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Perspective

Pepstatin-Derived Inhibitors of Aspartic Proteinases. A Close Look at an Apparent Transition-State Analogue Inhibitor[1]

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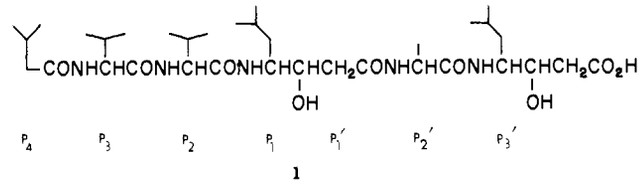
Physical studies of enzyme–inhibitor complexes have provided an increasingly precise picture of factors contributing to enzyme binding and mechanism. New concepts for enzyme catalysis have resulted, and the design of inhibitors of therapeutically important enzymes, such as the antihypertensive drugs captopril[2] and enalapril,[3] has been stimulated. These studies have been significantly influenced by the transition-state analogue inhibitor hypothesis,[4,6] one aspect of which states that stable structures resembling the transition state for an enzyme reaction will be bound more tightly than the substrate for the enzyme-catalyzed reaction. Other tight-binding inhibitors are often assumed, because of their low dissociation constants, to resemble a reaction pathway intermediate, such as the transition state, when X-ray crystal data are used to unravel enzyme catalytic mechanisms. If not examined carefully, these intertwined assumptions can become circular arguments, leading to misconceptions about enzyme catalytic mechanisms and to faulty design of inhibitors of new enzymes, many of which offer considerable potential for future drug discovery.[7] One way to approach this problem is to study naturally occurring tight-binding inhibitors of enzymes, many of which possess structural features not easily reconciled with our mechanistic preconceptions.

Pepstatin, a putative transition-state analogue inhibitor[8,9] isolated by Umezawa et al.,[10] provides a good example of the information that can be gained from detailed study of a naturally occurring inhibitor by a variety of physical methods. Pepstatin (Iva-Val-Val-Sta-Ala-Sta, 1),



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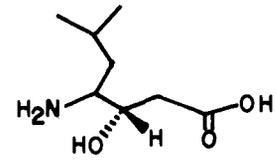
which contains the novel amino acid statine [(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid, **2**, Sta], inhibits most aspartic proteinases with dissociation constants in the range of 0.1–1 nM except for renin.[11] Synthetic manipulations of pepstatin's structure have led to the discovery of novel, potent renin and other aspartic proteinase inhibitors. In addition, molecular modeling based on X-ray crystal data and kinetic studies have led to new examples of biological isosterism and suggested alternate explanations for postulated transition-state analogue mechanisms. ^{13}C NMR studies of ketone analogues of substrates and inhibitors derived from pepstatin have been used to clarify the catalytic mechanism of aspartic proteinases. This



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(1) Abbreviations used follow the IUPAC–IUB commission on Biochemical Nomenclature recommendation. Additional abbreviations are as follows: Sta, 3-hydroxy-4-amino-6-methylheptanoic acid; AHPPA, 3-hydroxy-4-amino-5-phenylpentanoic acid; Sta^P, phosphastatine (ref 21); AHPBA, 2-hydroxy-3-amino-4-phenylbutanoic acid; DAHOA, (3*S*,4*S*)-4,8-diamino-3-hydroxyoctanoic acid; Me^{3}Sta, 3-hydroxy-4-amino-3,6-dimethylheptanoic acid; Sto, 3-oxo-4-amino-6-methylheptanoic acid; Leu$\overset{K}{-}$Ala, the ketomethylene analogue; Leu$\overset{OH}{-}$Ala, the hydroxyethylene analogue.

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Table I. Inhibition of Aspartic Proteases by Pepstatin Analogues

No.	Compound[a]	K_i, nM: Porcine Pepsin	Penicillopepsin
1	Iva-Val-Val—Sta—Ala-Sta-OH	.056	.15
3	Iva-Val-Val—dSta—Ala-dSta-OH[b]	210	
4	Iva-Val-Val-(3R,4S)—Sta—Ala-Iaa	2000	
5	Iva-Val-Val—Sta—OEt	10	47
7	Iva-Val—Sta[P]—Ala-Iaa	<0.1	
8	Iva-Val—Sta—Ala-Iaa	3	7600
9	Iva-Val—Sto—Ala-Iaa[c]	56	
10	Iva-Val—Leu[K]Ala-Ala-Iba[d]	110	
11	Iva-Val-Val—Sto—Ala-Iaa[c]	10	85
13a	Iva-Val-Val-Me[3]Sta—Ala-Iaa (3S)	1200	>5000
b	(3R)	1.5	80
15 a	Iva-Val-Leu[OH]Ala—Iaa (4S)[e]	27	
16 a	Iva-Val-Leu[OH]Ala—Ala-Iba[e,f] (4S)	3	
b	(4R)	700	
17	Iva-Val-Leu[OH]Ala—Gly-Iba[e,f]	53	
18	Iva-Val—Sta—Gly-Iaa	56	
19	Iva-Val-AHPPA—Ala-Iaa	0.9	
22	Iva-Val-Val-DAHOA—OEt[g]	>1000	0.4
23	Iva-Val-Val-DAHOA(Z)—Phe-OMe		500
41	Iva-Val-Leu[OH]Gly—Ala-Iaa	50	
42	Iva-Val-Leu[OH]Phe—Ala-Iaa	2.5	

[a] All statine analogues have the 3*S*,4*S* configuration. [b] dSta, 4-amino-6-methylheptanoic acid. [c] Sto, 4-amino-3-oxo-6-methylheptanoic acid. [d] K = $-COCH_2-$. [e] OH = $-CHOHCH_2-$. [f] Iba, isobutylamide. [g] DAHOA, (3*S*,4*S*)-4,8-diamino-3-hydroxyoctanoic acid.

Perspective summarizes these discoveries and examines how the emerging data may suggest revisions in the mechanism of other putative transition-state analogue inhibitors.

Is Pepstatin a Transition-State Analogue Inhibitor?

The fact that pepstatin efficiently inhibits most aspartic proteinases and that the 3(*S*)-hydroxyl group appeared structurally related to a hydroxyl group in the tetrahedral intermediate for amide hydrolysis led to early proposals that pepstatin acts as a transition-state analogue inhibitor (cf. Figure 1B vs. 1A).[8,9] This proposal was widely accepted in spite of the fact that a hydroxyl group is not a good replacement for an oxyanion which must develop in the transition state for hydrolysis nor would the hydroxyl protonate as readily as the carboxyl oxygen in the tetrahedral intermediate. In addition, steric hindrance between inhibitor C-3 proton and substrate water might be expected to occur (Figure 1B). Thus an alternative role appeared possible for the 3(*S*)-hydroxyl group.

Existing data indicate that the 3(*S*)-hydroxyl group (or an equivalent species) is needed for maximal inhibition. Thus, pepstatin analogues lacking a *pro-S* C-3 hydroxyl group, e.g., dideoxypepstatin (**3**) and the 3*R*,4*S* diastereomer **4** (Table I) are much weaker inhibitors of aspartic proteases than inhibitors containing the *pro-S* hydroxyl group found in natural statine.[12,13] The importance of this hydroxyl group is reinforced by the X-ray crystal structure of the complex between pepstatin and *Rhizopus chinensis* aspartic protease which places the C-3 hydroxyl group within hydrogen-bonding distance of the carboxyl groups of the catalytically essential Asp-32 and Asp-220 residues of the enzyme.[14] A virtually identical structure is found for the complex between Iva-Val-Val-Sta-OEt and penicillopepsin, which includes hydrogen bonds from the inhibitor hydroxyl group to the carboxyl groups of Asp-33 and Asp-213.[15]

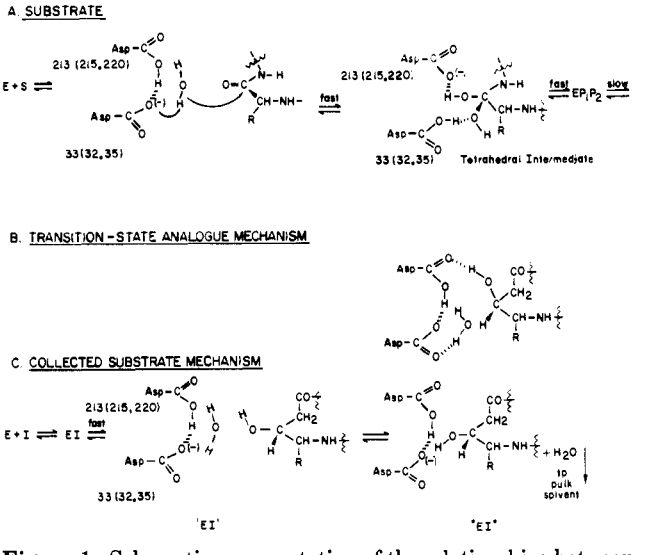

Figure 1. Schematic representation of the relationships between proposed catalytic and inhibitory mechanisms. (A) General acid–general base catalyzed mechanism for substrate hydrolysis by an aspartyl protease. The water molecule indicated is extensively hydrogen bonded to both aspartic acid residues plus other sites in the active site (see ref 17 for details). Hydrogen bonds to water are omitted here. (B) Transition-state analogue mechanism proposed in ref 10 and 11. (C) Postulated collected-substrate mechanism for inhibition of aspartic proteinases by statine-derived inhibitors. The *pro-S* hydroxyl group of statine displaces the enzyme-immobilized water molecule shown in Figure 1A. Variable aspartyl sequence numbers refer to penicillopepsin (pepsin, *Rhizopus* pepsin), respectively.

The function of the 3(*S*)-hydroxyl group is suggested by comparing the location of the 3(*S*)-hydroxyl group in the penicillopepsin–inhibitor **5** complex with the location of an enzyme-bound water, thought to be substrate water, in the native enzyme. High-resolution refinements of these

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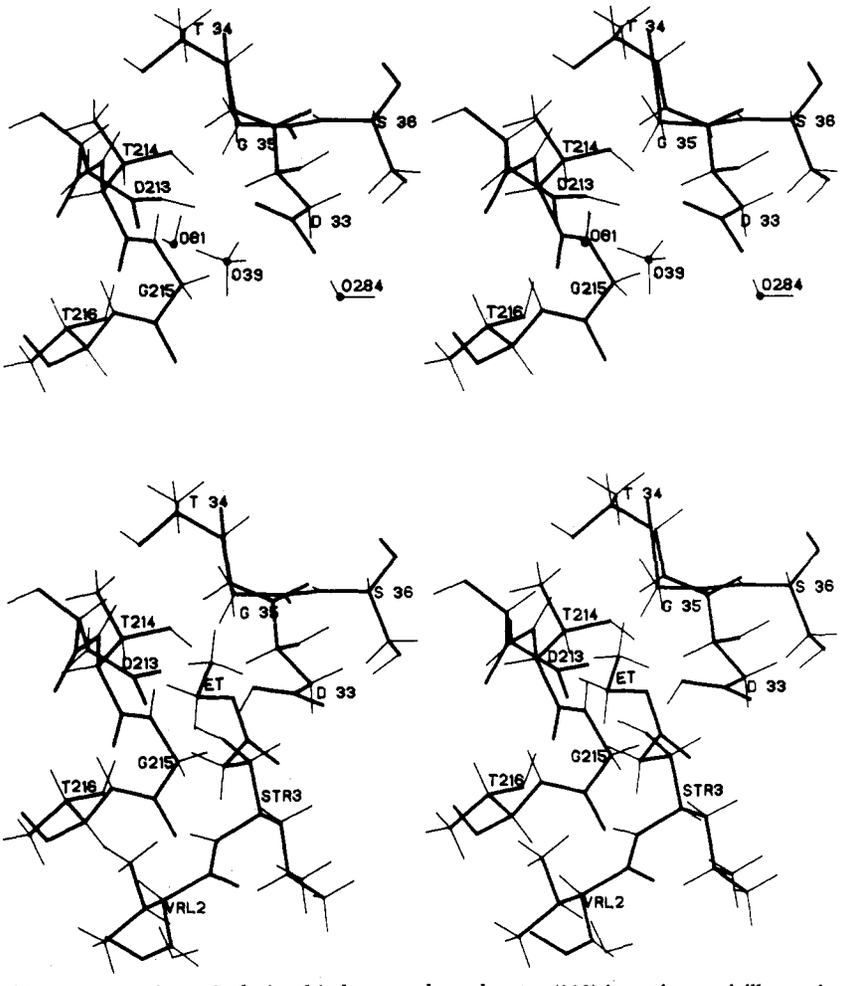

Figure 2. Computer graphic representations of relationship between bound water (039) in native penicillopepsin (A) and the 3(*S*)-hydroxyl group in Iva-Val-Val-Sta-OEt complexed to penicillopepsin (B) on the basis of 1.8 Å resolution crystal data of James and Sielecki. Reprinted with permission from ref 17.

data[16] have shown that the position of the hydroxyl group in tripeptide **5** within the complex is nearly identical with the site occupied by a water molecule oxygen that is hydrogen bonded to Asp-33 and Asp-213 in native penicillopepsin (Figure 2);[17] thus inhibitor binding must be accompanied by displacement or extrusion of an enzyme-bound water molecule, a process illustrated schematically in Figure 1C.

The significance of the extruded water molecule becomes apparent when one considers the extent to which the positive entropy change associated with water displacement will contribute to the strength of inhibitor binding. Jencks[18] has estimated that the return of a "bound" water molecule to bulk solvent increases entropy from 10–16 eu to produce 3–5 kcal of energy favorable to inhibitor binding, which is essentially the difference between pepstatin and the dideoxypepstatin analogue. It should be emphasized that the hydrogen bonds formed between the statine hydroxyl group and the enzyme must be considered only as replacements for the hydrogen bonds between the bound water molecule and the native enzyme, so that the net *enthalpic* change for the water displacement process would be comparatively small.

Thus, pepstatin is, in part, a collected-substrate inhibitor (that is, a stable collection of both substrates, water, and peptide) because the statine *pro-S* hydroxyl group replaces the enzyme-bound water molecule. The dissociation constant for dideoxypepstatin, **3** ($K_i = 10^{-7}$ M), is about 10–100-fold smaller than K_s for comparable substrates, and addition of a *pro-S* hydroxyl group to C-3 contributes an additional 1000–4000-fold to inhibitor binding (Table I, cf. 1 vs. **3**). Much of the tighter binding of **3**, compared with substrates, is attributable to the tetrahedral geometry of C-3, as predicted by transition-state analogue theory; and, to the extent that steric interactions between a proton on C-3 of the central deoxy Sta residue and the bound water might interfere with optimal binding of **3**, the contribution of tetrahedral geometry to the binding of inhibitors (e.g., pepstatin) which displace bound water (and thus do not encounter steric interference) could be greater. Consequently, it is difficult to assign precisely the degree to which entropic considerations are responsible for the considerably tighter binding of **1** compared with that of **3**; but as discussed above, this contribution is likely to be substantial.

Although X-ray data are not yet available for complexes of aspartic proteinases with inhibitors containing the hydroxyethylene isostere **6**,[19,20] it is probable that the above

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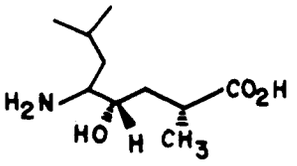
analysis applies to this type of inhibitor. Thus, the 4-



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(*S*)-hydroxyl groups of the hydroxyethylene analogues **15**–**17** are likely to bind to the enzyme so as to displace the bound water molecule. The phosphinic statine analogue **7** (Sta^P) (Figure 4) reported by Bartlett and Kezer binds to pepsin at least 20 times more tightly than the corresponding statine derivatives.[21] The tighter binding could be due to an additional hydrogen bond to the enzyme aspartic carboxyl group over and above those formed by the water extrusion mechanism.

Extrusion of solvent water also provides a possible explanation for the slow binding of pepstatin and several related analogues to pepsin and other aspartic proteinases.[22] Slow binding at saturating inhibitor concentrations (Figure 3) has been found for a number of enzyme inhibitors but is not necessarily related to enzyme mechanism. For example, the binding of methotrexate to dihydrofolate reductase is low,[23] but the inhibitor is not bound in the active site in the same way as is substrate.[24] All that slow binding at saturating inhibitor concentrations establishes is that the rate of formation of product from substrate is much faster than the rate constant for the slow binding process.

For the pepstatin–pepsin system, slow binding has been analyzed in terms of a postbinding transition from one enzyme–inhibitor complex to another.[22] The difference between initial binding and tightened binding is substantial (>500–1000 for some inhibitors). However, the apparent rate constant for the slow first-order transition (0.022 s^{-1}) for this system is too slow to be consistent with an enzyme-catalyzed formation of a tighter complex. Fruton has measured with stopped-flow kinetic methods the rates of amide bond cleavage of peptide substrates and determined that the rate constant for the cleavage step exceeds 430 s^{-1}.[25] A second, much faster, first-order step for binding of *N*-acetylpepstatin to pepsin (greater than 1000 s^{-1} at pH 4) has been detected by stopped-flow kinetics methods[26] so that the interaction between pepstatin and pepsin proceeds via the minimal mechanism presented in eq 1. Formation of the "intermediate" complex in eq 1 is as fast or faster than the fastest measured steps for cleavage of an amide bond ($sp^2 \rightarrow sp^3$) as would be expected for an inhibitor that can bind to an enzyme without requiring $sp^2 \rightarrow sp^3$ distortion.[27] (See also Figure 7B.)

$$E + I \rightleftharpoons EI \rightleftharpoons {}^*EI^* \rightleftharpoons \text{"EI"} \qquad (1)$$

collision intermediate tightened

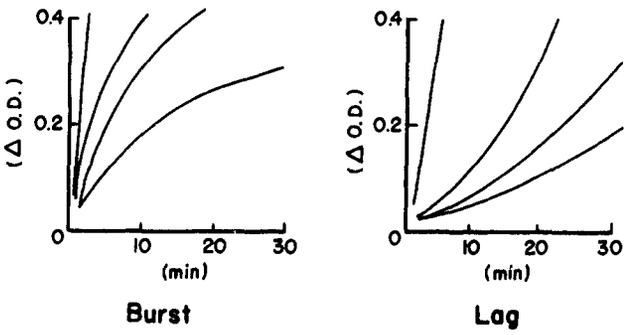
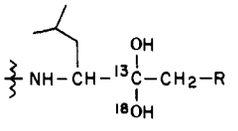


Figure 3. Change in reaction velocity for product formation in the presence of slow-binding inhibitor. (Left) Reaction initiated by addition of enzyme to mixture of inhibitor and substrate. (Right) Reaction initiated by addition of substrate to solution of enzyme and inhibitor. Time units greater than seconds are arbitrary.

Other fairly strong pepsin inhibitors bind to the enzyme much faster than pepstatin does. Ketone analogues of pepstatin (**9**) and of a possible substrate (**10**) bind quickly even though the carbonyl group is converted from sp^2 to sp^3.[28,29] Ketones **9** and **10** have been shown by ^{13}C NMR to be converted to the tetrahedral *gem*-diol adducts **11** and **12** upon binding to pepsin via an enzyme-catalyzed addition of water (shown by ^{18}O shifts of the ^{13}C NMR resonance for the *gem*-diol).[29] The addition is too fast to be detected kinetically (or in the NMR spectra) without resorting to stopped-flow methods so that the upper limit for the half-life for the addition of water is ≪20 s.



11, R = CO–

12, R = CH(CH$_3$)CO–

Thus both the fast first-order process detected in the binding of pepstatin to pepsin and the addition of water to ketomethylene peptide isosteres in the active site of pepsin proceed much more quickly than the slow binding step found for pepstatin binding to pepsin. The fast step probably involves closure of the "flap" region of the enzyme. X-ray crystal data of a complex between the aspartic proteinase, penicillopepsin, and Iva-Val-Val-Sta-OEt (**5**) show that, upon inhibitor binding, the "mobile" flap region, comprising protein amino acids 71–83, moves more than 2 Å relative to native enzyme to close the active site about the inhibitor.[15] Again kinetic data for substrates and difference UV and CD kinetic data for inhibitors[30] show that this process occurs faster (k_{app} > 10–15 s^{-1}) than the slow process for pepstatin binding to pepsin.

What Causes the Slow Binding?

With the exception of the closure of the "flap" region of aspartic proteinases already described, the protein backbone atoms in enzyme–pepstatin complexes remain in the positions found in the native, uncomplexed en-

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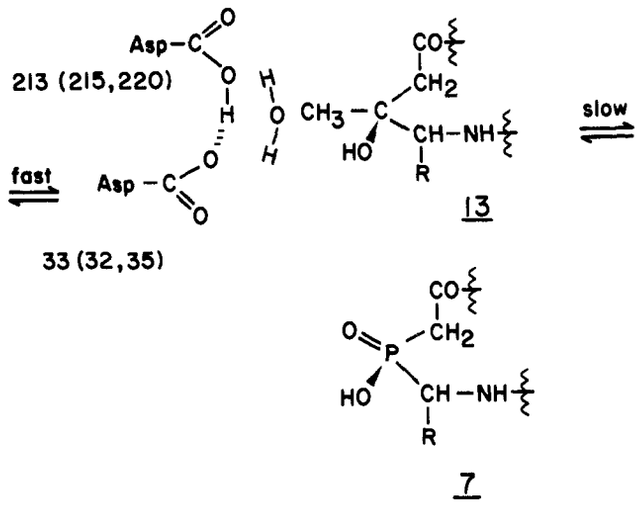

Figure 4. Relationships of binding of (3*R*)-Me^{3}Sta (A) and phosphastatine (B) containing inhibitors to statine-derived inhibitors (cf. Figure 1C).

zyme.[15] Small changes in conformation are detectable, but these involve simple rotations about single bonds in protein amino acid side chains, processes which should be very fast. Thus, the X-ray data are consistent with the bulk of the protein remaining conformationally stable, yet the kinetic data indicate some slow process occurs when the inhibitor binds which stabilizes binding several thousand fold. One possible explanation for slow binding to aspartic proteinases is that the displacement of water by the statine 3(*S*)-hydroxyl group is a slow process that occurs within the pepsin–pepstatin intermediate complex (Figure 1C). As envisioned in Figure 1C, the intermediate complex between pepsin and pepstatin would be stabilized by the normal peptide side chain and amide bond interactions plus a weak hydrogen bond to the statine hydroxyl group which occupies the amide carbonyl site. As described previously, the complex is metastable and possibly destabilized by the steric interactions between the C-3 proton of statine and the enzyme-bound water molecule. As water is replaced by the statine 3(*S*)-hydroxyl group, the "tightened complex" forms and is stabilized further by the favorable entropic process, return of water to bulk solvent. It is also possible, and perhaps even likely, that subtle repositioning of the inhibitor within the active site leads to tighter side chain and amide bond interactions although the entropic contribution from displacement of enzyme-bound water appears sufficient to account for the tighter binding observed (cf. **1** vs. **3**), and difference UV and CD spectra do not reveal such repositioning.

If water extrusion is the slow-binding process, then additional substituents on C-3 of statine which retard or even prevent water loss could slow binding even further. Statine derivatives in which the proton on C-3 of 3*R*-Sta analogues has been replaced with a methyl group [(3*R*)-Me^{3}Sta][31] (e.g., **13**:) are slower binding inhibitors than the corresponding statine analogue.[29] Thus analogue **13** has a half-life for binding 10 times longer than does pepstatin (>300 s vs. 31 s), but the opposite hydroxyl configuration of this surprisingly potent inhibitor (cf. **13a** and **4**) requires a new, as yet undefined, interaction between enzyme and inhibitor. Even more interesting with respect to possible water extrusion is the report that peptide **7**, which contains a phosphinic acid analogue of statine in place of statine,

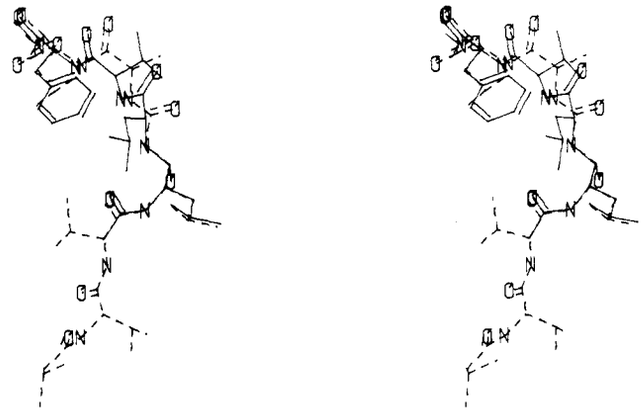

Figure 5. Ac-Leu*Leu-Val-Phe-NH_2 (solid), where an asterisk denotes a tetrahedral carbonyl (–CHOH–NH–), matched onto isovaleryl-Val-Val-Sta-Ala-Sta, **1**, (dashed, pepstatin) as in the complex with *R. chinensis* aspartic protease. Reprinted from ref 33. Copyright Pierce Chemical Co., 1983.

binds exceptionally slowly to pepsin ($T_{1/2}$ = 155 min).[21] The second oxygen on phosphorus may slow the replacement of water in the active site as depicted in Figure 4, although the formation of an anhydride between enzyme and inhibitor has not been excluded.

Statine Is an Analogue of a Dipeptide. The structure of statine has provided us with an unanticipated isosteric replacement for a dipeptide unit. The structures of statine and the tetrahedral intermediate for amide hydrolysis, compared in Figure 1, illustrate that statine can be isosteric with the tetrahedral intermediate only from C-3 through C-7. Because of atoms C-1 and C-2, statine is either two atoms too long to be isosteric with a normal α-amino acid or one atom too short to be isosteric with a dipeptide. Powers suggested on the basis of an extensive comparison of pepsin substrate sequences to pepstatin that statine might better resemble a dipeptide.[32] Using the X-ray data for pepstatin bound to *R. chinensis* aspartyl protease, Boger proposed a precise model in which statine is an analogue of an enzyme-bound dipeptide in its tetrahedral intermediate form.[33] A comparison between enzyme-bound pepstatin and the tetrahedral form of -Leu*-Leu-Val-Phe- generated by molecular modeling is shown in Figure 5. Pepstatin residues are indicated by dashed lines and the tetrahedral intermediate of -Leu*-Leu-Val-Phe- is indicated by solid lines. It is clear that in this conformation the isobutyl and hydroxyl groups of statine and the first Leu residue in substrate can bind to the same enzyme site (S1) while, at the same time, the isobutyl group of the second statine and the benzyl group of the substrate Phe can bind to the S3′ enzyme site, a steric "match" possible only if statine serves as a dipeptide replacement. Thus, statine is a replacement for a dipeptide unit but only for a restricted conformation of dipeptidyl unit. It is possible that other enzyme inhibitors with unusual amino acid constituents either those found in nature, e.g., AHPBA in bestatin,[34,35] or designed ones will replace one or more amino acids to mimic restricted conformations in

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Table II. Substrate-Derived Renin Inhibitors

Compound No.	Structure	IC_{50} (M), Human Plasma Renin
	6 7 8 9 10 11 12 13	
24	H His Pro Phe His Leu Leu Val Tyr OH	$(5.5 \times 10^{-5})^a$
25	H Leu-Val-Ile-His-OH	
26	Pro- Phe-Phe	7×10^{-6}
27	D-His Leu^{R}Leu	1×10^{-6}
28	Pro- Leu^{R}Leu	1×10^{-8}
29	Iva-His —Sta—Ile-Phe-NH_2	1.9×10^{-9}
30	Iva-His —Sta—Ile-Phe-OMe	0.6×10^{-9}
31	Boc-His Leu^{OH}Val-Ile-His	0.7×10^{-9}
32	Boc-Phe-His——Sta—Ala-Sta-OMe	2.7×10^{-8}
1	Iva-Val-Val——Sta—Ala-Sta	22×10^{-6}

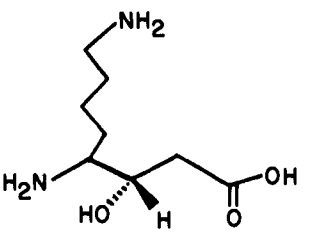
[a] K_m value for substrate. R = $-CH_2NH-$, OH = $-CH(OH)CH_2-$.

reaction-pathway intermediates for enzyme-catalyzed reactions.

The concept that statine is a dipeptide replacement was utilized to generate the series of potent renin inhibitors **29**, **30** shown in Table II in which statine replaces the Leu-Val unit in peptides derived from various renin angiotensinogen sequences.[36] Szelke et al. recently described the synthesis of a new series of renin inhibitors, e.g., **31**, derived from the hydroxyethylene isostere for a dipeptide.[19] As shown in Table II, replacement of the Leu-Leu or Leu-Val sequences in peptides derived from renin angiotensinogen sequences by the hydroxyethylene isostere also produces very potent renin inhibitors, and the close agreement between the hydroxyethylene **31** and statine-based inhibitors **29**, **30** is consistent with statine serving as a dipeptide analogue. A similar pattern is evident when the hydroxyethylene analogues **15** and **16** (Table I) are compared with statine-containing inhibitor **8** for inhibition of porcine pepsin.[29,37] Replacement of statine with a dipeptide isostere (e.g., **16**) leads to essentially equipotent inhibitors. Other parallels in enzyme inhibitory properties support the conclusion that isostere-containing and Sta-containing peptides are acting by similar mechanisms. Hydroxyethylene analogue **16** exhibits slow-binding properties similar to those observed for the more potent Sta-containing analogues, including **18**. Moreover, replacement of Ala with Gly in **16** (to give **17**) and in **8** (to give **18**) produces similar losses of binding potency and loss of the slow-binding properties of both inhibitors. Finally, a change in stereochemistry at the hydroxyl-bearing carbon produces dramatic losses of binding potency for both Sta and hydroxyethylene analogues (cf. **1** vs. **4** and **16a** vs. **16b**).

Specificity. Statine Side Chain. Two new statine analogues, 4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA)[1,39] and 4,5-diamino-3-hydroxyoctanoic acid (DAHOA),[1,37] are variations of statine modified so that the Phe side chain more closely approximates the side chain of a good substrate for the target enzyme. Replacement of statine with AHPPA produces the inhibitor, Iva-Val-AHPPA-Ala-Iaa, **19**, which is a slightly stronger inhibitor of pepsin than the parent compound **8** (Table I). The difference between the two inhibitors is not as great as would be expected from the 10-fold differences in k_{cat} or k_{cat}/K_m for substrates in which Phe replaces a Leu residue in the P1 position.[39]

A much more remarkable example of side-chain specificity is found with inhibitors derived from the lysine side chain analogue of statine, DAHOA, **20**,[1] which was designed to mimic fungal protease specificity for lysine. The best penicillopepsin substrate, N-Ac-Ala-Ala-Lys-Phe-(NO_2)-Ala-Ala-NH_2, **21**, is cleaved by penicillopepsin at the Lys–Phe(NO_2) bond.[40] A specific interaction between the positively charged ϵ-amino group of lysine and some negatively charged group on the enzyme is indicated because penicillopepsin cleaves Lys–Phe bonds faster than amide bonds between hydrophobic residues and because the kinetic parameters for cleavage of this substrate, K_m and k_{cat}, depend on pH.



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Replacement of (3*S*,4*S*)-statine in **5** with (3*S*,4*S*)-DAHOA leads to tripeptide **22** in which K_i has been decreased by a factor of about 100 (Table I).[37] The decrease in K_i for **22** relative to **5** corresponds to an increase binding interaction of 2–3 kcal, a value close to the interaction expected for a solvated ion pair.[41] An ionic interaction also is suggested by the fact that the Cbz-protected DAHOA tripeptide **23**, which cannot form an ion pair at the terminal nitrogen, is a much poorer inhibitor of penicillopepsin. The K_i of DAHOA tripeptide **22** is greater than 1000 nM on porcine pepsin even though the corresponding statine analogue **5** has a K_i of 10/ nM on porcine pepsin. This very weak binding of **22** to porcine pepsin relative to **5** suggests the positively charged ion may actually be repelled from the active site of pepsin.

Renin Inhibitors. Inhibition of the renin–angiotensin system via converting enzyme inhibitors is now a well-established approach for treatment of hypertension. Inhibition of the first enzyme in this sequence, renin, has yet to yield a therapeutically useful drug, although several very potent inhibitors of renin have been developed. Burton and co-workers[42,43] developed the first potent renin inhibitors, e.g., compound **26**, by replacing the leucine residues in the minimal porcine renin substrate, His-Pro-Phe-His-Leu-Leu-Val-Tyr, **24**, with phenylalanine (Table II). Szelke et al. obtained potent competitive inhibitors of canine renin by replacing the Leu-10–Leu-11 peptide bond in pig angiotensinogen(6–13) octapeptide **24** with the

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methylene amino isostere ($-CH_2NH-$), formed by reduction of the amide group.[44] The improved binding of compounds **27** and **28** relative to substrate **24** is thought to result from the tetrahedral geometry of the methylene group. Boger et al. devised potent renin inhibitors by replacing the Leu-10-Val-11 and Leu-10-Leu-11 dipeptide units in human and pig angiotensinogens **25**, **24** with statine and obtained both extremely potent and selective renin inhibitors **29** and **30** that have demonstrated hypotensive activity in animal model systems.[33,36] Szelke et al. very recently reported the synthesis of renin inhibitors designed by replacing the Leu-10–Val-11 bond in human angiotensinogen octapeptide **25** with the hydroxyethylene isostere of Leu-Val, i.e., Leu$\underline{\overset{OH}{}}$Val, as in compound **31**.[19] The potency of this class of inhibitor is virtually identical with that of the corresponding statine-derived renin inhibitors. Evin et al. also obtained good renin inhibitors by replacing the C-terminal tetrapeptide in the porcine angiotensin octapeptide sequence **24** with Sta-Ala-Sta to form **32**.[45] All of these compounds are much better inhibitors of human and animal renins than pepstatin and most are enzyme selective.

Thus very potent inhibitors of renin have been designed by means of two independent approaches. The approach of Szelke began with an assumed enzymatic mechanism and a known substrate sequence to generate analogues of a tetrahedral intermediate. The approach of Boger et al. began with a highly promising natural product which was modified by incorporating the renin substrate sequences to produce the desired selectivity and potency. The similarity between the structures generated by either strategy is remarkable.

Determination of the Catalytic Mechanism of Aspartic Proteases by ^{13}C NMR. The presence of two catalytically active aspartic β-carboxyl groups in the active sites of aspartic proteases was deduced from pH dependence and alkylation experiments.[39] These residues were subsequently assigned as Asp-32 and Asp-215 in the sequence of porcine pepsin.[46] The results of transpeptidation and ^{18}O-exchange studies led to numerous proposals for a catalytic mechanism for aspartic proteases which usually featured direct nucleophilic attack by an active-site carboxylate on a peptide substrate carbonyl with the intermediacy of either an "amino enzyme" (usually formulated as a carboxamide)[47-52] or of an acyl enzyme (formulated as a mixed carboxylic anhydride) (Figure 6).[53,54] However, amino-transfer transpeptidation is observable only for specific substrates, suggesting that proposed mechanisms involving covalent amino enzyme intermediates are inconsistent with a general mechanism for peptide hydrolysis.[55] Later studies of ^{18}O exchange during transpeptidation reactions convincingly discounted a carboxamide derived from an enzyme carboxyl group and an amine product as a credible intermediate.[56] The possibility that a general acid–base mechanism could be operable, that is, one in which water attacks the carbonyl of the scissile peptide bond with the active-site carboxylates mediating the appropriate proton transfers, resurfaced after it was recognized[39] that the resynthesis of peptide bonds from final hydrolysis products was an energetically reasonable process under the appropriate conditions.[56] Thus, most of the results of transpeptidation experiments could be rationalized by invoking the microscopic reversibility of a general-acid–general-base catalyzed forward reaction, which includes a structure-dependent ordered release of hydrolysis products.[39,57] Further evidence in support of the latter mechanism as opposed to a covalent mechanism involving an acyl enzyme intermediate includes the failure to trap an activated enzyme carboxyl group with nucleophiles[58] and X-ray data which argue against a covalent mechanism on spatial grounds.[14,15]

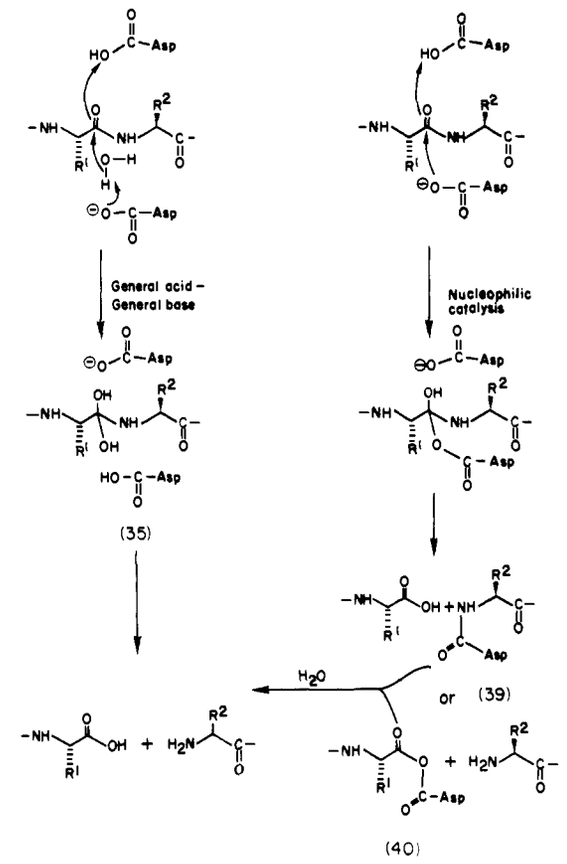

Figure 6. Schematic representation of the relationships between proposed catalytic mechanisms for action of aspartic acid proteinases in converting substrate to products. Nucleophilic catalysis (right) leads to covalent intermediates **39** or **40**. Direct addition of water (left) leads to tetrahedral intermediate **35**.

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Results from ^{18}O-exchange studies[55,59,60] are most consistent with a general acid–base mechanism; however, all possibilities consistent with covalent catalysis are not rigorously excluded, and more conclusive evidence is required to support the general acid–base mechanism.

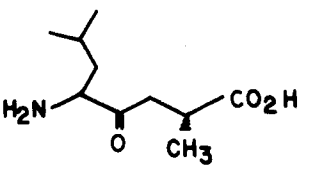
We have approached this problem by studying the interactions between pepsin and ketones with structures based on that of pepstatin. Our strategy was to design ketones which would serve as pseudosubstrates, that is, be subject to the catalytic action of the enzyme but only to the point of formation of a tetrahedral intermediate which, because of the increased stability of a C–C vs. a C–N bond, would not break down to products. Such a stable tetrahedral intermediate would then, in principle, be amenable to study by the appropriate physical methods. ^{13}C NMR appeared to be an ideal method since changes in hybridization of the susceptible carbonyl carbon could be followed readily. The results we obtained provided the first direct observation of a tetrahedral adduct in the active site of a protease. In addition, the transformation from trigonal to tetrahedral geometry is shown to be an enzyme-catalyzed process, and the added nucleophile is identified as water. Our data thus provide strong support for the general acid–general base catalytic mechanism. Some of the methods described here have been applied to the study of mechanisms of other proteases.[61,62]

The statone (4-amino-3-oxo-6-methylheptanoic acid) containing peptide Iva-Val-Sto-Ala-Iaa, **9**, is a good inhibitor of pepsin (K_i = 56 nM).[63] The C-3 of ^{13}C-3-Sto analogue **9** is found to be >95% trigonal in aqueous buffer by ^{13}C NMR spectroscopy. After the inhibitor binds to pepsin, the ^{13}C chemical shift (99 ppm) indicates that the geometry at C-3 is tetrahedral and that the added atom is oxygen.[28] The line widths (5–10 Hz in H_2O) are consistent with a tetrasubstituted carbon binding to an enzyme of 35 000 molecular weight.

The transformation from trigonal to tetrahedral geometry is an enzyme-catalyzed process, as opposed to one in which the ketone is hydrated in solution followed by binding to the enzyme. Thus, when statone analogue **9** was incubated with pepsin in 99% $H_2{}^{18}O$ for 3 h, recovered ketone contained <10% ^{18}O at C-3 as determined by mass spectral analysis.[15] In a mechanism involving ketone hydration prior to binding, ^{18}O incorporation in recovered inhibitor should be at least 50%, a value corresponding to that expected for a single cycle of nonstereospecific addition/nonstereospecific elimination of water to the ketone carbonyl. The actual results then indicate that addition–elimination is a highly stereospecific process and thus enzyme catalyzed.

The origin of the oxygen nucleophile that adds to the Sto C-3 carboxyl group was established by measuring the ^{13}C chemical shift for the C-3 carbon of statone peptide **9** in $^2H_2{}^{18}O$.[29] A ^{13}C NMR experiment carried out in $^2H_2{}^{18}O$ gave a 0.05-ppm upfield shift in the resonance for the C-3 carbon relative to the carbon resonance in $^2H_2{}^{16}O$ itself shifted upfield 0.36 ppm relative to H_2O. The additive upfield shift in the carbon resonance produced by both isotopes establishes that the oxygen nucleophile that adds to the C-3 carbonyl group when **9** binds to pepsin must come from water. These labeling results are not consistent with the addition of the Asp-32 carboxyl group to the carbonyl group to form a covalent tetrahedral species as would occur during nucleophilic catalysis because exchange of ^{18}O into the carboxyl groups is slow.[34]

The addition process observed for **9** in the active site of pepsin is analogous to that occurring with peptide substrates. The ^{13}C NMR experiments were repeated with peptide **10**, which contained the ketomethylene dipeptide isostere **34**, 99% ^{13}C enriched at C-4. The same ~100-ppm upfield shift of the carbonyl resonance (from C-4 in compound **10**) was observed. In ^2H_2O, the C-4 carbon resonance was shifted farther upfield by 0.30 ppm relative to a sample run in H_2O and shifted farther upfield 0.04 ppm when the ^{13}C NMR spectrum was obtained in $^2H_2{}^{18}O$ compared to that in $^2H_2{}^{16}O$.[29]



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These data establish unambiguously that pepsin catalyzes the stereospecific addition of water to ketone isosteres related to peptide substrates. Isotope effects on ^{13}C chemical shifts establish the formation of an ^{18}O–^{13}C bond in the *gem*-diol tetrahedral adduct and thus exclude the possibility that the observed tetrahedral adduct might have resulted from the direct attack of an enzyme carboxylate on the ketone carbonyl. Thus, given the reasonable assumption that pepsin-catalyzed hydration of apeptide substrate to the tetrahedral intermediate **35** (Figure 6) occurs in a process parallel to the observed pepsin-catalyzed hydration of pseudosubstrate ketones **9** and **10** to *gem*-diols **11**, **12**, then pepsin catalyzes the hydrolysis of peptides by a general acid–general base mechanism and not by a nucleophilic mechanism. It follows that a slow, structure-dependent, ordered release of products more reasonably accounts for data which earlier had been interpreted as evidence for covalent intermediates.

Can Transition-State Analogue Inhibition Be Proven?

Pauling[4] and later Wolfenden[k] and Lienhard[6] recognized that an analogue that closely approximates the structure and geometry of the transition-state should be bound exceedingly tightly by the enzyme. The traditional reaction pathway profiles for the catalyzed vs. the uncatalyzed reactions are represented in Figure 7A. The corresponding profile (excluding the positive free energy changes required for bond rehybridizations) is given for the enzyme substrate interactions in Figure 7B. Binding of a transition-state analogue inhibitor to the enzyme would follow along the pathway depicted in Figure 7B to end up in a complex related to ES* because the analogue of the transition state would not require energy to rehybridize. Note also that Figure 7B suggests transition-state analogues should bind more rapidly than substrates. The scheme is useful for illustrating why these compounds are expected to bind to the enzyme more tightly than substrate but is oversimplified in that second substrate water is ignored. Even the simplest proteolytic hydrolysis requires several steps (eq 2), each with a corresponding transition-state and low-energy complex and thus the transition-state analogue

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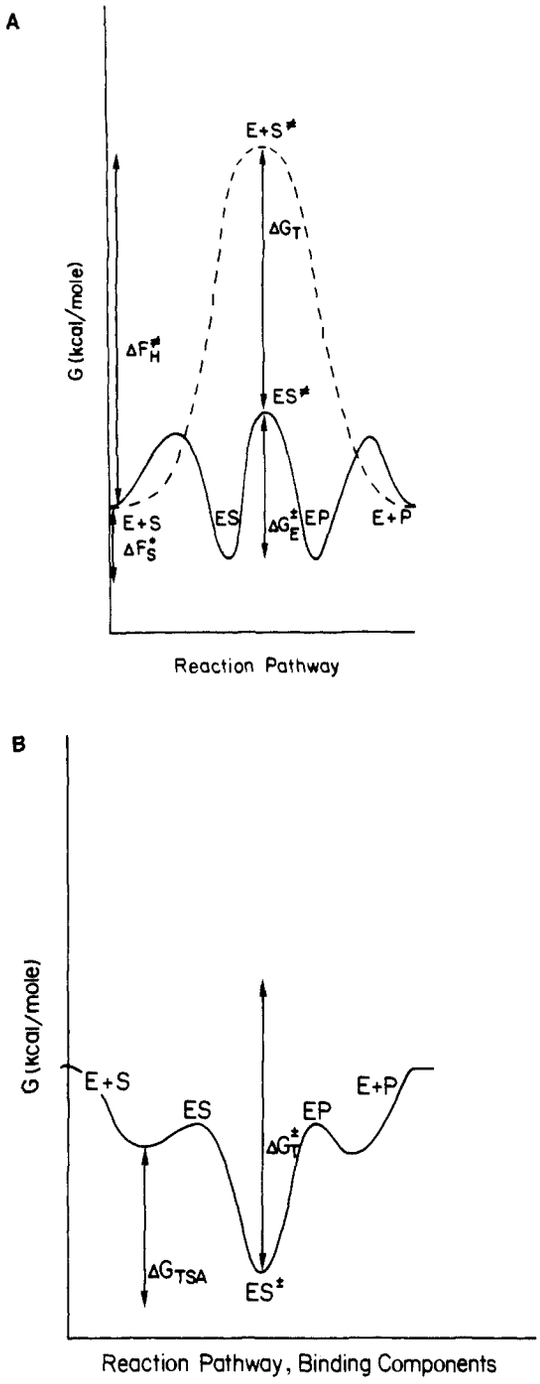

Figure 7. (A) Schematic representation of reaction pathway profile for nonenzymatic (---) and enzyme catalyzed hydrolysis of amide bond (—). (B) Schematic representation of binding profile for reaction pathway illustrated in A. Valley beneath ES‡ represents binding of peptide in the absence of unfavorable enthalpic and entropic processes such as bond rehybridization or reduction of molecular entropy. Low points are predicted by transition-state analogue theory.[6-8] Note also low activation energy for formation of enzyme–inhibitor complex.

could mimic any of at least four complexes, not just the tetrahedral intermediate (X) for the amide cleavage step.

$$E + A + B \rightleftharpoons EAB \rightleftharpoons EX \rightleftharpoons EPQ \rightleftharpoons EQ + P \rightarrow E + Q \quad (2)$$

Few compounds that successfully mimic a transition state have been prepared; most are collected-substrate or product inhibitors. One reason usually given for this is that the chemical instability of the transition state precludes synthesizing exact isosteres so that, at best, only a fraction of the binding energy available to the transition state will be realized by the analogue. Neither superficial resemblance to a transition state nor tight binding per se establish transition-state analogue inhibition. Tight-binding inhibitors, e.g., methotrexate[24] and captopril,[2] have been found to bind to enzymes in mechanistically related complexes that do not resemble transition states for a reaction. Other compounds have been accepted as approximate analogues of the transition state primarily because no alternative rationalization for the tighter binding relative to substrates existed. In general, these inhibitors are assumed to mimic the bond-breaking step in catalysis in spite of the fact that product release is rate limiting in many enzymatic reactions.

Thompson[64] and Bartlett[65] have proposed equations to relate K_i to K_m and k_{cat}/K_m as diagnostics for transition-state analogue inhibitors of elastase and thermolysin. Both methods gave excellent correlations of K_i with k_{cat}/K_m as predicted for transition-state analogue inhibitors.

The inhibition of pepsin by statine and hydroxyethylene inhibitors does not correlate with either K_m or k_{cat}.[66] As noted, replacement of the isobutyl group in the P_1 position (Sta) by the benzyl group (AHPPA; **19**) does not lower K_i as much as predicted by the 10-fold increase in k_{cat} or k_{cat}/K_m for the corresponding substrates.[39] Hydroxyethylene analogues in which the substitutent in the P_1 position is changed from H (**41**) to CH_3 (**16**) to benzyl (**47**) also do not correlate with the effects these substituents produce on k_{cat} and K_m for pepsin substrates (Table I). The dissociation constants of the Leu$\overset{OH}{—}$Ala and Leu$\overset{OH}{—}$Phe isosteres **41** and **42** are nearly identical.[66] Thus increased steric bulk in either the P_1 or $P_{1'}$ positions of statine- or hydroxyethylene-derived inhibitors has not yet lowered K_i to the extent predicted by the effects on V/K for substrates. These data could be consistent with the collected-substrate mechanism or may simply reflect the complexity of even the most simple mechanism for proteolysis (eq 2), which predicts multiple transition states separating substrate from product. Fruton's work has confirmed that multiple intermediates are detectable in proteolysis by aspartic proteinases. Stopped-flow methods established that k_{cat} includes at least two distinct steps, one step incorporating cleavage of the amide bond and one step incorporating release of a product (eq 2).[39] However, because the mechanism in eq 2 can be analyzed only in terms of two rate-limiting *segments*, the first which ends in the release of the first product and the second which releases the second product Q[67,68] (the first segment including both the transition state for amide cleavage and the release of the first product), it is not possible to separate individual steps by *steady-state methods*. All three steps appear in the expression for k_{cat}. Thus, equations that relate K_i with K_m and k_{cat}/K_m obtained from steady-state methods cannot distinguish whether the affected steps are single or multiple transition states for amide bond cleavage or for release of products. The lack of correlation shown by the hydroxyethylene analogues probably suggests that P_1 and $P_{1'}$ substituents accelerate product release by destabilizing enzyme–product interac-

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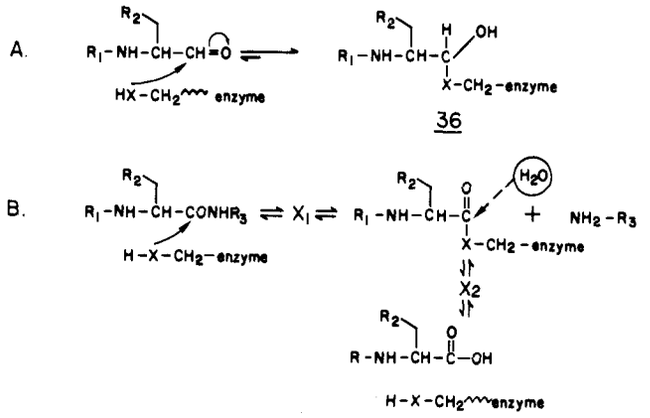


Figure 8. (A) Formation of hemiacetal in active sites of serine (X = O) and cysteine (X = S) proteases from peptide aldehydes. (B) hydrolysis of amide bonds by serine and cysteine proteases showing attack intermediate of water on acyl enzyme. X_1 and X_2 are tetrahedral intermediates. Note possible replacement of hemiacetal hydroxyl at nucleophilic water site.

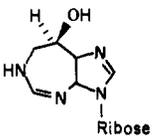
tions. These structural modifications could lead to improved substrates only if K_s also were not comparably increased. Thus, the P_1 and $P_{1'}$ substituents must, in part, affect product binding differently than substrate binding. Differential effects of structure on the stability of different enzyme–inhibitor (or substrate–enzyme) complexes would make these correlations multivariant and uninterpretable. The Thompson[64] and Bartlett and Marlowe[65] examples may succeed because a single step dominates the transformations, although this step need not be the amide cleavage step.

Other Tetrahedral Inhibitor Systems

Entropy-driven water extrusion may stabilize other proteinase–inhibitor complexes and deaminase–inhibitor complexes which are loosely related to the transition state for the chemical reaction. For example, it is well-known that peptide aldehydes related to leupeptin are potent inhibitors of serine and cysteine proteases.[69] These inhibitors are known to add the enzyme nucleophile (serine hydroxyl or cysteine thiol) to the aldehyde in the active site of the enzyme to generate the hemiacetal species **36** (Figure 8)[70,71] which has been observed by ^{13}C NMR.[61,62] Thus, the hemiacetal derived from peptide aldehydes is generally thought to be an analogue of the transition state or of a tetrahedral intermediate in proteolysis by serine and cysteine proteases.

It is possible that the hemiacetal species **36** formed in the enzyme active site displaces a molecule of water to stabilize the enzyme–inhibitor complex by water extrusion. The overall reaction pathway for hydrolysis of substrates by chymotrypsin and other serine proteases proceeds via a covalent intermediate (acyl enzyme) formed by attack of the enzyme nucleophilic groups. However, conversion of the acyl enzyme to product requires *a second transfer step*, this time to water. Addition of water to the ester carbonyl approaches along the vector for a formation of a tetrahedral species.[72] Thus, it is reasonable for the hydroxyl group of the hemiacetal **36** to displace enzyme-bound water and to inhibit, in part, as a collected-substrate analogue of the deacylation step, as opposed to the amide cleavage step. If water extrusion is a factor in the stabilization of complexes between serine and cysteine proteases and peptide aldehydes, then the estimates of the intrinsic binding energy between enzymes and peptide aldehydes due to transition-state geometry have been overestimated.[18]

Coformycin[73] **37** and the tetrahydrouridine[74] **38** are two other examples of apparent transition-state analogue inhibitors which resemble transition states derived by addition of water to a trigonal center. For the reasons cited above, it is possible that much of the stabilization between enzyme and inhibitor is due to the expulsion of enzyme-bound water by the chiral hydroxyl group in each inhibitor. Thus either inhibitor may be a collected-substrate inhibitor as much as an analogue of the transition state. It is interesting to note that both inhibitors are slow-binding inhibitors with rate constants for the lag transient much slower than expected for an enzyme-catalyzed deamination reaction.



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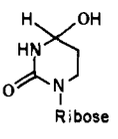

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The X-ray data of the pepstatin analogue–aspartic proteinase complexes have suggested that extrusion of substrate water is a factor that can stabilize enzyme–inhibitor complexes through an entropic rather than an enthalpic process. If pepstatin is a partial collected-substrate inhibitor, the enzyme active-site geometry in aspartic protease–pepstatin complexes may differ from the geometry in the transition state for hydrolysis. Deductions of catalytic mechanisms from these complexes may be complicated further by the fact demonstrated for aspartic proteinases that both rate-limiting segments include product release steps (eq 2). Some additional point of reference is needed in order to interpret the X-ray data unambiguously. One mechanistic revision, suggested by the proposed collected-substrate mechanism, is that the C-3 hydroxyl group of statine occupies the water site and not the substrate carbonyl site as was assumed in transition-state modeling based on coordinates of the *R. chinensis* pepsin–pepstatin complex.[75] It seems certain that continued characterization of aspartic proteinase–inhibitor complexes by physical methods will lead to a clearer understanding of the catalytic mechanism and to more potent and selective inhibitors.

Summary and Projections

To date, three new structural concepts have been identified as a result of the detailed analysis of the pepstatin–aspartic proteinase system: (1) statine replaces a conformationally constrained dipeptidyl unit; (2) the 3-(*S*)-hydroxyl group of statine mimics substrate water; and (3) pepstatin resembles a collected substrate more than the transition state. This last statement may have to be modified further because our recent data indicate that pepstatin is not an analogue of a substrate but rather an enhanced version of an aspartic proteinase inhibitor. Thus synthetic peptides such as Iva-Val-Val-Leu-Leu-Ala-Iaa,

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formed by replacing Sta with Leu-Leu (or Leu-Phe), are not substrates for pepsin or penicillopepsin but rather fairly good (1–10 μM) inhibitors of these enzymes.[75] Naming this type of inhibition should be deferred until a better understanding of the mode of inhibition is gained, but it should be apparent to the reader that these data complicate attempts to unravel aspartic proteinase catalytic mechanism from statine-derived inhibitor–enzyme complexes. Undoubtedly, these complexes serve to locate binding pockets within the enzyme active site, but the orientation of the catalytic groups to amide substrates may differ significantly from their positions during catalysis. How many other mechanism-based enzyme inhibitors are enhanced versions of non-transition-state complexes?

For aspartic proteinases, it appears the best models for observing the transition state or tetrahedral intermediate by physical methods will be found from nonhydrolyzed ketone pseudosubstrates, derived from established substrate sequences, that can be shown by ^{13}C NMR to be converted to tetrahedral adducts in the active site by an enzyme-catalyzed process. Hydrated carbonyl mimics, e.g., Sta^P **7** peptide derivatives will also be valuable models for tetrahedral intermediates when derived from established substrate sequences, but comparisons of K_i to K_s (or K_m) should be corrected for the possible contribution of entropic factors stabilizing EI complexes that cannot stabilize ES complexes.

Finally, the idea to use the transition-state analogue concept as a point of departure for designing novel enzyme inhibitors remains a valuable approach, especially when all reactants in the mechanism are considered. Clearly if an enzyme is constructed so as to force water and substrate to within covalent bond distances, then intrusion of added atoms on the inhibitor, even as small as a proton, must prevent attainment of geometry identical with that formed in transition state (cf. Figure 1B). This problem would appear to face all tetrahedral transition-state mimics modeled after hydrated trigonal bonds (Figure 1B, **37**, **38**). Detailed enzyme kinetics to establish the order of addition of substrates or release of products on either side of the anticipated transition state being modeled are vital to the rational design of mechanism-based inhibitors because this information establishes if collected-substrate or collected-product inhibitors are feasible. It would seem many challenges remain before this field is fully understood, but the potential benefits make the effort well worthwhile.

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Articles

Structure–Activity Relationships of C-Terminal Tri- and Tetrapeptide Fragments That Inhibit Gastrin Activity

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A series of tri- and tetrapeptide derivatives, analogues of the gastrin C-terminal region with no phenylalanine residue, were synthesized. These peptides were tested for their ability to inhibit gastrin-stimulated acid secretion in vivo as well as binding of [^{125}I]-(Nle^{11})-HG-13 to gastric mucosal cell receptors in vitro. Most of the peptides tested exhibited gastrin antagonist activity in vivo and in vitro. Most active derivatives were 20–30 times more potent than the well-known gastrin antagonist derivatives proglumide and benzotript and had 20–200 times more binding affinity. The smallest fragment exhibiting antagonist activity was the tripeptide Boc-L-tryptophyl-L-methionyl-L-aspartic acid amide.

Early work on structure–activity relationships of gastrin, particularly those of Morley,[1] showed that all the diverse biological activities of the gastrins were found to reside in the C-terminal 14–17 portion of the molecule L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide. Replacement of L-tryptophan, L-methionine, or L-phenylalanine residues led to agonists of varying potency, whereas even small changes at the L-aspartic acid residue resulted in inactive analogues. Many analogues of the type L-tryptophyl-L-methionyl-X-L-phenylalanine amide were prepared, but they were devoid of antisecretory activity. Some antigastrin peptides were recently proposed: Boc-

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