Mode of Inhibition of Acid Proteases by Pepstatin*

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Four derivatives of pepstatin, each of which contains the unusual amino acid 4-amino-3-hydroxy-6-methylheptanoic acid (statine) have been prepared. All four are potent porcine pepsin inhibitors. Both N-acetylstatine and N-acetyl-alanyl-statine are competitive inhibitors for pepsin with $K_i$ values of $1.2 \times 10^{-4}$ M and $5.65 \times 10^{-6}$ M, respectively. The $K_i$ value for N-acetyl-valyl-statine is $4.8 \times 10^{-6}$ M. These statyl derivatives, therefore, are very strong inhibitors. The $K_i$ value for N-acetyl-statine is 600-fold smaller than that of its structural analog N-acetyl-leucine. The derivative which contains two statyl residues in a tetrapeptide exhibits inhibitory properties which approach those of pepstatin itself. Other acid proteases, human pepsin, human gastricsin, renin, cathepsin D, the acid protease from Rhizopus chinensis and bovine chymosin, also are inhibited by pepstatin and its derivatives. It is suggested that the statyl residue is responsible for the unusual inhibitory capability of pepstatin and that statine is an analog of the previously proposed transition state for catalysis by pepsin and other acid proteases.

Pepstatin is a low molecular weight, potent inhibitor specific for acid proteases. Since its discovery by Umezawa and co-workers (2) in the culture filtrates of various species of Actinomyces, it has been shown to inhibit nearly all acid proteases including porcine pepsin, renin, cathepsin D (3), bovine chymosin (4), human pepsin, human gastricsin, protease B from Aspergillus niger, and several acid proteases of microbial origin (5). The stringent specificity of pepstatin toward acid proteases clearly has been demonstrated by the lack of inhibition for the neutral and alkaline proteases (6).

The unusual potency of pepstatin toward acid proteases is indicated by its $K_i$ which was reported by Kunimoto et al. (7) to be about $1 \times 10^{-18}$ M for porcine pepsin. Due to this remarkable property, pepstatin has been widely used as a research tool in the studies of enzyme mechanisms (8) and biological functions (9) and in affinity chromatography (10, 11). It also has been tested as a therapeutic agent for the experimental control of gastric ulcer (2, 6), carrageenin edema (2), and hypertension (12, 13).

At present, the mode of inhibition by pepstatin is not known. The chemical structure was determined by Morishima et al. (14) to be essentially a hexapeptide which contains 2 residues of an unusual amino acid, 4-amino-3-hydroxy-6-methylheptanoic acid (statine). The complete structure of pepstatin is isoamyric acid (statine). It conforms with the general nomenclature of amino acids and is rooted in its source, pepstatin. The term statyl therefore is used to describe the statine residue linked in peptide bonds.

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t-phenylalanilyl-t-diacidotryosine (Ac-Phe-Tyr(Val)) and bovine hemo-
globin were products of Nutritional Biochemical Corp. The protein
component (globin) of hemoglobin was prepared by extraction of
the hemoglobin precipitate with 1% HCl (by volume) in acetone. The pro-
cedure was repeated until the supernatant did not extract any color.
The globin then was suspended in water at pH 6, dialyzed against three
changes of water at pH 6, and lyophilized.

**Methods**

**Assay for Proteolytic Activity**—Three assay methods were used to
determine the activity of pepstatin and other acid proteases. First, with
hemoglobin substrate the proteolytic activity was determined from the
optical density of trichloroacetic acid-soluble peptides (30, 31). Sec-
ond, the rate of hydrolysis of globin substrate was followed in a pH stat
at pH 2.1. (The apparatus consisted of a standard Radiometer assembly
equipped with a 0.25-ml burette.) Globin was dissolved in distilled
water and centrifuged. The concentration of globin in the supernant
was determined by hydrolysis and amino acid analysis. The optical
density of the supernatant was measured at 280 nm. From these values
the rate, was calculated to be 16.1. This extinction coefficient was used
to adjust the globin concentration in subsequent experiments. A final
adjustment to pH 2.1 was required after dilution. In the case of inhibition
studies, an aliquot of inhibitor solution, which had previously been adjusted to pH 2.1, was added to the substrate. (In no instance did preincuba-
tion of enzyme and inhibitor alter the measured hydrolytic rate.)

The hydrolsate was initiated by the addition of an aliquot of pepstatin solution. The initial rate of hydrolysis was determined
from the plot of acid consumption versus time on the recorder.

**Preparation of Tetrapeptide, Valyl-sta-alanyl-sta-sta**—Pepstatin
was dissolved to a saturated solution at 50°C in 0.2 M N-ethylmorpho-
loline acetate buffer, pH 8, containing 5% methanol. After cooling to 37°C,
about a 0.05 molar ratio of α-lytic peptide was added. The mixture
was left standing at 37°C for 40 h and then was lyophilized. The dry
residue was extracted with the pH 2 buffer to be used in the subse-
quent high voltage electrophoresis and centrifuged to remove insoluble
material. The supernatant was applied as a 40-cm band on a full sheet
of Whatman No. 1 paper which was subjected to electrophoresis (14).
The structure Val- Ala- Sta was assigned to this peptide based on the
above evidence and on the known structure of pepstatin (14).

**Acetylation**—Acetylation of statin or statyl peptides isolated from
pepstatin was carried out using a 100-fold excess of acetic anhydride
with slight modification of the procedure described by Ridon and Vallee
(38). The reaction was carried out in an aqueous solution, pH 7.2. Small
amounts of acetic anhydride and powdered NaHCO₃ were added in turn to the reaction solution. The pH, which was monitored with a pH meter, was maintained within the range of pH 6.5 to 7.5. At

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1 The abbreviations used are: Ac-Phe-Tyr(Val), N-acetyl-t-phenyl-
alanyl-t-diacidotryosine; Sta, statine, which is 4-amino-3-hydroxy-6-
methylheptanoic acid; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.

![Fig. 1. Electrophoretic pattern of pepstatin fragments. High voltage electrophoresis was carried out at pH 2, 60 V/cm, 60 min.](http://www.bj.org/)

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the end of the reaction, the pH of the solution was adjusted to above 8 and the solution was allowed to stand at the room temperature for about 60 min in order to hydrolyze any O-acetate which may have been formed. The solution was then adjusted to pH 2 and used directly in the kinetic experiments. The completeness of the acetylation of statine was demonstrated by the absence of statine in an amino acid analysis. The acetylated peptides were tested in high voltage paper electrophoresis. No original peptide with a significant fluorescamine positive spot could be found. The acetyl peptides were quantified by hydrolysis and amino acid analysis.

Calculation of Kinetic Constants—After plots of the data (reciprocal velocity versus inhibitor concentration, a Dixon plot (39), and reciprocal of the fractional inhibition versus reciprocal of the inhibitor concentration, a Webb plot (40)) showed the inhibition to be competitive, direct calculation of the constants $K_i$, $k_a$, and $K_e$ was undertaken. As suggested by Cleland (41), the data were fit to the equation

$$v = \frac{k_2 E}{1 + \frac{K_m}{S} \left(1 + \frac{I}{K_i}\right)} \quad (1)$$

where velocity ($v$), enzyme concentration ($E$), substrate concentration ($S$), and inhibitor concentration ($I$) were observed and the values of the constants $K_m$, $k_a$, and $K_i$ were calculated. Because Equation 1 is not linear, a Taylor's expansion was used and the least squares refinement actually determined shifts from estimates of the constants. This method is advantageous (a) because unit weights are appropriate since the errors in the velocity determinations can be assumed dominant and equal and (b) because the values of all constants can be determined simultaneously. Expected deviations in the constants can be calculated from the regression analysis. Calculations were carried out on an IBM 360/40 computer.

In the case of one inhibitor N-Ac-Val-Sta, only enough material for a few points was available. So the inhibition was assumed to be competitive and previously determined values of few points was available. So the inhibition was assumed to be competitive, direct calculation of the constants $K_i$, $k_a$, and $K_e$ was undertaken. As suggested by Cleland (41), the data were fit to the equation

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RESULTS

Inhibition of Pepsin by N-Acetyl-statine—The results of N-acetyl-statine inhibition of porcine pepsin are shown in Fig. 2. Fig. 2a shows the Dixon (39) plot (reciprocal of velocity versus inhibitor concentration) at three concentrations of glutathione. The rate of hydrolysis was followed in a pH-stat at pH 2.1, 37°C. The rate of hydrolysis of Ac-Phe-Tyr($I_2$) is expressed as millimoles of diiodotyrosine produced/min/ml of assay volume at pH 2.1, 37°C. Inset, Webb plot (1/I versus 1/[$I$]) of the same data where $I$ = 1 - ($v/v_c$) and $v_i$ is the velocity for the uninhibited sample and $v_c$ is the velocity for an inhibited sample.
substrate are measured using the method of Green and Work (42). As shown in Table I, the $K_v$ value for the N-acetyl-tetrapeptide is about $6 \times 10^{-9}$ M and the $K_v$ for pepstatin is about $1 \times 10^{-8}$ M.

Fig. 4 shows the relationship between increasing inhibitor concentration and peptic activity. Fig. 4a is a plot of free pepsin versus increasing pepstatin concentration at three pH values. The initial portions of the lines are obviously linear. Extrapolation of these linear portions to the abscissa gives rise to enzyme/pepstatin ratios. At pH 2.1, the ratio is 1.96; at pH 3.1, it is 1.33; and at pH 3.65, it is 1.13.

Fig. 4b shows that, when Ac-Phe-Tyr(1,4) is the substrate and N-acetyl-Val-Sta-Ala-Sta is the inhibitor, the enzyme/inhibitor ratio varies randomly with pH. At pH 2, this ratio is 1.8 (line pH 2.1a). A decrease of about 4-fold in substrate concentration yields a ratio of 1.9 (line pH 2.1b). When the pH is increased to 3.5, the ratio is 1.9. Further increase to pH 4.5 results in a ratio of 1.8. Therefore, although the enzyme/inhibitor ratio approaches 1.0 as the pH increases for hemoglobin substrate, the ratio remains constant (1.8 ± 0.1) as the pH increases for Ac-Phe Tyr (1,4) substrate.

The results cannot be explained by irreversible inhibition, that is, irreversible in the sense that a pepsin molecule binds to a pepstatin molecule to form a covalent complex even if that complex could then bind to and inhibit a second pepsin molecule. A solution containing pepsin and pepstatin was

![Kinetic plots of the inhibition of porcine pepsin by N-acetyl-alanyl-statine. Dixon plot for two concentrations of Ac-Phe-Tyr(I,4): $S_1 = 10.5 \times 10^{-5}$ M (□); $S_2 = 3.0 \times 10^{-4}$ M (○). The concentration of pepstatin was $0.84 \times 10^{-8}$ M. Inset, Webb plot of the same data.](image)
Inhibition of Acid Proteases by Pepstatin and N-Acetyl-Tetrapeptide

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% Inhibition by Pepstatin</th>
<th>% Inhibition by N-Acetyl-Val-Sta-Ala-Sta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine pepsin</td>
<td>100 (1.2)</td>
<td>79 (1.4)</td>
</tr>
<tr>
<td>Human pepsin</td>
<td>100 (1.0)</td>
<td>65 (1.5)</td>
</tr>
<tr>
<td>Human gastricsin</td>
<td>9 (1.3)</td>
<td>2 (1.6)</td>
</tr>
<tr>
<td>Acid protease from R. chinensis</td>
<td>10 (1.6)</td>
<td>0 (1.6)</td>
</tr>
<tr>
<td>Bovine chymosin</td>
<td>46 (1.6)</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* The % inhibition was determined using Assay I. The pepstatin concentration was $5 \times 10^{-7}$ M and the N-acetyl-valyl-statyl-alanyl-statine concentration was $1.2 \times 10^{-7}$ M.

† The form of the data is per cent inhibition (enzyme concentration $\times 10^{-7}$ M).

‡ N.D. means not determined.

DISCUSSION

Although N-acetyl-statine is a weaker inhibitor than pepstatin, it is nevertheless a relatively strong inhibitor as compared to its structural analog N-acetyl-leucine. The inhibition constant of N-acetyl-statine ($1.2 \times 10^{-7}$ M) is almost 600-fold less than that of N-acetyl-leucine ($7 \times 10^{-7}$ M). This drastic difference in $K_i$ between the closely related structures (statine and leucine) suggests that N-acetyl-statine is a transition state analog of peptic catalysis and is the source of the inhibitory potency of pepstatin. This proposal is supported by several findings. First, the difference in $K_i$ values between N-acetyl-statine and N-acetyl-leucine, a product of peptic hydrolysis, is similar to that found for a transition state analog of elastase, peptidyl alanol (46). Second, the presence of two binding sites on pepstatin under some conditions suggests that each statine binds to a pepsin molecule. Third, the critical importance of the statyl residue in the inhibition is supported by the findings of Kunimoto and co-workers (15) that acetylation of the hydroxyl groups in pepstatin greatly reduces the inhibitory activity. Fourth, an examination of the transition state for porcine pepsin catalysis, for example as proposed by Hartsuck and Tang (20) and reviewed by Tang,3 reveals a high degree of structural similarity to a statyl residue. Fig. 5 shows such a comparison. It can be seen that in the proposed model of the transition state, both the carbon atom in the carbonyl group and the nitrogen in the

* J. Tang, submitted for publication.
amide group must be in the tetrahedral configuration. (This is in contrast to the planer configuration in a normal peptide bond.) The equivalent atoms in statine would be the carbons at positions 2 and 3, which are both in tetrahedral configuration (Fig. 5).

Although other investigators, Gregerman et al. (47), observed noncompetitive inhibition of renin by N-acetyl-statine and the N-acetyl-statyl dipeptides. We did not attempt to determine the type of inhibition associated with pepstatin or with any pepstatin peptides containing 2 statyl residues, because the $k_i$ values for molecules with 2 statyl residues are so small as to make it less favorable for the competitiveness test. The "competitiveness" and "transition state hypothesis" are both supported by x-ray crystallographic results (48) of R. chinensis bond.) The equivalent atoms in statine would be the carbons at positions 2 and 3, which are both in tetrahedral configuration (Fig. 5).

It is interesting to note that pepstatin and the tetrapeptide inhibit several other acid proteases with considerably less potency. It is possible that the orientation of the active site groups and the secondary binding sites of these proteases is slightly different, thus the binding strength is affected. However, it is likely that pepstatin inhibits these acid proteases by means of the same mode of action, presumably as a transition state analog. This is supported by the findings that the catalytic mechanisms of the acid proteases are nearly identical. First, the active center directed specific pepsin inhibitors, such as diazo compounds (16, 17) and epoxides (18-20), also inhibit other acid proteases (21-27). Second, the amino acid sequences of these enzymes are highly homologous suggesting a common evolutionary origin (50). These arguments support the idea that it may be possible to design specific sequences of statyl peptides for specific inhibition of a given acid protease.

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