A structural comparison of 21 inhibitor complexes of the aspartic proteinase from *Endothia parasitica*

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Abstract

The aspartic proteinases are an important family of enzymes associated with several pathological conditions such as hypertension (renin), gastric ulcers (pepsin), neoplastic disease (cathepsins D and E), and AIDS (HIV proteinase). Studies of inhibitor binding are therefore of great importance for design of novel inhibitors for potential therapeutic applications. Numerous X-ray analyses have shown that transition-state isostere inhibitors of aspartic proteinases bind in similar extended conformations in the active-site cleft of the target enzyme. Upon comparison of 21 endothiapepsin inhibitor complexes, the hydrogen bond lengths were found to be shortest where the isostere (P_1 - P_1') interacts with the enzyme's catalytic aspartate pair. Hydrogen bonds with good geometry also occur at P_2' , and more so at P_3 , where a conserved water molecule is involved in the interactions. Weaker interactions also occur at P_2 , where the side-chain conformations of the inhibitors appear to be more variable than at the more tightly held positions. At P_2 and, to a lesser extent, P_3 , the side-chain conformations depend intriguingly on interactions with spatially adjacent inhibitor side chains, namely P_1' and P_1 , respectively. The tight binding at P_1 - P_1' , P_3 , and P_2' is also reflected in the larger number of van der Waals contacts and the large decreases in solvent-accessible area at these positions, as well as their low temperature factors. Our analysis substantiates earlier proposals for the locations of protons in the transition-state complex. Aspartate 32 is probably ionized in the complexes, its charge being stabilized by 1, or sometimes 2, hydrogen bonds from the transition-state analogues at P_1 . The detailed comparison also indicates that the P_1 and P_2 residues of substrate in the ES complex may be strained by the extensive binding interactions at P_3 , P_1' , and P_2' in a manner that would facilitate hydrolysis of the scissile peptide bond.

Keywords: aspartic proteinase; inhibitor complexes; transition-state analogues

Aspartic proteinases provide excellent subjects for studying molecular recognition because they bind a range of peptide substrates and inhibitors with varying degrees of specificity. Catalysis stems from 2 invariant aspartate residues, at positions 32 and 215 in porcine pepsin, which are thought to activate a bound water molecule to hydrolyze the scissile bond of the substrate. X-ray analyses have shown that these enzymes have deep and extended active-site clefts in which there are binding subsites [S_6 - S_1'] for 9 amino acid residues of the ligand (see Davies, 1990, for review). Inhibitors bind in an extended conformation making β -sheet hydrogen bonds with the enzyme (James et al., 1982; Foundling et al., 1987; Suguna et al., 1992). This class of enzymes includes not only the pepsin-like enzymes with approxi-

mately 330 amino acids, but also the retroviral proteinases, which are less than half the size and form noncovalent dimers, each monomer containing 1 essential aspartate (e.g., Lapatto et al., 1989; Wlodawer et al., 1989). In contrast, the structures of the pepsin-like aspartic proteinases are bilobal with a local 2-fold axis passing through the plane containing the active-site aspartates (Tang et al., 1978). The secondary structure comprises 5 distorted β -sheets, one of which is shared between the lobes of the enzyme (e.g., Blundell et al., 1985, 1990).

Major advances in the design of aspartic proteinase inhibitors stem from attempts to mimic the putative tetrahedral noncovalent transition state ($-C(OH)_2-NH-$). Studies of pepstatin indicated that the inhibitory character of the statine was attributable to its main-chain hydroxyl group ($-CHOH-CH_2-CO-$), which was shown, by X-ray analysis, to hydrogen bond to the central aspartates of the enzyme (Bott et al., 1982; James et al., 1982). Inhibitors with a statine as the central residue in a sequence that is otherwise similar to angiotensinogen proved to be useful inhibitors of renin (Rich et al., 1980). Substitution of a hydroxy-

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ethylene isostere (-CHOH-CH₂-) resulted in a very highly potent inhibitor (H-261) with an inhibition constant of 0.7 nM for renin (Szelke, 1985). In this compound, the -CHOH- function is assumed to mimic one hydroxyl of the geminal diol intermediate and the -CH₂- function mimics its tetrahedral nitrogen. Ketone analogues (-CO-CH₂-) are also highly potent (Szelke et al., 1982) and were further modified to difluoroketone compounds (-CO-CF₂-), which have even higher potency (Gelb et al., 1985) due to hydration to a geminal diol (-C(OH)₂-CF₂-) that mimics the transition state very closely.

X-ray analyses of 3 reduced peptide bond inhibitors complexed with endothiapepsin defined interactions clearly at P₁' to P₃' for the first time (Blundell et al., 1987; Cooper et al., 1987; Foundling et al., 1987). Our studies on statine-containing inhibitors confirmed that statine acts as a dipeptide analogue in the binding cleft (Cooper et al., 1989). Further details of inhibitor-enzyme interactions and their dependence on the nature of the transition-state isostere have come from more recent X-ray analyses of phosphostatine (Lunney et al., 1993), glycol (Lunney et al., 1993), norstatine (Dhanaraj et al., 1992), and aminoalcohol analogues (Cooper et al., 1988). The X-ray structures of 4 hydroxyethylene-containing renin inhibitors have been determined (e.g., Veerapandian et al., 1990; Cooper et al., 1992), as have the structures of 2 difluorostatone inhibitors (CP-81,282 and PD-135,040) as complexes with endothiapepsin (Veerapandian et al., 1992; Badasso, 1994) and yeast proteinase A (C. Aguilar, unpubl.). Both hydroxyls of these hydrated fluoroketone analogues interact with the catalytic apparatus in a manner indicating how the catalytic intermediate would be stabilized by the enzyme. It has been suggested that the tetrahedral intermediate is uncharged because, if the carboxyl of Asp 32 carries a negative charge instead, the latter can be stabilized by a full complement of hydrogen bonds donated by the geminal diol and surrounding protein atoms (Veerapandian et al., 1992). The structures of 7 statine-based inhibitors in which the statine side chain has been replaced with bulky phenylalanyl and cyclohexylmethyl groups to optimize affinity for renin have also been determined (Cooper et al., 1992; Bailey et al., 1993).

In this paper we compare the structures of 21 endothiapepsin inhibitor complexes solved at high resolution (see Fig. 1), facilitating a detailed description of their bound conformations, interactions with the enzyme, and implications for the catalytic mechanism. Most inhibitors in Figure 1 bind to endothiapepsin with *K_i* values in the 10–100 nM order of magnitude range. Structures and kinetic data for most of these inhibitors have been published in detail separately (see above and Fig. 1 caption). A thorough analysis of the nature and extent of conformational changes that occur in the enzyme on inhibitor binding has been made by Šali et al. (1989, 1992) who showed that the enzyme could be divided into 2 rigid bodies that undergo a small movement on complex formation. Structural comparisons of inhibitor complexes of other aspartic proteinases have been published previously (James et al., 1992; Suguna et al., 1992). Until now, a broad-based comparison of all the known endothiapepsin-inhibitor structures has not been made. In this paper we compare the hydrogen bond geometry, torsion angles, non-bonded interactions, temperature factors, and solvent accessibilities of the inhibitor residues. These analyses provide a measure of the extent of interaction with the enzyme at individual subsites and the degree to which the bound conformation of each inhibitor is restrained by the subsite residues and neighboring

residues in the inhibitors themselves. There are important implications, too, for the catalytic mechanism, because the interactions and conformations of these inhibitors suggest how substrates and transition-state intermediates may bind to the enzyme.

Results

Accuracy of the structures

The resolutions of most of the refined inhibitor structures vary from 2.3 to 1.6 Å (Fig. 1). All the structures have been least-squares refined to convergence using RESTRAIN (Haneef et al., 1985). The method of Read (1986) was used to estimate the RMS coordinate errors based on the X-ray data. RMS errors in the range of 0.2–0.3 Å were obtained, which are acceptable values for structures of this resolution range. One of the highest resolution inhibitor structures, H-261, solved at 1.6 Å, has an estimated coordinate error of 0.1 Å (Veerapandian et al., 1992) as measured by the method of Luzzati (1952). The electron density in these analyses is best for inhibitor residues between P₄ and P₂'. Residues beyond these, i.e., P₆, P₅, and P₃', are very well defined in some of the structures, such as H-261, but are poorly defined by the electron density in the lower resolution complexes. Hence, it is inevitable that the structures will differ in these outer pockets due to small errors of interpretation. Therefore, the most accurate comparisons can only be made for residues in the P₄ to P₂' range. Discussion of differences between inhibitor residues in the outer pockets (i.e., P₆, P₅, and P₃') is restricted to only those complexes that have good electron density for these residues.

General features of inhibitor binding

All of the inhibitors, solved as complexes with endothiapepsin, adopt an extended β-strand conformation (see Fig. 2) in the active-site cleft and make hydrogen bonds, involving their main chains, to each lobe of the enzyme. The side chains alternate on either side of the main chain and make hydrogen bonds and van der Waals interactions with the enzyme thus defining subsites, S₆–S₃'. An antiparallel 2-stranded β-hairpin loop comprising residues 71–82, known as the "flap," interacts with the central region of the inhibitor and shields the active site from the solvent region.

Hydrogen bond interactions

Conserved hydrogen bonds to the enzyme are observed between P₃ and P₃' of the inhibitors, whereas interactions at P₆–P₄ and P₄' do not appear to be extensive. The conserved interactions are shown in Figure 3 with a graph indicating their geometry at the inferred positions of the hydrogen atoms. A good hydrogen bond will have a donor-acceptor atom distance of between 2.6 Å and 3.0 Å. For a hydrogen bond involving an amide NH group, it is possible to calculate the position of the hydrogen atom using standard molecular geometry. The angle subtended by the donor and acceptor atoms at the hydrogen is another measure of the strength of the interaction because the closer this angle is to 180°, the stronger the interaction. Where possible, the geometries of the hydrogen bonds are indicated in Figure 3 (see caption).

'Reduced Bond'	Code (PDB)	Resolution (R-factor)	Ki (nM)
H-256 (2ER6)	2.0 (20%)	60
H-77 (1ER8)	2.0 (20%)	-
H-142 (4ER4)	2.1 (19%)	160
'Hydroxyethylene'
H-261 (2ER7)	1.6 (14%)	<1
CP-71,362 (3ER3)	2.0 (19%)	81
PD-125,967 (4ER1)	2.0 (14%)	242
'Aminoalcohol'
BW624 (5ER1)	2.0 (21%)	960

Fig. 1. The chemical formulae of the 21 renin inhibitors solved with endothiapepsin; the scissile bond surrogates are indicated by asterisks. The 3-dimensional structure analyses of the following complexes have been presented elsewhere: H-256 (Cooper et al., 1987); H-142 (Foundling et al., 1987); H-261 (Veerapandian et al., 1990); PD-125,967 (Cooper et al., 1992); BW624 (Cooper et al., 1988); pepstatin (Bailey et al., 1993); H-189 (Bailey et al., 1993); L-363,564 (Cooper et al., 1989); PD-130,693 (Lunney et al., 1993); L-364,099 (Cooper et al., 1989); PD-125,754 (Cooper et al., 1992); CP-69,799 (Šali et al., 1989); PD-130,328 (Lunney et al., 1993); CP-81,282 (Veerapandian et al., 1992); PD-135,040 (Badasso, 1994); PD-133,450 (Lunney et al., 1993). The following unpublished structures: H-77, CP-71,362, CP-80,794, PS1, and PS2 (S. Al-Karadaghi, M. Crawford, and A.M. Hemmings) are also included in the analysis. (*Continues on the next 2 pages.*)

'Statines'	Code (PDB)	Resolution (R-factor)	Ki (nM)
Pepstatin A (4ER2)	2.0 (16%)	0.5
H-189 (3ER5)	1.8 (15%)	1
L-363,564 (2ER9)	2.2 (17%)	40
PD-130,693	1.9 (16%)	69
'Phenylstatine' and 'Cyclohexylstatine' Analogues
L-364,099 (2ER0)	3.0 (28%)	420
PD-125,754 (1EED)	2.0 (15%)	16180
PS1	2.0 (19%)	-
PS2	1.6 (17%)	-

Fig. 1. Continued.

Other Statine Analogues		
Chemical Structure	Code (PDB)	Resolution (R-factor) Ki (nM)
	CP-69,799 (5ER2)	1.8 (16%) 270
	PD-130,328 (1ENT)	1.9 (17%) 110
	CP-81,282	2.0 (18%) 11
	PD-135,040	2.3 (18%) -
	PD-133,450	1.9 (17%) 6
	CP-80,794	1.6 (17%) -

Fig. 1. Continued.

The shortest hydrogen bonds made are those involving the -OH of the transition-state isostere, which interacts with the inner oxygen of Asp 32 and the outer oxygen of Asp 215. These distances are significantly shorter than the separation of the 2 aspartate carboxyl inner oxygens (Fig. 3). The other distances from the inhibitor -OH to the aspartate oxygens are significantly longer, and those to the outer oxygen of Asp 32 are comparable to some of the notably weak hydrogen bond interactions made at P₂ with the active-site flap of the enzyme. It is highly likely that protons reside between the closest oxygens. In the uncomplexed enzyme, similar interactions are observed with the bound active-site water molecule, suggesting that it too is held by hydrogen bonds with the outer oxygen of Asp 215 and the inner oxygen of Asp 32. For inhibitors with a single hydroxyl group, in the transition-state analogue, the -OH hydrogen is probably oriented toward Asp 32, which may be negatively charged in the complex. The outer oxygen of Asp 215 would then be protonated and could donate a hydrogen bond to the

transition-state analogue hydroxyl oxygen. An alternative tautomeric form in which the hydroxyl's hydrogen is oriented toward the outer oxygen of Asp 215 is geometrically less satisfactory because it involves an unfavorable torsion angle about the hydroxyl's C-O bond. The interactions made by the geminal diol analogues (CP-81,282 and PD-135,040) indicate that they probably donate 2 hydrogen bonds to the negatively charged Asp 32 carboxyl. Information of this nature has been used to develop detailed mechanistic proposals (Suguna et al., 1987; James et al., 1992; Veerapandian et al., 1992). In terms of interactions with the aspartate diad, the most notable outliers are the phosphostatine (Lunney et al., 1993) and aminoalcohol analogues (Cooper et al., 1988), which are not thought to be very close analogues of the transition state. With the 3 reduced bond inhibitors, there appears to be a geometrically good hydrogen bond formed between the isostere nitrogen and the outer oxygen of Asp 215. This suggests that a similar interaction between the nitrogen of a substrate's scissile bond and the as-

Fig. 2. A superposition of the 21 inhibitors solved with endothiapsin. The inhibitor complexes were least-squares superimposed using the program XS6 (Šali & Blundell, 1990) to optimize the fit of the enzyme C α atoms. The structures are most highly conserved in the region P₄–P₂' inclusive. The P₂ histidine side chains adopt a range of conformations depending mainly on the nature of the P₁' side chain.

partate pair may play an important role in stabilizing the catalytic intermediate. It has also been suggested (Suguna et al., 1987) that a strained hydrogen bond between the P₂ carbonyl and the scissile bond nitrogen may stabilize the intermediate. In the reduced bond inhibitors, the equivalent interaction is rather variable but has reasonable geometry at the inferred proton position.

The P₃ position of the inhibitors forms strong hydrogen bonds with Thr 219 and to a well-ordered water molecule. These 3 interactions (see Fig. 3) are highly conserved and have good geometry in most complexes including that of PD-125,754, where the P₃ >C=O is replaced by a >CHOH function. The importance of the bound water in this interaction is suggested by one inhibitor (PD-135,040), where a blocking group at P₄ prevents the P₃ residue from interacting strongly with Thr 219, but a strong hydrogen bond to the water remains. This water is held in place by 2 hydrogen bonds to the enzyme, one of them involving the main-chain nitrogen of residue 220. This water interaction is also present in inhibitor complexes with other aspartic proteinases such as mouse renin (DeAlwis et al., 1994). In contrast, the P₂ position forms much weaker bifurcated interactions with the active-site flap of the enzyme. As can be seen from Figure 3, these interactions are less well conserved than those at P₃, and their geometries are often poor, both in terms of donor–acceptor distance and the angle subtended at the inferred proton position. For example, the hydrogen bond between the side chain of Asp 77 and the main-chain >NH at P₂ only occurs in 9 of the 21 complexes and has bad geometry in most of these. This is partly because the side-chain Asp 77 appears to adopt different conformations in the complexes. For example, in the complex with inhibitor PD-125,967, which has a bulky naphthyl moiety at P₃, steric effects cause the Asp 77

side chain to move significantly from its position in the native enzyme. In contrast, 2 well-conserved and pseudo-diad-related hydrogen bonds involving the main-chain carbonyls of Gly 34 and Gly 217 interacting with the >NH groups of P₁ and P₂' have reasonable donor–acceptor distances in all complexes. However, the interaction involving P₂' and Gly 217 appears to have consistently better geometry at the inferred proton position. The interaction between the P₁' carbonyl and the main-chain NH of Gly 76, in the flap, also appears to have better geometry than interactions between the flap and the P₂ carbonyl. Because most inhibitors do not extend to the P₃' position, the interaction between >C=O of Ser 74 and the >NH at P₃' has only been defined in 6 complexes. However, this interaction appears to have particularly good geometry at the inferred hydrogen position (see Fig. 3). Clearly, because substrates will extend to P₃' and beyond, interactions on the prime side of the active-site cleft may play a more significant role in stability of the complex than previously thought.

Main-chain conformation

The torsion angles for residues at P₁ and P₁' are not strictly comparable to standard amino acids due to the novel chemistry of the transition-state analogues. The main-chain ϕ and ψ torsion angles of the inhibitors all lie within the β -sheet region of the Ramachandran plot (Fig. 4A), reflecting their extended conformations. The only exceptions arise at the P₁' positions of statine residues, which appear to occupy a disallowed region of the Ramachandran plot with positive ϕ angles. Here the main-chain methylene group of statine has been treated as a distinct P₁' residue because we have previously shown that statine is a dipeptide analogue extending from P₁ to P₁' (e.g., Cooper et al., 1989). Pseudo- ϕ , ψ , and ω torsion angles were therefore measured for all statine inhibitors at the P₁' position by treating the main-chain methylene as a C α atom. Consequently, P₁ ω angles have not been measured for statine. Interestingly, the P₂' part of the statine residue mimics glycine, which obviously can occupy regions of the Ramachandran plot disallowed to other L-amino acids.

For inhibitor residues at the periphery of the active site, such as P₅ and P₃', which may be loosely bound and/or poorly defined in the X-ray analysis, the ϕ and ψ angles are quite spread out in the β -sheet region. However, the ϕ and ψ angles for most residues of the inhibitors seem to cluster, often quite tightly, in distinct regions of the Ramachandran plot, depending on their position in the inhibitor. This is shown in Figure 4B, where the mean ϕ and ψ angles for P₅ to P₃', together with their standard deviations, are shown. Presumably, this reflects the constrained environments of the inhibitor residues imposed by hydrogen bonding and van der Waals interactions with the active-site cleft. In addition, there appear to be 2 groups of inhibitor residues that have significantly different ϕ and ψ angles: one formed by P₄, P₃, P₁', and P₂' (group 1), and the other formed by P₅, P₂, P₁, and P₃' (group 2) (see Fig. 4B). Group 1 (mean ϕ , ψ = -86.3° , 143.2°) includes P₄, which is dominated by proline in the inhibitors and therefore restricted to ϕ and ψ angles close to the ideal poly-L-proline values. Group 2 residues (mean ϕ , ψ = -123.5° , 75.5°) lie in part of the β -sheet region, which, for proteins in general, is quite sparsely populated. In contrast, residues in group 1 reside in a commonly populated area and are closer to the theoretical energy minimum of the β -sheet region than those

Fig. 3. Conserved hydrogen bond interactions with inhibitors are shown (top) with a graph showing the donor-acceptor distances for the 21 inhibitor complexes (+ indicates an O...H-N interaction, ⊕ indicates an O...H-N interaction with an angle at the proton greater than 160°, and x indicates an O...H-O interaction). Some of the interactions with the aspartate carboxyls will be van der Waals contacts rather than hydrogen bonds (see text for discussion of proton locations).

in group 2, which are nearer the "bridging region" between α -helix and β -sheet. The apparently less favorable ϕ and ψ angles can be explained for the P₁ position because these residues have transition-state isosteres instead of standard peptide chemistry. These analogues have a higher degree of torsional freedom due to the absence of a planar peptide group. It has been suggested that this torsional freedom is essential to optimize interactions with the catalytic center of the enzyme (Cooper et al., 1987; Pearl, 1987; Suguna et al., 1987) and that during cataly-

sis the enzyme may strain bound substrates toward the geometry found in complexes with these transition-state isosteres. The observation that the P₂ residues occupy the same part of the β -sheet region suggests that they may also be strained to some extent upon binding. For P₅ and P₅' residues, which also appear to occupy this region, the torsion angles may contain significant errors due to poor definition and/or weak binding, as reflected in the large spread of ϕ and ψ angles. However, the P₂ position is always well defined in the electron density, suggesting that this

Fig. 4. **A:** A Ramachandran plot for the 21 inhibitors with residue position codes as shown. **B:** The mean ϕ, ψ angles for each position of the inhibitors with their standard deviations shown. **C:** The ω angles for each residue position of the inhibitors with vertical bars indicating the standard deviations and circles showing the mean values.

residue may indeed be strained in complex. This was also inferred from the observation that the ω angle for the P_2 residue can deviate significantly from 180° (Šali et al., 1989). Of the residues that are least strained (group 1), those at P_3 , P_1' , and P_2' are always held by hydrogen bonds and numerous van der Waals interactions with the enzyme (see Fig. 3). It may be that strong binding at these pockets is essential for distortion of the scissile bond at P_1 and the more loosely bound residue at P_2 .

It is conceivable that the distribution of inhibitor ϕ and ψ angles may reflect the most stable structures of the ligands themselves rather than conformations imposed by the surrounding cleft. There are several crystal structures for uncomplexed peptides that mimic the P_5 – P_2 residues of renin's natural substrate (Precigoux et al., 1989). Such peptides form type-I β -turns,

whereas the equivalent residues are extended when bound to the aspartic proteinase cleft. There are also crystal structures for peptide inhibitors extending along both sides of the transition-state analogue, but the statine residue at P_1 adopts a turn conformation (Precigoux, 1991) that is different from its more extended structure in the enzyme complexes. Hence, the global energy minimum for an uncomplexed inhibitor may involve a turn conformation because this will maximize the intramolecular hydrogen bonding.

The main-chain ω angles for all 21 inhibitors are shown in Figure 4C. The P_1 pseudo ω values deviate grossly from 180° , again reflecting the nonpeptide nature of the transition-state isosteres. At all other residues of the inhibitors the mean ω angle is within 1 standard deviation of 180° , although distortions of the pep-

tide groups are consistently in the same direction at each residue position. Omega angles are usually restrained in refinement to maintain planarity of the peptide bond. Thus, for a comparison of ω angles to be meaningful, all structures should be at the same resolution and have had the same geometric restraints applied during refinement. Because these structures have been solved at resolutions close to 2.0 Å, using similar refinement strategies, these criteria are largely, but not fully, met. At the P_2 position, the distribution of ω values is bimodal, with 1 group having ω values close to 180°. The other group has a mean ω value of around -167°. The group of P_2 ω values very close to 180° probably has resulted from the use of strong planarity restraints during refinement or rebuilding. The group of ω values, which deviate by around 13° from the ideal value, may conceivably be due to strain occurring within the bound inhibitor at the P_2 position, as suggested by the ϕ and ψ values (see preceding paragraph). However, inspection of high-resolution structures of inhibitor complexes of other aspartic proteinases does not indicate distortion of the P_2 peptide planarity. In addition, the RMS deviation of ω from 180° in the endothiapepsin moiety of the complexes analyzed here can be as high as 8–10°. Previously, it had been suggested that distortion of the peptide bond at P_2 will improve the hydrogen bond between the P_2 carbonyl and the leaving amino group at P_1' (Šali et al., 1989). The analogous hydrogen bond occurs in reduced bond inhibitor complexes where the $-\text{CH}_2\text{-NH}_2^+$ amino group do-

nates a proton to the $P_2 >\text{C}=\text{O}$ group, often with good geometry (see Fig. 3). Hence, strain imposed on the P_2 residue could have a direct effect at the catalytic center of the enzyme. However, the magnitude of deviations in ω is not sufficient to prove that strain at P_2 is occurring, but the consistent way in which the peptides appear to be distorted (Fig. 4C) suggests that slight twisting of the ligand's peptide groups may occur on binding.

Side-chain conformation

In general, the χ_1 angles for the inhibitors are highly conserved from P_4 to P_2' (Fig. 5). Many inhibitors have proline at P_4 and therefore have a very restricted range of χ_1 and χ_2 angles. In the outer regions of the inhibitors there is a wide distribution of χ_1 and χ_2 angles, which reflects both the disorder in the structures and, in the case of P_3' , the wide range of amino acid residues incorporated at this position. Very tight conservation of χ_1 angles occurs at the P_3 and P_1 positions, whereas those of P_2 and P_1' are much less conserved.

The residues incorporated at P_1 of the inhibitors usually have leucine-like or cyclohexylmethyl side chains. These have been found to give good affinity for the S_1 pocket of renin, which is very hydrophobic. This pocket in endothiapepsin makes the most extensive interactions with the inhibitors, there being typically 40 van der Waals interactions with cyclohexylmethyl residues (distance less than 4.0 Å) and the capacity to form a

Fig. 5. A: The distribution of χ_1 angles observed for each position of the inhibitors. B: The distribution of χ_2 angles.

number of hydrogen bonds with the catalytic aspartates and to Gly 217 $>C=O$ (see Fig. 3). Not surprisingly for such a tightly bound residue, the P_1 χ_1 and χ_2 angles are highly conserved for all types of amino acid with mean values of -58° ($\pm 14^\circ$) and 173° ($\pm 13^\circ$).

At P_3 the high degree of conservation of χ_1 is largely due to phenylalanine residues that are present in 15 of the 21 inhibitors. Branched side chains at this position, such as Thr and Val, adopt rather different χ_1 values, although one of the $C\beta$ substituents is always oriented in the same direction as the phenylalanines. The S_3 pocket of endothiasepsin is filled rather well by phenylalanine, which forms numerous van der Waals interactions with the enzyme. The mean χ_1 value for phenylalanine at P_3 is -78° with a standard deviation of only 7° . In contrast, the χ_2 angle varies considerably more (standard deviation 40°). Experimental error cannot be responsible for this effect because the orientation of the aromatic ring is always well defined by the electron density. This intriguing effect in such a closely packed binding pocket is probably due to interactions with the adjacent side chain at P_1 . In complexes where the S_1 pocket is occupied by a leucine side chain, the P_3 phenylalanyl group is oriented such that the plane of the ring points toward the P_1 residue. However, when the S_1 pocket is occupied by a cyclohexylmethyl group, the phenylalanyl ring at P_3 adopts a different range of orientations about χ_2 to minimize unfavorable contacts with the bulky P_1 side chain.

Despite the P_2 residue being histidine in 11 of the 21 complexes there is an apparent lack of conservation of χ_1 at this position. Instead, there are indications of a bimodal distribution with mean χ_1 angles of -49° ($\pm 7^\circ$) and -165° ($\pm 22^\circ$), which correspond to the *gauche*⁺ and *trans* conformations, respectively. This effect is apparently due to an interesting interaction with P'_1 . In general, when the S'_1 pocket is occupied by small side chains such as valine or is left empty such as with statine analogues, which have the equivalent of glycine at P'_1 , the histidine at P_2 adopts a *trans* conformation, allowing its imidazole ring to partly occupy the adjacent S'_1 subsite. However, when the P'_1 residue has a larger side chain, such as a leucyl group, the histidine at P_2 has the *gauche*⁺ conformation and is oriented away from the S'_1 pocket into a more exposed, polar environment, where it can interact with the side chain of Asp 77. The electron density at P_2 of inhibitor CP-69,799 was bifurcated, indicating that both the *trans* and *gauche*⁺ conformations were occurring in dynamic or static equilibrium. This compound has an interesting urea-like planar group in which the P'_1 $C\alpha$ atom is replaced by a trigonal nitrogen. This reduces the extent to which the leucine side chain at P'_1 fills the S'_1 pocket (Šali et al., 1989) and may cause the 2 conformers of the P_2 side chain to have similar stabilities. The nature and hence positioning of the transition-state analogue with respect to the catalytic aspartates may also have an effect on the P_2 conformation. This is suggested by 2 compounds, H-142 (reduced bond analogue) and H-261 (hydroxyethylene analogue) which, between P_3 and P'_3 , differ only in their transition-state isosteres. With H-142, the P_2 histidine is in the *gauche*⁺ conformation, whereas in H-261, it is *trans*, i.e., pointing toward the S'_1 pocket. Because with reduced bond analogues the interactions with the catalytic aspartates are primarily with the isostere's $>NH_2^+$ function, these compounds appear to be shifted in the aminoterminal direction relative to hydroxyethylene compounds where interactions are mainly with the isostere's $>CHOH$ function.

Hence, despite there being a small side chain at P'_1 , the histidine at P_2 of H-142 may be unable to interact with the S'_1 pocket due to overall positioning of the P_1 - P'_1 residues causing the observed *gauche*⁺ conformation to be more favorable. In contrast, the *trans* conformation observed with the H-261 complex may arise from the S'_1 pocket being less well occupied by the P'_1 valine due to a shift of the P_1 - P'_1 unit in the primeward direction. These effects are only possible due to the loose binding environment provided by the S_2 pocket. This is exemplified by the inhibitor PD-130,693, where a modified lysine at P_2 occupies a position almost exactly midway between the 2 conformations adopted by the histidine residues. Interestingly, the same bifurcation also occurs with glutamate at P_2 . One inhibitor (H-256) has a bulky phenylalanine at P'_1 , which appears to force the P_2 glutamate away from the S'_1 pocket. In contrast, with a phenylstatine-containing inhibitor (PS1), the glutamate at P_2 is clearly oriented toward the vacant S'_1 subsite.

Van der Waals contacts

The strength of van der Waals contacts made between the enzyme and inhibitor moieties will depend on the number of interactions in the binding subsites, the contact distances, and the polarity of the interacting atoms. An analysis of the number of van der Waals contacts made by inhibitor residues with either the enzyme or other residues of the inhibitor excluding contacts with water molecules was made (Fig. 6). For most inhibitors, the P_1 residue makes the largest number of van der Waals interactions, there being typically 40 contacts shorter than $4.0 \text{ \AA}$ at this subsite. This is in accordance with the hydrophobic nature of the ligand side chain and some S_1 subsite residues (Table 1). At P_1 the inhibitors have either leucyl-like side chains or the 6-membered ring systems of the phenylalanyl or cyclohexylalanyl analogues. Inhibitors with such bulky cyclic side chains at P_1 make between 30 and 40 contacts shorter than $4.0 \text{ \AA}$, whereas the smaller leucyl-like side chains make between 20 and 30 such contacts. Hence, the bulkier side chains make a slightly larger number of contacts with the enzyme primarily due to extra interactions with the side chain of Phe 111. The spatially adjacent S_3 pocket provides a smaller number of interactions than S_1 but a significantly greater number than the S_2 or S'_1 pockets, which have a more solvent-accessible character in the complexes. For inhibitors with histidine at P_2 , there appears to be some correlation between the conformation adopted by the side chain about χ_1 and the number of van der Waals contacts formed. In complexes where the histidine side chain is oriented toward S'_1 , a slightly greater number of interactions, on average, are made than when the side chain adopts the more exposed *gauche*⁺ conformation. Surprisingly, the P_4 and P'_2 residues appear to make a relatively small number of contacts with the enzyme and residues bound at the outer extremities of the active-site cleft can interact more extensively. Clearly, in terms of van der Waals interactions, binding to the enzyme is dominated by the P_3 - P'_1 region of the ligand with P_1 and P_3 playing the most dominant role due to the hydrophobic nature of both enzyme and ligand at these subsites.

Solvent-accessible contact areas

Binding to the enzyme results in a loss of solvent-accessible contact area (calculated by the Lee & Richards [1971] algorithm)

Table 1. Residues that constitute the enzyme pockets of endothiapepsin

S ₆	S ₅	S ₄	S ₃	S ₂	S ₁	S' ₁	S' ₂	S' ₃
Leu 10	Leu 10	Asp 12	Ile 7	Tyr 75	Asp 30	Gly 34	Ser 35	Tyr 75
Phe 275	Asp 12	Thr 219	Asp 12	Gly 76	Asp 32	Tyr 75	Ile 73	Ile 299
Gly 276		Leu 220	Ala 13	Asp 77	Tyr 75	Gly 76	Ser 74	
Pro 277		Tyr 222	Asp 114	Gly 217	Asp 77	Ile 213	Tyr 75	
Ile 278			Ile 117	Thr 218	Ser 79	Asp 215	Leu 128	
Phe 284			Gly 217	Tyr 222	Phe 111	Gly 217	Thr 130	
			Thr 218	Ile 297	Leu 120	Thr 218	Phe 189	
			Thr 219	Ile 301	Asp 215			
					Gly 217			
					Thr 218			

at all residue positions of the inhibitors (Fig. 7A). There is an obvious bimodal distribution at the P'₁ position. This arises from the statine-containing inhibitors, which appear to lose much less accessible area than others on binding. For these inhibitors, the P'₁ moiety of statine mimics glycine and would therefore lose less accessible area than other amino acids on binding. The greatest loss of solvent-accessible area occurs at the P₁ position where very nearly 100% of the free ligand's solvent-accessible area is lost upon formation of the complex (see Fig. 7B) due to this residue being completely surrounded by the enzyme and other inhibitor residues. At this position, there are indications of a bimodal distribution in the lost accessible area (Fig. 7A). This arises because P₁ residues with cyclohexylalanyl or phenylalanyl side chains lose around 60 Å² of solvent-accessible area on binding compared with around 50 Å² for residues with leucyl side chains. There also appears to be a bimodal distribution at the P₂ position, which arises from the different orientations adopted by the histidine side chains at this position. When the P₂ histidine binds in the *trans* conformation it partly

occupies the S'₁ pocket and around 40 Å² of solvent-accessible area is buried. This compares with a loss of typically 30 Å² when the P₂ histidine adopts the more exposed *gauche*⁺ conformation (see above).

The P'₂ position seems to make a very significant contribution to the binding in terms of buried surface-accessible area. At this position there appears to be a bimodal distribution of the solvent-accessible area in the complex (Fig. 7C). Most inhibitors have small hydrophobic side chains at this position such as leucine, valine, and isoleucine. For inhibitors that possess a P'₃ residue, certain residues at P'₂ (Leu, Ile, and Val) are well buried in the complex and have solvent accessibilities close to or lower than 10 Å². In contrast, when a P'₃ residue is not present in an inhibitor, the solvent accessibility of the P'₂ residue is significantly higher (typically greater than 20 Å²). Some inhibitors have lysine or arginine residues at P'₂, which retain the hydrophobic interactions with the enzyme through their aliphatic moieties, yet have accessibilities in excess of 20 Å² due to the exposed positions of their polar side-chain groups.

Typically 80% of the solvent-accessible area is lost for the P₃–P'₂ positions upon complex formation. In contrast, much smaller percentage losses occur at the P₅, P₄, and P'₃ positions, reflecting their lesser contribution to the binding energy. Surprisingly, P₆ residues bury a larger amount of surface-accessible area on binding due to interactions with a hydrophobic patch in the C-terminal domain consisting of Phe 275, Ile 278, and Phe 284, although the inhibitor electron density at these “outer” subsites is often poor and the coordinate errors will be largest for these residues.

Temperature factors

Refined temperature factors, or *B*-values, in high-resolution X-ray structures indicate the extent of local static or dynamic disorder within the molecules of the crystal. Figure 8 shows the temperature factors for the inhibitor amino acids segregated into residue-averaged main-chain and side-chain *B*-values. As can be seen, the P₁ position has the lowest mean *B*-values, an effect that is very pronounced for the buried side chain of this residue (Fig. 8C). In contrast, the P₆, P₅, and P'₃ positions have very high *B*-values for both main-chain and side-chain atoms, reflecting the weaker binding interactions at these subsites. Low main-chain *B*-values are observed for positions P₃–P'₁, probably due to their extensive hydrogen bonding interactions with the en-

Fig. 6. The number of van der Waals contacts shorter than 4.0 Å at each residue position in the inhibitors.

Fig. 7. **A:** The reduction in solvent-accessible contact area (Å²) on formation of the complex, calculated using the Lee and Richards (1971) algorithm. The accessibility changes are calculated by subtracting the values for the complex from those of free ligand, i.e., it is assumed that binding to the enzyme occurs without conformational change of the ligand. **B:** The reduction in solvent-accessible area expressed as a percentage of the free ligand's accessibility. **C:** The solvent-accessible area of inhibitor residues in the complexes (Å²).

zyme. The P₃ and P₂ positions display a much larger scatter in the side-chain temperature factors, which is in accord with the diversity of side-chain conformations observed at these positions (see above). However, there appears to be no clear correlation between the mean side-chain *B*-value and the conformation adopted about χ_1 and χ_2 at these positions. In summary, the P₃–P₂' residues, in particular their main-chain atoms, appear to be highly ordered in the complexes with the P₁ side chains having by far the lowest mean side-chain *B*-values.

Discussion

Fungal aspartic proteinases have a relatively low substrate specificity, in accordance with their open and extended active-site clefts. In contrast, human renin has only 1 known natural substrate (angiotensinogen) and peptide mimics of this have to be a minimum of 8 residues in length for hydrolysis to occur. Dhanaraj et al. (1992) showed that the active-site cleft of renin encloses bound ligands to a greater extent than its fungal homo-

logues. This was attributed to domain movements and shifts in secondary structure elements and loops, some of which are rigidified by substitution of proline residues. In the present analysis, we have compared the binding of peptide ligands to a relatively nonspecific aspartic proteinase, endothiapepsin. All inhibitors adopt strikingly similar main-chain conformations, particularly in the central region of the active-site cleft (P₄–P₂'), due to the strongly directional hydrogen bond interactions made with the enzyme. At some subsites, the side-chain conformation of the bound ligand is tightly restrained, whereas at others, subtle interactions between inhibitor residues cause a range of different side-chain conformations to be adopted.

Between residues P₃ and P₃', with the exception of P₂, the hydrogen bond interactions with the enzyme have good geometry. At P₃, the interactions involve a water molecule that is conserved in all other aspartic proteinase inhibitor complexes currently in the Brookhaven Protein Data Bank (Bernstein et al., 1977) due to its interactions with the main chain of the enzyme and the inhibitor. Hydrogen bond interactions at P₃' have good

Fig. 8. **A:** The mean temperature factors ($\text{\AA}^2$) for each residue of the inhibitor complexes. **B:** The mean main-chain temperature factors ($\text{\AA}^2$). **C:** The mean side-chain temperature factors ($\text{\AA}^2$).

geometry in the complexes but are rarely seen because most inhibitors are truncated at P_2' . Hydrogen bonds involving the P_1 - P_1' isostere have particularly good geometry, e.g., hydrogen bond lengths as short as 2.6 $\text{\AA}$ are consistently observed for all inhibitors having a hydroxylic transition-state analogue. This hydroxyl binds with interactions that are apparently similar to those of the active-site water molecule in the native enzyme, i.e., hydrogen bonds are most probably made with the inner oxygen of Asp 32 and the outer oxygen of Asp 215. For geminal diol inhibitors, the extra hydroxyl forms another short hydrogen bond with the outer oxygen of aspartate 32. Veerapandian et al. (1992) proposed that in the transition-state complex, these hydrogen bonds would stabilize a negatively charged Asp 32 carboxyl. This mechanism was based on the key suggestion by Suguna et al. (1987) that, although the transition-state analogues displace the active-site water molecule, the more weakly bound substrate may not. Instead, as the substrate binds, the water molecule may be partly displaced to an appropriate position for nucleophilic attack on the scissile bond carbonyl. It is proposed that the Asp 215 carboxyl is negatively charged in the ground

state and may polarize the active-site water to nucleophilically attack the substrate to form the geminal diol intermediate. This would be stabilized by 2 hydrogen bonds with the Asp 32 carboxyl, which is negatively charged in the transition-state complex (James et al., 1992; Veerapandian et al., 1992). Concomitant with the initial activation of the active-site water, Asp 215 would become protonated. This proton can be transferred to the amino leaving group after inversion of the scissile bond nitrogen, allowing fission of the main-chain C-N bond. Simultaneously, Asp 32 will receive a proton from the geminal diol as the tetrahedral carbon reverts to the sp^2 configuration in the leaving carboxyl group.

In the 21 complexes, the P_4 , P_3 , P_1' , and P_2' residues are found to have main-chain torsion angles that are close to the theoretical energy minimum for β -sheets. In contrast, P_2 and P_1 occupy a region of the Ramachandran plot that is less commonly populated in globular proteins, presumably due to its higher potential energy. This effect can be explained for P_1 due to the nonstandard chemistry of the transition-state isosteres, but at P_2 , it may reflect strain on the ligand originating from

residues at P_4 , P_3 , P'_1 , and P'_2 , which are held in more stable conformations by extensive interactions with the enzyme. It has been shown that, when a substrate binds, it would be impossible for it to adopt the same conformation as an inhibitor without distortion of the P_1 - P'_1 peptide bond (Cooper et al., 1987; Pearl, 1987). Hence, strain exerted on the scissile peptide bond may play an important role in aspartic proteinase catalysis. Our analysis indicates that strain may occur at the loosely bound P_2 position, too. Šali et al. (1989, 1992) have suggested that strain exerted at the P_2 peptide group will improve the geometry of the hydrogen bond between the P_2 CO group and the leaving amino group.

Analyses of the van der Waals contacts, isotropic temperature factors, and buried solvent-accessible area indicate consistently that residues P_3 - P'_2 dominate the binding interactions with the enzyme, with the P_2 position making fewer contacts than either P_3 or P'_1 . The flexible nature of the binding interactions at S_2 is further indicated by our analysis of torsion angles, which shows that side chains occupying this pocket (principally histidine) may adopt very different conformations about χ_1 depending mainly on the bulk and positioning of the P'_1 residue. A similar effect, whereby the conformation about χ_2 of phenylalanines at P_3 depends on the size of the P_1 side chain, is also observed.

For endothiapepsin, the least potent inhibitor (see K_i values in Fig. 1) is PD-125,754, which lacks a P_2 side chain and has a hydroxyethylene isostere in place of the P_3 - P_2 peptide bond. Although the above analysis suggests that interactions at P_2 are less extensive than at other subsites, the absence of a side-chain and main-chain nitrogen at this position appears to weaken the binding quite significantly. The best inhibitors are generally those with hydroxyethylene, statine, glycol, or fluoroketone analogues at P_1 . Thus H-261, H-189, pepstatin, PD-133,450, and CP-81,282 all have binding constants in the 1–10 nM range. The phosphostatine and amino-alcohol analogues, PD-130,328 and BW624, respectively, have poorer binding constants, presumably because they are poor mimics of the transition state. BW624 also lacks residues in the P_4 - P_2 region, which are important for strong binding. H-142 has essentially the same amino acid sequence as H-261 (a hydroxyethylene analogue) from P_5 to P'_3 and yet has a 160-fold worse potency. H-142 possesses a reduced bond isostere, which is probably a poorer mimic of the catalytic intermediate due to the lack of a hydroxyl function. The inhibitors L-363,564 (a statine-based inhibitor) and L-364,099 differ only in their P_1 side chains, L-364,099 having a cyclohexylmethyl group at this position. We have noted that bulky side chains at P_1 appear to make the spatially adjacent side chain at P_3 twist around, perhaps to relieve unfavorable van der Waals contacts. Because L-364,099 has a 10-fold worse binding constant than L-363,564, the presence of a cyclohexylmethyl side chain at P_1 may have a detrimental effect on binding strength in endothiapepsin due to the interaction with P_3 (Cooper et al., 1989). In contrast, for renin, cyclohexylmethyl at P_1 improves the potency relative to smaller side chains. CP-69,799 and CP-71,362 differ only in that the P'_1 C α atom of CP-71,362 is replaced by a trigonal nitrogen in CP-69,799. This introduces a 7-membered plane into the inhibitor's main chain and reduces the extent to which the leucine side chain at P'_1 can occupy its binding pocket (Šali et al., 1989). In this inhibitor complex, the P_2 histidine apparently compensates for the poorer occupancy of S'_1 by adopting 2 conformations and in one of

these it partly fills the S'_1 pocket. Nonetheless, the binding constant for CP-69,799 is almost 4 times worse than that of CP-71,362.

In these analyses we have studied the interactions responsible for inhibitor and, by inference, substrate binding by endothiapepsin. The very strongly conserved nature of the ligand conformation and interactions in the central hydrophobic pockets of the binding cleft (S_4 , S_3 , S_1 , S'_1 , S'_2) are consistent with the fact that the substrate specificity of aspartic proteinases is usually greatest in these subsites. In contrast, the S_2 subsite is known from numerous studies to be of lower specificity, in accordance with its exposed character and the diversity of ligand side-chain conformations observed in our analyses. Similar effects have been observed in complexes of other fungal aspartic proteinases (James et al., 1992; Suguna et al., 1992) but the extent to which the enzymes of greater specificity, such as human renin, restrain the conformations of bound ligands remains to be established.

Methods

All inhibitor structures have been solved and least-squares refined at the Laboratory of Molecular Biology, Department of Crystallography, Birkbeck College (University of London) and details of most structure analyses have been published elsewhere (see Fig. 1 caption for references). As can be seen in Figure 1, most of the inhibitor complexes have been solved at resolutions of 2.0 Å or better and the coordinates for all complexes have been submitted to the Brookhaven Protein Data Bank (Bernstein et al., 1977). Solvent accessibilities were calculated using the Lee and Richards (1971) algorithm and graphical inspection of the structures was performed using FRODO (Jones, 1978) on an Evans and Sutherland PS390.

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