Kinetics of the Inhibitory Effect of Pepstatin on the Reaction of Hog Renin with Rat Plasma Substrate

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ABSTRACT
The reaction between hog renin and rat angiotensinogen followed Michaelis-Menten kinetics. A Km value of 1,109 ng angiotensin/ml was determined for the rate of angiotensin formation at pH 7.4 and 37°C. The pH optimum for the reaction of hog renin with rat angiotensinogen was between 6.8 and 7.0. Pepstatin inhibited the reaction in a noncompetitive way. The maximum inhibitory effect occurred at approximately pH 6; at values above pH 8.0, pepstatin had no inhibitory effect. The effect of pepstatin was reversible, since renin activity could be restored by 24 hours of dialysis against distilled water. A 50% inhibition of the enzymatic activity of 0.01 dog units of hog renin was obtained with 0.45 ng pepstatin/ml.

KEY WORDS hog renin, rat angiotensinogen, enzyme kinetics, enzyme inhibitor, angiotensin, noncompetitive inhibition

Pepstatin, a pentapeptide isolated from the culture filtrate of actinomycetes strains (1) and initially characterized as an inhibitor of pepsin and other acid proteases (2, 3), has also been shown to inhibit the reaction of hog renin with rat plasma substrate at a neutral pH. The interaction of renin with heterologous plasma substrate has been demonstrated both in vitro (4) and in vivo in rats (5, 6). Although various authors have confirmed the inhibitory effect of pepstatin on renin, no extensive studies on the kinetics of this reaction have been published. Recent studies (6, 7) have claimed that competitive antagonism occurs, but evidence for such a claim is not convincing. Preliminary studies in our laboratory have indicated a noncompetitive type of inhibition. Therefore, the present investigation attempted to elucidate the mechanism underlying the inhibitory effect of pepstatin on the release of angiotensin from rat plasma substrate by hog renin.

Methods

RENIN
Solutions of hog renin (0.1 dog units/ml) were prepared in 0.067M Soerensen phosphate buffer, pH 7.4, and human albumin (final concentration 1 mg/ml) was added to the solutions, which were then stored at -25°C.

SUBSTRATE
The substrate was prepared according to the method of Haas et al. (8) and Boucher et al. (9) from the plasma of rats subjected to a bilateral nephrectomy 24 hours earlier. The lyophilized substrate preparation contained an amount of angiotensinogen equivalent to 100 ng angiotensin/mg protein.

PEPSTATIN
Pepstatin1 was dissolved in a phosphate-buffered (pH 8) 0.9% saline solution in a concentration of 4 µg/ml and stored at -25°C. Mixtures of enzyme, substrate, and inhibitor were incubated at pH 7.4 and 37°C for 10 minutes. For determinations of the pH optimum, the incubation period was extended to 20 minutes to obtain more distinct differences in the amounts of angiotensin formed at various values of pH. When the substrate concentration was varied, the duration of incubation was reduced to 5 minutes in the experiments with a low concentration of substrate to keep substrate consumption below 10% of the initial amount. The incubate contained an amount of angiotensinogen equivalent to 4,500 ng angiotensin/ml solution. Each milliliter of solution contained 0.067M phosphate buffer, 0.01M Na, ethylenediaminetetraacetate (EDTA), 0.02% neomycin, 0.1 ml of hog renin (0.01 dog units) solution, and 0.1 ml of pepstatin (0.4 µg) or, in control experiments, 0.1 ml of 0.9% saline. At the end of the incubation period, the reaction was stopped by the addition of 0.2 ml of 1N hydrochloric acid followed by boiling in a water bath for 5 minutes. Then 0.7 ml of 0.067M NaOH was added to correct the pH, and the samples were frozen and stored at -25°C. Details of the method have been described previously (10).

DETERMINATION OF ANGIOTENSIN
To ascertain the Michaelis constant (Km) and the inhibitor constant (Ki), the concentration of angiotensin in the samples was estimated by a radioimmunoassay specific for angiotensin I, which was similar to that described by Haber et al. (11). In control experiments, care was taken to ensure that only angiotensin I was

1 Kindly supplied by Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan.
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formed. In all other experiments, angiotensin concentra-
tions were measured by bioassay in the rat (10).

Results

pH DEPENDENCE

For the reaction between hog renin and rat plasma substrate, the pH optimum was between 6.8 and 7.0. At a pH above 8.0, the activity of hog renin rapidly declined (Fig. 1). The inhibitory effect of pepstatin on the hog renin–rat substrate reaction was most marked in the acid pH range. At a pH above 6.8 pepstatin lost activity, and at pH values greater than 8 no inhibitory effect was demonstrated.

BINDING OF PEPSTATIN TO ANGIOTENSINOGEN

Since binding of the pepstatin molecule to the substrate might interfere with the enzyme-sub-
strate interaction, the question of whether rat substrate in the presence of pepstatin can be converted quantitatively to angiotensin by an excess of hog renin was raised. Various amounts of substrate (434–3,472 ng angiotensin/ml) with and without pepstatin were incubated with 1 dog unit of hog renin (Fig. 2). The results of this experiment showed that pepstatin was not irreversibly bound to an active site of the substrate, since the amount of angiotensin cleaved from the substrate in the presence of pepstatin was the same as that cleaved in the absence of pepstatin.

BINDING OF PEPSTATIN TO RENIN

To determine whether the binding of pepstatin to renin was reversible, a mixture of renin (0.1 dog units/ml) and pepstatin (4 µg/ml) was dialyzed against distilled water for 24 hours at 0°C. The renin activity in the dialyzed, lyophilized, and reconstituted sample was compared with that in a similarly treated control sample which contained no pepstatin and also with that in nondialyzed control samples. The renin activity in the sample containing pepstatin was 82 ng angiotensin formed before and 135 ng angiotensin formed after dialysis, whereas the renin activity in the sample without pepstatin was 134 ng angiotensin formed before and 132 ng angiotensin formed after dialysis. Therefore, the inhibition of the renin-substrate reaction was completely reversible.

DETERMINATION OF Kₐ AND Kᵢₚ

The pepstatin concentration that produced a 50% inhibition of the activity of hog renin (0.01 dog units) was ascertained by the method of Dixon and Webb (12). By using two substrate concentrations (1,060 ng angiotensin/ml and 4,690 ng angiotensin/ml) and varying the pepstatin concentration between 0 and 0.8 µg/ml, a Kₐ value of 0.455 µg pepstatin/ml was obtained (Fig. 3). The finding that the two lines intersected the abscissa at virtually the same point indicated a noncompetitive inhibition.

To verify this finding and to further characterize the type of inhibition, the reaction between hog renin and rat substrate was studied at various substrate concentrations between 222 and 4,000 ng angiotensin/ml with and without the addition of

\[\text{Or} \text{dinate} = \text{angiotensin released after a 20-minute incubation, and abscissa} = \text{pH values.}\]
Determination of $K_\text{m}$, $S_\text{m}$, = substrate equivalent to 1,060 ng angiotensin/ml, $S_\text{s}$ = substrate equivalent to 4,690 ng angiotensin/ml, ordinate = reciprocal plot of angiotensin produced by 0.01 dog units of hog renin (ng$^{-1}$ x 10$^{-4}$), and abscissa = concentration of pepstatin (µg/ml). Each point represents the mean of three radioimmunological determinations of duplicate incubations.

pepstatin (0.4 µg/ml). The values measured for angiotensin were plotted by the Lineweaver-Burke method (12). A $K_\text{m}$ of 1,109 ng angiotensin/ml was found at pH 7.4 and 37°C, and the maximum rate of angiotensin formation ($V_\text{max}$) was 17.4 ng angiotensin/min with 0.01 dog units of hog renin. When pepstatin (0.4 µg/ml) was added, $V_\text{max}$ fell to 8.4 ng angiotensin/min. Hence, by means of the Lineweaver-Burke plot, a noncompetitive type of inhibition was also observed. Pepstatin reduced the $K_\text{m}$ value from 1,109 ng angiotensin/ml to 804 ng angiotensin/ml (Fig. 4).

**Discussion**

The results obtained in this study clearly indicate that pepstatin inhibits the reaction between hog renin and rat plasma substrate in a noncompetitive way. This finding contrasts with the observations reported by other investigators (6, 7), who have proposed a competitive type of inhibition. An interpretation of these divergent findings is not possible, since no detailed information about the method used or the results obtained is available. Originally, pepstatin was described as a noncompetitive inhibitor of pepsin (1). Kunimoto et al. (2) then found a competitive inhibition of pepsin for diacetylpepstatin when a special substrate, N-acetyl-L-phenylalanyl-L-diodotyrosine, was used. These authors (2) concluded that, under certain conditions, pepstatin can also act as a competitive inhibitor of pepsin. Our data do not permit conclusions as to whether the renin-substrate reaction is inhibited in a noncompetitive way under all conditions, but recently Bath and Gрегerman have found a noncompetitive inhibition of the reaction between human renin and a polymeric tridecapeptide substrate (13) by pepstatin (personal communication).

Whether the differences in the $K_\text{m}$ values that were calculated with and without the addition of pepstatin are due to an increase in the affinity of the enzyme-inhibitor complex for the substrate or merely reflect variations in measurement is not clear. Further studies are necessary to answer this question. The fact that pepstatin can be completely removed from a mixture with renin by dialysis demonstrates that the binding of pepstatin to renin is reversible. The same phenomenon has been shown for the binding of pepstatin to pepsin (2).
The observation that the extent of inhibition by pepstatin is pH dependent corresponds to former data obtained with rat renin and homologous substrate (10). This pH effect probably reflects differences in the degree of dissociation at the sites of pepstatin that are responsible for the binding to renin.

References
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