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The Renal Membrane Dipeptidase (Dehydropeptidase I) Inhibitor, Cilastatin, Inhibits the Bacterial Metallo-β-Lactamase Enzyme CphA

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The *Aeromonas hydrophila* AE036 chromosome contains a *cphA* gene encoding a metallo-β-lactamase which is highly active against carbapenem antibiotics such as imipenem. Here we show that the *cphA* gene product shares inhibitory similarities with a mammalian zinc peptidase, membrane dipeptidase (MDP; dehydropeptidase I). Both enzymes are able to hydrolyze imipenem and are inhibited by cilastatin. The active site similarities of these enzymes are not reflected in any significant primary sequence similarity.

Thienamycin (11) and related carbapenem antibiotics are rapidly hydrolyzed and inactivated in vivo in humans by renal or membrane dipeptidase (MDP; EC 3.4.13.19), also commonly referred to as dehydropeptidase I, which is a zinc metalloenzyme located in the microvilli of kidney proximal tubular cells (2, 10, 13). MDP exhibits versatile substrate specificity, hydrolyzing dipeptides and dehydropeptides, as well as β-lactam antibiotics of the *trans*-conformation, such as imipenem (2). Cilastatin (MK0791; {Z-S-[6-carboxy-6-(2,2-dimethyl-(S)-cyclopropyl) carboxy)-amino-5-hexenyl]-L-cysteine}) was developed as a reversible, competitive inhibitor of MDP (50% inhibitory concentration = 0.1 μM) on the basis of the structural similarities between the scissile bonds in imipenem and dehydropeptides (10) (Fig. 1).

Bacterial zinc metallo-β-lactamases are typified by the β-lactamase II of *Bacillus cereus* (9, 14) and the *cfiA* gene product of *Bacteroides fragilis* (20) and are capable of hydrolyzing different classes of β-lactam compounds, including oxyiminocephalosporins, cephamycins, penicillins, and carbapenems (1, 7, 18). Some of these metalloenzymes pose a considerable threat to antibiotic therapy, particularly since carbapenems show an exceptionally wide spectrum of antibacterial activity. The recently described *cphA* gene product of *Aeromonas hydrophila* (16) shares a lower degree of sequence similarity with the *B. cereus*, *B. fragilis*, and *Xanthomonas maltophilia* (16, 21) metallo-β-lactamases and appears to fall into a different molecular subclass within the class B metalloenzymes. It can hydrolyze carbapenems efficiently, but it is unable to cleave with very high efficiency the *cis*-conformation of the β-lactam ring seen in the classical penicillins and cephalosporins (5, 6, 17). Since MDP is also a Zn^{2+}-dependent carbapenem-specific β-lactamase, we have examined possible similarities in structural features and catalytic mechanisms between the mammalian and bacterial enzymes and the possibility that the *A. hydrophila* enzyme may be inhibited by cilastatin.

MDP was purified to homogeneity from porcine kidney cortex by affinity chromatography on cilastatin-Sepharose (15), and the bacterial CphA enzyme was purified from an *Escherichia coli* DH5 strain (pAA20R) containing the *cphA* gene as described previously (17). The assessment of enzyme activity towards imipenem was performed spectrophotometrically by monitoring the decrease in A_{299} with 0.1 mM imipenem as a substrate (17). The hydrolysis of Gly-D-Phe or glycyldehydrophenylalanine (Gly-dh-Phe) (3) was monitored by a high-performance liquid chromatography (HPLC) method (8). Purified MDP (13 ng) or CphA enzyme (10 μg) was incubated in 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM Gly-dh-Phe or 3 mM Gly-D-Phe with or without cilastatin (0.3 mM) in a final volume of 100 μl. After incubation at 37°C, samples were boiled for 4 min and centrifuged at 12,000 × *g* for 10 min at 4°C. The products, phenylpyruvate or D-Phe, were analyzed by reverse-phase HPLC on a μBondapak C_{18} column by using a linear gradient of 4.5 to 30% acetonitrile in 0.08% H_3PO_4 with detection of the product at 214 nm.

The rates of enzyme-catalyzed hydrolysis of imipenem by MDP and CphA in the presence of a range of concentrations of cilastatin (0.01 M to 1.0 nM) were compared (Fig. 2). Imipenem hydrolysis by both enzymes was seen to be cilastatin sensitive. The 50% inhibitory concentrations were 0.3 ± 0.01 and 178 ± 11 μM (n = 8), respectively. Although cilastatin is substantially less potent against the CphA enzyme and therefore unlikely to be of clinical value, it does provide a useful lead for development of more potent compounds. Hydrolysis of imipenem by partially purified *B. fragilis* metallo-β-lactamase (7) was found to be inhibited partially by high concentrations of cilastatin, with 60% inhibition being seen at 0.01 M cilastatin (data not shown). With the *X. maltophilia* L1 enzyme and imipenem (300 μM) or nitrocefin (30 μM) as the reporter substrate, no inhibition by cilastatin was seen at concentrations below 10 mM. Thus, cilastatin has a narrow spectrum of activity against bacterial metallo-β-lactamases. Cilastatin was not hydrolyzed by the metallo-β-lactamases. We further investigated the possible hydrolysis by the CphA enzyme of the two characteristic substrates of MDP, Gly-D-Phe and Gly-dh-Phe. Hydrolysis products were resolved by HPLC and identified by coelution with the appropriate standards (D-Phe and phenylpyruvate). Phenylpyruvate was also confirmed as a product of Gly-dh-Phe hydrolysis by MDP by its reduction to phenyllactate in the presence of lactate dehydrogenase and NADH. Figure 3 illustrates that only MDP is able to hydrolyze the dipeptide and dehydropeptide substrates. Even prolonged in-

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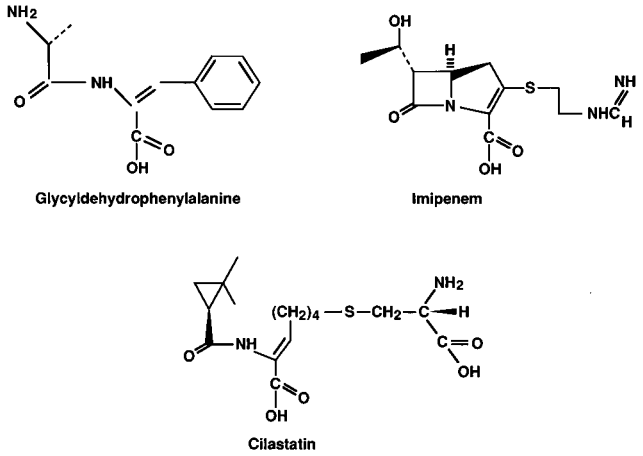

FIG. 1. Structures of glycyldehydrophenylalanine, imipenem, and cilastatin.

cubation with CphA enzyme (10 μg of protein, 16 h) failed to result in peptide hydrolysis (Fig. 3c and f).

Thus, mammalian MDP and the bacterial CphA enzyme are both zinc metalloenzymes which share the ability to hydrolyze imipenem, and both are inhibited by cilastatin, although with differing sensitivities. Imipenem was more efficiently hydrolyzed by CphA enzyme than MDP (specific activities, 26 and 0.2 μmol/min/mg of protein for the purified, homogeneous enzymes, respectively). However, the CphA enzyme is unable to hydrolyze dipeptides or dehydropeptides that are efficient substrates of MDP. Comparison of the predicted amino acid sequences of MDP and cphA enzyme by using the GCG program (4) fails to reveal any significant sequence similarity. Crystallographic structure analysis of the β-lactamase II (Blm) from *B. cereus* (19) has revealed that the zinc ion is coordinated to three histidine residues and a cysteine with the motif -HXH-(79 residues)-C-(41 residues)-H-. This motif is conserved in the *B. fragilis* CfiA enzyme (20). However, in the CphA enzyme the first histidine of the motif is replaced by asparagine (16) and in the *X. maltophilia* L1 enzyme (21) the three histidines are conserved but the cysteine is substituted by a serine. Directed mutagenesis is required to confirm the essential residues in all these enzymes. In MDP a critical histidine residue (His-219) has been pinpointed by site-directed mutagenesis (12), but other zinc ligands have not been identified.

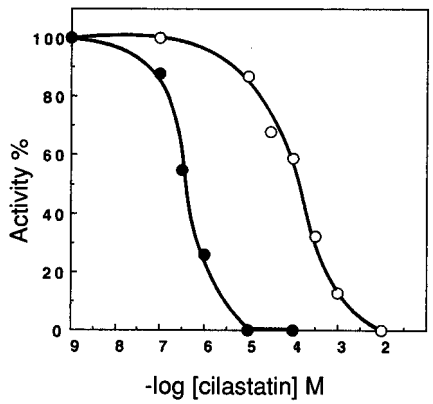

FIG. 2. Inhibition of imipenem hydrolysis by cilastatin. CphA enzyme (500 ng) (○) or purified MDP (10 μg) (●) was incubated in a total volume of 1 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing cilastatin at a final concentration as indicated on the abscissa. The activity was monitored spectrophotometrically (A_{299}) with imipenem (0.1 mM) as the substrate. The maximum activity (100%) represents 26 ± 1.5 μmol/min/mg of protein (CphA enzyme) and 0.2 ± 0.01 μmol/min/mg of protein (MDP) respectively.

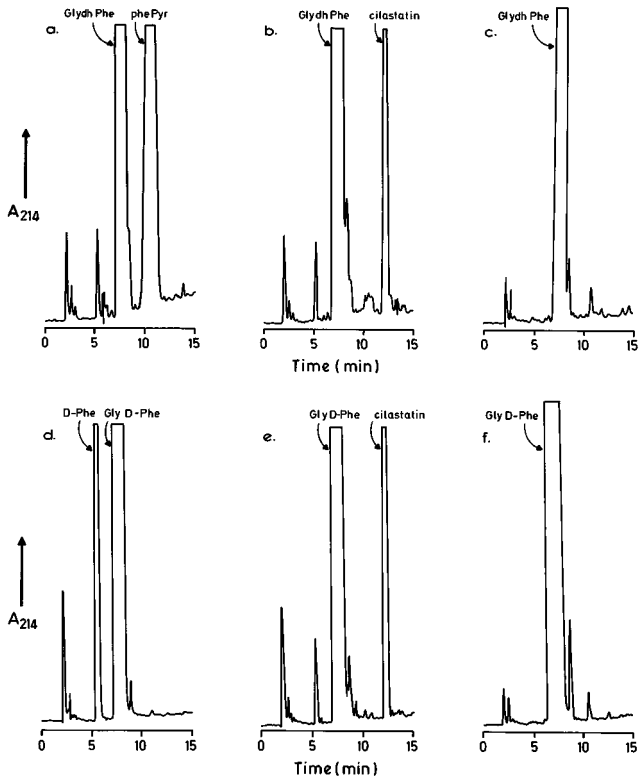

FIG. 3. HPLC analysis of Gly-D-Phe and glycyldehydrophenylalanine hydrolysis by MDP and CphA enzymes. Gly-dh-Phe and Gly-D-Phe were incubated with MDP (13 ng; 10 min) and with CphA enzyme (10 μg; 16 h), respectively, at 37°C in the presence or absence of cilastatin (0.3 mM). Products were resolved by HPLC and quantified by measurement of A_{214}. The metabolism of Gly-dh-Phe is shown by MDP (a), MDP in the presence of cilastatin (b), and CphA enzyme (c). The metabolism of Gly-D-Phe is shown by MDP (d), MDP with cilastatin (e), and CphA enzyme (f).

Comparative structural studies of MDP and CphA enzyme may provide insight into mechanistic aspects of this class of β-lactamase enzymes and could be helpful in the development of novel and specific inhibitors of bacterial metallo-β-lactamases. Cilastatin or analogs may be useful to distinguish between different subclasses of metallo-β-lactamases.

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