Pepstatin Inhibition of Human Renin

KINETIC STUDIES AND ESTIMATION OF ENZYME PURITY

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SUMMARY

Pepstatin has been shown to be an extremely potent inhibitor of human renin \( (K_i = 1.3 \times 10^{-10} \text{M}) \). Kinetic analyses by several techniques show reversible interaction of pepstatin with renin and classical noncompetitive inhibition. The high affinity of pepstatin for renin allows kinetic determination of the molarity of the enzyme in partially purified preparations. This method has been validated by parallel studies of pepstatin inhibition of pure porcine pepsin. A widely used preparation of human renin has been shown to contain about 0.1% of the pure enzyme. The maximum theoretical specific activity of human renin has been calculated from this value at 118 Goldblatt units per mg of enzyme protein.

Pepstatin is a pentapeptide of microbial origin with the sequence iso-valeryl-Val-4-amino-3-hydroxy-6-methylheptanoyl-Ala-4-amino-3-hydroxy-6-methylheptanoic acid. The peptide inhibits the acid proteases pepsin and cathepsin D (1) and the procozyme enzyme renin (2, 4). Recently a number of other small to medium sized peptide inhibitors of renin have been described (5). Although some of these peptides are also quite potent \( (K_i = 10^{-5} \text{ to } 10^{-4} \text{M}) \), the unique structure and effectiveness of pepstatin raised the question of whether its mechanism of action differs from that of other peptide inhibitors. The present study explores the kinetics of pepstatin inhibition of human renin and compares the results with those recently reported elsewhere for the conventional peptide inhibitors.

EXPERIMENTAL PROCEDURES

Materials

The human renin, specific activity 0.13 Goldblatt unit per mg (Lot 13), was obtained from E. Haas (6). The pepstatin (specific activity 2883 units per mg), of porcine origin, was obtained from Worthington Biochemical, Freehold, N. J. H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan) provided the pepstatin. Tridecapeptide renin substrate (Lot y2169) was from Schwarz BioResearch, Orangeburg, N. Y. All other materials for synthesis of \(^{125}\text{I}\)-polymeric renin substrate were similar to those described earlier (6). The reagents used for the inhibition and kinetics experiments were from sources previously presented (5).

Methods

Enzyme assays were performed using the \(^{125}\text{I}\)-labeled polymeric substrate described for renin by Bath and Gregerman (6). Assays were performed at pH 5.5 in 0.1 M sodium phosphate buffer containing 1 mg per ml of lysozyme. Diisopropyl fluorophosphate (0.0027 M) and EDTA (0.015 M) were added to prevent nonspecific peptidase activity. Lysozyme had to be omitted in the pepstatin studies due to formation of a white suspension, but this omission did not affect the assay results. The final concentration of renin was \( 1 \times 10^{-4} \text{ units per ml} \). The amount of pepstatin required to generate the same net counts per min as \( 1 \times 10^{-4} \text{ units per ml} \) of renin was determined by performing parallel titrations of pepstatin and renin in the labeled polymeric assay. The equivalent final concentration of pepstatin was \( 2.89 \times 10^{-8} \text{ M (0.1 \mu g per ml)} \). The total reaction volume for both enzyme determinations was 1.0 ml. Reactions were initiated by addition of enzyme and terminated following 1-hour incubation (37°) by boiling the tubes for 10 min. The pH was adjusted to 7 to 8 with 0.5 ml of 0.2 M sodium phosphate, pH 7.5, prior to extraction with 1-butanol (6).

Kinetic Studies

Kinetic studies were performed by a modification of the assay as described by Workman et al. (6). This technique was used for three separate types of kinetic analysis:

(a) Test for Reversibility using Method of Ackerman and Potier—For an enzyme-inhibitor system with a very high affinity of enzyme for inhibitor and a small dissociation constant, the possibility exists that the inhibition is not readily reversible in which case Michaelis-Menten kinetic equations do not apply. Such irreversible or pseudo-irreversible type of inhibition can be recognized graphically by plotting the reaction rate versus different enzyme concentrations in both the presence and absence of the inhibitor (7, 8).

The reversibility of inhibition of human renin by pepstatin was tested by this method by varying the renin concentration in the absence of pepstatin and with three concentrations of inhibitor. A plot of reaction velocity versus renin concentration was made for the four situations (Fig. 2).

(b) Three Linear Transformations of Michaelis-Menten Equation—These data were generated by varying the substrate concentration from 0.1 to 0.75 \( \mu \text{M} \) in the absence and presence of a single inhibitor concentration. The concentration of pepstatin used for the inhibitor curves was \( 2.36 \times 10^{-10} \text{ M at which 50% inhibition (I_50) was evident (9). The values of S, V, and V_i, where V is the reaction rate without inhibitor and V_i the velocity with inhibitor, were used for plots of 1/V versus 1/S, V/S versus V, and S versus S/V (9, 10). The lines were calculated by the method of least squares. Values for K, were calculated in the usual manner for noncompetitive results.}

(c) Dixon and Webb Plots—These analyses were made by determining the reaction velocity at two widely separated substrate concentrations from 0.1 to 0.75 \( \mu \text{M} \) in the absence and presence of a single inhibitor concentration. The concentration of pepstatin used for the inhibitor curves was \( 2.36 \times 10^{-10} \text{ M at which 50% inhibition (I_50) was evident (9). The values of S, V, and V_i, where V is the reaction rate without inhibitor and V_i the velocity with inhibitor, were used for plots of 1/V versus 1/S, V/S versus V, and S versus S/V (9, 10). The lines were calculated by the method of least squares. Values for K, were calculated in the usual manner for noncompetitive results.}

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concentrations, $S_1$ and $S_0$, with concentrations of inhibitor in the $I_{50}$ range. The inhibitor concentration for pepstatin inhibition of human renin was varied from $1.1 \times 10^{-10}$ M to $9.4 \times 10^{-8}$ M. A plot of $1/V_1$ versus $[I]$ (Dixon plot) was made for each substrate level. Since a control is included with no inhibitor present for both $S_1$ and $S_0$, the fractional inhibition, $i = 1 - V_1/V_0$, where $V_0$ is the velocity of the control, can be calculated for each concentration of inhibitor. Subsequently, a Webb plot of $1/i$ versus $1/I$ can be produced from the same experimental data.

**Determination of Molarity of Renin (and Pepsin)**—High affinity inhibitors can be used to determine enzyme concentration. Such inhibitors have $K_i$ values on the order of $10^{-8}$ M (11). In such cases the molarities of inhibitor and enzyme are essentially the same. Since the amount of inhibitor bound to the enzyme is no longer a negligible fraction of the total, the Michaelis-Menten equations expressed in terms of free inhibitor are not valid. Strauss and Goldstein (12) have developed the following equations expressed in terms of total inhibitor concentration:

$$ I = \frac{K_i}{1 - i} + iE $$

where $K_i/1 - i$ is the concentration of free inhibitor; $K$ is the dissociation constant of the enzyme-inhibitor complex; $i$ is the fractional inhibition; $iE$ is the molar concentration of combined inhibitor; and $E$ is the enzyme concentration. Dividing Equation 1 by $i$ yields:

$$ \frac{I}{i} = K_i \frac{1}{1 - i} + E $$

A plot of $I/i$ versus $1/1 - i$ will give a straight line of slope $K_i$ and $y$ intercept equal to $E$, the concentration of the enzyme in the system.

The values of $I/i$ and $1/1 - i$ for the graphical determination were obtained from the kinetics experiment (c) which yielded the Dixon plot and the Webb plot. The equation of the line giving the slope ($K_i$) and $y$ intercept ($E$) was calculated by the method of least squares.

The validity of this procedure for renin was tested using pure pepsin of known concentration. Dixon and Webb plots were also made from the data used to determine the molarity of pepsin. The two substrate levels used were the same as for renin and the concentration of pepstatin was varied from $0.4i \times 10^{-9}$ M to $3.7i \times 10^{-9}$ M.

**RESULTS**

**Inhibition of Human Renin by Pepstatin**—The inhibition of human renin was monitored over a wide range of pepstatin concentrations (Fig. 1). Fifty percent inhibition ($I_{50}$) was observed at $2.36 \times 10^{-10}$ M. Complete loss of enzymatic activity was approached at $2.36 \times 10^{-8}$ M pepstatin which produces greater than 92% inhibition. Under certain assay conditions, complete inhibition of pepsin and cathepsin has been reported to occur at approximately equimolar concentrations of pepstatin and enzyme (13, 14). If one assumed that the same were true for the human renin-pepstatin interaction, the molarity of the enzyme corresponding to $1 \times 10^{-4}$ units per ml could be read from the inhibition curve of Fig. 1. The value obtained, $1 \times 2 \times 10^{-8}$ M, proves to be a considerable overestimate of the maximum renin concentration. The absolute renin molarity is actually close to $1 \times 10^{-10}$ M as shown below.

**Inhibition of Pepsin by Pepstatin**—A rate of cleavage of the labeled polymeric substrate similar to that produced with $1 \times 10^{-8}$ units per ml of human renin was seen with $0.1 \mu g$ per ml of pepstatin ($2.89 \times 10^{-9}$ M). However, the amount of pepstatin required to inhibit this concentration of pepsin was nearly ten times greater than that required for this amount of renin. Nearly complete inhibition of $2.89 \times 10^{-8}$ M pepsin occurs at a concentration of pepstatin which is close to $10^{-8}$ M (Fig. 1; see below). Thus even with pepsin this technique cannot be used to estimate enzyme concentration with accuracy.

**Reversibility of Inhibition of Renin by Pepstatin**—A plot of reaction velocity versus renin concentration with no pepstatin (control) and at three inhibitor concentrations produced a family of lines intersecting at the origin (Fig. 2). The slopes of the lines with the inhibitor present decreased with increasing inhibitor concentration corresponding to the graphical representation expected for a reversible reaction. Barrett and Dingle (15) also performed this type of experiment for pepstatin inhibition of cathepsin D. With that enzyme quite different results were ob-
**FIG. 3.** Kinetic representation for the inhibition of human renin by pepstatin. Data plotted by method of (a) Lineweaver-Burk (1/V versus 1/S); (b) Eadie-Hofstee (V versus V/S); (c) Hanes (S/V versus S). Control, no inhibitor present (○); inhibitor present at 2.36 × 10^{-10} M (●). The intersection on the x axis of the inhibitor and control lines for the Hanes plot was obtained using negative values for x in the calculated (least squares) equations of the lines. V, reaction velocity, counts per min generated per min of incubation; S, micromolar substrate concentration.

**TABLE I**

<table>
<thead>
<tr>
<th>Method or Molarity</th>
<th>Ki values obtained from kinetic studies of pepstatin inhibition of human renin and porcine pepsin</th>
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<td></td>
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<tr>
<td><strong>Renin</strong></td>
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<tr>
<td>Lineweaver-Burk (1/V vs 1/S)</td>
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<tr>
<td>Eadie (V/S vs V)</td>
<td>2.6 × 10^{-10}</td>
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<tr>
<td>Hanes (S/V vs S)</td>
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<tr>
<td>Dixon (1/V vs 1/I)</td>
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<tr>
<td>Webb (1/i vs 1/l)</td>
<td>2.9 × 10^{-10}</td>
</tr>
<tr>
<td>Molarity of renin (I/i vs 1/1 - i)</td>
<td>1.9 × 10^{-10}</td>
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<tr>
<td><strong>Pepsin</strong></td>
<td></td>
</tr>
<tr>
<td>Dixon (1/V vs 1/I)</td>
<td>1.7 × 10^{-4}</td>
</tr>
<tr>
<td>Webb (1/i vs 1/l)</td>
<td>1.2 × 10^{-4}</td>
</tr>
<tr>
<td>Molarity of pepsin (I/i vs 1/1 - i)</td>
<td>1.2 × 10^{-4}</td>
</tr>
</tbody>
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Kinetics of Inhibition of Renin by Pepstatin—Variation of the substrate with the inhibitor concentration held constant yielded data which are presented as plots of 1/V versus 1/S (Fig. 3a; Lineweaver-Burk), V versus V/S (Fig. 3b; Eadie-Hofstee), and S/V versus S (Fig. 3c; Hanes). All three graphical representations illustrate classical noncompetitive inhibition (9). Values for Ki are presented in Table I. The limitations of the familiar Lineweaver-Burk plot generally used for kinetic studies should be kept in mind. This type of plot has been shown to be markedly inferior to those of Hanes and Eadie (Hofstee) for determination of kinetic parameters (10).

Two additional kinetic plots were obtained by varying the inhibitor concentration from 1.1 × 10^{-10} M to 9.4 × 10^{-10} M at two substrate concentrations (S1, 0.74 μM and S2, 2.96 μM) and measuring the reaction velocities. A Dixon plot of 1/V versus [I] illustrates noncompetitive kinetics (Fig. 4a, top; (9)) since the lines of S1 and S2 intersect on the x axis to the left of the origin at -Ki. Although the Dixon plot may fail under some circumstances to distinguish between competitive and mixed inhibition (16), the present data are typically noncompetitive and in agreement with the other kinetic analyses. A Webb plot of 1/i versus 1/[I] results in two separate lines if the inhibition is competitive while noncompetitive inhibition generates two lines superimposed upon each other which intersect the y axis at 1 and the x axis at -1/Ki (9). The Webb plot (Fig. 4b, top) for renin inhibition by pepstatin corresponds to noncompetitive kinetics. Values of Ki for the Dixon and Webb plots are shown in Table I.

Kinetics of Inhibition of Pepsin by Pepstatin—The kinetic data obtained for pepsin inhibition by pepstatin are presented using two graphical methods. The Dixon plot (1/V versus [I]; Fig. 4a, bottom) and the Webb plot (1/i versus 1/[I]; Fig. 4b, bottom) both illustrate noncompetitive inhibition and are very similar to those obtained with renin. Values of Ki are presented in Table I. Both noncompetitive and uncompetitive inhibition have been reported for pepstatin inhibition of pepsin. The results depended on the substrate used but detailed data have not been presented (1).

Although the labeled polymeric substrate has not been previously used for measurement of pepsin activity and the pepsin cleavage product was not characterized, it is presumably the same tetrapeptide which results from renin action on the labeled substrate. Pepsin is known to cleave selectively both the Leu-Leu bond of renin protein substrate and the synthetic tetradecapeptide renin substrate (17, 18). It seems most probable that the labeled polymeric substrate is similarly cleaved.

Determination of Molarity of Renin (and Pepsin)—The plot of 1/i versus 1/1 - i for the graphical evaluation of both Ki and E
porcine pepsin by pepstatin. (a) Dixon plot (1/Vi versus I); inhibitor

i, the fractional inhibition, is 1 - Vi/V, where V, is

the values for Ki (slope) and E (y intercept) directly (see "Kinetic

strate concentrations 81, 0.74 PM (O), and &, 2.96 pM (w).

the reaction velocity of the control (no inhibitor present). Sub-

tion (molarity) of human renin and porcine pepsin (inset). The

plot of Z/i versus l/l - i for pepstatin inhibition of renin yields

Studies"). The molarity of renin is determined to be 2.15 X 10-10 M. This value is

very close to the 1~ for pepstatin inhibition of renin. Therefore,

is shown in Fig. 5. The value of Ki, 1.9 X 10-19 M, agrees well

with the Ki values shown in Table I which were calculated from

the Michaelis-Menten kinetic analyses. The concentration of

renin read directly from the y intercept of the line calculated by

the method of least squares is 2.15 x lo-10 M. This value is

a maximal value. The true concentration of renin is un-

doubtedly much lower due to the presence of pseudorenin (see

"Discussion"). The applicability of this kinetic method for

renin is validated by the nearly perfect results obtained with pep-

statin inhibition of pepsin (Fig. 5, inset). This graphical method

is the only procedure known to us which allows estimation of the

concentration of an enzyme in a crude preparation prior to avail-

ability of pure enzyme. Since pepstatin will undoubtedly prove to

inhibit a number of other proteases, this sensitive and relatively

simple procedure may find additional applications in the purifica-

tion of this group of enzymes.

DISCUSSION

Detailed kinetic studies of pepstatin inhibition of renin have not

been previously published. A preliminary report indicated that

the pepstatin effect on hog renin acting upon natural protein

substrate is competitive (21) but further considerations suggest

noncompetitive inhibition.2 Our data clearly indicate that pep-

statin inhibits human renin at concentrations some four orders of

magnitude lower than those required with the most potent pep-

zyme producing prolonged elevation of blood pressure. In a

preliminary report inhibition of this enzyme by pepstatin was

noted to be of the competitive type (21), but later data showed

that the inhibition was noncompetitive (Lineweaver-Burk; Eadie,

Hanes; Miller, personal communication).
tide inhibitors of the enzyme heretofore studied (5, 22, 23). Nonetheless, the kinetic analyses are very similar to those with the less potent peptide inhibitors in showing classical noncompetitive results (5). Since inhibition of renin by pepstatin is readily reversible, Michaelis-Menten kinetics are applicable despite the extremely high potency of this inhibitor.  

Substitution of one or more of the L-amino acids by n-amino acids in several synthetic renin substrate analogs produces inhibitors which appear to act by a mechanism qualitatively different from that of conventional peptides and pepstatin. Inhibition of renin by these n-amino acid-substituted peptides is reported to be of the competitive type (22, 23), but it should be noted that the data are limited. In one investigation the inhibition was studied only by a Dixon plot (22); in the other only a Lineweaver-Burk analysis was used and the data were not presented in graphical form (23).

The calculation presented for the purity of the human renin preparation illustrates the problem which will be encountered during future attempts at purification of the enzyme. From our calculations the crude renin preparation used for these experiments (Haas Lot 13) contains no more than 0.58% renin by weight. Evidence from work with highly purified pseudorenin-free human renin indicates that only 20% of the activity of Lot 13 measured in the labeled polymeric substrate assay under our present conditions is due to renin; the remainder is attributable to pseudorenin. Thus the preparation may contain only about 0.12% renin and a 1000-fold purification of this material will be required to obtain pure enzyme. The practical problem of isolation is made more difficult due to the small amount of renin in the kidney and the significant losses which accompany purification (20). The isolation of appreciable quantities of pure human renin will clearly remain a difficult task.

An estimate of the theoretical specific activity of human renin (Golblatt units per mg of protein) can be made from the present calculation of the purity of renin. The preparation containing 0.13 units per mg with a purity of 0.12% would have a specific activity of 118 units per mg at 100% purity. Waldhäuser et al. estimated the specific activity of their purest renin preparation at no more than 25 units per mg (20). Their material showed three components on gel electrophoresis, at least one of which was believed to be a major contaminant. If one assumes that one-third of the total material was pure renin then the maximum activity of renin would clearly remain a difficult task.

Note Added in Proof—Through the courtesy of Dr. Edgar Haber we have now been able to review the kinetics of inhibition of renin by the n-amino acid substituted peptides (23). These data were not presented in graphical form in the original publication. The Lineweaver-Burk plots, fitted by the authors (23), using a weighted point technique (10), show competitive inhibition. We have replotted the same data by the method of Hanes; competitive inhibition is again indicated. The data are not precise enough for meaningful plots by the method of Eadie. Thus, the n-amino acid substituted peptide inhibitors of renin (22, 23) can be considered competitive inhibitors which differ in action from protein and ordinary peptide inhibitors (5) and from pepstatin, all of which appear to inhibit noncompetitively.

Orth et al. have now published their studies on the kinetics of pepstatin inhibition of hog renin (26), and Kunimoto et al. have further studied the inhibition of pepsin (27).

REFERENCES

23. Poulsen, K., Burton, J., and Haber, E. (1973) Biochemistry 12, 3877-3880