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Protein Backbone Engineering through Total Chemical Synthesis: New Insight into the Mechanism of HIV-1 Protease Catalysis☆

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Abstract—Total chemical synthesis by convergent chemical ligation was used to prepare a 'backbone engineered' 202-residue covalent dimer *asymmetric* form of the HIV-1 protease molecule. The Gly49-Ile50 peptide bond backbone –N(H)– atom, critically involved in H-bonding to substrates, was specifically replaced by an –O– atom *in one flap only*. The resulting enzyme analogue retained *full intrinsic activity*, demonstrating that enzyme–substrate hydrogen bonding at the Gly49-Ile50 peptide bond in only a *single* flap is sufficient for normal catalytic function.

Introduction

Studies of protein function are acquiring new relevance in the emerging post-genome era. DNA sequence data specifying hundreds of thousands of ribosomally translated polypeptides is now known. Functions have been tentatively ascribed to many of the corresponding proteins based on analogy, i.e. by sorting coded proteins into known families based on amino acid sequence similarities. At the same time, the three-dimensional structures of a wide variety of proteins are being determined at an ever increasing rate. Despite this, numerous questions of fundamental significance remain: e.g. How does the polypeptide sequence define the unique folded three-dimensional structure of a protein? How does the protein's chemical structure give rise to its properties, such as specific ligand binding or enzyme catalysis? Questions such as these must be elucidated if we are ever to understand the chemistry of life.

Understanding the molecular basis of protein function is most directly achieved by systematic variation of chemical structure, correlated with the observed effects of such changes on protein function. Such an approach could be optimally undertaken if proteins were accessible by total chemical synthesis. This fundamental insight can be attributed to the great organic chemist Emil Fischer, early in the twentieth century;[1] since that time, the total chemical synthesis of proteins has been one of the primary goals of organic chemistry.[2] Despite notable early successes in

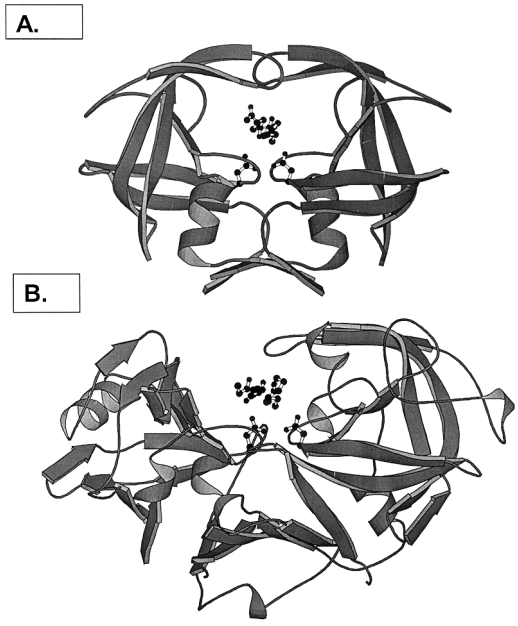

Figure 1. Role of the flap(s) in enzyme–ligand binding in aspartyl proteinases, as inferred from crystallography. (A) Molscript[47] representation of the HIV-1 protease bound to a substrate-derived inhibitor (from Ref. 10a). (B) Molscript representation of the crystallographic structure of a pepsin-like protease (rhizopuspepsin) bound to a reduced isostere inhibitor.[14a] This cell-encoded aspartyl proteinase is a single polypeptide chain, two domain molecule and has only a single flap which interacts directly with the active site ligand.

☆ The experimental work that forms the basis of this paper was performed while the authors were at The Scripps Research Institute, La Jolla, California.

Keywords: chemical protein synthesis; chemical ligation; unprotected peptide segments; thioester; backbone engineering; HIV-1 protease; enzyme mechanism; hydrogen bond.

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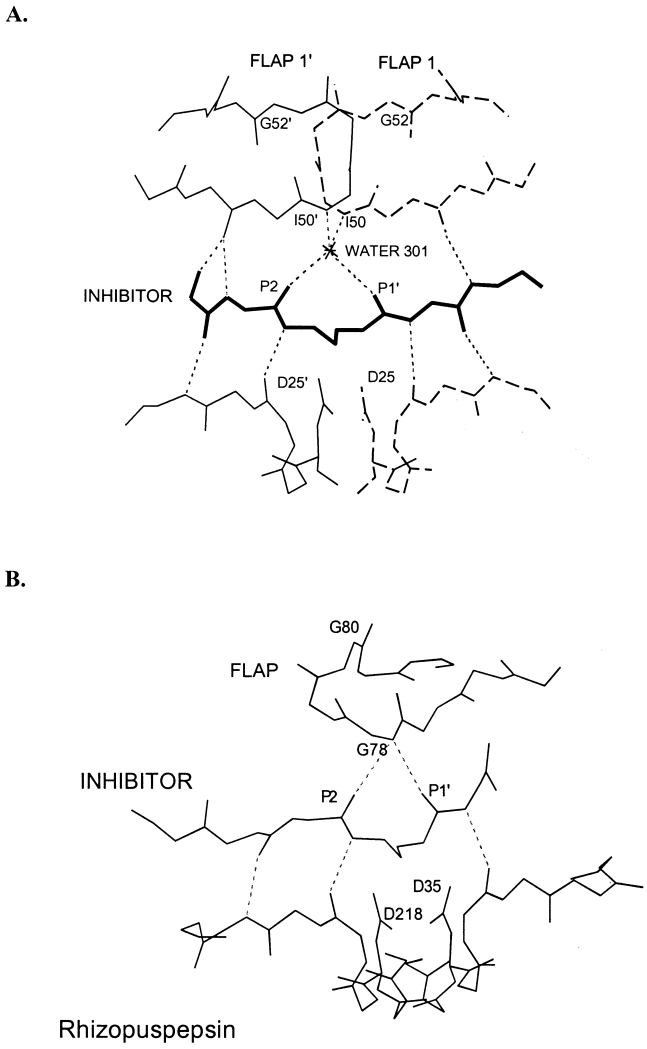

Figure 2. Detailed interactions. The flap structures contribute hydrogen bonds to the P2 and P1′ carbonyls of the inhibitor. (A) In HIV-1 PR, one enzyme amide (–NH–)-to-substrate (P2 or P1′) carbonyl hydrogen bond is contributed by each flap, and the hydrogen bonding is *mediated* by a specific internally bound water molecule.[10] These hydrogen bonds have been shown to play an important role in the catalytic mechanism of HIV-1 PR.[18] (B) In the pepsin-like cell encoded proteases, the single flap contributes both the hydrogen bonds to the substrate carbonyls, by direct interaction of the flap with the substrate. Shown is the pepsin-like protease from rhizopus.[14a]

isolated instances, the goal of routine, reproducible synthetic access to the world of proteins has only recently become a practical reality, thanks to major innovations in protein chemistry.

A quantum leap in the ability to produce proteins by total synthesis has occurred based on the use of the 'chemical ligation' principle: the chemoselective reaction of *unprotected* peptide segments.[3] Chemical ligation has enabled the routine, reproducible total chemical synthesis of a variety of proteins[4] with molecular weights already in excess of 20 kDa.[5] At the same time, a complimentary protein analytical technique, electrospray mass spectrometry (ESMS),[6] has contributed to the full realization of the potential of the chemical ligation approach to total protein synthesis. Using ESMS, in a matter of minutes the molecular mass of unprotected polypeptides and synthetic proteins can be determined with extraordinary precision, thus routinely providing high accuracy covalent structural data in the course of a synthesis.

The HIV-1 protease molecule

One of the success stories in the application of total chemical synthesis to the elucidation of key aspects of the molecular basis of protein function has been the HIV-1 protease (HIV-1 PR). This virus-encoded enzyme is essential for the replication of the AIDS virus,[7] and for this reason HIV-1 PR has been the target of intensive efforts to develop enzyme inhibitors as therapeutic agents to treat AIDS.[8] Total chemical synthesis by highly optimized stepwise SPPS was used to prepare protein for the elucidation by X-ray crystallography of the original correct structure of the HIV-1 PR molecule[9] and the first structures of the enzyme complexed with examples of the canonical classes of inhibitors.[10a–c] These structural data were made widely available to the research community and were a key contribution to the development of the 'protease inhibitor' class of therapeutics, an essential component of the highly effective combination drug therapy for AIDS.[11]

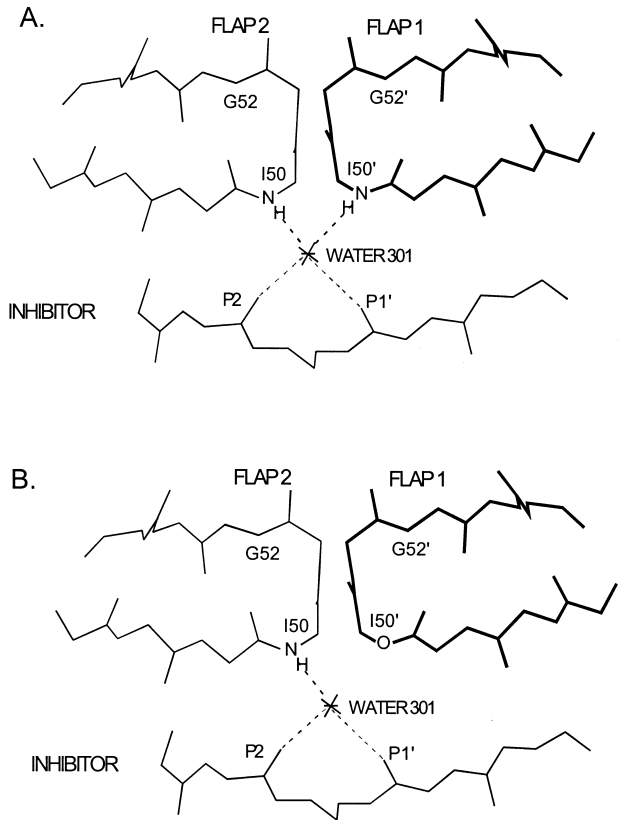

Figure 3. Design of the backbone-engineered enzyme to test the function of the flaps. (A) (Top) The flaps of the native backbone HIV-1 PR molecule, showing the experimentally observed[10] hydrogen bonds from the Ile50 amide –N(H)– in each flap to the unique Water301; (B) (Bottom) A hypothetical: what would be the effect of conversion of the Ile50 amide to an ester in one flap only?; clearly, it would result in the deletion of the hydrogen bond-donating ability of that moiety, but what effect would that have on enzymatic activity?

In the several years since the introduction of the protease inhibitor class of AIDS drugs, problems with viral resistance have emerged.[12] In light of these problems, it is important to improve our basic understanding of HIV-1 PR. In this paper we describe the application of chemical protein synthesis to the direct experimental elucidation of fundamental aspects of the mechanism of action of the HIV-1 PR enzyme. Results of the current study suggest new insights into the molecular basis of the action of this enzyme and may contribute to the development of more effective protease inhibitor drugs for AIDS therapy.

The role of the flaps in HIV-1 PR catalysis

One of the most distinctive features of the HIV-1 PR molecule is the pair of flexible β-hairpin loops, termed 'flaps', that are involved in enzyme–substrate interactions. The HIV-1 PR molecule is a homodimer of 99 residue polypeptide chains, and one flap is contributed from each of the identical subunits[9,13] (Fig. 1A). In a number of co-crystal structures with substrate-derived inhibitors, the two flaps appear to work together, closing over the substrate peptide chain in the active site of the enzyme, desolvating the bound peptide chain and providing other interactions that are thought to contribute both to substrate recognition and to catalysis of peptide bond hydrolysis.[10] Particularly notable is a pair of specific hydrogen bonds between backbone amides of the enzyme molecule and the carbonyls on either side of the 'scissile' bond in the substrate-derived inhibitor (Fig. 2). In HIV-1 PR, these hydrogen bonds are transmitted from enzyme to inhibitor (substrate) via a specific, tetrahedrally coordinated, internal water molecule ('water 301') (Fig. 2A). Such hydrogen bonds are believed to contribute to distortion of the scissile bond away from planarity.[14] This increases the electrophilic character of the scissile bond carbonyl. This effect is thought to make an important contribution to catalysis of peptide bond cleavage by a nucleophilic water molecule.[†]

By contrast, the corresponding enzymes in eukaryotes (the pepsin-like aspartyl proteinases[15]) are two-domain, single polypeptide chain molecules. Interestingly, they have only one flap (Fig. 1B) and this interacts with the substrate P2 and P1′ carbonyls by direct (i.e. not water-mediated) hydrogen bonding (Fig. 2B). This observed difference in enzyme-inhibitor hydrogen bonding between the cell-encoded and retroviral aspartyl proteinases has led to successful programs[16] for the design, based on the presumed mechanistic relevance of the unique internally coordinated water301, of inhibitors specific for HIV-1 PR rather than cell-encoded aspartyl proteinases. Furthermore, it has been proposed[10] that enzyme–substrate hydrogen bonds mediated by water 301 play an important role in the catalytic mechanism of the HIV-1 protease.[17] Indeed, our own work has shown that specific deletion of just these two hydrogen bonds leads to a drastic decrease in the catalytic efficiency of the enzyme.[18]

Backbone engineering—design of the experiment

Given these intriguing differences between the retroviral and pepsin-like aspartyl proteinases and yet the evolutionary, structural, and presumed mechanistic relationships of these two classes of enzymes,[19] it was important to experimentally determine whether the HIV-1 PR required functional H-bonding from one or from both flaps for normal catalytic activity. To that end, a 'backbone engineered'[18,20] HIV-1 PR analogue was designed in which the Gly49-Ile50 amide bond –N(H)– [Fig. 2A] would be replaced in *one flap only* by an oxygen atom not capable of acting as a hydrogen bond donor (Fig. 3).

The HIV-1 protease is a homodimeric protein made up of two non-covalently associated identical 99-residue polypeptide chains.[9,13] In order to carry out asymmetric modification of just one of the two monomers within the protein molecule, it was necessary to make a 'tethered dimer' construct, effectively making a single polypeptide of the two monomer chains. Covalent dimer analogues of HIV-1 PR have been produced by recDNA expression in bacterial systems and display identical kinetic properties to the native two-chain homodimeric enzyme.[21] In the covalent dimer form of HIV-1 PR prepared by total chemical synthesis

[†] Water301 is NOT the nucleophilic water proposed in the mechanism of Suguna et al.;[14] Water301 is located *anterior* to the scissile bond, while the presumed nucleophilic water and the catalytic aspartyl side chain carboxyls are located on the *posterior* face of the scissile peptide bond, away from the flaps.

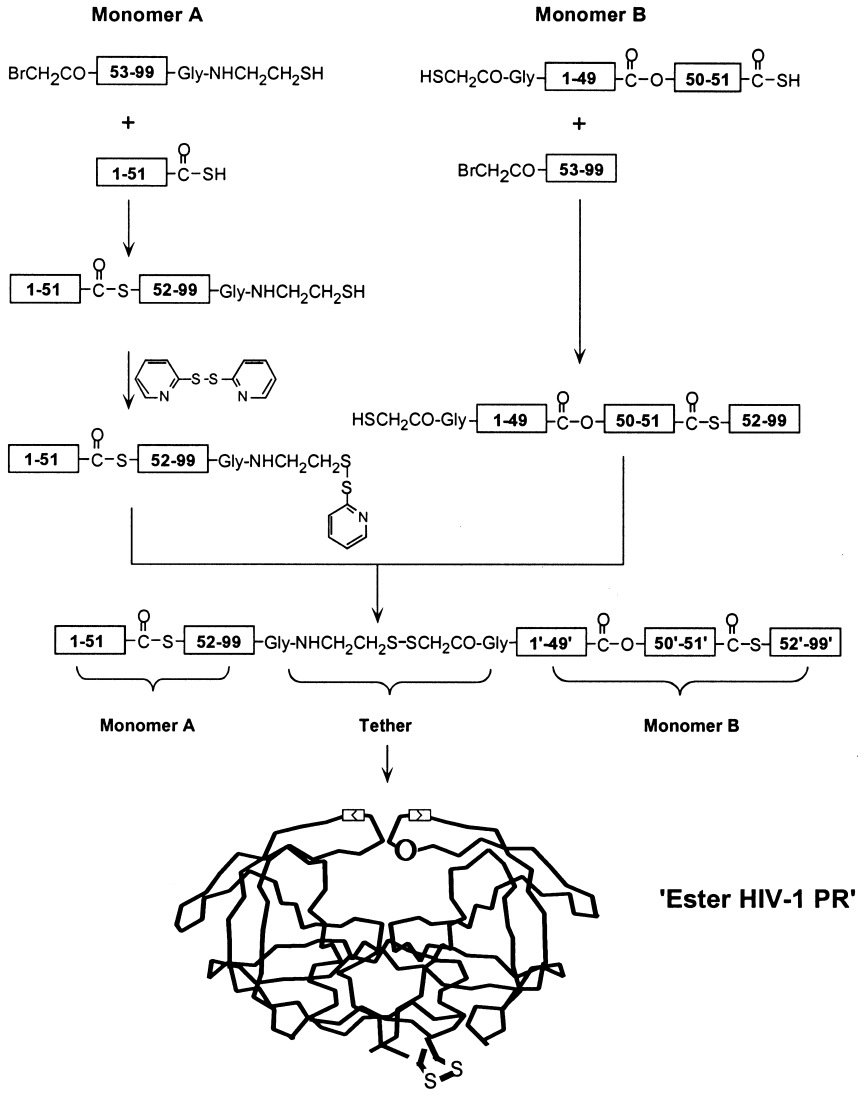

Figure 4. Synthetic scheme for preparation of the Ester HIV-1 PR (adapted from Ref. 5b). The target tethered dimer HIV-1 PR construct has a site-specific single atom backbone substitution in one flap only. The scheme presented here is identical to that for synthesis of the Control HIV-1 PR,[5b] except for the single ester-for-amide backbone substitution at Gly49′[COO]Ile50′. This ester linkage was incorporated as the depsidipeptide Boc-Gly-[COO]-Ile·OH in Monomer B during the stepwise assembly of the polypeptide chain. Synthetic peptide segments[24,42] were chemically ligated via thioester-forming chemoselective reaction.[3a] After reaction of the C-terminal cysteamine residue of monomer A with 2,2′-dipyridyl disulfide to form the *S*-(2-pyridylsulfenyl)cysteamine derivative, the desired disulfide-linked heterodimer was formed by thiolysis with mercaptoacetyl-Monomer B. The tethered dimer comprised the two 99-amino acid subunits of HIV-1 PR (residues 1–99 and 1′–99′), covalently linked by a four residue intersubunit disulfide bridge. A single backbone ester bond was present in one subunit only, between residues 49′–50′ in Monomer B, and both subunits contained a backbone thioester bond between residues 51–52. The synthetic Ester HIV-1 PR tethered dimer construct was folded[5b] to give the catalytically active enzyme.

that is the subject of the work reported here, the two normally identical 99-amino acid polypeptides are covalently joined in a similar fashion by means of a short linker structure between the C-terminus of one subunit and the N-terminus of the other.

If, as predicted based on crystallographic observations,[10,22] each of the two flaps contributes one catalytically relevant hydrogen bond from a backbone amide to the substrate carbonyls, then an HIV-1 PR analogue backbone-engineered to delete mechanistically relevant enzyme–substrate H-bonding in *one flap only* (Fig. 3) would be expected to exhibit a substantially reduced catalytic efficiency. Based on our previous work,[18] in which we deleted the relevant H-bonding in both flaps, this asymmetric backbone engineered construct would be expected to display a rate (k_{cat}) reduction of ~100-fold compared with native HIV-1 PR. This anticipated k_{cat} reduction is a combination of two effects: a 2.5 kcal/mol increase in the activation energy of enzyme-catalyzed substrate hydrolysis (i.e. half the ~5 kcal/mol observed for deleting both H-bonds[18]), corresponding to an ~50-fold reduction in the intrinsic catalytic efficiency in the E–S complex; and, a further two-fold rate reduction because of the newly introduced asymmetry of the covalent dimer form of the enzyme modified in only one flap.[‡]

[‡] That is, only one of the two orientations of bound substrate can be catalytically productive; in terms of the kinetic treatments, this would effectively halve the turnover per unit concentration of protein and thus reduce k_{cat} by a factor of two.

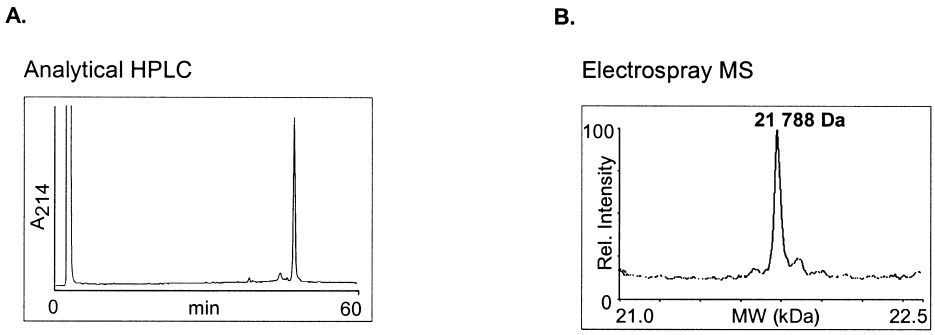

Figure 5. Chemical characterization of Ester HIV-1 PR. (A) (Left) Analytical reverse-phase HPLC of the synthetic enzyme. A linear gradient of 0–60% acetonitrile/0.1% TFA versus 0.1% TFA was used. The single sharp peak corresponding to the tethered dimer Ester HIV-1 PR was collected and analyzed by electrospray mass spectrometry as shown in the right panel (B). The minor peaks are autocatalytic degradation products that arise from the HIV-1 PR proteolyzing its own polypeptide chain.[48] The experimentally determined mass (21,788±3 Da) is identical to the calculated mass of 21,788 Da (average isotopes) confirming the correct covalent structure of the tethered dimer Ester HIV-1 PR construct.

However, if both the enzyme–substrate P2–P1′ carbonyl hydrogen bond(s) can be donated from a single-flap (as in the pepsin-like proteinases (Fig. 2B)), no change in intrinsic catalytic activity would result from deletion of the essential H-bonding element in only one of the two flaps. In this event, only a two-fold rate (k_{cat}) reduction would be expected (because only one productive substrate binding mode would be possible in the now-asymmetric enzyme).

Results

Chemical protein synthesis

In order to experimentally explore these aspects of flap function, we prepared a backbone-engineered covalent dimer HIV-1 PR molecular construct by total chemical synthesis using a convergent chemical segment ligation strategy,[5b,23] as shown in Fig. 4. It can be seen from Fig. 1 that the N- and C-terminal residues of different monomers are adjacent to one another in the folded enzyme molecule, and can be linked by structures as short as two amino acids.[21] In the work reported here, the tethered dimer comprised the two 99-amino acid subunits of HIV-1 PR (i.e. residues 1–99, 1′–99′), covalently linked by a four residue intersubunit disulfide bridge. Synthetic peptide segments,[24] after deprotection and purification, were chemically ligated via thioester-forming chemoselective reaction.[3a] Thus, both subunits contained a backbone thioester bond between residues 51 and 52, a modification previously shown to have no effect on HIV-1 PR enzymatic activity.[3a] After reaction of the C-terminal cysteamine residue of monomer A with 2,2′-dipyridyl disulfide to form the *S*-(2-pyridylsulfenyl)cysteamine derivative, the desired disulfide-linked heterodimer was formed by directed disulfide formation by thiolysis with the Monomer B N-terminal mercaptoacetyl group. The 202-residue synthetic tethered dimer construct was then folded to give the catalytically active enzyme molecule.[5b]

The first covalent dimer construct ('Control HIV-1 PR') contained the native backbone amide structure [–CONH–] at Gly49-Ile50 in both flaps; total chemical synthesis of this Control HIV-1 PR has been previously described.[5b] The second chemically synthesized tethered dimer construct ('Ester HIV-1 PR'), the subject of the present work, had the Gly49-Ile50 backbone amide bond replaced, in one flap only, by an ester [–COO–] moiety not capable of donating a hydrogen bond to the substrate carbonyl. This ester linkage was incorporated as the depsipeptide Boc-Gly-[COO]-Ile-OH in Monomer B during stepwise SPPS assembly of that segment of the polypeptide chain (Fig. 4). The simultaneous preparation of Ester HIV-1 PR and Control HIV-1 PR by the same method provided a reference enzyme molecule, differing in only a single backbone atom.

Analytical characterization of the chemically synthesized Ester HIV-1 PR is shown in Fig. 5. Reverse phase HPLC of the 202-residue synthetic enzyme gave a single sharp peak that was collected and analyzed by electrospray mass spectrometry. The experimentally determined mass of 21,788±3 Da was identical to the calculated mass of 21,788 Da (average isotope composition), confirming both the high purity and the correct covalent structure of the asymmetric tethered dimer Ester HIV-1 PR construct.

Enzymatic activity

The catalytic activity and substrate specificity of both the Control and Ester HIV-1 PR enzyme constructs were evaluated by cleavage of synthetic peptide substrates. As expected, synthetic peptide analogues of the viral *gag-pol* polyprotein corresponding to the matrix/capsid and capsid/nucleocapsid junctions were cleaved at the -Tyr–Pro- (matix/capsid peptide) and -Met–Met-, -Leu–Ala- residues

Table 1. Kinetic properties for cleavage of fluorogenic substrate by HIV-1 protease analogues

Enzyme	Kinetic parameter	
	k_{cat} (s^{-1})	K_M (μM)
Control HIV-1 PR	21	15
Ester HIV-1PR	9.8	81
$[COS]_2$ HIV-1 PR[a]	0.0086	5.3
Native HIV-1 PR[46]	24	21

[a] HIV-1 PR analogue with the Gly49-Ile50 backbone –CON(H)– amide bond replaced with an isoteric thioester –COS– bond in *both* flaps.[18] This data obtained at I1.0; all the other data obtained at I0.1.

(capsid/nucleocapsid peptide).[9a] These enzymatic properties were consistent with the observed properties of native backbone homodimeric HIV-1 PR and with the *gag-pol* processing events observed in viral maturation in vivo.

Kinetic parameters of both enzymes were determined for cleavage of a fluorogenic substrate[25] and the data are shown in Table 1. As previously reported,[5b] the k_{cat} value for Control HIV-1 PR ($21\ s^{-1}$) is typical of values reported for hydrolysis of similar substrates by both native homodimeric HIV-1 PR[26] and by covalent dimer HIV-1 PR produced by recombinant means.[21] The full activity of the covalent dimer Control enzyme prepared by total chemical synthesis demonstrates that the non-genetically encoded elements of covalent structure had no adverse effect on enzymatic function. Unnatural elements of covalent structure present in the Control HIV-1 PR were: the Gly51[COS]Gly52 structure in both flaps;[3a] the disulfide-bridged tether linking the two subunits;[5b] and, multiple non-coded amino acids including the cysteamine at the C-terminal of Monomer A,[5b] the mercaptoacetic acid at the N-terminal of Monomer B,[5b] and L-α-amino-*n*-butyric acid substituted for each of the four Cys residues.[9a]

Most significantly, the observed k_{cat} for the Ester HIV-1 PR ($9.8\ s^{-1}$) was reduced only 2-fold relative to Control HIV-1 PR. This minor effect on k_{cat} should be contrasted with the ~100-fold effect expected if, as discussed above, both of the Ile50 NH flap-substrate hydrogen bonds contributed equally to catalysis in the E–S complex (Fig. 3).[18] Thus deletion of the flap hydrogen bond donor at Gly49-Ile50 in *one flap only* has essentially *no effect* on the intrinsic catalytic efficiency of the enzyme. The observed ~2-fold reduction of the turnover number in the E–S complex can be attributed to the catalytic non-equivalence of the two subunits in the covalent tethered dimer Ester enzyme, consistent with only one productive substrate orientation on binding to the now-asymmetric enzyme molecule.

Discussion

Does the HIV-1 PR use only one flap in catalysis?

The observation that critical flap(amide) hydrogen bonding is required from only one flap for full enzymatic activity in HIV-1 PR has important implications. On the basis of crystallographic observations, it had been assumed[10,22] that both flaps were intimately involved in the action of the enzyme. It was further assumed that the unique tetrahedrally coordinated water301, and the two hydrogen bonds it mediates, from the Gly49-Ile50 peptide bond –N(H)– moiety in each flap to the substrate carbonyls on either side of the scissile bond, played a key role in the catalytic mechanism, stabilizing the distortion of the scissile amide bond from planarity and thus increasing its susceptibility to nucleophilic attack.[14] Indeed, we had previously established that hydrogen bonds from the flaps to the substrate are in fact important for the enzymatic activity of HIV-1 PR.[18] Deletion of the hydrogen bonding potential at the –N(H)– of Gly49-Ile50 peptide bonds of *both* flaps led to a 3000-fold *decrease* in intrinsic catalytic activity, corresponding to an overall increase in the activation energy of ~5 kcal/mol in the rate-limiting step. The magnitude of this effect was interpreted as consistent with deletion of two hydrogen bonds.[18]

This dramatic decrease in activity, of the HIV-1 PR backbone-engineered to precisely delete from the tip of *both* flaps only the two –N(H)– sites involved in enzyme–substrate hydrogen bonds, also unequivocally demonstrated that other potential H-bond donors within the flap(s) cannot effectively take up the deleted interactions with the P2–P1′ carbonyls of the substrate to restore enzymatic activity.[18] This serves as an important control for our current observations.

The results reported in the current studies suggest that in the action of the HIV-1 PR the catalytically productive flap-substrate interactions involve hydrogen bonding from the Gly49-Ile50 amide –N(H)– of only one flap to effect hydrolytic cleavage of the bound substrate. Yet an abundance of data shows that the predominant binding mode for substrate-derived inhibitors to the HIV-1 PR molecule involves *both* flaps and 'water301',[22] even in solution.[27] The most straightforward interpretation of this fact together with our current observations is that the two-flap/water301 enzyme–substrate complex is simply a favored but unproductive binding mode, a situation that is commonly encountered in enzymatic catalysis.[28] It should be borne in mind that physical techniques such as NMR and X-ray crystallography deal with bulk properties of an enzyme–ligand system. By contrast, kinetic studies provide more direct information about catalytically relevant features even if these are minor constituents of the whole system.

Although the precise nature of the HIV-1 PR flap-substrate hydrogen bonding interactions is not revealed by our results, the situation in the single flap cell-encoded pepsin-like proteinases (Fig. 1B) is suggestive. Water-mediated hydrogen bonding from the enzyme flap to the P2 and P1′ carbonyls of a substrate-derived inhibitor has not been observed in any pepsin-like proteinase–inhibitors complex.[15] As seen by X-ray crystallography, in some of those complexes with substrate-derived inhibitors both the P2 and the P1′ inhibitor carbonyls appear to interact directly with a single backbone amide –N(H)– moiety of the pepsin-like proteinases[14] (Fig. 2B), while other structures suggest that two backbone amide –N(H)– moieties in the single flap may be involved.[29]

Based on these observations and the full intrinsic catalytic activity of the Ester HIV-1 PR found in the current work, we suggest that the HIV-1 PR uses only a single flap in catalysis in a manner analogous to the pepsin-like proteases (Fig. 6). The native backbone flap is 'closed down' over the ligand in a face-on orientation, rather than edge-on orientation seen in crystal structures,[10,22] providing both hydrogen bonds to the P2 and P1′ carbonyls of the substrate, analogous to the ligand binding mode for the single-flap pepsin-like aspartyl proteinases.[14,29] That a single flap can provide the desolvation of the substrate and other features of the active site necessary for substrate hydrolysis is of course evidenced by the efficacy of the pepsin-like proteinases.

Although this suggestion may seem a radical departure from

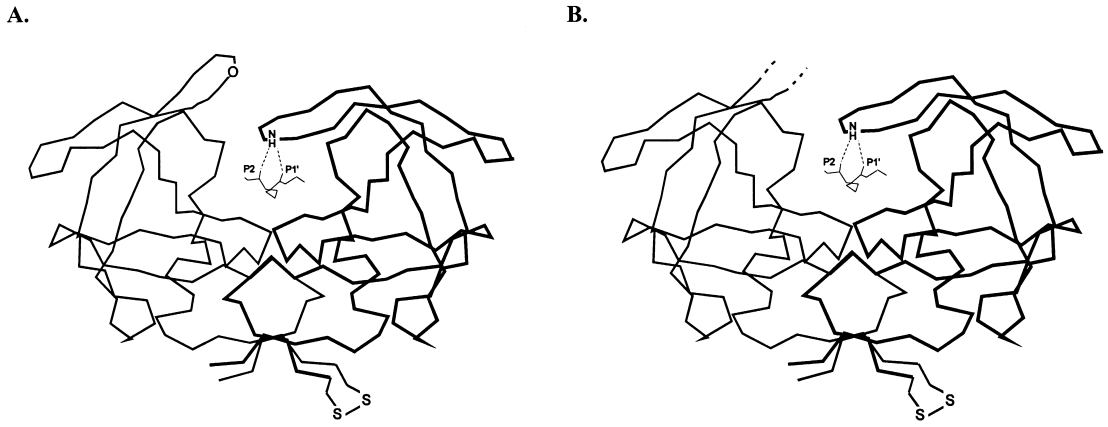

Figure 6. Model of a hypothetical productive Ester HIV-1 PR ligand-binding mode as inferred from the results of the current study. The native backbone flap is closed down over ligand, face-on rather than edge-on, providing both hydrogen bonds to the P2 and P1′ carbonyls of substrate/inhibitor by direct interaction (analogous to the ligand binding mode for the single-flap, pepsin-like aspartyl proteinases[14,15]). The second ester-containing enzyme flap is not involved in the catalytic action of the enzyme molecule, and is hypothesized to be in a 'raised' position (A) it is likely that this flap would be highly mobile,[30d] and that would lead to a situation (B) analogous to the 'open' flaps of the RSV and SIV unliganded proteases.[30c] Because of the full intrinsic catalytic activity of the ester-containing covalent dimer HIV-1 PR construct, we hypothesize that native HIV-1 PR functions in an analogous manner.

the accepted wisdom, it is not an unreasonable suggestion: it is based on analogy with the cell-encoded single flap enzymes,[15] and on direct kinetic investigation of the distinct catalytic role of the two flaps in the HIV-1 PR protein molecule (this work). Even the recombinant tethered dimer constructs that have been engineered in a less subtle way by deleting entire residues in one flap only, while generally inactive have also revealed surprising catalytic efficiency under some conditions.[21c] Everything reported here is consistent with a pepsin-like flap mechanism of action for the homodimeric HIV-1 protease and critical observations, summarized above, cannot be easily explained in any other way.

The second flap (i.e. either flap in the native enzyme depending on the orientation of the bound substrate; or, the [–COO–]-containing flap in the Ester enzyme construct) is not involved in the catalytic action of the enzyme molecule, and we hypothesize that it would be in a 'raised' position as shown in Fig. 6, although it is likely that in such an arrangement this flap would be highly mobile.[30]

Inhibitor studies

The functional relevance of flap-ligand hydrogen bonds in the action of inhibitors on the enzyme was examined by inhibitor studies on the tethered dimer HIV-1 PR constructs (Table 2). Representative HIV-1 PR inhibitors were compared for their effects on the Control and Ester tethered dimer enzymes. Inhibition by the substrate-derived, reduced isostere inhibitor MVT-101[10a] was essentially unaffected by the loss of a single hydrogen bond (this work) or loss of both hydrogen bonds (previously reported[18]) to the Gly49-Ile50 backbone amide in the flap(s), despite the clear presence of this interaction with both flaps in the crystal structure of the MVT-101 complex with native enzyme.[10a,31]

Table 2. Inhibition data for HIV-1 protease analogues

Enzyme	Inhibitor (K_i in nM)		
	MVT-101	DMP 323	JG-365[a]
Ester HIV-1 PR	833	0.25	1830
Control HIV-1 PR	345	0.86	71
Native[b] HIV-1 PR	780[10a]	0.27[16]	0.24[10b]

[a] Desmethyl form[40] of this inhibitor.
[b] Native backbone, homodimeric HIV-1 PR (identical data were obtained from chemically synthesized or recombinant enzyme).

Even more surprising at first glance were the results obtained for inhibition by the cyclic urea DMP-323.[16] This inhibitor molecule was expressly made to incorporate a carbonyl group designed to act as a water 301 surrogate and to take up the hydrogen bonding interactions with both flaps, as observed in the complexes between HIV-1 PR and substrate-derived inhibitors. The sub-nanomolar potency of DMP-323 and related inhibitors and their high selectivity for HIV-1 PR over cell-encoded pepsin-like proteinases was attributed to formation of two flap-inhibitor hydrogen bonds, one with each flap.[16] Crystallography of an HIV-1 PR complex with a DMP323-related cyclic urea inhibitor appeared to confirm the existence of these hydrogen bonds.[16] Based on this, we expected that the deletion in the current study of one of these two proposed hydrogen bonds would lead to a significant reduction (1–3 kcal/mol) in the binding affinity of this inhibitor. Contrary to this expectation, our results showed that binding of DMP-323 to the Ester HIV-1 PR analogue is actually slightly stronger than the binding of DMP-323 to the Control HIV-1 PR or to native HIV-1 PR (Table 2).

Thus, for these two classes of inhibitors, our data show that the crystallographically observed hydrogen bonds from inhibitor carbonyl(s) to the Ile50 –N(H)– moiety in each flap make no net contribution to binding. This simply means that there is no net free energy difference between the hydrogen bonding interactions in the enzyme–inhibitor complex compared with hydrogen bonding interactions that occur in the uncomplexed enzyme and the inhibitor free in solution.

Implications for protease inhibitor drug design

Putative mechanistic differences between the retroviral and eukaryotic enzymes, based on crystallographic observations and centering on the presumed role of water 301 which is found exclusively in the retroviral proteinases, has been a key focus of structure-based drug design targeted at retroviral proteinases.[16] The work reported here suggests that while Water301 evidently plays a role in inhibitor binding, it does not play a role in the catalytic action of the enzyme.[§] The role of Water301 in the action of the HIV-1 PR is till the subject of active investigation by others.[35]

Clearly, the co-crystal structures of HIV-1 PR with substrate-derived inhibitors[10,22] have been a fruitful basis for inhibitor design, and features apparently unique to HIV-1 PR have played an important role in the development of AIDS therapeutics.[16,36] Clinical data have unequivocally demonstrated that protease inhibitors targeted at HIV-1 PR dramatically reduce viral loads in infected individuals,[37] and several protease inhibitors have proven to be highly effective in HAART combination drug regimes that effectively combat AIDS.[38] However, a serious problem has become evident: the rapid development of inhibitor-resistant forms of HIV-1 PR in individuals treated with protease inhibitors.[39] It may be that inhibitors targeted more explicitly at catalytically relevant aspects of HIV-1 PR action would be less susceptible to the development of resistance mutants.

In the context of escape mutants and improved inhibitor design, an interesting situation is presented by the action of the *S*-isomer of Ac-Ser-Leu-Asn-[CH(OH)CH_2N]Pro-Ile-Val-OH on the Control and Ester HIV-1 PR constructs studied in the present work (Table 2). This is the desmethyl form[40] of the potent substrate-derived inhibitor JG-365.[41] This class of hydroxyethylamine isostere-containing inhibitors has proven to be one of the most effective in clinical use. As shown in Table 2, deletion of a single flap-inhibitor hydrogen bond has the effect of reducing the affinity of this inhibitor for the enzyme by ~25-fold (K_i 1830 nM vs. 71 nM). In light of the negligible change between the Ester and Control enzymes in either substrate kinetic constants or in K_i values for the other inhibitors studied (Table 2), this result is surprising. It should be noted that the potency of this inhibitor on Control HIV-1 PR (K_i 71 nM) is itself significantly compromised relative to the native HIV-1 PR (K_i 0.24 nM), perhaps due to the change in flap geometry induced by the thioester bonds in each flap at the Gly51-Gly52 sequence.[32]

Thus, intriguingly, for the highly potent hydroxyethylamine isostere inhibitor JG-365, the tethered dimer constructs of HIV-1 PR represent unusual inhibitor-resistant mutants. Both Control and Ester HIV-1 PR analogues retain full enzymatic activity on a range of *gag-pol* cleavage sites, yet each is inhibited several orders of magnitude less by JG-365 than is the native HIV-1 PR. Further study of this phenomenon[‖] could contribute to our understanding of the basis of inhibitor-resistant mutants, currently a very important topic for the clinical efficacy of HIV-1 PR inhibitors.[39]

Conclusions

Precise, single atom backbone-engineering[18,20] through total protein synthesis by chemical ligation[3] has provided a novel insight into the role of the flaps in HIV-1 PR catalysis of the hydrolysis of peptide substrates. The results of the current work show that the two flap/water301-mediated enzyme–substrate P2–P1′ hydrogen bonding model[10,22] is not a correct picture of the catalytically relevant action of the flaps. This insight may serve as a basis for the design of improved, mechanism-based HIV-1 PR inhibitors less susceptible to the evolution of viral resistance.

The construction of a backbone-engineered HIV-1 PR analogue in which a single atom has been replaced in a unique position in the molecule exemplifies the manner in which chemical synthesis has added new dimensions to the study of proteins. By applying recent innovations in the chemical ligation of unprotected peptide segments,[3,4] proteins of typical size are now amenable to total synthesis. The design and construction of protein analogues is thus no longer restricted to the building blocks afforded by the genetic code. This promises an exciting and fruitful new era in protein engineering in which we can hope to both productively challenge existing beliefs and successfully tackle unresolved issues in protein structure and function.

Experimental[¶]

Materials and methods

Boc-amino acids and HBTU were obtained from Novabiochem (San Diego, CA). Boc-aminoacyl-OCH_2Pam-resins and diisopropylethylamine were purchased from Applied Biosystems (Foster City, CA). *N*,*N*-Dimethylformamide was obtained from Mallinckrodt Chemical Co.

[§] Other data which are not consistent with a functionally relevant role for water301 includes: (1) the crystal structure of the fully active enzyme [(COS)51–52,51′–52′]HIV-1 PR complexed with MVT101[32] does *not* show water301, which is clearly present in the corresponding complex between native backbone HIV-1 PR and MVT101;[10a] rather, two poorly ordered structural water molecules are observed, one associated with each of the P2 and P1′ carbonyls of the inhibitor; (2) a complex of the homologous HIV-2 PR with a substrate-derived inhibitor has been crystallized in which Water301 was absent; the relative flap conformations in this complex differed from one another, with one flap 1 Å closer to the inhibitor than the other;[33] (3) the related SIV PR has been crystallized ligand-free in an alternate crystal packing arrangement to that observed for the HIV-1 PR studies;[34] despite the absence of bound ligand, this structure shows the flaps in a 'closed' conformation, completely analogous to that seen in the ligand-bound form of the HIV-1 PR complex; furthermore, electron density was observed for a bound water molecule in a site equivalent to water301 and within hydrogen-bonding distance of the Ile50 in each flap; the similarity of this structure indicates that ligand binding is not a prerequisite for closure of the flaps,[30c,34] nor for specific internal binding of a Water301.

[‖] Such studies would include: kinetic studies of the effects on larger numbers and more diverse types of inhibitors on the enzymatic activity of the Ester HIV-1 PR, to gain an understanding of the profile of occurrence of this resistance phenomenon in the backbone-engineered enzyme; the chemical synthesis of other backbone engineered flap variant forms of the HIV-1 PR molecule, and similar studies of their inhibitor kinetics; structural studies of key {backbone engineered enzyme}–inhibitor complexes by crystallography, and also by NMR methods[30d] to explore a possible correlation of the 'resistance' with flap flexibility.

[¶] Adapted from the published synthesis of the Control HIV-1 PR construct.[5b]

(Paris, KY). HPLC grade acetonitrile was purchased from EM Science (Gibbstown, NJ) and all other reagents were reagent grade from Aldrich Chemical Co.

Preparative HPLC was performed on a Waters Delta-prep 4000 HPLC system. Analytical and semipreparative HPLC were performed either on a Rainin HPXL dual pump system with detection on a Dynamax UV detector, or on an integrated Hewlett Packard 1050 system. Preparative HPLC was run on a Vydac C18 column (15 μm, 50×250 mm) at a flow rate of 30 mL/min; semipreparative HPLC was run on a Vydac C18 column (10 μm, 10×250 mm) at 3 mL/min; and, analytical HPLC was run on a Whatman C18 (5 μm, 4.0×140 mm) or Vydac C4 column (5 μm, 4.6×150 mm) at 1 mL/min. All runs used linear gradients of 0.1% aqueous TFA (solvent A) versus 90% acetonitrile, 0.1% TFA (solvent B). Mass analysis of all synthetic peptides and ligation products was performed on a Sciex API-III triple quadrupole electrospray mass spectrometer. Calculated masses were obtained using the program MacProMass (Sunil Vemuri and Terry Lee, City of Hope, Duarte, CA)

Solid phase peptide synthesis

All peptides were synthesized by stepwise solid phase methods according to the in-situ neutralization/HBTU activation protocol for Boc chemistry as previously described.[42] Synthesis of the HIV-1 PR fragments was performed on a modified Applied Biosystems 430A peptide synthesizer. The HIV-1 PR fragment BrAc(53–99)Gly-$NHCH_2CH_2SH$ was prepared on a N^{α}-Boc-cysteamine-*S*-benzyl-4-(oxyacetamidomethyl)-resin (P. Alewood, personal communication). Peptide α-thiocarboxylic acids ($^{\alpha}$COSH) were constructed on a Gly-thioester support, yielding peptides with C-terminal Gly-$^{\alpha}$COSH after cleavage.[43] All other peptides were synthesized on appropriate Boc-aminoacyl-OCH_2Pam-resins. Where required the bromoacetyl group was introduced at the N-terminal of peptides by coupling the preformed symmetric anhydride. The terminal mercaptoacetyl residue on the HIV-1 PR fragment $HSCH_2CO$-Gly(1–51) $^{\alpha}$COSH was coupled as the preformed symmetric anhydride of the *S*-(4-methylbenzyl)- protected derivative. In all cases side-chain protecting groups were removed and the peptides cleaved from the resin by treatment with liquid HF containing 4% anisole for 1 h at 0°C. After evaporation of the HF, crude peptide products were precipitated and washed with diethyl ether, then dissolved in aqueous acetic acid (10–50%) and lyophilized.

Purification and characterization of peptide segments

Crude lyophilized peptides were dissolved in either acidic aqueous buffers or 50% aqueous acetic acid, and purified by preparative or semipreparative reverse phase HPLC. Purified peptides were stored as lyophilized powders at −20°C. The purified peptide segments were characterized by ESMS, and all had observed masses within experimental error of the calculated masses.

Preparation of Boc-Gly-[COO]-Ile·OH

(2*S*,3*S*)-2-hydroxy-3-methyl valerate was converted to the benzyl ester derivative by reaction with benzyl bromide and the product was purified by silica chromatography. A portion of this product (1.0 g; 4.5 mmol) was dissolved in dichloromethane (20 mL) and combined with the symmetric anhydride of Boc-glycine (22.5 mmol), previously prepared by the reaction of Boc-glycine (7.88 g, 45 mmol) and diisopropylcarbodiimide (3.6 mL; 22.5 mmol). 4-Dimethylaminopyridine (275 mg; 2.25 mmol) was added as a catalyst, and the reaction mixture was stirred for 10 h at room temperature. This was then extracted with 5% aq. $NaHCO_3$ and 10% aq. $KHSO_4$ and the solution dried with anhydrous $MgSO_4$. The Boc-Gly-[COO]-Ile.OBzl product was purified by silica chromatography and characterized by ^{1}H NMR. Removal of the benzyl ester protecting group was effected by hydrogenation to yield the desired Boc-Gly-[COO]-Ile·OH product.

Synthesis of covalent dimer analogues of HIV-1 protease

The synthesis of the Control HIV-1 PR molecule has been reported previously.[5b] Synthesis of the Ester HIV-1 PR molecule, shown schematically in Fig. 4, was essentially the same as Control HIV-1 PR except for the single ester-for-amide backbone substitution (-Gly49′[COO]Ile50′-). This ester linkage was incorporated as the depsidipeptide Boc-Gly-[COO]-Ile·OH in Monomer B during the stepwise assembly of the polypeptide chain (Fig. 4). All subsequent steps in the preparation of the Ester HIV-1 PR molecule were identical to that for Control HIV-1 PR.[5b]

Substrate specificity

Substrate specificity was determined by cleavage of synthetic peptide analogues of the viral *gag-pol* polyprotein matrix/capsid and capsid/nucleocapsid junctions,[9a] and by cleavage of the fluorogenic substrate Abz-Thr-Ile-Nle-Phe(NO_2)-Gln-Arg·amide (Nle=norleucine; Abz=anthranilic acid; Phe(NO_2)=4-nitrophenylalanine).[25] As anticipated, the matrix/nucleocapsid peptide was cleaved at -Tyr–Pro- and the capsid/nucleocapsid peptide at the expected -Met–Met- and -Leu–Ala- junctions,[9a] while the fluorogenic substrate was cleaved at -Nle–Phe(NO_2)-.[25] No additional cleavages were observed in any of these peptides even upon prolonged incubation with enzyme preparation.

Enzyme kinetics

Steady state kinetic parameters were determined for cleavage of the fluorogenic substrate Abz-Thr-Ile-Nle–Phe(NO_2)-Gln-Arg·amide. Conditions of the assay were: pH 5.5, ionic strength 0.1, 37°C. Enzyme concentrations were: Control HIV-1 PR=78 pM; Ester HIV-1 PR=219 pM. Initial velocities were measured at different substrate concentrations, and the kinetic constants k_{cat} and K_m were computed by fitting the experimental data to the Michaelis–Menten equation using non-linear regression analysis. Data for ($[COS]_2$49–50)HIV-1 PR is from previous work[5b] and was obtained under identical assay conditions to those described above, except for ionic strength 1.0, which has been shown to affect K_M but not k_{cat}.[46]

Inhibitor studies

Inhibition of the enymes Ester HIV-1 PR (219 pM) and Control HIV-1 PR (78 pM) by Ac-Thr-Ile-Nle-[CH2NH]-Nle-Gln-Arg·amide[10a] (MVT-101), Ac-Ser-Leu-Asn-[CH(OH)CH2N]Pro-Ile-Val·OH[40] (desmethyl JG-365, *S*-isomer), and the cyclic urea DMP-323,[16] all three of which are well characterized competitive inhibitors of the native enzyme, was assessed in a chromatographic assay[5b] at pH 5.5, ionic strength 0.1. Except for DMP-323 inhibition of the Ester HIV-1 PR, a fixed fluorogenic substrate concentration of 50 μM was used and IC_{50} values were determined by least squares analysis using Dixon plots.[45] These values were converted into inhibitor dissociation constants (K_i) by use of the expression $K_i=IC_{50}/(1+[S]/K_m)$. The K_i for inhibition of Ester HIV-1 PR by DMP-323 was determined by the method of Henderson.[44]

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