Instruments, Inc., Fullerton, Calif.) at 30°C. The rate of enzyme hydrolysis was determined from the initial density over a 1-min reaction period. The *K*~i~ value was calculated from the apparent *K*~m~ value obtained from a Lineweaver-Burk plot of the initial hydrolysis rate at various substrate concentrations in the presence of subactam.

The *K*~i~ value for inhibition by subactam of the penicillinase was determined with benzylpenicillin used as a substrate. The conditions for the enzyme reaction were the same as those used for the cephalosporinase, except for the substrate concentrations (15, 20, 30, 40, 50, or 70 μM), the inhibitor concentrations determined by a microiodometric method (11) with modifications (18).

**Measurement of the progressive inactivation of β-lactamase.** β-Lactamase (1 μM protein) was incubated with subactam (10, 20, or 50 μM) in 50 mM sodium-potassium phosphate buffer (pH 7.0) at 30°C. At different time intervals, samples of the mixture were removed and the amount of hydrolyzed subactam was determined by a microiodometric method (11) with modifications (17).

**Measurement of the progressive inactivation of β-lactamase.** Inactivation of β-lactamase by subactam was measured by a modification of the method of Fisher et al. (5). β-Lactamase (final concentration, 1 μM) was preincubated at 30°C in 200 μl of 50 mM sodium-potassium phosphate buffer (pH 7.0). Ten or 20 μl of subactam (see Fig. 3 for final concentration) was added to the enzyme solution and incubated at 30°C. At different time intervals, 5 μl of the reaction mixture was withdrawn and residual enzyme activity was determined spectrophotometrically with an excess of substrate (100 μM cephalothin or 200 μM benzylpenicillin).

**Measurement of recovery of cephalosporinase activity.** One hundred microliters of a 10 μM cephalosporinase solution was mixed with the same volume of 4 mM subactam in a test tube, and the mixture was incubated at 30°C for 10 or 120 min. The reaction was stopped by rapid cooling to 0°C, and the mixture was immediately applied to a Sephadex G-25 column (0.98 by 15 cm) which was developed with 50 mM sodium-potassium phosphate buffer (pH 7.0) at 4°C. The pooled enzyme fraction was incubated at 30°C and the recovery of enzyme activity was followed spectrophotometrically as described above. As a control, the enzyme was treated in the same way, except that it was incubated without subactam.

**RESULTS**

**Competitive inhibition of cephalosporinase by subactam.** Figure 1 shows the time course of the decrease in absorption at 265 nm caused by cephalosporinase hydrolysis of cephalothin after the simultaneous addition of subactam and cephalothin. It was confirmed that the absorption of subactam at this wavelength did not change after its hydrolysis by the enzyme (data not shown). The gradual decrease in the rate of hydrolysis is explained by progressive inactivation (see below). The reaction rate measured over the first minute was defined as the initial rate of enzyme hydrolysis (tangent to the curve). The Lineweaver-Burk plot obtained from the initial rate of substrate hydrolysis in the presence or absence of subactam (Fig. 2) indicated that the initial inhibition of the cephalosporinase by subactam was competitive. The affinity of subactam for the cephalosporinase (*K* = 500 μM) was significantly lower than its affinity for the penicillinase (*K* = 0.5 μM) (Table 1).

**Progressive inactivation of the cephalosporinase by subactam.** To measure the progressive

![FIG. 1. Time course of the change in optical density at 265 nm for hydrolysis of cephalothin in the presence of subactam. The optical density was measured continuously at 30°C after the addition of 20 μl of 0.12 μM cephalosporinase into 3 ml of 50 mM sodium-potassium phosphate buffer (pH 7.0) containing 100 μM cephalothin and 500 μM subactam. The broken line indicates the initial rate of the optical density change (v0).](http://aac.asm.org/download/FIG.1.jpg)

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(i.e., time-dependent) inactivation of the cephalosporinase by sulbactam, the activity remaining after preincubation of the enzyme with the indicated sulbactam concentrations was assayed by the method of dilution with excess cephalothin, as described in Materials and Methods. The fraction of enzyme activity remaining was plotted against the time of incubation with sulbactam (Fig. 3A). Progressive inactivation of the penicillinase was measured in the same way, except benzylpenicillin was used as a substrate (Fig. 3B). Sulbactam produced more powerful progressive inactivation of the cephalosporinase than of the penicillinase (Fig. 3B).

The rate constant of inactivation was determined from a double reciprocal plot of the initial rates of the enzyme inactivation at various concentrations of sulbactam. The rate constant of inactivation for the cephalosporinase and the penicillinase were 0.48 and 0.058 min⁻¹, respectively (Table 1).

The mode of penicillinase inactivation by sulbactam appeared to be essentially the same as that by clavulanic acid; i.e., the inactivated enzyme fraction consisted of a reversibly inhibited form and an irreversibly inactivated form. Therefore, the activity of the inactivated enzyme could be partially restored by prolonged incubation (Fig. 3). The level of inactivated enzyme found after this partial reactivation probably represents the irreversibly inactivated form. The level of irreversible inactivation which was determined after 120 min of incubation with sulbactam was proportional to the inhibitor/enzyme molar ratio (Fig. 4B). The number of hydrolytic events per enzyme mole-

![Graph of Lineweaver-Burk plot](https://example.com/graph.png)

**Fig. 2.** Lineweaver-Burk plot of cephalothin hydrolysis by cephalosporinase in the presence or absence of sulbactam. The rate of initial enzyme hydrolysis was measured with cephalothin as substrate in the presence of 0 (○), 100 (●), or 300 (△) μM sulbactam.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Rate constant (min⁻¹)</th>
<th>Kᵢᵥ, (μM)</th>
<th>Turnover no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kₑᵃᵗ</td>
<td>Kᵢⁿᵃᶜᵗ</td>
<td>Kᵢⁿᵉᵃᵏᵗ</td>
</tr>
<tr>
<td><em>C. freundii</em> cephalosporinase</td>
<td>29</td>
<td>0.48 (1.4)</td>
<td>0.005 (140)</td>
</tr>
<tr>
<td>Type Ib penicillinase</td>
<td>114</td>
<td>0.058 (12)</td>
<td>0.059 (12)</td>
</tr>
</tbody>
</table>

*a* Kₑᵃᵗ, Hydrolysis rate determined from a double-reciprocal plot of the initial rate of hydrolysis of sulbactam by the enzyme; Kᵢⁿᵃᶜᵗ, inactivation rate determined from a double-reciprocal plot of the initial rate of inactivation of the enzyme at different initial concentrations of sulbactam; Kᵢⁿᵉᵃᵏᵗ, reactivation rate; X, unstable complex; Y, pseudo-irreversible complex; ND, not determined. Half-lives (in minutes) are shown in parentheses.

TABLE 1. Kinetic constants for sulbactam inactivation by *C. freundii* cephalosporinase and type Ib penicillinase
Cule before complete irreversible inactivation of the enzyme was estimated by extrapolation of the plot to the x intercept. The value obtained, termed the turnover number, represents the effectiveness of suicide inactivation. A value of 5,200 was estimated for the penicillinase (Table 1).

In contrast, the mode of cephalosporinase inactivation by sulbactam was quite different. The enzyme activity was not recovered even after 180 min of incubation. However, when the inactivated enzyme was incubated for 20 h, most of the enzyme activity was recovered even at the initial inhibitor/enzyme molar ratio of &ge;50 (data not shown). This result suggested that inactivation of the cephalosporinase by sulbactam was mainly caused by the formation of a very long-lived inhibitor-enzyme complex, designated the pseudo-irreversible complex. The turnover number before the formation of pseudo-irreversible complex was 80 (Fig. 4A).

**Sulbactam hydrolysis by cephalosporinase.** To examine the possibility that the extremely slow recovery of inactivated cephalosporinase was due to intact sulbactam remaining in the medium, the time course of sulbactam hydrolysis by cephalosporinase was followed by micro-iiodometric assay described in Materials and Meth-

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**FIG. 3.** Progressive inactivation of β-lactamase activity by sulbactam. Enzymes (1 μM) were incubated with the indicated concentrations of sulbactam. At different time intervals, enzyme activity remaining was measured as described in the text. (A) Cephalosporinase; (B) penicillinase.
CEPHALOSPORINASE INACTIVATION BY SULBACTAM

complex formed between sulbactam and the cephalosporinase was followed at 30°C after removal of excess sulbactam by gel filtration. The semilogarithmic plot of the inactive enzyme fraction versus incubation time is shown in Fig. 6A. Enzyme was preincubated with a 400-fold molar excess of sulbactam for 120 min at 30°C before gel filtration. The results indicated that the degradation process obeyed good first-order kinetics; the rate constant was estimated to be $1.3 \times 10^{-3}$ min$^{-1}$ (half-life, 550 min).

When the preincubation time was reduced to 10 min, the degradation kinetics of inactivated complex were biphasic (Fig. 6B). The rate constants for the first and the second phase were $5.0 \times 10^{-3}$ min$^{-1}$ (half-life, 140 min) and $1.5 \times 10^{-3}$ min$^{-1}$ (half-life, 462 min), respectively. The latter, slower rate was in agreement with the rate constant obtained in the case of the 120-min preincubation period. Furthermore, the faster degradation process observed in Fig. 6B suggests the existence of another unstable enzyme-inhibitor complex.

DISCUSSION

The present investigation revealed that sulbactam is a powerful progressive inactivator of C. freundii cephalosporinase but not of type Ib penicillinase. However, the inhibitor showed lower affinity for the cephalosporinase than for the penicillinase. In other words, sulbactam was

FIG. 4. Determination of the number of hydrolytic events per enzyme molecule before complete inactivation of the enzyme (turnover number). The fractional enzyme activity remaining after incubation for 120 min in the presence of different concentrations of sulbactam was obtained from Fig. 3 and was plotted against the molar ratio of sulbactam to each enzyme. (A) Cephalosporinase; (B) penicillinase.

ods (Fig. 5). All of the sulbactam in the medium was hydrolyzed within 50 min, even at the initial concentration of 50 μM sulbactam (enzyme concentration, 1 μM) (Fig. 5). Therefore, this possibility could be ruled out.

Hydrolysis of sulbactam by the penicillinase was also measured (data not shown). The results strongly suggested that the rapid recovery of the penicillinase activity occurred just after completion of the hydrolysis of sulbactam in the medium.

Recovery of cephalosporinase activity after gel filtration. Degradation of the pseudo-irreversible

FIG. 5. Hydrolysis of sulbactam by cephalosporinase. β-Lactamase reaction was carried out in 3 ml of 50 mM sodium-potassium phosphate buffer (pH 7.0) containing different concentrations of sulbactam (a, 50 μM; b, 20 μM; c, 10 μM). The final enzyme concentration was 1 μM.
a strong progressive inactivator but a weak competitive inhibitor for the cephalosporinase. The minimum scheme for the inactivation process of the cephalosporinase by sulbactam is shown in Fig. 7. At least two distinct inactive complexes are assumed, i.e., X and Y. The half-life of Y, which is termed a pseudo-irreversible complex, is 550 min. This is one of the slowest degradation rates for a reversible inhibitor–β-lactamase complex except for that of the monobactam derivative SQ 26,917 reported by Bush et al. (2). The half-life of the complex formed by SQ 26,917 and the E. cloacae P99 cephalosporinase is 18.5 h; however, inactivation involved a nonbranched mechanism different from the branched mechanism of inactivation by sulbactam.

Retsema et al. (15) reported that sulbactam acts as both a competitive and a noncompetitive inhibitor for type III penicillinase. Their observation that the non-competitively inhibited form of the enzyme was very stable or almost irreversible is consistent with our results. Although they suggested inhibition by a nonbranched
mechanism, we proposed a branched mechanism for sulfactam inhibition of the C. freundii β-lactamase. This mechanism was based on the following reasons: (i) The turnover number for sulfactam by the enzyme before its complete inactivation was 80. A turnover number of more than 1 could not be explained by a nonbranched mechanism. (ii) The rate of sulfactam hydrolysis by the enzyme was much larger than the rate of reactivation of the enzyme (Table 1). If a nonbranched mechanism were involved, the rate of inhibitor hydrolysis would be the same as the rate of reactivation of the enzyme at least after one cycle of enzyme catalysis.

The \( K_v \) value and the degree of progressive inactivation of the cephalosporinase by sulfactam were quite different from the corresponding values for the C. freundii 2732 cephalosporinase reported by Fu and Neu (6). These authors reported 3.8 μM for the \( K_v \) value and described sulfactam as a relatively weak progressive cephalosporinase inactivator. The enzyme of Citrobacter 2732 is inducible, whereas that of C. freundii GN346 is produced constitutively at 30°C. Although their cephalosporinase may be different from ours, the possibility that the differences observed between the two enzymes can be attributed to the differences in experimental conditions cannot be ruled out: the apparently low \( K_v \) value might be due to the long reaction time (5 min) used by Fu and Neu (6) for measuring enzyme activity in the presence of sulfactam. Our findings indicated that significant progressive inactivation had already occurred within 2 min (Fig. 1).

The turnover number is a good measure of the effectiveness of suicide inactivation (4). However, it should be noted that the turnover number depends on the purity of the enzyme used. When crude enzyme was used, the apparent turnover number appears to be smaller than the true value. Even when an electrophoretically homogeneous enzyme was used, this number might decrease with age because of partial denaturation of the enzyme. In this study, we employed enzyme preparations stored at −80°C until use, and the thawed enzyme solution was not refrozen. It was confirmed that the specific activity of the enzymes used in this study was maintained throughout the experiment.

In this study we showed that sulfactam had apparently conflicting characteristics as an inactivator for the β-lactamases, that is, a weak competitive inhibitor and a strong progressive inactivator for the cephalosporinase and the reverse for the penicillinase. It is interesting to speculate which characteristic is dominant in the expression of synergistic activity of the suicide inhibitor when it is administered with a β-lactamase-sensitive β-lactam. In preliminary studies, sulfactam showed relatively weak synergistic activity for both E. coli ML1410 RGN823 and C. freundii GN346, whereas it showed very strong synergistic activity for Proteus vulgaris, which produces a cephalosporinase with broad substrate specificity (16) having both a low \( K_v \) value and a small turnover number for sulfactam. This suggests that both characteristics, competitive and progressive inactivation, are necessary for sulfactam to exert its synergistic activity in combination with other β-lactams.

**LITERATURE CITED**


