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Kinetic Studies on the Inhibition of *Proteus vulgaris* β-Lactamase by Imipenem

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Imipenem was found to inhibit *Proteus vulgaris* β-lactamase in a progressive manner. Kinetic experiments confirmed that the inactivated enzyme was not completely recovered after intact imipenem had been exhausted.

Recently developed β-lactamase inhibitors such as clavulanic acid and penicillanic acid sulfone have proved to be suicide inactivators as well as competitive inhibitors of certain types of β-lactamases (1, 2, 6, 8, 14, 16). Since the discovery of thienamycin (7), many detailed kinetic studies have appeared describing reversible inhibition of β-lactamase by various kinds of carbapenem compounds, such as PS-5 (9), olivanic acid derivatives (3, 5), and 5,6-*cis*-carbapenem compounds (10). Our previous study showed that imipenem (formerly imipemide, *N*-formimidoyl thienamycin, or MK 0787) is a progressive inhibitor of several types of β-lactamases (14). In the present investigation, we elucidate the mechanism of inhibition of *Proteus vulgaris* cephalosporinase by imipenem.

Imipenem was supplied by Merck Sharp & Dohme Research Laboratories, Rahway, N.J. Cephalothin was a commercial product of Shionogi Pharmaceutical Co., Osaka, Japan.

P. vulgaris GN76/C-1, a constitutive mutant with respect to the production of cephalosporinase, was derived from the wild-type strain GN76 (13, 15). The β-lactamase from *P. vulgaris* was purified as described previously (13); purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4, 11). The specific activity of the enzyme was 319 U/mg of protein. One unit of β-lactamase was defined as the amount of enzyme which hydrolyzed 1 μmol of cephalothin per min at pH 7.0 and 30°C.

β-Lactamase inactivation by imipenem was measured by a modification of the dilution method of Fisher et al. (6). The K_i of imipenem for the β-lactamase was determined by the procedure described previously (14). The K_m(inact) and the rate constant of inactivation were determined from a double-reciprocal plot of the initial rate of inactivation of the enzyme at different initial inactivator concentrations (3). The rate constant of hydrolysis k_{cat} was determined from the initial velocity of imipenem hydrolysis with the cephalosporinase at a concentration of imipenem sufficiently higher than the K_i. The hydrolysis of imipenem was determined by measuring the change of absorption at 298 nm. The rate constant of reactivation (k_{react}) was determined by measuring the recovery of the enzyme activity after gel filtration (6).

The progressive inhibition by imipenem of *P. vulgaris* cephalosporinases and the hydrolysis of imipenem by the β-lactamase are shown in Fig. 1. *P. vulgaris* cephalosporinase was rapidly inactivated by imipenem several minutes after preincubation, although further incubation led to restoration of enzyme activity to some extent, depending on the inhibitor-to-enzyme ratio. The number of hydrolytic events per enzyme molecule before complete and irreversible inactivation of the enzyme by imipenem was 110 (the turnover number), estimated by extrapolation of the plot of the irreversible part of inactivation versus the imipenem-to-β-lactamase ratio to the intercept on the abscissa. The rate constant of inactivation and K_m(inact) were estimated to be 0.70 min^{-1} and 0.38 μM, respectively. The K_m(inact) was approximately equal to the K_i, 0.59 μM within experimental error.

Partial reactivation in Fig. 1 started after complete consumption of imipenem. The k_{cat} was estimated to be 1.51

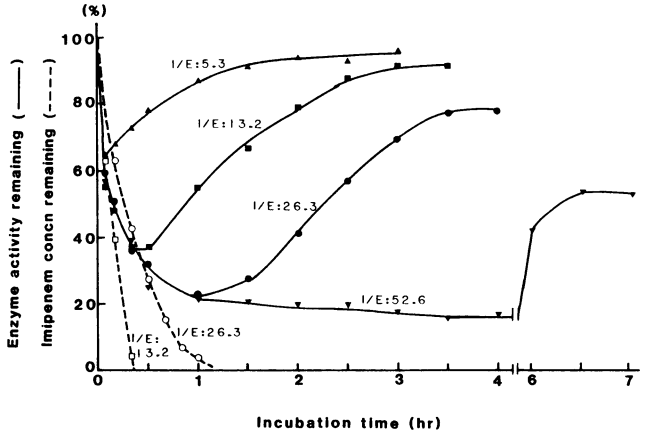

FIG. 1. Effect of preincubation of *P. vulgaris* β-lactamase with imipenem on enzyme activity and hydrolysis of imipenem by β-lactamase. Three-hundred microliters of β-lactamase (E) (final concentration of active enzyme, 380 nM) was preincubated with 15 μl of 2 (▲), 5 (■), 10 (●), and 20 (▼) μM imipenem (I) in 50 mM sodium-potassium phosphate buffer (pH 7.0) at 30°C for an appropriate period of time. After incubation, a 5-μl portion of the mixture was removed and immediately added to 3.0 ml of prewarmed phosphate buffer containing 100 μM cephalothin. Remaining β-lactamase activity was measured for the first 2 min of the reaction at 30°C by a spectrophotometric method at a wavelength of 265 nm. β-Lactamase inactivation was expressed as the percent activity remaining compared with the enzyme activity assayed in the absence of imipenem. The reaction of hydrolysis was carried out at 30°C in 3.0 ml of the same buffer containing 12.5 (□) and 25 (○) μM imipenem with 950 nM enzyme. Hydrolysis of imipenem was monitored by spectrophotometry at a wavelength of 298 nm.

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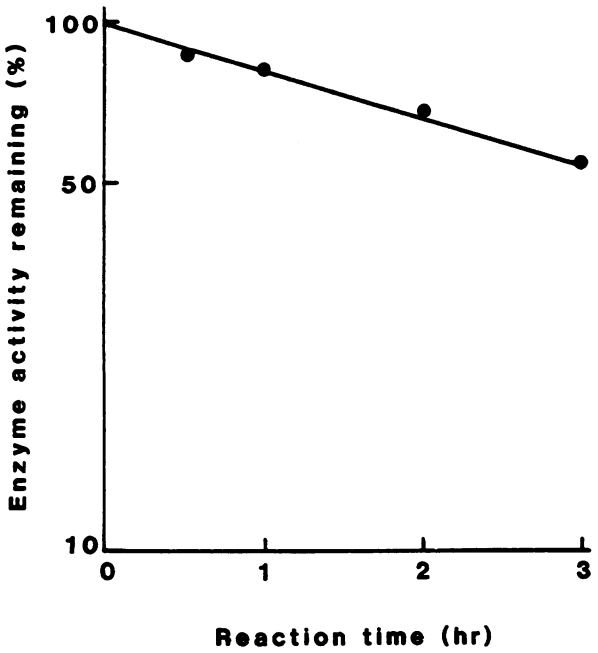

FIG. 2. Determination of the rate constant for irreversible inactivation of *P. vulgaris* cephalosporinase by imipenem. The enzyme preparation (final concentration, 3.8 μM) was preincubated with 10 mM imipenem in 100 μl of 50 mM sodium-potassium phosphate buffer (pH 7.0) at 37°C for 30, 60, 120, and 180 min. After the reaction was stopped by rapid cooling in an ice bath, the reaction mixture was immediately applied to a Sephadex G-25 column (0.9 by 8.0 cm), which was developed at 4°C with the same buffer. Pooled fractions containing the enzyme were quickly incubated at 30°C, and reactivated enzyme activity was measured by the spectrophotometric assay. The fraction of enzyme recovered after incubation was calculated by comparison with a control reaction in which imipenem was absent.

min^{-1} from the initial velocity of hydrolysis. However, the rate of hydrolysis progressively decreased for the duration of the incubation period (Fig. 1, dotted line), although the concentration of imipenem was sufficiently higher than the K_i. This finding implies that active enzyme was constantly transformed to an inactivated form. Figure 1 also shows that complete recovery of enzyme activity was not attained. To investigate the irreversible inactivation of the enzyme by imipenem, reactivation was tested after the imipenem-β-lactamase complex had been separated from excess imipenem by gel filtration. The k_{react} was estimated to be 0.031 min^{-1}, independent of the inactivation time of the experiment, whereas the maximum reactivation was dependent on the inactivation time, and the rate constant for irreversible

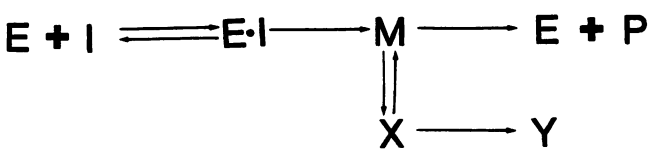

FIG. 3. Possible minimal scheme for the inactivation of *P. vulgaris* cephalosporinase by imipenem. E · I is the Michaelis complex. M is the acyl-intermediate. X and Y correspond to the unstable complex and irreversible complex, respectively. P represents hydrolyzed imipenem.

inactivation was found to be 0.003 min^{-1} from first order kinetics (Fig. 2).

Richmond reported that imipenem is a potent irreversible inhibitor of β-lactamases from *Enterobacter cloacae* and *Bacteroides fragilis* (12). However, apparent irreversibility may be due to the presence of unhydrolyzed imipenem in the reaction mixture (competitive inhibition). We observed that an acceleration of hydrolysis of good substrates by the inactivated enzyme of *P. vulgaris* occurred within a few seconds after dilution by excess good substrates (data not shown).

In kinetic studies on the interaction of olivanic acid and its derivatives with TEM-2 penicillinase (3, 5) and of PS-5 with *P. vulgaris* cephalosporinase (9), it was demonstrated that full recovery of the enzyme activity occurred but only after disappearance of intact inhibitor from the reaction medium. However, irreversible inhibition of the enzyme by imipenem was evident in our study.

On the basis of the results described above, we propose the minimum scheme for the inactivation of the *P. vulgaris* cephalosporinase by imipenem (Fig. 3). In this scheme, Y is sequentially formed from X, since the irreversible inactivation kinetics were apparently monophasic. If Y was formed directly from M, we should have observed a rapid initial loss of activity, as reported in the case of clavulanic acid (6).

LITERATURE CITED

1. **Brenner, D. G., and J. R. Knowles.** 1981. Penicillanic acid sulfone: an unexpected isotope effect in the interaction of 6α- and 6β-monodeuterio and of 6,6-dideuterio derivatives with RTEM β-lactamase from *Escherichia coli*. Biochemistry **20**:3680–3687.
2. **Charnas, R. L., J. Fisher, and J. R. Knowles.** 1978. Chemical studies on the inactivation of *Escherichia coli* RTEM β-lactamase by clavulanic acid. Biochemistry **17**:2185–2189.
3. **Charnas, R. L., and J. R. Knowles.** 1981. Inhibition of the RTEM β-lactamase from *Escherichia coli*. Interaction of enzyme with derivatives of olivanic acid. Biochemistry **20**:2732–2737.
4. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404–427.
5. **Easton, J. E., and J. R. Knowles.** 1982. Inhibition of the RTEM β-lactamase from *Escherichia coli*. Interaction of the enzyme with derivatives of olivanic acid. Biochemistry **21**:2857–2862.
6. **Fisher, J., R. L. Charnas, and J. R. Knowles.** 1978. Kinetic studies on the inactivation of *Escherichia coli* RTEM β-lactamase by clavulanic acid. Biochemistry **17**:2180–2184.
7. **Kahan, J. S., F. M. Kahan, R. Goegelman, S. A. Currie, M. Jackson, E. O. Stapley, T. W. Miller, A. K. Miller, D. Hendlin, S. Mochales, S. Hernandez, H. B. Woodruff, and J. Birnbaum.** 1979. Thienamycin, a new β-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties. J. Antibiot. **32**:1–12.
8. **Labia, R., V. Lelievre, and J. Peduzzi.** 1980. Inhibition kinetics of three R-factor-mediated β-lactamases by a new β-lactam sulfone (CP-45899). Biochim. Biophys. Acta **526**:572–579.
9. **Okamura, K., M. Sakamoto, and T. Ishikura.** 1980. PS-5 inhibition of a β-lactamase from *Proteus vulgaris*. J. Antibiot. **33**:293–302.
10. **Okonogi, K., S. Harada, S. Shinagawa, A. Imada, and M. Kuno.** 1982. β-Lactamase inhibitory activities and synergistic effects of 5,6-*cis*-carbapenem antibiotics. J. Antibiot. **35**:963–971.
11. **Reisfeld, R. A., U. J. Lewis, and D. E. Williams.** 1962. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature (London) **195**:281–283.
12. **Richmond, M. H.** 1981. The semi-synthetic thienamycin derivative MK-0787 and its properties with respect to a range of β-lactamases from clinically relevant bacterial species. J. Antimicrob. Chemother. **7**:279–285.

13. **Sawai, T., M. Kanno, and K. Tsukamoto.** 1982. Characterization of eight β-lactamases of gram-negative bacteria. J. Bacteriol. **152:**567–571.
14. **Sawai, T., and K. Tsukamoto.** 1982. Cefoxitin, N-formimidoyl thienamycin, clavulanic acid, and penicillanic acid sulfone as suicide inhibitors for different types of β-lactamases produced by gram-negative bacteria. J. Antibiot. **35:**1594–1602.
15. **Sawai, T., T. Yoshida, K. Tsukamoto, and S. Yamagishi.** 1981. A set of bacterial strains for evaluation of β-lactamase-stability of β-lactam antibiotics. J. Antibiot. **34:**1318–1326.
16. **Yamaguchi, A., T. Hirata, and T. Sawai.** 1983. Kinetic studies on inactivation of the *Citrobacter freundii* cephalosporinase by sulbactam. Antimicrob. Agents Chemother. **24:**23–30.