X-ray-crystallographic studies of complexes of pepstatin A and a statine-containing human renin inhibitor with endothiapepsin

David BAILEY,* Jon B. COOPER,* Balasubramanian VEERAPANDIAN,* Tom L. BLUNDELL,*§ Butrus ATRASH,† Dave M. JONES† and Michael SZELKE†

*Laboratory of Molecular Biology, Department of Crystallography, Birkbeck College, University of London, Malet Street, London WC1E 7HX, and †Fehring Research Institute, Southampton University Research Centre, Chilworth, Southampton SO1 7NP, U.K.

H-189, a synthetic human renin inhibitor, and pepstatin A, a naturally occurring inhibitor of aspartic proteinases, have been co-crystallized with the fungal aspartic proteinase endothiapepsin (EC 3.4.23.6). H-189 [Pro-His-Pro-Phe-His-Sta-(statyl)-Val-Ile-His-Lys] is an analogue of human angiotensinogen. Pepstatin A [Iva(isovaleryl)-Val-Val-Sta-Ala-Sta] is a blocked pentapeptide which inhibits many aspartic proteinases. The structures of the complexes have been determined by X-ray diffraction and refined to crystallographic R-factors of 0.15 and 0.16 at resolutions of 0.18 nm (1.8 Å) and 0.2 nm (2.0 Å) respectively. H-189 is in an extended conformation, in which the statine residue is a dipeptide analogue of P₁ and P₁'

as indicated by the conformation and network of contacts and hydrogen bonds. Pepstatin A has an extended conformation to the P₂' alanine residue, but the leucyl side chain of the terminal statine residue binds back into the S₁' subsite, and an inverse γ -turn occurs between P₁' and P₂'. The hydroxy moiety of the statine at P₁ in both complexes displaces the solvent molecule that hydrogen-bonds with the catalytic aspartate residues (32 and 215) in the native enzyme. Solvent molecules originally present in the native structure at the active site are displaced on inhibitor binding (12 when pepstatin A binds; 16 when H-189 binds).

INTRODUCTION

Renin (EC 3.4.23.15) is a mammalian aspartic proteinase whose only known physiological function is to catalyse the removal of an N-terminal decapeptide from angiotensinogen. This biologically inactive decapeptide (angiotensin I) (Tewkesbury et al., 1981) has its C-terminal dipeptide removed by angiotensin converting enzyme (EC 3.4.15.1) to give angiotensin II, which initiates vasoconstriction and stimulates the formation and release of aldosterone, leading to an increase in blood pressure. Since the rate-limiting step in this cascade involves renin, inhibitors against renin may be clinically useful. Renin inhibitors are also of value for delineating the role of renin in normal blood pressure and homeostasis.

Endothiapepsin (EC 3.4.23.6) is a 330-residue microbial aspartic proteinase from the chestnut-blight fungus (*Endothia parasitica*). This family of enzymes was characterized initially by active-site-directed diazoacetamido compounds (Rajagopalan et al., 1966), diazoketones (Delpierre & Fruton, 1966) and certain epoxides (Tang, 1971), and by a non-covalent inhibitor, the microbial compound pepstatin A (Umezawa et al., 1970).

The three-dimensional structures of several fungal enzymes have been determined by X-ray-crystallographic studies [endothiapepsin (Blundell et al., 1990) penicillopepsin (James and Sielecki, 1983), rhizopuspepsin (Bott et al., 1982), yeast proteinase

A (C. Aguilar, M. Badasso, J. B. Cooper, M. P. Newman and T. L. Blundell, unpublished work), trichodermapepsin (M. Crawford and J. B. Cooper, unpublished work), *Mucor pusillus* pepsin (Newman et al., 1993)], as have several mammalian ones [porcine pepsin (Abad-Zapatero et al., 1990; Sielecki et al., 1990; Cooper et al., 1990), its precursor pepsinogen (James and Sielecki, 1986; J. A. Hartsuck and S. J. Remington, unpublished work), human renin (Sielecki et al., 1989; Badasso et al., 1992; Dhanaraj et al., 1992), mouse renin (C. DeAlwis, C. Frazao, J. B. Cooper, M. Badasso, B. L. Sibanda, S. P. Wood, T. L. Blundell, I. J. Tickle, and H. Driessen, unpublished work) and bovine chymosin (Gilliland et al., 1990; Strop et al., 1990; Newman et al., 1991). There are also crystal structures for the retroviral proteinases of Rous-sarcoma virus (Miller et al., 1989) and human immunodeficiency virus I (Lapatto et al., 1989; Wlodawer et al., 1989).

Although many of the pepsin-like enzymes show pairwise sequence identities over only one third of the molecule (Foltmann and Pedersen, 1977), structural comparisons show that they have similar bilobal tertiary structures comprised mainly of β -sheet. Each lobe of the pepsins and each subunit of the retroviral proteinases contribute one of the two catalytic aspartic acid residues Asp-32 and Asp-215 (pepsin numbering), which lie in the centre of the active-site cleft.

Natural inhibitors can be isolated from culture filtrates of

Abbreviations used: Sta, statine [4-(S)-amino-3-(S)-hydroxy-6-methylheptanoic acid]; Iva, isovaleryl [3-methylbutanoic acid (valine with no amino group)]; tBoc, tertiary butoxycarbonyl; Bom, π -benzyloxymethyl; HOBt, hydroxybenzotriazole.

§ To whom correspondence should be addressed.

Table 1 Sequences of the four inhibitors aligned to the natural substrate human angiotensinogen

The structure of tBoc is $(\text{CH}_3)_3\text{C}-\text{O}-\text{CO}-$, that of cyclohexylstatine (Chs) is $-\text{NH}-\text{CH}(\text{CH}_2\text{C}_6\text{H}_{11})-\text{CH}(\text{OH})-\text{CH}_2-\text{CO}-$ and that of Sta is $-\text{NH}-\text{CH}[\text{CH}_2\text{CH}(\text{CH}_3)_2]-\text{CH}(\text{OH})-\text{CH}_2-\text{CO}-$. Phe^N, Phe-NH₂.

Inhibitor	Enzyme subsite ...	Sequence										
		S ₆	S ₅	S ₄	S ₃	S ₂	S ₁	S' ₁	S' ₂	S' ₃	S' ₄	S' ₅
Angiotensinogen		Ile	His	Pro	Phe	His	Leu	Val	Ile	His	Asn	
H-189		Pro	His	Pro	Phe	His	Sta		Val	Ile	His	Lys
L364,099		Iva	His	Pro	Phe	His	Chs		Leu	Phe ^N		
L363,564		Boc	His	Pro	Phe	His	Sta		Leu	Phe ^N		
Pepstatin A				Iva	Val	Val	Sta		Ala		Sta	

various actinomycete species by screening for anti-pepsin activity (Morishima et al., 1970; Umezawa et al., 1970). These so-called 'pepstatins' contain two statine [Sta; 4-(*S*)-amino-3-(*S*)-hydroxy-6-methylheptanoic acid] residues, and the potency of pepstatin A as an inhibitor of pepsin has been ascribed to it being a transition-state analogue of the tetrahedral intermediate of peptide-bond cleavage (Marciniszyn et al., 1976).

Potent inhibitors to renin can be formed by modification of the scissile bond of human angiotensinogen (Szelke et al., 1982a,b; Natarajan et al., 1983). For example, in H-189 the P₁ residue and its scissile bond are replaced by statine (Tree et al., 1983) to give the sequence:

Pro-His-Pro-Phe-His-Sta-Val-Ile-His-Lys

X-ray-diffraction studies of such enzyme-inhibitor complexes are the best way of delineating the binding subsites of these enzymes. Successful X-ray studies have been performed with many scissile-bond mimetics. The 'reduced-bond' inhibitor complexes ($-\text{CONH}-\rightarrow-\text{CH}_2\text{NH}-$) include several with endothiapepsin (Foundling et al., 1987; Cooper et al., 1987a) and rhizopuspepsin (Suguna et al., 1987). There are those where the leucine residue at P₁ has been replaced with statine or variants of statine. These have been complexed with rhizopuspepsin [Bott et al., 1982 (statine)], with penicillopepsin [James et al., 1982; (statine)] and with endothiapepsin [Blundell et al., 1987 (statine); Šali et al., 1989 (azahomostatine); Cooper et al., 1989 (cyclohexylstatine); M. Crawford, J. B. Cooper, C. Humblet and E. A. Lunney, unpublished work (phenylstatine); C. DeAlwis, J. B. Cooper, A. Beveridge, T. L. Blundell, E. A. Lunney, C. Humblet, W. T. Lowther and B. M. Dunn, unpublished work (phosphostatine) and Veerapandian et al., 1992 (difluorostatone)]. There have also been X-ray studies on complexes of endothiapepsin with an 'amino-alcohol' inhibitor [$-\text{CH}(\text{OH})-\text{CH}_2\text{NH}-$] (Cooper et al., 1987b) and a 'hydroxy-isostere'

Table 2 Cell constants for native and complexed forms of endothiapepsin

Endothiapepsin	a (nm)	b (nm)	c (nm)	β (°)
Native	5.36	7.41	4.57	110
Complexed with				
H-261	4.30	7.57	4.29	97
H-189	4.31	7.54	4.28	97
Pepstatin A	4.31	7.56	4.29	97

Figure 1 $2|F_o| - |F_c|$ electron-density maps of (a) H-189 (P₄→P₂) and (b) pepstatin A (P₄→P₂)

Figure 2 Stereo views of (a) pepstatin A ($P_4 \rightarrow P_4'$, -----) and (b) L363,564 ($P_4 \rightarrow P_3'$, -----) superimposed on the extended conformation of H-189 ($P_4 \rightarrow P_3'$, —) after least-squares fitting of the complexes on to the native structure

inhibitor [$-\text{CH}(\text{OH})\text{CH}_2$] with a subnanomolar K_i for human renin (Veerapandian et al., 1990).

In the present paper we report the X-ray analyses to resolutions of 0.18 nm (1.8 Å) and 0.2 nm (2.0 Å) of endothiapepsin complexes of H-189 and pepstatin A (Table 1). The structural features of these complexes are described and compared with other complexes with statine residues at P_1 .

METHODS AND MATERIALS

Synthesis of Inhibitors

H-189 was synthesized by the solid-phase method for peptide synthesis (Merrifield, 1963) as follows. Chloromethylated polystyrene resin cross-linked with 1% divinylbenzene was obtained from the Pierce Chemical Co., Rockford, IL, U.S.A. Esterification of butoxycarbonyl(Boc)-lysine to the resin was performed by the method of Horiki et al. (1978), giving a substitution of 0.2 mmol/g of resin. All amino acids were coupled as their N^t -Boc derivatives (Protein Research Foundation, Osaka, Japan). Boc-statine was prepared as described by Rich et al. (1978). The histidine side chain was protected with the π -benzyloxymethyl (Bom) group. Each residue was coupled as a pre-formed hydroxy-benzotriazole (HOBt) ester, with a standard protocol being used for all the intermediate steps (washing, de-protection, neutralization etc.) in the synthesis. The peptide was cleaved from the resin by treatment with anisole/(liquid)HF (1:9, v/v) at 0 °C for 1 h. After removal of the HF under nitrogen, the crude peptide was extracted with acetic acid/water (1:1, v/v) and freeze-dried. This crude peptide was then purified first by gel chromatography (Sephadex G-25; acetic acid/water, 1:1, v/v) and then by ion-exchange chromatography (CM-52, linear gradient 0.05–1.00 M ammonium acetate buffer) to give, after repeated freeze-drying, a chromatographically homogeneous white solid. Amino acid analysis after hydrolysis in 6 M HCl containing 0.1% phenol at 110 °C for 40 h gave the following ratios: His, 3.10; Ile, 0.85; Lys, 1.01; Phe, 0.96; Pro, 1.93; the peptide content was 77%, the rest being water and acetic acid. Pepstatin A was obtained commercially from the Peptide Institute, Kyoto, Japan.

Crystallization

Co-crystals of endothiapepsin with H-189 and pepstatin A were grown using a modified method of Moews and Bunn (1970). Freeze-dried enzyme and inhibitor were dissolved in an 0.1 M acetate buffer, pH 4.5, to give 60 μM and 600 μM solutions respectively. The insolubility of pepstatin A meant that propan-2-ol had to be added to dissolve it. After 24 h the propan-2-ol

Figure 3 Graph of isotropic temperature factor (B_{iso}) after normalization of the 'flap' region (71→82) against residue number for native endothiapepsin complexes with inhibitors H-189, pepstatin A and L363,564

Figure 4 Hydrogen-bonding diagrams showing interactions between inhibitor and enzyme (a) H-189 and (b) pepstatin A

was dialysed off and the resulting solution Millipore-filtered. After dissolution, finely powdered $(\text{NH}_4)_2\text{SO}_4$ was then added to give a 2.2 M (55% satd.) solution, which was Millipore-filtered. Turbidity was removed by the addition of a few drops of acetone. Crystals of the endothiapepsin-H-189 complex appeared after approximately 3 months, whilst only a few good crystals of the endothiapepsin/pepstatin A complex appeared after about 2

years. The crystals of both complexes were of spacegroup $P2_1$, but were non-isomorphous with the native crystals (Table 2).

X-ray analyses

X-ray data to a resolution of 0.18 nm were collected from four crystals of the endothiapepsin-H-189 complex using an Enraf-

Nonius CAD4F diffractometer. After applying corrections for absorption, Lorentz-polarization and radiation decay, 36927 reflections were merged to give 23224 unique reflections with a merging R of 3.9% (Sheldrick, 1982). The observed structure factor amplitudes, $|F_o|$, were scaled to calculated amplitudes, $|F_c|$, from the endothiapepsin-H-261 complex (Veerapandian et al., 1990), the crystals of which were isomorphous with these statine-containing complexes. Difference-Fourier calculations with coefficients, $|F_o| - |F_c|$ and $2|F_o| - |F_c|$ were calculated and displayed on an Evans and Sutherland PS300 graphics system using the FRODO program (Jones, 1978). A model of H-189 was then built into the density and refined by a stereochemically restrained least-squares procedure implemented in the program RESTRAIN (Haneef et al., 1985). Cycles of refinement and model building into weighted $2|F_o| - |F_c|$ and $|F_o| - |F_c|$ maps gave a final R factor of 15.2% for reflections in the range 1.0–0.18 nm [$F > 2\sigma(F)$, 21972 reflections]. Only one crystal of the endothiapepsin-pepstatin A complex was usable. X-ray data were collected using an Enraf-Nonius FAST system to a resolution of 0.20 nm. The batches of data were scaled and merged using the method of Fox and Holmes (1966). 60968 reflections were merged to give 16933 unique reflections with a merging R of 4.2%. Refinement of this complex gave a final R factor of 15.8% for reflections in the range 1.0–0.20 nm [$F > 2\sigma(F)$ 16895 reflections].

RESULTS

Electron density and general structure of H-189 and pepstatin A

The electron density for H-189 is clearly defined from the P_4 Pro to the P'_3 Ile (Figure 1a) [the numbering (P_n) of the peptide inhibitors, given in Table 4 below, assumes that the statine residue is a dipeptide analogue]. There is no electron density for the P_6 Pro and the P'_5 Lys, and that for the P_5 His and the P'_4 is also very weak. For pepstatin A at 0.2 nm (2.0 Å) the electron density is continuous from the terminal isovaleric acid residue at P_4 to the C-terminal statine residue (Figure 1b). H-189 binds in the active-site cleft as an extended β -strand. Pepstatin A exists in a similar conformation down to P'_1 (Figure 2a). However, an inverse γ -turn is found between the two statine residues. Both inhibitors participate in a network of hydrogen bonds with the main chain and some side chains of the proteinase, as well as with several water molecules. The two inhibitors also make numerous van der Waals contacts with the enzyme. The P_1 statine hydroxy group interacts closely with the two aspartate residues, displacing the solvent molecule usually present in the native structure. Residues 71–82, which form an anti-parallel β -hairpin, known as the 'flap', shield the active site from solvent. The flap forms hydrogen bonds to the inhibitors, and has van der Waals contact from P_2 to P'_4 with pepstatin A and from P_2 to P'_3 with H-189. These hydrogen bonds probably account for the lower isotropic temperature factors of the flap when the inhibitor complexes are compared with the native enzyme (Figure 3).

Hydrogen bonds

Figures 4(a) and 4(b) show the hydrogen bonds in the inhibitor complexes. H-189 makes 14 hydrogen bonds with the enzyme and three with solvent compared with 16 and two respectively for pepstatin A. A pepstatin fragment bound to penicillopepsin (James et al., 1982) shows a similar hydrogen bonding pattern to P_1 . In both complexes Thr-219 forms two good hydrogen bonds to the P_3 inhibitor residue, one with its side-chain hydroxy group to the P_3 NH, and one with its NH to the P_3 CO. Pepstatin A in addition forms a hydrogen bond between the O^H of Thr-219

Figure 5 Stereo view of active site 'flap' covering the P_1 - P'_1 section of H-189

Figure 6 The inverse γ -turn in pepstatin A

Inverse γ -turn statistics*	Found in pepstatin A
Mean CO...HN = 0.219 ± 0.018 nm	0.23 nm
Mean O $\cdots$ HN angle = $137^\circ \pm 10^\circ$	130°
Mean CO $\cdots$ H angle = $103^\circ \pm 7^\circ$	105°

*Data taken from Baker and Hubbard, 1984

Figure 7 Stereo view of the active-site residues of the H-189 inhibitor complex (—) and the native structure (----) superimposed after least-squares fitting

and P_3 CO. A solvent molecule forms a hydrogen bond to the P_3 CO in both complexes, a situation also occurring with inhibitor L363,564 (Cooper et al., 1989). The O^H of the carboxy side chain of Asp-77 hydrogen-bonds to the P_2 main-chain NH and the P_2 CO forms two hydrogen bonds to the main chain of the flap, one

Table 3 van der Waals contacts of (a) H-189 and (b) pepstatin A

(a) H-189

Subsite...	P ₆	P ₅	P ₄	P ₃	P ₂	P ₁ -P ₁ '	P ₂ '	P ₃ '
Sequence...	Pro	His	Pro	Phe	His	Sta	Val	Ile
Condition of electron density (P ₄ ' and P ₅ ' very weak)...	Weak van der Waals contacts [$d \leq 0.4$ nm (4 Å)]							
	Asp-12 Leu-10	Asp-12 W208 Asp-114 W195	Thr-219 W1 Asp-12	Thr-219 W1 Thr-218 W4 W219 Asp-12 Gly-217 W5 W195 Ile-117 Asp-77 Ile-7 Asp-114	W223 Asp-77 Gly-76 Thr-218 Ile-301 Tyr-75 Gly-217 Ile-297	Asp-32 Asp-215 Gly-76 Tyr-75 Gly-217 Thr-218 Asp-77 W4 Gly-34 Asp-30 Leu-120	Gly-34 Ser-74 W7 Ile-73 Phe-189 Tyr-75 Leu-128 W223	W7 Tyr-75 Ser-74 Gly-76
Total number of contacts of each inhibitor residue...	7	13	7	35	30	50	15	15
Number of water molecules in contact...	0	2	1	5	1	1	2	1
Total number of water contacts...	0	2	1	11	3	1	2	1

(b) Pepstatin A

Subsite...	P ₄	P ₃	P ₂	P ₁ -P ₁ '	P ₂ '	P ₃ '-P ₄ '
Sequence...	Iva	Val	Val	Sta	Ala	Sta
Condition of electron density...	Good van der Waals contacts [$d \leq 0.4$ nm (4 Å)]					
	W1 Thr-219 W20 Asp-12 Tyr-222	Thr-219 Thr-218 W1 W4 Asp-77 W220 W5 Gly-217 Asp-12	Gly-76 Asp-77 Thr-218 Tyr-75 W1	Asp-215 Asp-32 Gly-76 Tyr-75 Gly-217 Thr-218 W148 Gly-34 W4 Asp-30 Leu-120 Asp-77 Ser-79	Gly-34 Ser-74 Phe-189 Tyr-75	W166 Ser-74 W175 Gly-76 Tyr-75 Ile-297 Phe-189
Total number of contacts to each inhibitor residue...	7	24	19	51	6	15
Number of water molecules in contact...	2	4	1	2	0	2
Total number of water contacts...	2	6	1	2	0	55

to the NH of Asp-77 and the other to the NH of Gly-76 in both pepstatin A and H-189 complexes (Figure 5). A unique hydrogen bond in H-189 exists between the imidazole N^{ε2} of P₂ His and a solvent molecule.

The P₁ statine residue binds in a similar way in both complexes.

The statine NH forms a bifurcated hydrogen-bonded system with the Gly-217 CO and the side-chain hydroxy group of Thr-218. Two hydrogen bonds are formed between the statine hydroxy group and the two catalytic aspartate residues. Short O^{δ1}-statine-hydroxy-group distances indicate that the same hydrogen bonds

Table 4 ϕ , ψ , ω and χ angles for (a) H-189 ($P_6 \rightarrow P_3$) and (b) pepstatin A ($P_4 \rightarrow P_3$)

All angles are in $^\circ$. For statine residues, ϕ was calculated as $\text{CO}_{P_2}-\text{N}_{P_1}-\text{C}_{P_1}^\alpha-\text{CH}(\text{OH})_{P_1}$, ψ as $\text{N}_{P_1}-\text{C}_{P_1}^\alpha-\text{CH}(\text{OH})_{P_1}-\text{CM}_{P_1}$ and ω as $\text{CM}_{P_1}-\text{CO}_{P_1}-\text{N}_{P_1}-\text{C}_{P_1}^\alpha$

(a) H-189

Residue	Angle...	ϕ	ψ	ω	χ_1	χ_2	χ_3	χ_4
Pro P_6			98	-176	7	5	-15	20
His P_5		-3	96	178	-152	-164		
Pro P_4		-90	154	-172	0	35	-53	57
Phe P_3		-105	153	174	-79	-102		
His P_2		-150	96	-167	174	-163		
Sta $P_1-P'_1$		-127	64	180	-61	-179		
Val P'_2		-79	147	177	68			
Ile P'_3		-90	122	176	-54	145		

(b) Pepstatin A

Residue	Angle...	ϕ	ψ	ω	χ_1	χ_2	χ_3	χ_4
Iva P_4				179				
Val P_3		-134	150	178	-52			
Val P_2		-129	107	-177	-179			
Sta $P_1-P'_1$		-127	66	180	-52	170		
Ala P'_2		-87	82	-179				
Sta $P'_3-P'_4$		-114	68		-43	166		

(Figures 4a and 4b) exist in each complex. If one compares the hydrogen-bonding pattern in these two complexes with that of H-142 (Pro-His-Pro-Phe-His-Leu/[CH₂NH]Val-Ile-His-Lys; Foundling et al., 1987), which possesses the same standard amino acids as in the natural renin substrate, it is clear that the central statine residue spans two standard residues. The hydrogen bond between the statine CO and the Gly-76 NH is equivalent to the one formed via the P₁ valine CO in H-142. Furthermore, there is a hydrogen bond at P₂' with Gly-34 in both H-189 and H-142. The statyl CO forms a strong hydrogen bond to the NH of Gly-76. Pepstatin A hydrogen-bonds through P₁' CO to P₃' NH to form an inverse γ -turn (Figure 6). At P₃' H-189 forms two hydrogen bonds: from NH to Ser-74 CO and from CO to a solvent molecule. This interaction is absent in pepstatin A, owing to the different conformation of the terminal statine residue. The C-terminus forms three hydrogen bonds: the first weakly with a solvent molecule, the second more strongly with Ser-79 O^H, and the third an intramolecular hydrogen bond between the O^r of the statine residue and the carboxy group of the same statine residue.

Solvent molecules

Displacement of solvent bound in the active site to bulk solvent is entropically favourable to inhibitor binding (Jencks, 1975). To calculate how many solvent molecules were displaced, the co-ordinates of endothiapepsin were 'least-squares fitted' to the native co-ordinates. The contacts [$d \leq 0.4$ nm (4.0 Å)] between the inhibitors and the native solvent molecules were then calculated; 12 solvent molecules are displaced in the pepstatin A complex, and 16 in the H-189 complex. The solvent molecule bound by the aspartate residues in the native structure is displaced in both complexes. Examination of the superposed structures shows that the statine hydroxy group lies ~ 0.01 nm (~ 0.1 Å) from the native solvent molecule (Figure 7 shows superposition of statine of H-189 with the native co-ordinates). Of the eight

solvent molecules in van der Waals contact with H-189, five have equivalent positions in the pepstatin A complex and are in van der Waals contact with pepstatin A. The other three solvent molecules do not contact pepstatin A.

The γ -turn in pepstatin A

When the CO of a residue, i , hydrogen bonds to the NH of another residue, $i+2$, a γ -turn is formed. There are two types: the *classic* usually (but not always), involves a reversal of main chain direction, whilst the *inverse*, although rarely associated with a chain reversal, 'kinks' the main chain (Milner-White et al., 1988). In pepstatin A an inverse γ -turn occurs, with a hydrogen bond between the CO and NH of the two statine residues occupying P₁-P₁' and P₃-P₄' respectively. Figure 6 shows a stereoscopic view of the turn, together with the mean values of the parameters associated with such turns (Baker and Hubbard, 1984).

Specificity subsites

In H-189, P₅ His packs against the P₃ Phe aromatic ring, in a favourable orientation. The closest approach distance of C ^{ϵ} 1 P₅ to C ^{ζ} P₃ is 0.34 nm (3.4 Å). The P₅ imidazole N ^{δ} 1 is 0.34 nm (3.4 Å) from the solvent molecule W208 and N ^{δ} 2 is 0.34 nm (3.4 Å) from the P₄ CO, which does not usually participate in the hydrogen-bonding network of these inhibitors. The P₄ residues, Pro and isovaleric acid (Iva), make relatively few contacts with the enzyme (Tables 3a and 3b), perhaps because of their small size. The P₃ Phe ring in H-189 and the inhibitor L363,564 adopt similar conformations (Figure 2b), but in L364,099 the Phe ring is almost perpendicular to the orientation found in the other two. It has been suggested that this is correlated with the existence of a cyclohexylalanine side chain at P₁, which occurs both in L364,099 and in an azahomostatine inhibitor complex (Šali et al., 1989). The P₃ Phe has van der Waals contacts with Ile-117 and Ile-7, and also with three aspartate residues, 12, 77 and 114. The pH at which this complex was crystallized may result in the carboxy groups on these residues being protonated. Two of these carboxy groups are close to the P₃ ring edge, giving a favourable interaction between the oxygen atoms and the ring-hydrogen partial positive charge (Thomas et al., 1982; net free-energy difference favouring the ring edge is ~ 4 kJ.mol⁻¹). Pepstatin A has a valine at P₃, which is less deeply buried in the S₃ pocket than the Phe side chain of H-189. Consequently there are no contacts with the two isoleucine residues.

The inhibitor complexes with histidine at P₂ show two orientations of the imidazole ring. In one of these the imidazole is close to the S₁' binding pocket as in H-189, ($\chi_1 = 174^\circ$ and in the other, where the side chain is flipped about χ_1 , it lies close to the P₄ binding pocket, as in H-142 ($\chi_1 = -45^\circ$) (Foundling et al., 1987).

The P₁ conformational angles in the statine-containing complexes are similar (Tables 4a and 4b), with the exception of χ_2 of the cyclohexyl statine in L364,099. The P₁ ϕ angles for H-189 and pepstatin A are similar, but the inverse γ -turn in pepstatin A shows up in the differing ψ angles. The conformation of the leucine side chain allows extensive contacts to be made with the relatively hydrophobic S₁ binding pocket, and the hydroxy group is hydrogen-bonded to Asp-32 and Asp-215. The configuration of the hydroxy branch point is critical for potent inhibition. Pepstatin-type inhibitors (Rich et al., 1978) with a (3S) configuration are approx. 3000 times more potent than the corresponding (3R) enantiomorphs towards pepsin. Similarly Boger

Table 5 Inhibition constants (K_i) for H-189 and pepstatin A

Endothiapepsin assays were carried out at 37 °C and pH 3.1 using chromogenic substrates. For renin, unless otherwise stated, assays carried out at pH 7.2 with angiotensinogen as substrate. The reaction rate determined by measurement by immunoassay of the amount of angiotensin I released.

Inhibitor	K_i (nM)		Reference
	Endothiapepsin	Human renin	
H-189	1.0	12	Leckie (1985) (IC_{50})
Pepstatin A	0.5	13000§	Boger (1985)
L363,564	40	2.3‡	Cooper et al. (1989)
L364,099	420	0.16‡	Cooper et al. (1989)

et al. (1983) have given details of two angiotensin analogues incorporating statine showing that the (3*R*) enantiomorph is approx. 500 times less potent than the (3*S*) enantiomorph towards pig kidney renin. These observations are consistent with our structural study which indicates that fewer favourable hydrogen bonds are possible with an (*R*) configuration at the statine.

Statine is a dipeptide analogue and occupies the P_1 - P'_1 and P_3 - P'_4 residue positions in pepstatin A. The *C*-terminal statine residue of pepstatin A should therefore interact with the S'_3 - S'_4 specificity subsites. However, in the endothiapepsin complex the leucine side chain of the terminal statine residue 'binds back' into the vacant S'_1 subsite and makes a van der Waals contact with the P_2 valine side chain [Leu $C^{\alpha 2}$ →Val $C^{\gamma 1}$ distance is 0.36 nm (3.6 Å)]. This effect was first modelled by Boger (1985), leading to the design and synthesis of conformationally restricted inhibitors containing a disulphide cross-link from P_2 to P'_3 .

Against human renin H-189 is ~1000 times more potent than pepstatin A (Table 5). H-189 possesses a high degree of residue identity with renin's natural substrate, angiotensinogen, whilst pepstatin A is a microbial inhibitor evolved as a defence against fungal predators.

Pepstatin A has been modelled into mouse renin (D. Bailey, unpublished work). The model shows that, although the side chains of pepstatin A can be accommodated, they leave voids in the S_3 , S_2 and S'_2 subsites. This is due to the shorter side chains in pepstatin A (Val, Val and Ala at P_3 , P_2 , and P'_2 respectively) in comparison with H-189 (Phe, His and Val). This may explain the weaker potency of pepstatin A against renins compared with pepins.

We thank the Medical Research Council for financial support for D.B. We are grateful to Chris DeAlwis for providing mouse renin-inhibitor complex co-ordinates, Ray Jupp for kinetic measurements, and Professor John Kay, Dr. Stephen Wood and Dr. Ian Tickle for helpful advice and discussion.

REFERENCES

- Abad-Zapatero, C., Rydel, T. J. and Erickson, J. (1990) *Proteins* **8**, 62–81
- Badasso, M., Frazao, C., Sibanda, B. L., Dhanaraj, V., DeAlwis, C., Cooper, J. B., Wood, S. P., Blundell, T. L., Murakami, K., Miyazaki, H. et al. (1992) *J. Mol. Biol.* **223**, 447–453
- Baker, E. N. and Hubbard, R. E. (1984) *Prog. Biophys. Mol. Biol.* **44**, 97–179
- Blundell, T. L., Jenkins, J. A., Sewell, B. T., Pearl, L. H., Cooper, J. B., Tickle, I. J., Veerapandian, B. and Wood, S. P. (1990) *J. Mol. Biol.* **211**, 919–941
- Boger, J., Lohr, N. S., Ulm, E. H., Poe, M., Blaine, E. H., Fanelli, G. M., Lin, T.-Y., Payne, L. S., Schorn, T. W., LaMont, B. I. et al. (1983) *Nature (London)* **303**, 81–84
- Boger, J. (1985) in *Aspartic Proteinases and their Inhibitors* (Kostka, V., ed.), pp. 401–420, de Gruyter, Berlin
- Bott, R., Subramanian, E. and Davies, D. (1982) *Biochemistry* **21**, 6956–6962
- Cooper, J., Foundling, S., Hemmings, A., Blundell, T., Jones, D. M., Hallett, A. and Szelke, M. (1987a) *Eur. J. Biochem.* **169**, 215–221
- Cooper, J. B., Foundling, S. I., Blundell, T. L., Arrowsmith, R. J., Harris, C. J. and Champness, J. N. (1987b) in *Topics in Medicinal Chemistry* (Leeming, P. R., ed.), pp. 308–313, Royal Society of Chemistry, London
- Cooper, J. B., Foundling, S. I., Blundell, T. L., Boger, J., Jupp, R. A. and Kay, J. (1989) *Biochemistry* **28**, 8596–8603
- Cooper, J. B., Khan, G., Taylor, G., Tickle, I. J. and Blundell, T. L. (1990) *J. Mol. Biol.* **214**, 199–222
- Delpierre, G. R. and Fruton, J. S. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1817–1822
- Dhanaraj, V., Dealwis, C., Frazao, C., Badasso, M., Sibanda, B. L., Tickle, I. J., Cooper, J. B., Driessen, H. P. C., Newman, M., Aguilar, C. et al. (1992) *Nature (London)* **357**, 466–472
- Foltmann, B. and Pedersen, V. B. (1977) in *Acid Proteases* (Tang, J. ed.), pp. 3–22, Plenum Press, New York
- Foundling, S. I., Cooper, J., Watson, F. E., Cleasby, A., Pearl, L. H., Sibanda, B. L., Hemmings, A., Wood, S. P., Blundell, T. L. and Valler, M. J. et al. (1987) *Nature (London)* **327**, 349–352
- Fox, G. C. and Holmes, K. C. (1966) *Acta Crystallogr.* **20**, 886–891
- Gilliland, G. L., Winborne, E. L., Nachman, J. and Wlodawer, A. (1990) *Proteins* **8**, 82–101
- Haneef, I., Moss, D. S., Stanford, M. J. and Borkakoti, N. (1985) *Acta Crystallogr.* **A41**, 426–433
- Horiki, K., Igano, K. and Inouge, K. (1978) *Chem. Lett.* 165–168
- James, M. N. G. and Sielecki, A. R. (1983) *J. Mol. Biol.* **163**, 299–361
- James, M. N. G. and Sielecki, A. R. (1986) *Nature (London)* **319**, 33–38
- James, M. N. G., Sielecki, A., Salituro, F., Rich, D. H. and Hofmann, T. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6137–6141
- Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol.* **43**, 219–490
- Jones, T. A. (1978) *J. Appl. Crystallogr.* **11**, 268–272
- Lapatto, R., Blundell, T., Hemmings, A., Overington, J., Wilderspin, A., Wood, S., Merson, J. R., Whittle, P. J., Danley, D. E., Geohagan, K. F. et al. (1989) *Nature (London)* **342**, 299–302
- Leckie, B. (1985) in *Aspartic Proteinases and their Inhibitors* (Kostka, V., ed.), pp. 443–461, de Gruyter, Berlin
- Marciszyn, J., Jr., Hartsuck, J. A. and Tang, J. J. N. (1976) *J. Biol. Chem.* **251**, 7088–7094
- Merrifield, R. B. (1963) *J. Am. Chem. Soc.* **85**, 2149–2154
- Miler, M., Jaskolski, M., Mohana Rao, J. K., Leis, J. and Wlodawer, A. (1989) *Nature (London)* **337**, 576–579
- Milner-White, E. J., Ross, B. M., Belhadj-Mostefa, R. I. K. and Poet, R. (1988) *J. Mol. Biol.* **204**, 777–782
- Moews, P. and Bunn, C. W. (1970) *J. Mol. Biol.* **54**, 395–397
- Morishima, H., Takita, T., Aoyagi, T., Takeuchi, T. and Umezawa, H. (1970) *J. Antibiot.* **23**, 263–265
- Natarajan, S., Condon, M. E., Nakane, M., Reid, J., Gordon, E. M., Cushman, D. W. and Ondetti, M. A. (1983) in *Peptides: Synthesis-Structure-Function* (Proceedings of the 8th American Peptide Symposium) (Gross, E. and Meienhofer, J., eds.), Rockford Pierce Chemical Co.
- Newman, M. P., Saffro, M., Frazao, C., Khan, G., Zdanov, A., Tickle, I. J., Blundell, T. L. and Andreeva, N. (1991) *J. Mol. Biol.* **221**, 1295–1309
- Newman, M. P., Watson, F., Roychowdhury, P., Jones, H., Badasso, M., Cleasby, A., Wood, S. P., Tickle, I. J. and Blundell, T. L. (1993) *J. Mol. Biol.*, in the press
- Rajagopalan, T. G., Stein, W. H. and Moore, S. (1966) *J. Biol. Chem.* **241**, 4295–4297
- Rich, D. H., Sun, E. T. O. and Ulm, E. (1980) *J. Med. Chem.* **23**, 27–33
- Šali, A., Veerapandian, B., Cooper, J. B., Foundling, S. I., Hoover, D. J. and Blundell, T. L. (1989) *EMBO J.* **8**, 2179–2188
- Sheldrick, G. (1982) in *Computational Crystallography* (Sayre, D., ed.), pp. 506–514, Clarendon Press Oxford
- Sielecki, A. R., Hayakawa, K., Fujinaga, M., Murphy, M. E. P., Fraser, M., Muir, A. K., Carilli, C. T., Lewicki, J. A., Baxter, J. D. and James, M. N. G. (1989) *Science* **243**, 1346–1350
- Sielecki, A. R., Fedorov, A. A., Boodhoo, A., Andreeva, N. S. and James, M. N. G. (1990) *J. Mol. Biol.* **214**, 143–170
- Singh, J. and Thornton, J. M. (1990) *J. Mol. Biol.* **211**, 595–615
- Strop, P., Sedlacek, J., Stys, J., Kalevskova, Z., Blaha, I., Pavlickove, L., Pohl, J., Fabry, M., Kostka, V., Newman, M. et al. (1990) *Biochemistry* **29**, 9863–9871
- Suguna, K., Padlan, E. A., Smith, C. W., Carlson, W. D. and Davies, D. R. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7009–7013
- Szelke, M., Leckie, B., Hallett, A., Jones, D. M., Sueiras, J., Atrash, B. and Lever, A. F. (1982a) *Nature (London)* **299**, 555–557
- Szelke, M., Jones, D. M. and Hallett, A. (1982b) *Eur. Pat. Appl. EP 45665*
- Tang, J. (1971) *J. Biol. Chem.* **246**, 4510–4517
- Tewkesbury, D. A., Dart, R. A. and Travis, J. (1981) *Biochem. Biophys. Res. Commun.* **99**, 1311–1315

- Thomas, K. A., Smith, G. M., Thomas, T. B. and Feldman, R. J. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4843–4847
- Tree, M., Atrash, B., Donovan, B., Gamble, J., Hallett, A., Hughes, M., Jones, D. M., Leckie, B., Lever, A. F., Morton, J. J. and Szelke, M. (1983) *J. Hypertens.* **1**, 399–403
- Umezawa, H., Aoyagi, T., Morishima, H., Matsuzaki, M., Harmada, M. and Takeuchi, T. (1970) *J. Antibot.* **23**, 259–262
- Veerapandian, B., Cooper, J. B., Šali, A. and Blundell, T. L. (1990) *J. Mol. Biol.* **216**, 1017–1029
- Veerapandian, B., Cooper, J. B., Šali, A., Dominy, B. W., Hoover, D. J. and Blundell, T. L. (1992) *Protein Sci.* **1**, 322–328
- Wlodawer, A., Miller, M., Jaskólski, M., Sathyanarayana, B. K., Baldwin, E., Weber, I. T., Selk, L. M., Clawson, L., Schneider, J. and Kent, S. B. H. (1989) *Science* **245**, 616–621

Received 14 May 1992; accepted 8 July 1992