Covalent Binding of Moxalactam to Cephalosporinase of Citrobacter freundii

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The inhibition of Citrobacter freundii cephalosporinase activity by moxalactam is shown to be due to the formation of a transiently stable covalent complex, probably acyl enzyme. The covalent complex formed was identified by coelution of $[^{14}C]$moxalactam with the enzyme by using Sephadex G-25 gel filtration in the presence of 5.7 M guanidine hydrochloride and by analytical isoelectric focusing. Both the side-chain carboxyl group and the $\alpha$-methoxy group of moxalactam were necessary to stabilize the complex. Moxalactam is racemic with respect to the $\alpha$ carbon of the $\beta$-acylamino side chain, and the complex with the $R$ epimer (half-life, 4.6 min) decomposed much more rapidly than that formed with the $S$ epimer (half-life, 130 min). For other $\beta$-lactam antibiotics that were stable to $\beta$-lactamases, the half-lives of enzyme-antibiotic complexes were less than 4 min.

The production of $\beta$-lactamase is the main cause of bacterial resistance to $\beta$-lactam antibiotics (5, 17). $\beta$-Lactamases catalyze the hydrolysis of the $\beta$-lactam ring, which renders the antibiotic inactive. The clinical need to overcome this $\beta$-lactamase-mediated resistance has prompted the development of $\beta$-lactam antibiotics that are stable against $\beta$-lactamases.

Moxalactam (Fig. 1), one of these $\beta$-lactam antibiotics (26), is stable against both cephalosporinases and penicillinases and offers improved antibacterial activities. The remarkable structural elements of moxalactam leading to $\beta$-lactamase stability are the $\alpha$-methoxy group, which stabilizes it against penicillinases and against the cephalosporinase of Proteus vulgaris, and the $\alpha$-carboxyl group of the $\beta$-acylamino side chain (side-chain carboxyl group), which confers a high affinity for cephalosporinases of bacteria other than P. vulgaris and in turn leads to enzyme inhibition (13, 25). To understand the stability of moxalactam at the molecular level, we examined its interaction with the cephalosporinase of Citrobacter freundii, keeping the two substituents described above in mind. We found that moxalactam inhibited the enzyme by forming a transiently stable covalent complex with it, thereby gaining stability against the enzyme, and that both the substituents and the configuration around the asymmetric carbon at the $\alpha$ position of the 7-$\alpha$-acylamino side chain affected the stability of the complex. We also compared the half-lives of the moxalactam complexes with those of other $\beta$-lactam antibiotics of current interest.

MATERIALS AND METHODS

$\beta$-Lactam antibiotics. $[^{14}C]$Moxalactam (12.2 mCi/mmol), which contained about equimolar amounts of $R$ and $S$ epimers (8), compound 1 (the demethoxy derivative of moxalactam), and compound 2 (the side-chain decarboxyl derivative of moxalactam), were synthesized, and aspenamycin A was isolated from a fermentation broth of Streptomyces sp. (20) at the Shionogi Research Laboratories. $[^{14}C]$Moxalactam contained $^{14}C$ at the phenylmalonyl acetate carbonyl carbon. The $R$ epimer of moxalactam diammonium salt was synthesized by the method of K. S. Yang of Eli Lilly & Co. Other compounds were obtained from commercial sources.

Purification of cephalosporinase. C. freundii SR19, which produces cephalosporinase constitutively, was grown without shaking overnight at 37°C in nutrient phosphate broth (Nissui, Tokyo, Japan). Cells (27 g [wet weight]) were washed in 1 liter of 0.85% NaCl, suspended in an appropriate volume of 0.01 M Na$_2$HPO$_4$-KH$_2$PO$_4$ (pH 6.6), and then subjected to ultrasonic disintegration for 5 min in an ice bath with a Sonifier 350 (Branson Sonic Power Co., Danbury, Conn.). Cell debris was removed by centrifugation at 33,000 × g for 30 min at 4°C, and the supernatant was dialyzed against the phosphate buffer described above (pH 6.6).

The dialyzed supernatant (75 ml) was applied to a carboxymethyl Sephadex C-50 column (2.6 by 34 cm) equilibrated with 0.01 M phosphate buffer (pH 6.6). The column was washed with 410 ml of the same buffer, and the cephalosporinase was eluted with a linear gradient constructed from 500 ml of 0.01 M phosphate buffer (pH 6.6) and 500 ml of 0.5 M phosphate buffer (pH 6.2). Pooled active fractions were dialyzed against distilled water and concentrated by lyophilization.

The concentrated enzyme solution (2 ml) was applied to and eluted from a Sephadex G-75 column (2.6 by 95 cm) equilibrated with 0.1 M potassium phosphate buffer (pH 7.0). A pooled active fraction containing 24 mg of enzyme protein was stored at −78°C until use.

The overall recovery of enzyme activity was 65%. The purified enzyme was more than 95% homogeneous based on the results of both polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and analytical isoelectric focusing on a thin layer of polyacrylamide gel.

Assay of $\beta$-lactamase. $\beta$-Lactamase activity was determined by spectrophotometric assay (14) with a Hitachi model 200-20 spectrophotometer into which water at 30°C was circulated.

The maximum hydrolysis rate ($V_{\text{max}}$) and Michaelis constant ($K_m$) of the $\beta$-lactam compounds were calculated from Lineweaver-Burk plots (11). To determine the inhibitor constant ($K_i$), 0.2 ml of the cephalosporinase solution was added to 2 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing an inhibitor and cephalothin as the substrate, and the initial hydrolysis rate was determined within 3 min. The
Both of these methods (i and ii) indicated similar half-lives for the complex formed with the R epimer of moxalactam. The concentration of intact \( \beta \)-lactam after dilution was always low enough so that its effect as an inhibitor was negligible.

**Analytical isolectric focusing.** Analytical isolectric focusing on a thin layer of polyacrylamide gel was performed with an LKB Multiphor apparatus according to the instructions of the manufacturer. Samples were run on an LKB Ampholine PAG plate (pH 3.5 to 9.5) for 2 h at a constant level of power. Proteins were stained with Coomassie brilliant blue R-250.

### RESULTS

**Kinetic properties of \( C. \) freundii cephalosporinase and its inhibition by moxalactam.** The \( \beta \)-lactamase of the \( C. \) freundii strain used in this study was more active against cephalosporins, such as cephalothin and cephaloridine, than against ampicillin and was inhibited by an excess of moxalactam (Table 1), as were the cephalosporinases of other bacterial species (10, 13, 16, 26). Even at lower molar ratios of moxalactam to the enzyme (1.0 to 2.0), the antibiotic inhibited enzymatic activity by 90 to 95% until the activity could recover (Fig. 2). When the molar ratio of antibiotic to enzyme was increased, a longer incubation period was required before the recovery of enzyme activity began.

**Specific covalent binding of moxalactam to the cephalosporinase.** After incubation of the cephalosporinase with \( ^{14} \text{C} \)moxalactam at a molar ratio of 10 (antibiotic to enzyme), the enzyme-antibiotic mixture was eluted from a Sephadex G-25 column in the presence of 5.7 M guanidine hydrochloride. A small amount of radioactive material was coeluted with the denatured enzyme (peak I in Fig. 3; peak II in Fig. 3 corresponds to free moxalactam). Even when the molar ratio of \( ^{14} \text{C} \)moxalactam to the fixed amount of enzyme was increased from 2 to 30, the amount of radioactivity in peak I did not change and accounted for approximately 0.78 mol of moxalactam per mol of the enzyme. Since the enzyme protein contained no cysteine residue (unpub-

<table>
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<th>( \text{Lactam compound} )</th>
<th>( V_{\text{max}} ) (( \mu \text{M} ))</th>
<th>( K_{\text{m}} ) (( \mu \text{M} ))</th>
<th>( K_{\text{i}} ) (( \mu \text{M} ))</th>
<th>Half-life' (min)</th>
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<td>ND</td>
</tr>
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</tr>
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<tr>
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*a* Hydrolysis rates were determined by spectrophotometric assay at 30°C. *b* \( V_{\text{max}} \) values are relative to an arbitrary value of 100 for cephalothin. *c* Half-lives of complexes formed with both epimers of moxalactam were calculated from the data presented in Fig. 6 and 7. For the other \( \beta \)-lactam compounds, half-lives were determined from each hydrolysis curve of the compound as described in the text. *d* ND, Not determined.
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FIG. 2. Recovery of cephalosporinase activity inhibited by moxalactam. Cephalosporinase (5 μM) was incubated with moxalactam at 30°C in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.01% gelatin. The molar ratios of moxalactam to the enzyme are given for each curve. At the indicated times, 10-μl portions were taken, and after appropriate dilution, the β-lactamase activities were determined with 100 μM of cephaloridine as the substrate.

FIG. 3. Gel filtration results with a mixture of cephalosporinase and [14C]moxalactam. After incubation of 1.9 nmol of cephalosporinase with 19 nmol of [14C]moxalactam at room temperature for 10 min in 100 μl of 0.1 M potassium phosphate buffer (pH 7.0), 200 μl of 8.5 M guanidine hydrochloride was added to denature the enzyme. Gel filtration and radioactivity counts were performed as described in the text. ○, Guanidine hydrochloride was added to the enzyme before its incubation with [14C]moxalactam. All other conditions were as described above.

FIG. 4. Change in the UV spectrum of moxalactam after incubation with cephalosporinase. Moxalactam (final concentration, 7.2 μM) was incubated with an equimolar amount of the enzyme at 30°C for 4 min in 0.1 M potassium phosphate buffer (pH 7.0). Curves: a, moxalactam before incubation; b, moxalactam after incubation with the enzyme.

FIG. 5. Isoelectric focusing of a mixture of cephalosporinase and moxalactam on a thin layer of polyacrylamide gel. The enzyme (final concentration, 13.2 μM) was mixed with moxalactam at molar ratios (moxalactam to enzyme) of 30, 10, 3, 1, 0.5, or 0 (lanes A through F, respectively). The pH was higher in the upper area of the gel.
moxalactam at 30°C and at time zero was diluted with the buffer to remove the inhibition by free moxalactam. The incubation was continued, and the rate of recovery of enzyme activity was monitored. When the enzyme was preincubated with moxalactam for 1 min, recovery of activity was biphasic, with an initial rapid phase followed by a slow phase (Fig. 6A). Decomposition of the complex in the slow phase followed first-order kinetics, and the half-life was 130 min. Extrapolation of the slow-phase slope to time zero showed that about 50% of the complex at time zero had a half-life of 130 min. When the preincubation time before dilution was 50 min, the rapidly decaying complex was almost lost (Fig. 6B). Because moxalactam contains an asymmetric carbon at the α position of the 7-acylamino moiety, moxalactam preparations usually contain approximately equimolar amounts of R and S epimers (9). The decomposition of complex formed with the R epimer, as measured in a preparation containing 97% R epimer, was monophasic and followed first-order kinetics with a half-life of 4.6 min (Fig. 7). Thus, the rapid and slow phases (Fig. 6A) evidently resulted from the decay of complexes with R and S epimers, respectively. The observation that at time zero about 50% of the complex was the slowly decaying type agreed with the fact that moxalactam preparations are composed of almost equimolar amounts of R and S epimers.

**Comparison of half-lives among enzyme-antibiotic complexes formed with various β-lactam compounds.** The half-lives of complexes formed with various β-lactam compounds known to be resistant to hydrolysis by β-lactamases are shown in Table 1. Though carbenicillin has an asymmetric carbon, its decomposition curve was not biphasic but monophasic (as in Fig. 7). Extrapolation of the curve indicated less than 50% inhibition at time zero. The half-life of complexes with one of the two epimers of carbenicillin was probably too short to measure, and Table 1 shows the value for the complex with the longer half-life. Of the β-lactam compounds listed in Table 1, the S epimer of moxalactam formed the most stable complex, followed by the moxalactam R epimer, carbenicillin, and cefotaxime. Complexes with compounds other than the S epimer, however, exhibited half-lives of only 3 to 4 min. Complexes of the other compounds were too labile to allow measurement of their half-lives.

**DISCUSSION**

Moxalactam reportedly inhibits cephalosporinases of many gram-negative bacteria (10, 13, 16, 26). In this study with the cephalosporinase of C. freundii, moxalactam was shown to inhibit the enzyme by forming a transiently stable covalent complex. The formation of the complex prevented the enzyme from attacking other moxalactam molecules. Bush et al. have reported the formation of moxalactam complexes with the cephalosporinase of Enterobacter cloacae (2). Thus, these complexes seem to form with the cephalosporinases of other bacterial species which moxalactam inhibits.

β-Lactamase inactivators such as clavulanic acid, 6β-bromopenicillanic acid, penicillanic acid sulfone, and carbapenems have been shown to irreversibly inactivate β-lactamases (3, 4, 6, 12, 15). In contrast, moxalactam did not irreversibly inhibit the enzyme, although a covalent complex was isolated. After the consumption of excess moxalactam, the enzyme activity gradually recovered, indicating decomposition of the complex. Because the enzyme-[¹⁴C]moxalactam complex was stable in the presence of 5.7 M guanidine hydrochloride, which completely denatures proteins (21), it is clear that enzyme activity is necessary for the decomposition of the complex. This fact, together with the observation that the β-lactam ring was opened upon

**FIG. 6.** Decomposition of the covalent complex formed with moxalactam. After preincubation of cephalosporinase with moxalactam at 30°C, free moxalactam was removed by dilution at time zero. The incubation was continued, and the enzyme activity was measured as a function of time. (A) A 5-M sample of the enzyme preincubated with an equimolar amount of moxalactam for 1 min. (B) A 5-M sample of the enzyme preincubated with 10 μM moxalactam for 50 min.

**FIG. 7.** Decomposition of the covalent complex formed with the R epimer of moxalactam. Cephalosporinase (5 μM) was preincubated with 5 μM R epimer at 30°C for 1 min. After removal of the free R epimer by dilution, the remaining enzyme activity was measured as a function of time.
formation of the complex, suggests that the covalent complex formed was acyl enzyme, which is an intermediate formed during the enzymatic hydrolysis of other hydrolyzable \( \beta \)-lactam compounds (1, 22). The intermediate of moxalactam was very stable, and its hydrolysis rates \( (k_{\text{err}}) \) were \( 5.3 \times 10^{-3} \text{ min}^{-1} \) and \( 1.5 \times 10^{-1} \text{ min}^{-1} \) for the \( S \) and \( R \) epimers, respectively.

There were two structural factors affecting the stability of the enzyme-moxalactam complex. One was the existence of both the 7a-methoxy group and the side-chain carboxyl group. Although compound 1 was shown by a conventional method to inhibit the enzyme and to be stable to it (Table 1), the half-life of its complex with the enzyme was less than 2 min. Compound 2 was hydrolyzed at a rate that was 29% that of cephalothin. In contrast, the moxalactam complex had a longer lifetime. Cooperation of the side-chain carboxyl group with the 7a-methoxy group was critical for the stability of the complex. Another factor affecting the stability was the configuration around the \( \alpha \) carbon of the 7-acylamino side chain. The \( S \) configuration stabilized the complex much more effectively than did the \( R \) configuration.

The \( R \) epimer of moxalactam has a stronger antibacterial activity against many bacterial strains than does the \( S \) epimer (23). However, the \( R \) epimer is less active against some cephalosporinase-producing strains (T. Yoshida, Y. Kameda, and Y. Kimura, unpublished data), probably because of its instability to the enzyme. In a preparation of moxalactam containing equimolar amounts of \( R \) and \( S \) epimer, the \( R \) epimer would be protected to some extent from cephalosporinase by the \( S \) epimer, and both epimers are likely to act synergistically against cephalosporinase-producing strains.

The isoelectric focusing pattern showed that two kinds of complexes were formed with moxalactam. The major and the minor bands were probably not derived from the \( S \) and \( R \) epimers, respectively, because the enzyme incubated with the \( R \) epimer also showed two very faint bands at the same position (presumably due to a minute amount of the \( S \) epimer in the preparation) and because the complex formed with the \( R \) epimer was too labile to be detected by isoelectric focusing. The difference between the two bands is unclear.

There have been several studies concerning the involvement of \( \beta \)-lactamase in bacterial resistance to \( \beta \)-lactamase-stable \( \beta \)-lactam antibiotics (19, 22, 24), and nonhydrolytic mechanisms such as the trapping of the compound by \( \beta \)-lactamase or the presence of a penetration barrier determined by \( \beta \)-lactamase have been proposed (18). Although hydrolysis could not be detected by conventional methods, all of the \( \beta \)-lactam compounds listed in Table 1 were hydrolyzed, and large differences in stability were found in terms of the half-life of the enzyme-antibiotic complex. Thus, any discussion of the participation of \( \beta \)-lactamase in the resistance to so-called nonhydrolyzable \( \beta \)-lactam antibiotics would require more precise assessments of such factors as the decomposition rate of the complex.

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LITERATURE CITED


