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MECHANISM OF INHIBITION OF PEPSIN BY PEPSTATIN

EFFECT OF INHIBITOR STRUCTURE ON DISSOCIATION CONSTANT AND TIME-DEPENDENT INHIBITION*

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Abstract—Investigations were made of the inhibition of pepsin by pepstatin and nine synthetic analogs of pepstatin. Analysis of progress curve data indicated that the inhibition produced by pepstatin (1) conforms to a mechanism in which a rapidly formed enzyme–inhibitor complex (collision complex) is transformed slowly to a more tightly bound complex (tightened complex). Dissociation constants for the collision complex (k_2/k_1) and first-order rate constants for formation (k_3) and reversal (k_4) of the tightened complex are reported for pepstatin and nine pepstatin analogs. The data show that at least three structural parameters are needed to induce the lag transient characteristic of the tight-binding inhibition of pepsin by pepstatin. These are: a 3(S)-hydroxyl group in the third residue of pepstatin, an isopropyl group or its equivalent in the first residue, and some portion of the C-terminal dipeptidyl group –Ala–Sta. The data indicate that these groups interacted cooperatively to induce the time-dependent increase in inhibition. Removal of any of these binding groups affected, predominatly, k_4, the first-order rate constant for return of tightened complex to collision complex. The proposal that pepstatin is an analog of the transition state for pepsin catalyzed hydrolysis of amide bonds is discussed.

Pepstatin, isovaleryl-L-valyl-L-valyl-(3S,4S)-4-amino-3-hydroxyl-6-methylheptanoyl-L-alanyl-(3S,4S)-4-amino-3-hydroxyl-6-methylheptanoic acid (1) (Fig. 1), is one of several enzyme inhibitors isolated by Umezawa *et al.* [1] in an effort to find naturally occurring inhibitors of therapeutically important enzymes [2]. Pepstatin strongly inhibits several carboxyl proteases, including pepsin and cathepsin D [3] and renin [3–7], but does not inhibit trypsin, chymotrypsin, plasmin, kallikrein, thrombin, thrombokinase or papain [3]. Pepstatin binds to the active sites of pepsin [5] and of *Rhizopus chinensis* acid protease [8] but exhibits non-competitive patterns for inhibition of renin and pepsin [4], and pseudorenin [9]. The tight binding ($K_i = 4.6 \times 10^{-11}$ M) [10] and the specificity for carboxylproteases led to suggestions that pepstatin may be a transition-state analog inhibitor of these enzymes [11, 12].

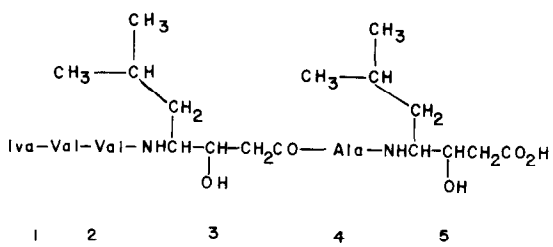

Fig. 1. Structure of pepstatin A. Numbers under each amino acid are residue numbers referred to in the text.

In spite of the widespread use of pepstatin in biological studies, relatively little is known about either the minimal structural features of pepstatin needed to produce an inhibitor with a very low dissociation constant or the origin of its specificity for carboxyl proteases. We report here the kinetics of inhibition of pepsin by pepstatin and by synthetic analogs of pepstatin. Our results show that tight-binding inhibition is characterized by a time-dependent phenomenon, or lag-transient, that is strongly dependent on inhibitor structure.

EXPERIMENTAL PROCEDURES

Materials

Synthetic analogs of pepstatin, prepared from optically pure synthetic t-butyloxycarbonyl-statine [13] using a stepwise solution strategy, are listed in Table 1. Each analog was fully characterized using amino acid analysis, microanalysis, mass spectrometry and nuclear magnetic resonance spectroscopy. Details of these syntheses have been reported separately [14].

Enzymatic studies

Heptapeptide substrate, Phe–Gly–His–Phe(NO)$_2$–Phe–Ala–Phe–OMe, was prepared as described previously [16]. Porcine pepsin was purchased from the Sigma Chemical Co., St. Louis, MO. Pepsin activity was assayed by spectrophotometric measurement of the hydrolysis of the –Phe(NO)$_2$– bond of the heptapeptide substrate at 25° in pH 4.0 formate buffer. Absorbance at 310 nm was monitored as described by Medzihradszky *et al.* [17]. The analogs were dissolved in methanol and

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Table 1. Structure of pepstatin analogs

Compound Number	Structure*†
1	Iva–Val–Val–(3S,4S)Sta–Ala–(3S,4S)Sta
2	Iva–Val–Val–DeoxySta–Ala–DeoxySta
3	Ac–(3S,4S)Sta–Ala–$NH^iC_5H_{11}$
4	Iva–(3S,4S)Sta–$\widetilde{Ala}$–$NH^iC_5H_{11}$
5	Iva–Val–(3S,4S)Sta–Ala–$NH^iC_5H_{11}$
6	Iva–Val–(3R,4S)Sta–Ala–$NH^iC_5H_{11}$
7	Iva–Val–DeoxySta–Ala–$NH^iC_5H_{11}$
8	Iva–Val–(3S,4S)AHPPA–Ala–$NH^iC_5H_{11}$
9	Iva–Val–Val–Sta
10	Ac–Sta
11	Ac–DeoxySta

* Abbreviations used: Sta, 3-hydroxy-4-amino-6-methyl heptanoic acid (statine); DeoxySTa, 4(S)-amino-6-methyl heptanoic acid; $NH^iC_5H_{11}$, isoamyl amide; Iva, isovaleryl; and AHPPA, 4(S) amino-3(S)hydroxy-5-phenyl pentanoic acid.

† R and S configurations are defined in Cahn *et al.* [15].

added to buffer. The final concentration of methanol in both sample and control was about 1.6%.

Reaction velocities expressed as ΔO.D./min were first analyzed graphically as double-reciprocal plots and were also processed on a digital computer according to the procedure described by Cleland [18]. Inhibition data were fitted to equations for both linear competitive and linear noncompetitive inhibition. The kinetic analysis of dideoxypepstatin (2) using this procedure has been described [16].

Dissociation constants for the relatively tight-binding analogs (5) and (8) were measured at steady state and estimated by fitting the data to the rate equations. The enzyme and inhibitor were equilibrated for 5 min and the reactions were initiated by addition of substrate. A lag transient was evident for reactions carried out in this manner (Fig. 4). Steady-state rate data were collected from the linear portion of the curve.

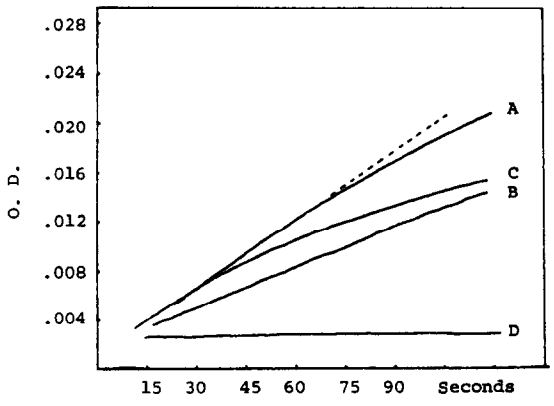


Fig. 2. Time-dependent inhibition of pepsin by pepstatin. Recorder tracings of initial velocity for hydrolysis of heptapeptide substrate catalyzed by pepsin. The incubation mixture was comprised of 0.61 ml of 0.04 M formate buffer, pH 4.0, containing 89.3 μM substrate, 1.93×10^{-8} M pepsin and various concentrations of inhibitors: (A) control, $[I] = 0$; (B) dideoxypepstatin, 3×10^{-7} M; (C) pepstatin, 2×10^{-8} M; and (D) pepstatin, 2×10^{-8} M. In A, B and C, the enzyme was added last. In D, pepstatin and pepsin were equilibrated for 5 min, and the substrate was added last.

RESULTS

Characterization of time-dependent inhibition of pepsin by pepstatin

Inhibition of pepsin by pepstatin depended on the length of time the enzyme and inhibitor were equilibrated (Fig. 2). Figure 2C shows the reaction progress curves when pepstatin (2×10^{-8} M) was used, without preincubation of enzyme with inhibitor, at a concentration slightly greater than that of pepsin. The initial velocity clearly was non-linear, and inhibition increased with time. The pepstatin inhibition was non-linear during the time period in which the initial velocities of control (Fig. 2A) and dideoxypepstatin (Fig. 2B) were linear. When this concentration of pepstatin was equilibrated with pepsin for 3–5 min before substrate was added, no product was formed (Fig. 2D). The latter result was also observed by Kunimoto *et al.* [5]. The data in Fig. 2 established that the binding of pepstatin to pepsin did not reach a steady state for at least 2–3 min.

The time-dependent increase in inhibition shown by pepstatin is consistent with the mechanisms depicted in Scheme 1 where k_3 and k_4 are slow, and $k_3 \gg k_4$.

$$\text{Mechanism A} \quad E + I \underset{k_2}{\overset{k_1}{\leftrightharpoons}} EI \underset{k_4}{\overset{k_3}{\leftrightharpoons}} {}^*EI^*$$

$$\text{Mechanism B} \quad E \underset{k_4}{\overset{k_3}{\leftrightharpoons}} E^* \underset{k_2}{\overset{k_1 I}{\leftrightharpoons}} {}^*EI^*$$

$$\text{Mechanism C} \quad E + I \overset{\text{slow}}{\leftrightharpoons} EI$$

Scheme 1

In Mechanism A, the time-dependent increase in inhibition is caused by slow changes in the initially formed collision complex (*EI*) leading to a new enzyme–inhibitor complex (**EI**) in which inhibitor is more tightly bound to the enzyme. The latter complex is termed the 'tightened' complex. In Mechanism B, the time-dependent increase in inhibition is caused by a slow conformational change from one free enzyme form to another form of the unbound enzyme prior to formation of the tightened complex (**EI**). It is evident from the data in Figs. 2 and 3A that reaction velocity from time zero is inhibited by pepstatin. This allows us to exclude Mechanism C in which pepstatin binds slowly to the enzyme. Mechanism A is consistent with a dependence of initial velocity on pepstatin concentration.

As a working hypothesis, we considered that the inhibition of pepsin by pepstatin follows Mechanism A where the approach to equilibirium requires 3–5 min for completion. Because the approach to equilibrium is slow (k_3 is small) and leads to increasing inhibition with time, k_4 is very small relative to k_3. When the reaction progress curve for the off reaction (Fig. 2D) is followed for several hours, no dissociation of the tightened complex between pepstatin and pepsin is observed. Thus, under the assay conditions

employed here $k_4 \simeq 0$ and the lag transient can be analyzed using Scheme 2.

Scheme 2 has been used to determine the kinetic parameters of irreversible inhibition of enzymes [19], and can be used to analyze the pepstatin results by plotting the reciprocal of the pseudo-first-order rate constant (k_{obs}) versus the reciprocal of the inhibitor concentration [I] according to equation 1.

$$E + I \underset{k_2}{\overset{k_1}{\leftrightharpoons}} EI \xrightarrow{k_3} {}^*EI^*$$

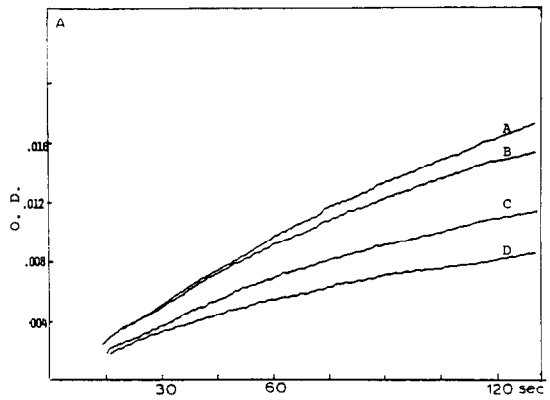
Scheme 2

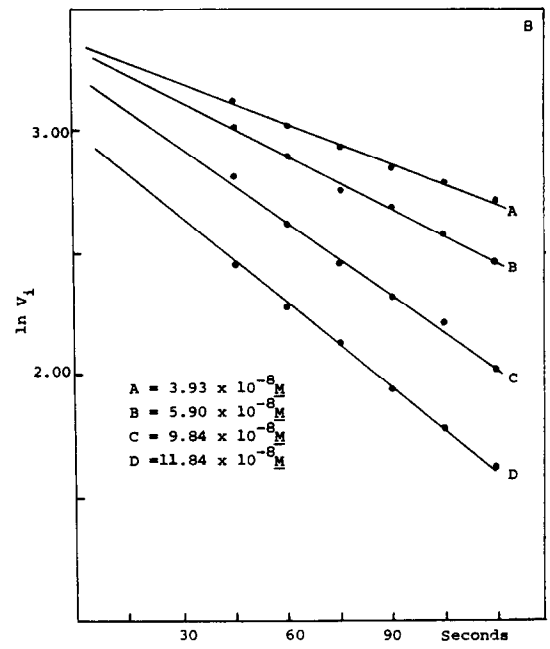
This analysis allows one to estimate values for the apparent dissociation constant of the collision complex (k_2/k_1) and for the rate constant for the lag transient (k_3).

$$\frac{1}{k_{obs}} = \frac{k_2/k_1}{k_3[I]} + \frac{1}{k_3} \qquad (1)$$

The analysis was carried out in the following manner. The initial velocity of hydrolysis of the heptapeptide substrate was measured at increasing concentrations of pepstatin; the data are given in Fig. 3A. Measuring the slope of each curve at time (T) gave the inhibited velocity (V_i) at time, T. The slope can be used because the final velocity at steady state is zero. A plot of ln inhibited velocity (V_i) vs time (T), is shown in Fig. 3B; the slope of each line gives k_{obs}. The plot of $1/k_{obs}$ vs 1/[I] is shown in Fig. 3C. From Fig. 3C and equation 1 the following constants are obtained: k_2/k_1(app) = 1.22×10^{-7} M, k = 0.022 sec^{-1} and $T_{1/2}$ = 31 sec. The value for k_2/k_1 after correction for substrate concentration ($1 + S/K_m$) gives $k_2/k_1 = 1.3 \times 10^{-8}$ M. The double-reciprocal plot ($1/k_{obs}$ vs $1/[I]$) obtained for the pepstatin–pepsin systems is linear, which is consistent with k_4 approaching zero under the assay conditions [20].

Mechanism A outlined in Scheme 1 represents the minimum mechanism that would produce the time-dependent phenomena. It is not known if the binding of pepstatin to pepsin produces other complexes in addition to those depicted in Scheme 1. Kinetic analysis of Mechanism A, expanded to include three (or more) complexes (e.g. $EI \leftrightharpoons 'EI' \leftrightharpoons "EI"$) would yield plots identical to Fig. 3A–C, but the limiting value for k_{obs} at infinite inhibitor concentration would be a function of several rate constants for individual steps and would not be interpretable in terms of a single transition between complexes.





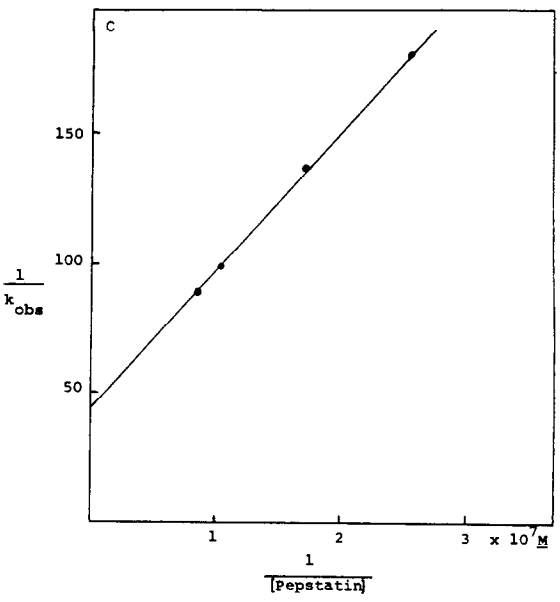


Fig. 3. Panel A: Initial velocity for hydrolysis of heptapeptide substrate in the presence of different pepstatin concentrations. The incubation mixture was comprised of 0.61 ml of 0.04 M formate buffer, pH 4.0, containing 340 μM substrate, 1.98×10^{-8} M pepsin, and various concentrations of pepstatin: (A) 3.93×10^{-8} M; (B) 5.9×10^{-8} M; (C) 9.84×10^{-8} M; and (D) 11.84×10^{-8} M. Reaction was initiated by the addition of enzyme. Panel B; Determination of k_{obs}. The natural logarithms (ln V_i) of inhibited velocity at time T were plotted against time. Panel C: Reciprocal plot of k_{obs} vs pepstatin.

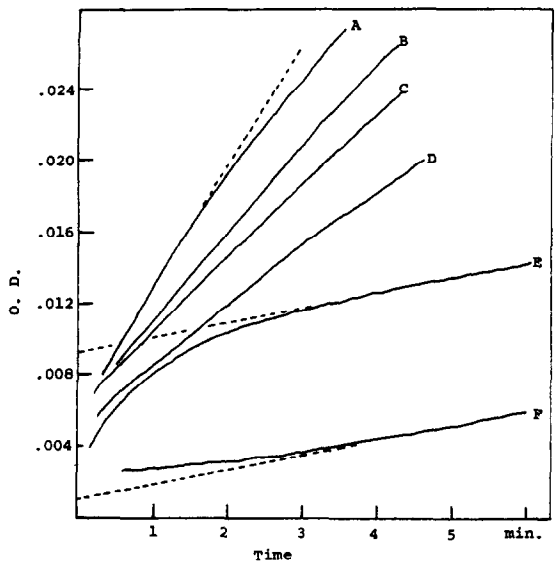


Fig. 4. Recorder tracings of initial velocity for hydrolysis of heptapeptide substrate in the presence of various pepstatin analogs. Substrate was 211 μM. Pepsin was 19 nM. (A) control, $I = 0$; (B) (3R,4S) tripeptide 6, 1.38 μM; (C) analog 4, 1.29 μM; (D) analog 3, 159 μM and (E and F) (3S,4S) tripeptide (5), 22.6 nM. In F, the inhibitor and pepsin were preincubated for 5 min before substrate was added. All other reactions were started by adding the enzyme.

Tripeptide (5) (Iva–Val–(3S,4S)–Sta–Ala–$NH^iC_5H_{11}$) was found to produce a measurable time-dependent increase in inhibition. However, as the reaction progress curve in Fig. 4F shows, this inhibitor–pepsin complex dissociated much faster than the pepsin–pepstatin complex. Since the complex of 5 dissociated (k_4 is finite), Scheme 2 could not be assumed to be valid for analyzing this system under the described assay conditions. Instead, the graphical method of Fridovich [21] was used to generate a series of concentration-dependent k_{obs}. Following the equations of Strickland *et al.* [20], k_4 was estimated to be 0.015 sec^{-1} and $(k_3 + k_4)$ to be about 0.07 sec^{-1} (Fig. 5). The other rate constants (k_2/k_1), however, could not be accurately determined because they could only be obtained at high concentrations of inhibitor and, under these conditions, the transient state was too short to be observed accurately without stopped-flow techniques.

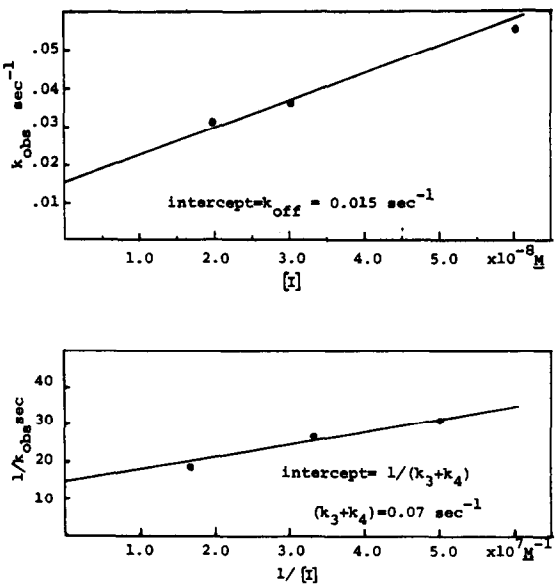


Fig. 5. Determination of k_{off} and $(k_3 + k_4)$ for (3S,4S) tripeptide (5). The method of Fridovich [21] was used to obtain the rate constant for the approach to steady state, k_{obs}. Substrate was 211 μM. Pepsin was 19 nM.

The kinetic parameters determined for each pepstatin analog are presented in Table 2. "Net" dissociation constant (K_i) refers to the kinetically determined dissociation constant and is defined in equation 2.

$$\text{net } K_i = K_D \times \frac{k_{off}}{k_{on}} = k_2/k_1 \times \frac{k_4}{k_3 + k_4} \qquad (2)$$

When $k_4 \ll k_3$, then net $K_i = K_2/k_1 \times k_4/k_3$ and $k_{obs} = k_3$. When $k_4 \gg k_3$, then net $K_i = k_2/k_1$ and $k_{obs} = k_3 + k_4$.

The net dissociation constant for the pepsin–pepstatin complex was determined by Workman and Burkitt [10]. For the remaining analogs, net dissociation constants were determined by fitting kinetic data obtained at steady state to programs for both competitive and non-competitive inhibition [18]. For the tight-binding analogs 5 and 8, these values are approximate values since the equations of Cleland do not correct for mutual depletion of inhibitor and enzyme due to tight binding [22]. Plots of V^{-1} vs S^{-1} at different inhibitor concentrations revealed little deviation from linearity when the rate data for 5 were plotted for competitive inhibition. The rate data for 5 also could be fitted to the computer program for competitive inhibition. Thus, the net K_i for 5 should be close to the kinetically measured K_i. The data for compound 8, however, could not be fitted to the competitive inhibition program, so the apparent net dissociation constant may be too large and the correct net K_i somewhat smaller than the value obtained kinetically.

With compounds showing increasing inhibition with time (1, 5 and 8), the collision dissociation constant (k_2/k_1) does not equal net K_i. The decrease in net K_i caused by the slow, first-order transient from the collision complex to the tightened complex is $k_4/(k_3 + k_4)$ (see equation 2). For pepstatin, $k_4/(k_3 + k_4)$ was calculated from the reported dissociation constant ($K_i = 4.57 \times 10^{-11}$ M) [10] and the value of k_2/k_1 determined here. Using these relationships and the value obtained for k_3, it was possible to calculate the value of k_4 shown in Table 2. For compounds 5 and 8 accurate values of k_2/k_1 could not be obtained directly from the graphical analysis for the reason cited previously. Values of k_2/k_1 were estimated by dividing net K_i by $k_4/(k_3 + k_4)$.

Competitive inhibiton patterns were obtained for all the analogs in Table 2 except pepstatin and compound 8. The non-competitive pattern obtained for pepstatin was seen because $k_4 \approx 0$ under our assay conditions and, therefore, the assay system is not at steady state. The theoretical basis for observing non-

Table 2. Effect of inhibitor structure on kinetic parameters

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} {}^*EI^*$$

Analog*	k_2/k_1 (M)	k_3 (sec^{-1})	k_4 (sec^{-1})	$k_4/(k_3 + k_4)$	"net" K_i (M)	Kinetic pattern†
1‡	1.3×10^{-8}	0.022	4.6×10^{-5}	2.1×10^{-3}	4.57×10^{-11}	N
2	2×10^{-7}	T.N.O.§			$1.99 \pm 0.05 \times 10^{-7}$	C
3	4.2×10^{-5}	T.N.O.			$4.19 \pm 0.78 \times 10^{-5}$	C
4	3.5×10^{-7}	T.N.O.			$3.50 \pm 0.63 \times 10^{-7}$	C
5	$\sim 6 \times 10^{-8}$	$(k_3 + k_4) \geq 0.07$	0.015	0.018	$1.06 \pm 0.19 \times 10^{-9}$	C
6	2.4×10^{-6}	T.N.O.			$2.39 \pm 0.24 \times 10^{-6}$	C
7¶	$\geq 2 \times 10^{-6}$	T.N.O.			$> 2 \times 10^{-6}$	
8	5×10^{-8}	$(k_3 + k_4) \geq 0.08$	0.015	0.02	$9.9 \pm 1.5 \times 10^{-10}$	N
9	$\sim 10^{-7}$	T.N.O.			$\sim 10^{-7}$	C
10		T.N.O.			1.2×10^{-4}	C
11		T.N.O.			3.0×10^{-3}	C

* Structures given in Table 1.
† C, competitive; N, non-competitive.
‡ k_2/k_1 (corr) is the dissociation constant obtained by correcting for substrate competition by multiplying $(1 + S/K_m)^{-1}$.
§ Transient was not observed.
¶ K_i was estimated. Analog 7 was sparingly soluble in the solvent system employed for kinetic studies. At concentrations of 10^{-7} M or lower, no inhibition of pepsin activity was detectable. At 1×10^{-6} M, slight inhibition was observed. However, direct measurement of K_i was not possible since the compound precipitated when the concentration was increased to $2–3 \times 10^{-6}$ M.

competitive inhibition patterns under non-steady state reaction conditions with tight-binding inhibitors has been presented [23–25].

In the case of the AHPPA analog 8, kinetic data were collected after the system had reached steady state. A plot of V^{-1} vs S^{-1} at different inhibitor concentrations gave a small intercept effect. The rate data also could be fitted to a computer program for non-competitive inhibition better than to a program for competitive inhibition. Thus, the apparent non-competitive inhibition pattern probably indicates that tight binding caused a significant curvature of the double-reciprocal plots due to mutual depletion of free enzyme and inhibitor concentrations as described by Morrison [22].

Effect of structure on inhibition

Compound 5 was used as the standard peptide for making a comparative analysis of the effect of structure on net K_i and on time-dependent inhibition. It is a tight-binding inhibitor that shows the time-dependent inhibition found with pepstatin. Compound 5 is a superior model for structure–activity work, however, because it is a readily reversible, competitive inhibitor of pepsin and, therefore, is more easily analyzed kinetically than pepstatin.

Hydroxyl group. Modification of the hydroxyl group in 5 was studied first. Compounds 6 and 7 illustrate that deleting the hydroxyl group or changing its chirality from 3(S) to 3(R) led to 1000- to 2000-fold weaker inhibitors of pepsin (Table 2). No time-dependent inhibition was found for either analog. Simple derivatives of statine are not tight-binding inhibitors of pepsin. The analog *N*-acetyl-statine (10) is only eighteen times more potent than *N*-acetyl-deoxystatine (11), in which the hydroxyl group is absent [26]. This is in sharp contrast to the 4000-fold difference in potency between pepstatin and dideoxypepstatin. These data indicate that the hydroxyl group must be incorporated within larger molecules in order to obtain a potent inhibitor.

Minimal size. Although modifications of the two C-terminal residues in pepstatin were not studied systematically here, several semi-synthetic analogs of pepstatin are known in which the C-terminal –Ala–Sta residues have been deleted [11, 27, 28]. These compounds are much weaker inhibitors than 1, 5 or 8, with dissociation constants between 10^{-5} and 10^{-7} M [14]. Therefore, certain portions of the –Ala–Sta residues are needed for tight-binding inhibition.

N-terminus. The data in Table 2 and Fig. 3 show that increasing the chain length by addition of hydrophobic residues to the N-terminus of the peptide chain (e.g. 3 → 5) increased binding 10,000-fold. In the transition of 4 → 5, the 300-fold tighter binding also was accompanied by the appearance of time-dependent inhibition (Fig. 4).

DISCUSSION

Characterization of time-dependent inhibition for the pepstatin–pepsin system

While studying pepstatin inhibition of pepsin, we observed a progress curve transient that depended on the length of time the enzyme and pepstatin were incubated (Fig. 2). When enzyme and inhibitor were mixed, and substrate added after 5 min to initiate the reaction, no transient was observed. When pepstatin and substrate were added to pepsin, however, a lag of inhibition occurred. This was especially obvious when preincubation of a slight stoichiometric

excess of pepstatin with pepsin completely inhibited the enzyme. The half-life for the onset of inhibition was 31 sec and the half-life for dissociation of the enzyme–inhibitor complex was more than 3 hr.

Several explanations for the time-dependent phenomena have been considered. The time-dependent increase in inhibition observed for pepstatin cannot be caused by a slow bimolecular collision between enzyme and inhibitor (Mechanism C). Rate constants for bimolecular collisions are dependent on the size (and shape) of both molecules and on diffusion. For molecules this size, k_1 ranges between 10^7 and $10^8\,M^{-1}sec^{-1}$ [29]. For a pepstatin concentration of 2×10^{-8} M, k_{obs} for the bimolecular association would be about $0.2\,sec^{-1}$, for which a half-life of 3.5 sec can be calculated. The half-life of the process shown in Fig. 2C is 31 sec and is, therefore, too long to reflect a bimolecular association. This calculation is supported by the kinetic properties of the structurally related inhibitor, dideoxypepstatin (2), which reaches a steady state with pepsin rapidly (Fig. 2B). Since both pepstatin (1) and dideoxypepstatin (2) have nearly identical molecular weights and shapes, they are likely to form bimolecular collision complexes with pepsin at nearly identical rates. A mechanism involving a slow isomerization of one unbound enzyme form to another (Mechanism B) prior to inhibitor binding must be excluded because reaction velocity is dependent on inhibitor concentration (Fig. 3B). The lag transient also cannot arise from multiple pepstatin binding sites on pepsin because pepstatin forms 1:1 complexes with pepsin [4] and with *Rhizopus chinensis* pepsin [18], and in the latter study the crystallographic data show the inhibitor bound to the active site of the enzyme.

The lag transient we observed for the pepstatin–pepsin interaction is consistent with Mechanism A (Scheme 1) in which the isomerization of collision complex (EI) to tightened complex ($^*EI^*$) is slow. One consequence of Mechanism A is that the rate of conversion of the initial complex to the tightened complex (k_{obs}) should be dependent on the structure of the inhibitor at saturating inhibitor concentrations. Indeed, our early inhibition data [16, 26] indicated that the rate constant for the time-dependent inhibition (k_{obs}) was dependent on inhibitor structure in that a lag transient for pepsin inhibition was not observed in the case of dideoxypepstatin (2). This could have occurred because there was no second (or tightened) complex formed, i.e. $k_3 \simeq 0$, or it could arise if $k_4 > k_3$. In the latter case, $k_{obs} = (k_4 + k_3)$ which would lead to a transient that is too fast to have been observed by the methods available to us when k_4 approaches $0.1\,sec^{-1}$. Furthermore, as $k_4 > k_3$, $K_i \rightarrow k_2/k_1$. This could explain why the apparent dissociation constant for the pepstatin–pepsin collision complex ($k_2/k_1 = 1.3 \times 10^{-8}$ M) was similar to the net dissociation constant for the dideoxypepstatin–pepsin complex (2×10^{-7} M). To further define this system a more comprehensive structure–activity study was initiated.

Effect of inhibitor structure on time-dependent inhibition

Kinetic analysis of the inhibition of pepsin by pepstatin using heptapeptide substrate Phe–Gly–His–Phe(NO_2)–Phe–Ala–Phe(OMe) provides a very sensitive method for probing the nature of the enzyme–inhibitor interaction. Using this assay, it is possible to characterize the inhibition produced by each analog in terms not only of net K_i as is usually done in enzyme inhibitor studies but also in terms of the constants for the lag transient, k_3 and k_4, and the apparent collision dissociation constant k_2/k_1. This allows us to determine which of these parameters is most affected by modifications of the structure of the inhibitor.

To carry out this comparative analysis we used analog 5 as the reference structure. Compound 5 formally corresponds to a pepstatin analog in which the C-terminal statine residue has been converted to an isoamyl amide group. This corresponds to replacement of the β-hydroxy propionic acid [$-CH(OH)CH_2CO_2H$] moiety by a hydrogen atom. Since Miyano *et al.* [30] showed that the hydroxyl and carboxyl groups in the C-terminal residue of pepstatin are not essential for potent inhibition of pepsin, the isoamyl amide group was expected to replace this statine without decreasing its contribution to inhibition. As shown in Table 2, analogs containing the C-terminal isoamyl amide group, 5 and 8, are good pepsin inhibitors with net dissociation constants approaching that of pepstatin. These analogs have the advantage of quickly reaching steady state with pepsin and, thereby, allow kinetic analysis via standard methods.

Our results show that at least three structural features are needed to induce the lag transient characteristic of the tight-binding inhibition of pepsin by pepstatin. These are: a 3(S) hydroxyl group in the third residue of pepstatin, an isopropyl group or its equivalent in the first residue of pepstatin, and some portion of the C-terminal dipeptidyl group –Ala–Sta. The minimum size of the C-terminal group has not been determined, but the potency of compound 5 shows that the β-hydroxy propionic acid group is not essential for tight-binding inhibition of pepsin. Some portion of the $-Ala-NH^tC_5H_{11}$ group in compounds 1 and 5 is essential because compounds like Iva–Val–Val–Sta (9), which lack this group, are weak inhibitors.

These structural requirements are illustrated in Fig. 6 where the structures of pepstatin and compound 5 are compared. The isovaleryl group in 5 (or 8) corresponds to a deaminovaline residue which can be postulated to bind to the same site on pepsin that the valine in residue one of pepsatin binds to. This appears to be an important binding site since removal of this group, as in compound 4, causes a 300-fold decrease in binding to pepsin and disappearance of the lag transient.

The data in Table 2 suggest that these groups interact cooperatively with pepsin after binding to effect the time-dependent increase in inhibition. When the 3(S) hydroxyl group is added to dideoxypepstatin (2) or compound 7, the increased binding is too much to be caused by simple hydrogen bonding. A 10- to 20-fold decrease in dissociation constant can be attributed to simple hydrogen bonding by the 3(S) hydroxyl group in this inhibitor system. For example, acetylstatine (10) binds to pepsin about eighteen times tighter than does acetyldeoxystatine

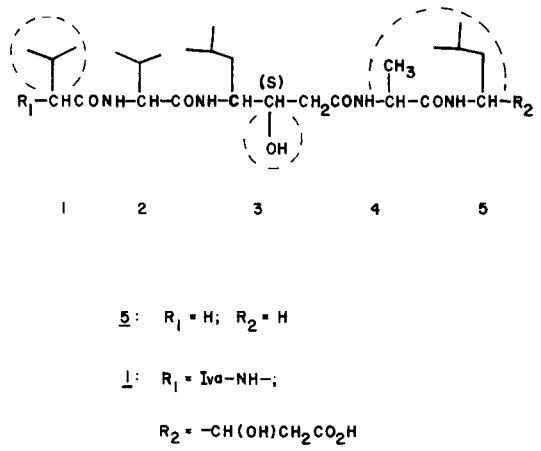


Fig. 6. Comparison of structures of tight-binding pepstatin analog 5 and pepstatin. The dotted lines represent probable binding sties on pepsin for the encircled groups. Numbers under amino acids designate residue number found in text.

(11). Addition of this 3(S)-hydroxyl to dideoxypepstatin decreases k_2/k_1 about 10-fold [26], a value close to that expected from acetyl statine and acetyldeoxystatine data. Both estimates of the effect of the hydroxyl group on the dissociation constant are consistent with the expected 10-fold decrease in dissociation constant for a hydrogen bond between enzyme and substrate in aqueous media in the absence of cooperative effects [31]. Our results suggest that further decreases in net K_i attributable to the 3(S) hydroxyl group develop in the transition from the collision complex to the tightened complex.

We interpret these results to indicate that the (3S) hydroxyl group on the statine residue, the elongated N-terminal acyl group on the statine residue, and portions of the C-terminal dipeptidyl unit act cooperatively to stabilize the tightened complex (*EI*) and, thereby, decrease k_4. The nature of the transition from $EI \rightarrow {}^*EI^*$ probably involves a conformational change, but additional data are needed to determine this. The data in Table 2 show that the decrease in net K_i produced by elongating the N-terminal substituents on the statine residue (e.g. compare compounds 3, 4 and 5) is caused primarily by a decrease in k_4. In the case of pepstatin, k_4 is very small and the half-life for dissociation from pepsin was more than 3 hr. In contrast, k_4 was about 300-fold greater in compound 5, and the dissociation half-life was on the order of seconds. The remaining individual rate constants (k_3, k_2/k_1) for these two compounds do not appear to differ by more than 5-fold.

The absence of time-dependent inhibition in analogs with shortened peptide chains (e.g. 3, 4 and 9) or in analogs lacking the (3S) hydroxyl group (e.g. 2, 6 and 7) can be rationalized in terms of a large k_4. It is clear that shortening the N-terminal portion of the peptide chain by one valine residue caused k_4 to increase 300-fold. A further shortening of the chain would be expected to continue this increase which would render $k_4 > k_3$. Since $k_{obs} = (k_3 + k_4)$ and k_4 approaches 0.1 sec^{-1}, the lag transient would become too fast to be observed without stopped-flow kinetic methods. Additional analogs with intermediate affinities for the tightened complex will be needed to test this interpretation; the synthesis of these analogs is in progress.

In some respects the kinetics of interaction of pepstatin analogs with pepsin parallel the interaction of pepsin substrates with pepsin [32]. The appearance of the lag transient with elongation of the N-terminal and C-terminal portions of pepstatin analogs appears to parallel the increase in k_{cat} for substrates modified in a similar manner. However, in contrast to the large increases in k_{cat} (> 10-fold) observed when substrate side-chains are changed from isobutyl groups to benzyl groups [31], replacement of the isobutyl side-chain in the statine residue in compound 5 with the benzyl side-chain derivative, AHPPA (8), did not alter substantially either net K_i or the first-order constant (k_{obs}) for the lag-transient (Table 2). Thus, the effect of structure on k_{obs} for the inhibitors is different from the effect of structure on k_{cat} for substrates.

One objective of this study has been to determine if the tight binding of pepstatin to acid proteases is related to the catalytic mechanism of carboxyl proteases, as suggested by others [11–12]. The structure of the statine portion of pepstatin is compared in Fig. 7 with a postulated tetrahedral intermediate for hydrolysis of an amide bond by pepsin. To mimic the transition state an inhibitor must bind to the active site of the enzyme. Enzyme alkylation experiments [5] and X-ray data [8] have established that pepstatin does bind to acid protease active sites. Because pepstatin and the heptapeptide substrate are too large to be bound simultaneously to the active-site of pepsin, pepstatin should give competitive inhibition patterns, and not the non-competitive patterns observed [4].

The non-competitive patterns probably are seen because of mutual depletion of enzyme and inhibitor at the enzyme concentrations used. Morrison [22] has shown that, at steady state, plots of V^{-1} vs S^{-1} are non-linear, concave downward hyperbolas for tight-binding inhibitors whether inhibition is competitive or non-competitive, and he noted that linear extrapolation of data obtained at low substrate concentrations can produce an apparent non-competitive pattern. Our data support this interpretation. As net dissociation constant for each analog (Table 2) decreases, inhibition patterns change from competitive to non-competitive. Compound 8, the most potent synthetic analog we have prepared, could not

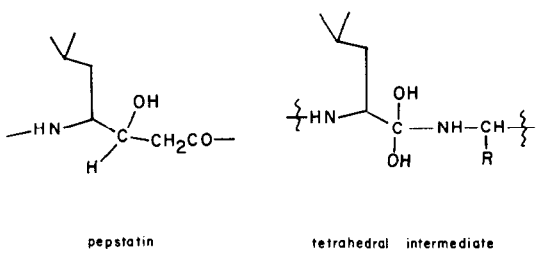


Fig. 7. Comparison of statine portion of pepstatin with structure of postulated transition state for amide bond hydrolysis.

be fitted to a standard program for competitive inhibition. Because of its limited solubility we could not increase the concentration of the heptapeptide substrate to levels sufficient to detect curvature in V^{-1} vs S^{-1} plots. Pepstatin, which has a smaller net dissociation constant, would also deplete enzyme concentration and double-reciprocal plots of velocity versus substrate should be non-linear when the analysis is carried out under steady-state conditions using hemoglobin as the substrate. In view of this analysis plus the X-ray crystallographic data [8], it is probable that both pepstatin and analog 8 are competitive inhibitors of pepsin, as are the other inhibitors in Table 2.

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