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Journal of Molecular Structure (Theochem) 423 (1998) 137–152

THEO CHEM

Probing the structural basis of the catalytic activity of HIV-1 PR through total chemical protein synthesis

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Received 2 October 1996; accepted 29 November 1996

Abstract

Historically, total chemical synthesis had been used to prepare native proteinase from the human immunodeficiency virus (HIV-1 PR) for structural studies by X-ray crystallography. More recently, several functionally-relevant analogues of HIV-1 PR have also been obtained by total chemical synthesis. The results of structural and biochemical studies of the backbone engineered analogues put in question the established belief of the importance of an internal, tetrahedrally coordinated water molecule (water 301) in mediating catalytically important flap–substrate interaction. An enzyme analogue in which the peptide bond between residues Gly^{51} and Gly^{52} was replaced by a thioester moiety displayed normal enzymatic activity, while the crystal structure of its complex with the inhibitor MVT101 (solved at 2.5 Å resolution as mirror image, D-enantiomer) did not show the presence of water 301. The enzyme analogue in which the ability to donate hydrogen bonds to substrate was deleted (by substitution of Ile^{50} –N(H)– by a sulfur atom) in *both flaps* was 2500-fold less active. By contrast, the covalent dimer form of the enzyme with the Gly^{49}–Ile^{50} peptide bond –N(H)– atom specifically replaced by an –O– atom *in one flap* only retained normal enzymatic activity. The combined data from these studies strongly indicate that flap–substrate hydrogen bonds from only one flap are sufficient for full enzymatic activity of the HIV-1 PR, and raise the possibility that the retroviral enzyme may make use of only one flap in catalysis. This result may have profound implications for drug design targeted at HIV-1 PR.

Keywords: HIV-1 proteinase; Structural engineering; Chemical protein synthesis; Catalytic mechanism; Structure-based drug design

1. Introduction

Successful chemical synthesis of complex polypeptides marked a milestone in the development of the field of protein chemistry. It opened not only numerous novel avenues for biotechnology and drug design, but also new possibilities for the study of physico-chemical and biological properties of the protein molecule itself. One of the best examples is the case of the retroviral aspartyl proteinase from human immunodeficiency virus HIV-1 (HIV-1 PR). It was the first biologically active enzyme obtained in reproducible fashion by total chemical synthesis [1], and the first example of the preparation of a complete

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PII S0166-1280(96)05022-1

enzyme molecule containing non-coded amino acids. Obtaining milligram quantities of a folded homogenous protein of that size was precedent-setting, and the synthesis came at a time when efficient procedures for recombinant expression of HIV-1 PR had not yet been developed. Because of this confluence of circumstances, total chemical synthesis of the HIV-1 PR molecule has had a profound influence on our understanding of this important molecule.

In early 1989, a low resolution X-ray structure of unliganded HIV-1 PR had been reported by the Merck group [2]. This was the first reported structure of this important target for drug design and revealed the essential features of the catalytic apparatus and the presumed substrate binding cleft at the interface between the two subunits. However, aspects of this original HIV-1 PR structure did not conform to expectations based on known structural data from the eukaryotic (the "pepsin-like") aspartic proteinases. Notably, there were significant differences with respect to a structural model of the HIV-1 PR based on homology modeling [3], and to the just then completed structure of the Rous sarcoma virus (RSV) proteinase [4]. These discrepancies were particularly pronounced in the dimer interface region involving the N- and C-terminal of each subunit polypeptide chain, and cast doubt on the mechanism originally proposed [2] for the autocatalytic release of the HIV-1 PR from the virally encoded gag-pol polyprotein.

Because of these questions, and because of the potential importance of the HIV-1 PR molecule for structure-based drug design, in early 1989 a collaborative effort was undertaken between two research groups, one at the California Institute of Technology (chemical protein synthesis) and the other at the NCI-Frederick (X-ray crystallography). The goal of this joint effort was to solve the molecular structure of the HIV-1 PR both unliganded and in complex with substrate-based inhibitors. The 99 amino acid residue polypeptide chain of the HIV-1 PR monomer was prepared by total chemical synthesis using highly optimized stepwise solid phase peptide synthesis (SPPS) [5]. The sequence of the SF2 isolate of the virus was used, with the cysteine residues at positions 67 and 95 of the chain replaced with the isosteric L-α-amino-*n*-butyric acid (i.e. replacing an –SH of the cysteine residue with a CH_3) [6]. The synthetic proteinase was crystallized (Fig. 1(a)) and X-ray diffraction studies provided the first correct crystal structure of the free enzyme [6], and the first co-crystal structure of HIV1-PR complexed with an inhibitor, the substrate-derived MVT101 [7] (Fig. 1(b)). Synthetic HIV-1 PR was also used to determine the structures of two other complexes with substrate-derived inhibitors; these were the first structures of the enzyme with canonical examples of the two most important classes of inhibitors of this enzyme, containing respectively hydroxyethylamine [8] and hydroxyethylene [9] isosteres.

These three sets of coordinates were made freely available to the research community and were used in modeling and theoretical studies [10–14], and also in many laboratories worldwide to solve by molecular replacement X-ray structures of the HIV-1 PR complexed with a variety of inhibitors. In these ways, the data obtained from the synthetic enzyme played a key role in the intense world-wide drug design effort focused on this molecule, with the aim of developing anti-AIDS therapeutics. The crystal structures of synthetic and recombinant HIV-1 PR complexed with different active-site directed ligands [15–17] established the mode of inhibitor binding by the dimeric retroviral enzyme (Fig. 1(c)). In some respects this was different from the binding mode observed for inhibitors of the pepsin-like cell-encoded aspartic proteinases (for a review, see [18]). The most intriguing difference between the two classes of enzymes was in the mode of interaction of the tip of the flap(s) with the inhibitor. The flaps are mobile, extended β-sheet regions that close down over the substrate, desolvating it, and contributing to the formation of the specificity pockets involved in substrate recognition. The cell-encoded aspartyl proteinases consist of a single polypeptide chain folded into two homologous domains and have only one flap. By contrast, the retroviral aspartyl proteinases are homodimeric and have two flaps, one from each monomer.

Based on the results of the structural studies of the HIV-1 PR, of similar studies on the cell-encoded aspartyl proteinases, and on the general body of knowledge of this class of proteolytic enzymes, a consensus has been reached on the mechanism of catalysis. The accepted mechanism has been described best by Suguna et al. [19], for the aspartic proteinase from *rhizopus chinensis*, and involves nucleophilic

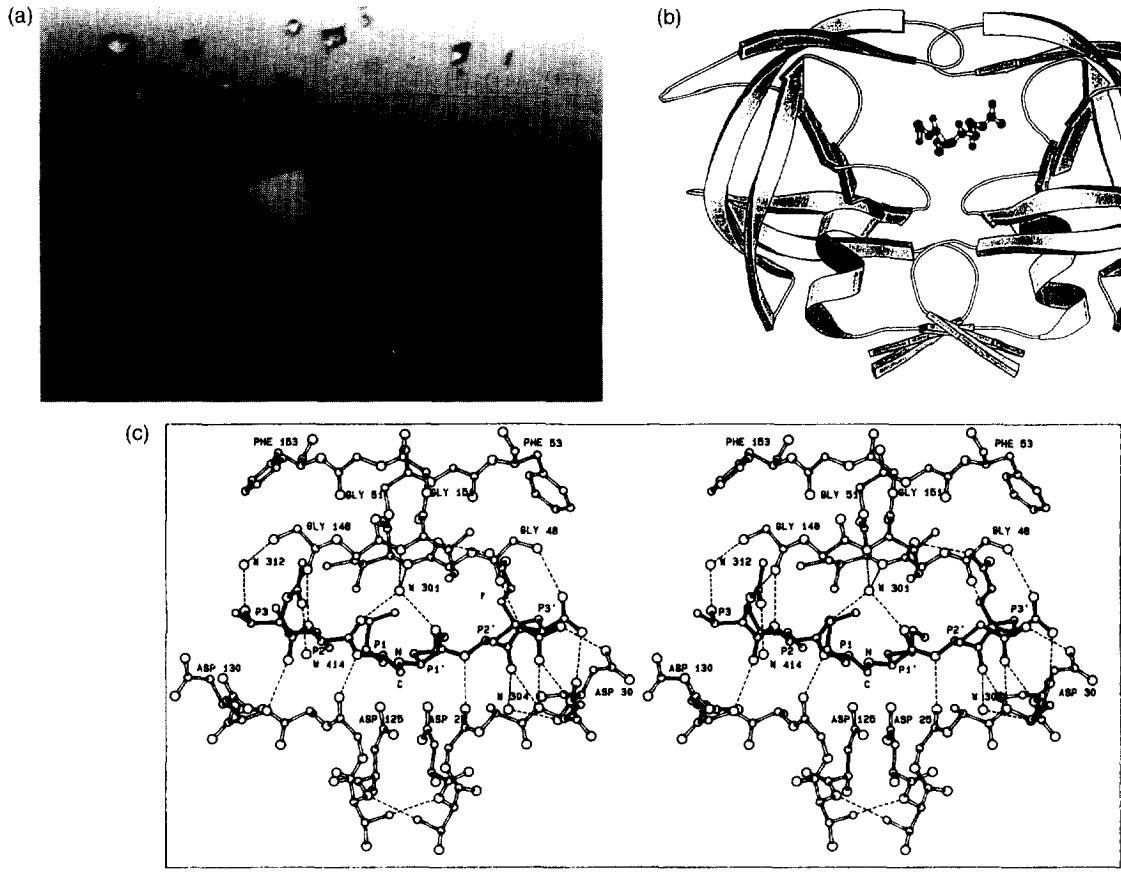

Fig. 1. (a) Crystal of HIV-1 PR prepared by total chemical synthesis, obtained as described in Ref. [6]. (b) The structure of the complex of synthetic HIV-1 PR with the MVT101 inhibitor. The picture was drawn using program MOLSCRIPT. (c) Stereo view of the MVT101 (solid line) inhibitor in the active site in its highly occupied state [34]. Hydrogen bonds are shown as dashed lines.

attack of a water molecule presumed to exist between the side chain carboxyl groups of the two catalytically essential aspartic acid residues. In the case of the retroviral proteinases, one catalytic Asp is contributed from each monomer [3]. The side chain carboxyls function as general acid–base catalysts for the attack by the water molecule. The broad features of this catalytic mechanism for the aspartyl proteinases are noncontroversial. The proposed mechanism is in general agreement with the few, but carefully done, kinetic studies of this class of enzyme [20,21]. Alternative mechanisms, such as covalent catalysis, have not been rigorously excluded but are not currently favored by the broad preponderance of evidence [22].

A mechanism essentially similar to that of Suguna et al. [19] has been discussed by Pearl [23] who pointed out that in the aspartyl proteinases the distortion of the scissile peptide bond, an out-of-plane rotation "stabilized by interactions of the substrate with the extended binding cleft", gives rise to the "apparent electrophilicity of the catalysis". This distortion would occur on binding and may be promoted by the observed H-bonding interactions between the interior side of the flap(s) and the substrate carbonyls on either side of the scissile bond. In crystal structures of both types of aspartic proteinases (Fig. 2), hydrogen bonds are observed between the protein backbone near the tip of the flap(s) and the carbonyl oxygens flanking the pseudo-scissile bond of bound substrate-based peptide inhibitor. As shown in Fig. 2, the single flap of the cell-encoded aspartyl proteinases closes down "flat" over the scissile bond, i.e., with the

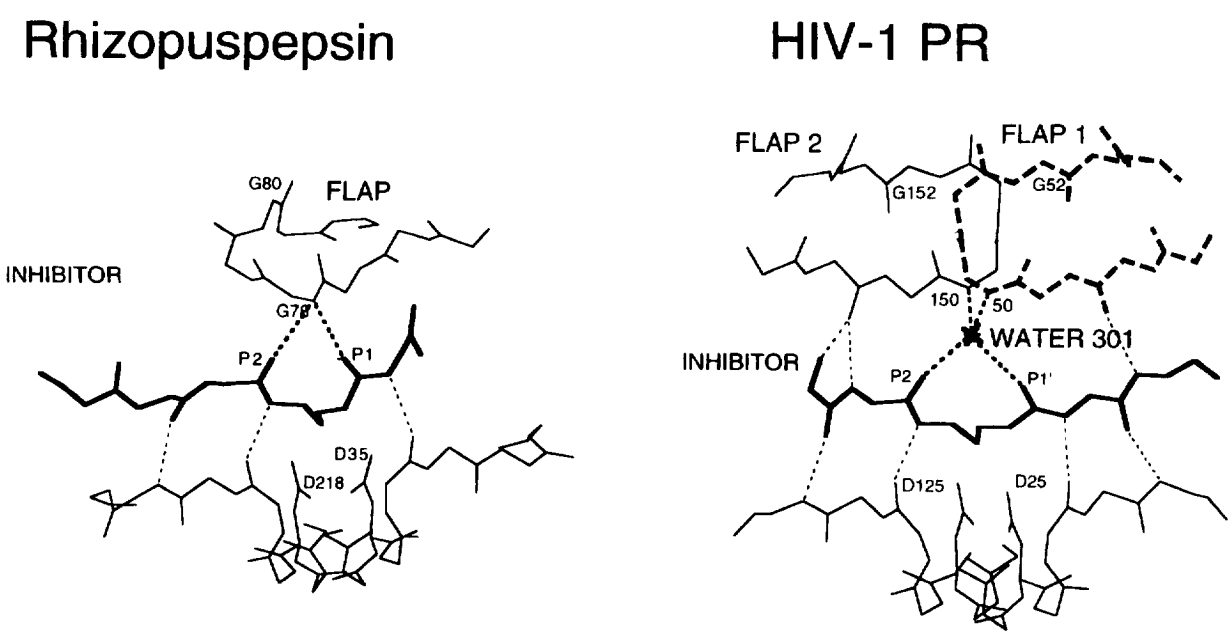

Fig. 2. Comparison of flap(s)–"substrate" interactions in cell encoded and HIV-1 proteinases.

plane of the reverse turn at the tip of the flap (nearly) parallel to the plane of the inhibitor peptide; direct H-bonding interactions occur between backbone peptide bonds in this flap and the substrate carbonyls. By contrast, the two flaps of the HIV-1 PR both close over the substrate-derived inhibitor, "edge on" (i.e. at ≈90°) to the plane of the substrate polypeptide chain.

Besides this major difference in the inhibitor–enzyme interactions, it should be mentioned that the amino acid sequences in the flap region of these two classes of enzymes are quite different.[2] Moreover, while the amide NH moieties of peptide bonds near the tip of the pepsin-like proteinase flap donate hydrogen bonds directly to the substrate, the corresponding hydrogen bonds in the HIV-1 PR are contributed one from each flap, and are mediated by a specific, tetrahedrally coordinated internal water molecule, "water 301", poised between the flaps and the inhibitor. Since first being observed in the HIV-1 PR–MVT101 complex [7] (see Fig. 1(c)), this water 301 has been observed in a wide variety of co-crystal structures of the HIV-1 PR with inhibitors [17], and has also been observed in solution by NMR studies of enzyme–inhibitor complexes [24].

The way in which the two flaps of the HIV-1 PR were observed to interact with the inhibitor was regarded as a key feature differentiating the mechanisms of the retroviral HIV-1 PR from the corresponding cell-encoded enzymes [25–27]. This key mechanistic difference has become a major target for structure-based drug design [28]. Several classes of inhibitors have been explicitly designed to take up the specific water 301-mediated H-bonding interactions with both flaps of the HIV-1 PR, and in this way are designed to exhibit specific inhibition of the retroviral enzyme over the cell encoded aspartyl proteinases.

Here we describe several examples making use of a total chemical protein synthesis approach in order to obtain functionally relevant enzyme analogs designed to investigate the structural basis for the enzymatic activity of HIV-1 PR. HIV-1 PR composed of D-amino acids was synthesized in order to show that a polypeptide composed entirely of D-amino acids is able to properly fold to form a functional molecule

[2] The observed role of the flaps in co-crystal structures of the HIV-1 PR with substrate-derived inhibitors is similar to that proposed in the homology model developed by Pearl and Taylor [3]. With respect to this and other features of the (now) known molecular structure of the HIV-1 PR, this work of Pearl and Taylor [3] stands out as one of the best examples of the prediction of the structure of a folded protein molecule.

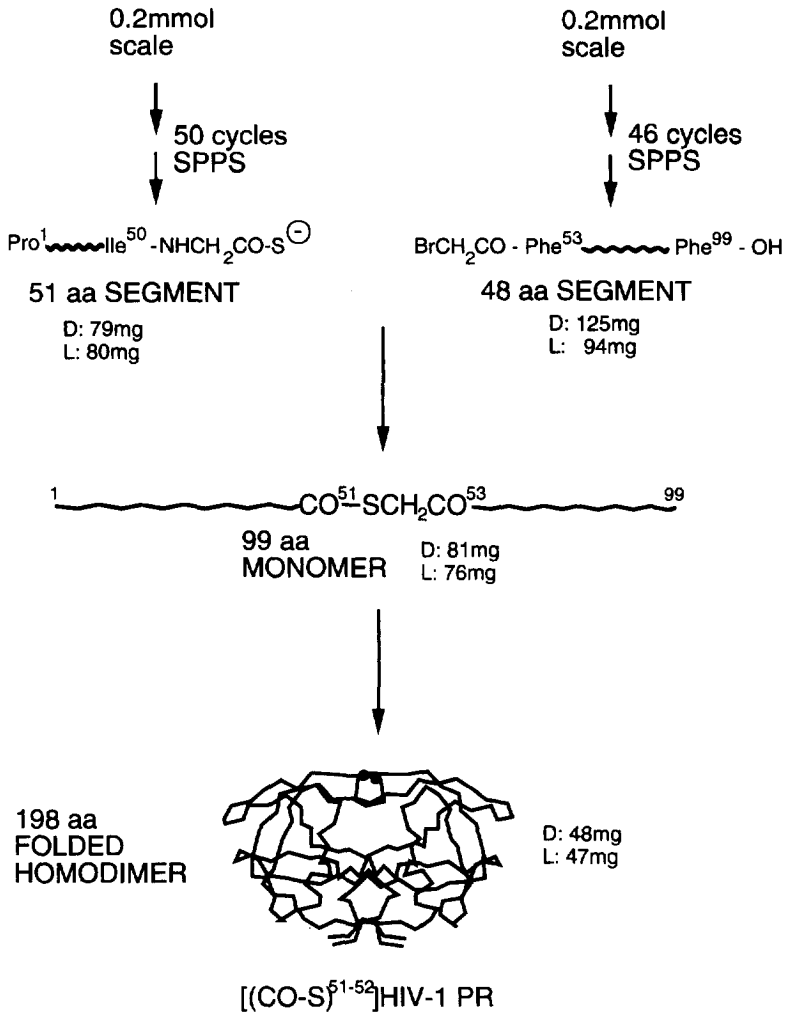

Fig. 3. A scheme for the total chemical synthesis of ([COS]$^{51-52}$)HIV-1 PR as described in Ref. [31].

and to investigate the chiral specificity of peptide substrates and inhibitors. Also, co-crystals of both enantiomers of the enzyme, if obtained in the centrosymmetric space group, would provide very high quality data for structural studies [29]. Additional backbone engineered analogues of the HIV-1 PR were prepared to delete a prominent enzyme(flap)–substrate hydrogen bond either from one or both flaps. The kinetic and structural properties of these synthetic enzyme analogues were investigated. These studies were aimed to answer the question whether the water-mediated hydrogens bonds from the flaps to substrate (inhibitor) observed by X-ray crystallography in the enzyme complexed with substrate-derived inhibitors are really important for the enzymatic activity of the HIV-1 PR.

2. Results

2.1. ([COS]$^{51-52}$)$_2$HIV-1 PR

The development of the chemical ligation method for the total synthesis of proteins [30] made possible the preparation of several backbone engineered HIV-1 PR analogues, each with precise single atom substitution

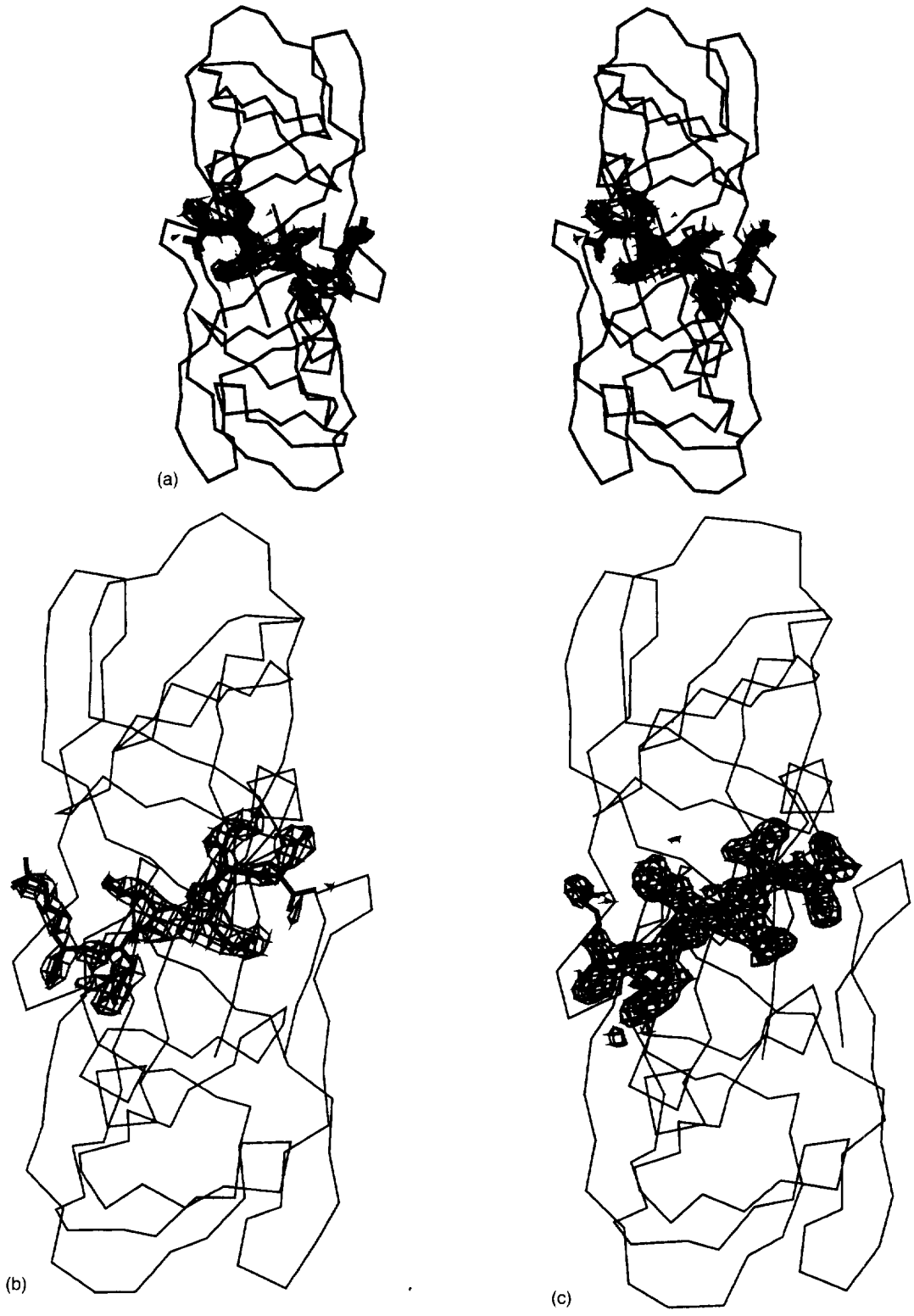

Fig. 4.

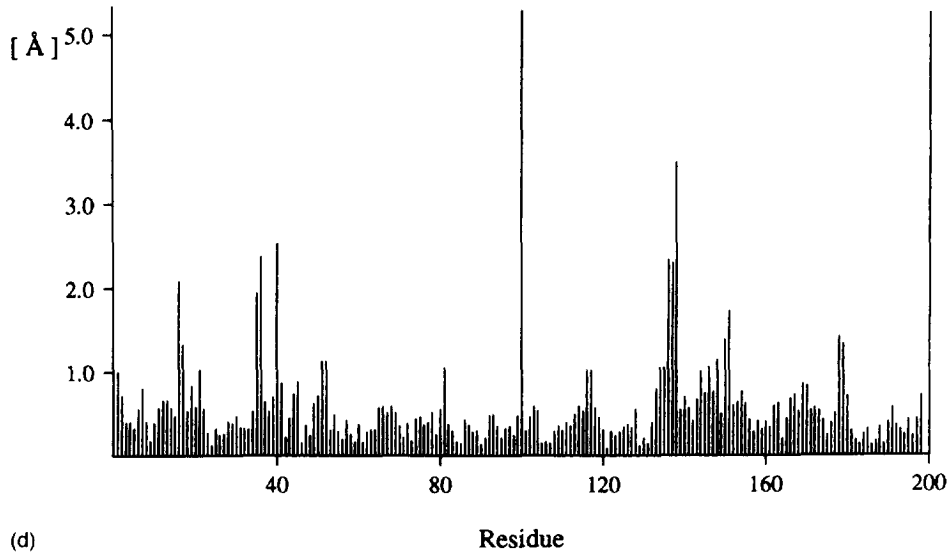

Fig. 4. (*continued*) (a) Stereo view of the C_α tracing of the ligated D-PR structure (see also Ref. [35]). Bound D-MVT101 inhibitor (thick line) is shown in its electron density map. (X-ray diffraction data to a resolution of 2.5 Å were collected from a single crystal using a Siemens area detector. The rotation and translation solutions were obtained using the program MADIRA developed by J.K. Mohana Rao and followed by a rigid body refinement. The structure was refined to *R*-factor of 0.188 in the resolution range of 10–2.5 Å). (b) Structure of D-(HIV-1 PR/MVT101) complex displayed as mirror image, for comparison with the L-enzyme. (c) Native backbone L-(HIV-1 PR/MVT101) complex, as described in Ref. [34]. Two possible alternative orientations with 70% and 30% occupancy are shown for the bound MVT101 hexapeptide. (d) Distance differences between equivalent C_α atoms of natural backbone L-PR and the mirror image of ligated D-PR.

in the polypeptide backbone. A fully active analogue of the enzyme was first prepared by this method, as outlined in Fig. 3. The ligation of the two unprotected peptides: HIV-1 PR(1–51)αCOSH and BrAcetyl(53–99)HIV-1 PR gave a 99 residue polypeptide corresponding to the HIV-1 PR monomer, with the peptide bond between Gly^{51}–Gly^{52} replaced by an isosteric thioester bond. The homodimeric form of the enzyme, $([COS]^{51-52})_2$HIV-1 PR, was fully active, thus demonstrating that replacement of the Gly^{51}–Gly^{52} amide bond by a thioester had no ill effect on the folding or catalytic function of the enzyme.

By the same approach, the D-enantiomer of the enzyme, D-$([COS]^{51-52})_2$HIV-1 PR was prepared and its enzymatic properties were determined [31,32]. The two mirror image enzyme molecules were equally active, but showed reciprocal chiral specificity in that the L-enzyme cleaved only the L-substrate whereas the D-enzyme cleaved only the corresponding D-substrate. Similarly, the enantiomeric forms of the inhibitor MVT101 [32] were effective only against the corresponding enantiomer of the enzyme; i.e., L-MVT101 inhibited the L-HIV-1 PR catalyzed reaction but not the D-HIV-1 PR catalyzed reaction, while D-MVT101 inhibited D-HIV PR but had no effect on the reaction catalyzed by L-enzyme.

Crystallographic studies were undertaken with the total D-enzyme. Synthetic D-$([COS]^{51-52})_2$HIV-1 PR co-crystallized with the D-MVT101 hexapeptide in the space group $P2_12_12_1$, $a=67.5$, $b=92.8$, $c=29.4$ Å. There were two monomers of the enzyme and one inhibitor in the crystallographic asymmetric unit. X-ray diffraction data to a resolution of 2.5 Å were collected from a single crystal. The structure was solved by molecular replacement [33], using the coordinates of the protein dimer from the structure of the complex of native backbone L-HIV-1 PR and MVT101 inhibitor [34] (in the absence of anomalous dispersion it is not possible to distinguish between L and D enantiomers using X-ray diffraction techniques). The same refinement protocol, that was used in the refinement of the L-complex [7,34] (using X-PLOR and PROFFT packages) was followed so the comparison between the two structures would be more meaningful. The structure was refined to an *R*-factor of 0.188 in the resolution range of 10–2.5 Å.

A stereo view of the backbone of the D-enantiomorph of HIV-1 PR and the bound hexapeptide inhibitor in its electron density map is shown in Fig. 4(a).

Comparison of the crystal structure of inhibitor–D-HIV-1 PR complex [35] with that of the natural-backbone synthetic L-amino acid enzyme [7,34] showed that the two molecules were in all respects the mirror image of each other, including the centers of asymmetry not directly determined by the chirality of C_α atoms in the polypeptide backbone The root mean square (rms) differences between equivalent C_α atoms when the mirror image of the D-PR dimer (Fig. 4(b)) was superimposed on the L-enantiomer model [34] (Fig. 4(c)) was 0.73 Å for 198 target pairs (Fig. 4(d)). The largest deviations are for residues involved in the crystal contacts in one or both the crystal forms. This result demonstrates that the structure of $([COS]^{51-52})_2$HIV-1 PR is essentially the same as that of the native backbone enzyme.

Despite the overall similarity, the crystal structure of the $([COS]^{51-52})_2$HIV-1 PR/MVT-101 complex revealed some important differences to the native backbone enzyme. Although the general mode of inhibitor binding is the same, there are notable deviations in the flap region of the enzyme. Instead of a single tetrahedrally coordinated water molecule, two distinct water molecules were observed to mediate hydrogen bonds between the Gly^{49}–Ile^{50} amide bond and inhibitor P2 and P1′ carbonyl groups. These two water molecules, were characterized by high thermal vibration factors and only partial (≈50%) occupancy. By contrast, the single water (Wat-301) in the structure of the natural backbone enzyme is highly ordered. The disorder of Wat-301 in the $([COS]^{51-52})_2$HIV-1 PR/MVT101 complex could not be attributed to any artifacts caused by crystal packing, since crystal structure of a recombinant HIV-1 PR/inhibitor complex in the same space group showed well defined density for Wat-301 (K. Appelt, personal communication). In addition to discrepancies in bound water, the flap regions of $([COS]^{51-52})_2$HIV-1 PR could not be unambiguously modeled to the electron density. The flap from monomer 1 was involved in crystal contacts, while the flap from monomer 2 was exposed to solvent and appeared to be disordered. As shown in Fig. 5, there were several breaks in the electron density of the flaps, even when contoured at 0.8σ level.

A further observation to arise from comparison of the flap conformations in natural backbone versus $([COS]^{51-52})_2$HIV-1 PR complexed with MVT-101 relates to the N–C_α dihedral angle (ϕ) for Ile^{50} at the tip of each flap. In the original HIV-1PR/MVT-101 complex, this angle was observed to be −65° in both subunits [7,34]. However, in the structure of the $([COS]^{51-52})_2$HIV-1 PR/MVT-101 complex, the Ile^{50} N–C_α dihedral angle was −110° in monomer 1 and −95° in monomer 2. To check that this discrepancy was not an artifact of crystal packing in the $([COS]^{51-52})_2$HIV-1 PR/MVT-101 complex, we also measured the Ile^{50} N–C_α dihedral angle in solution. For this, we prepared $([COS]^{51-52})_2$HIV-1 PR (using L-amino acids) containing a single site-specific ^{15}N label (95% enrichment) in the Ile^{50} residue in each subunit [36]. We measured the Ile^{50} N–C_α dihedral angle for the solution complex of this molecule bound to MVT-101 by NMR (Fig. 6) [36]. A series of *J*-modulated ^{15}N-edited HSQC experiments were recorded to obtain an estimate of the $^3J_{HN\alpha}$ coupling constant for residue Ile^{50} in each subunit of the enzyme. This coupling constant was measured as 9.0 Hz, consistent with $\phi = -100°$ or −140° (±5°) for Ile^{50} in each flap. The value of −100° correlates closely with values of −110° and −95° observed in the crystal structure. Thus, these data suggest that the conformation at the tips of the flaps observed in the $([COS]^{51-52})_2$HIV-1 PR/MVT-101 crystal structure is the same as that which exists in solution.

The structural differences observed in the flaps of native backbone HIV-1 PR compared to the backbone engineered $([COS]^{51-52})$ HIV-1 PR analogue are almost certainly related to the replacement of the Gly^{51}–Gly^{52} amide bond with a thioester. In the backbone-engineered $([COS]^{51-52})_2$HIV-1 PR, the Gly^{52}NH is replaced by the sulfur atom of the thioester isostere. Consequently, the hydrogen bond normally present between the NH of Gly^{52} and the carbonyl of Gly^{49} is disrupted. In the absence of this hydrogen bond, the Gly^{49}–Ile^{50} amide bond rotates ≈70° out of the plane of the flap and this necessarily affects the Gly^{49}–Ile^{50} N–H vector and thus water 301 binding. In the place of the ordered, tetrahedrally coordinated water 301 we observed two poorly defined water molecules.

Intriguingly, the $([COS]^{51-52})_2$HIV-1 PR is a fully

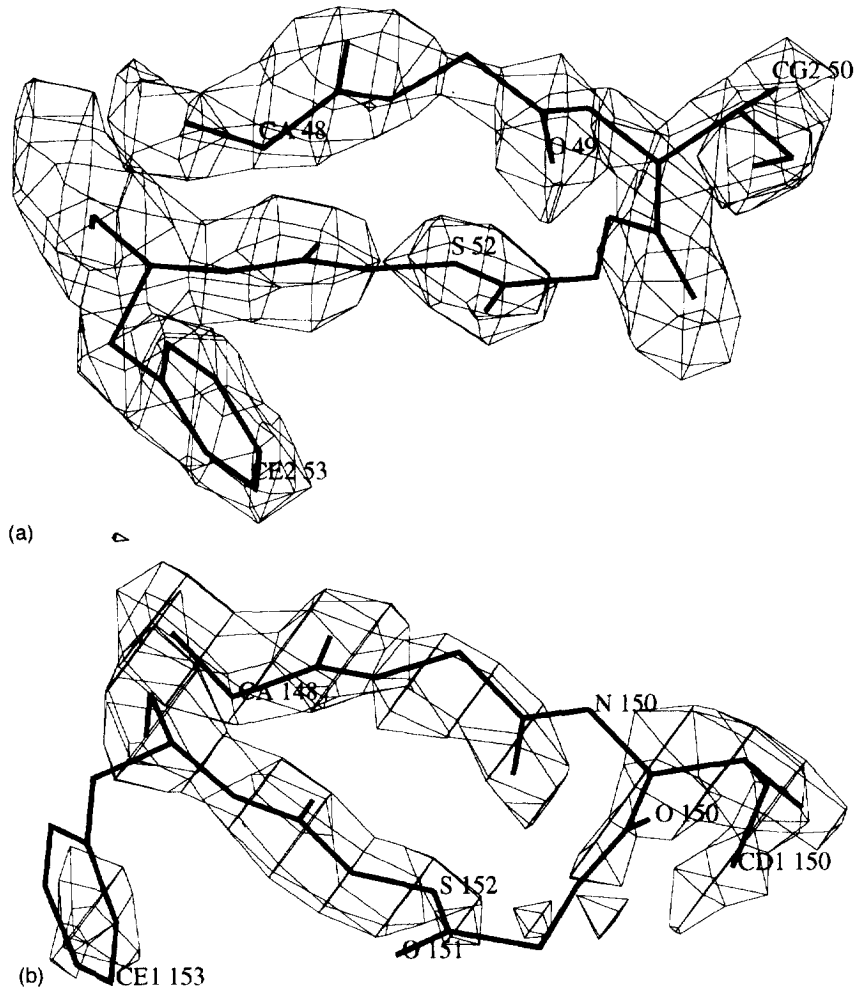

Fig. 5. (2Fo – Fc) electron density maps contoured at 0.8σ level for the model in flap regions.

active enzyme. The observation of a fully active HIV-1 PR analogue *without* water 301 in the crystal structure with a substrate-derived inhibitor casts doubt on the role of the crystallographic water 301 in HIV-1 PR enzymatic activity.

2.2. $([COS]^{49-50})_2$HIV-1 PR

To further investigate the role of flap–substrate hydrogen bonding in the activity of HIV-1 PR, we sought to specifically eliminate hydrogen bonds from the Ile^{50} NH in each flap to substrate P2 and P1′ carbonyl groups. To this end, a backbone-engineered HIV-1 PR analogue, $([COS]^{49-50})_2$HIV-1 PR, was prepared [37] in which the amide –CONH– bond between residues Gly^{49} and Ile^{50} in each monomer was replaced by a thioester –COS– (Fig. 7). Single atom substitution of the –NH– by an –S– removed the hydrogen bond donating ability at that position at the tip of each flap in this HIV-1 PR analogue. Analysis of the kinetic parameters for cleavage of a peptide substrate by $([COS]^{49-50})_2$HIV-1 PR indicated an identical K_m to the native backbone enzyme, but revealed a dramatic 2500-fold reduction in the turnover

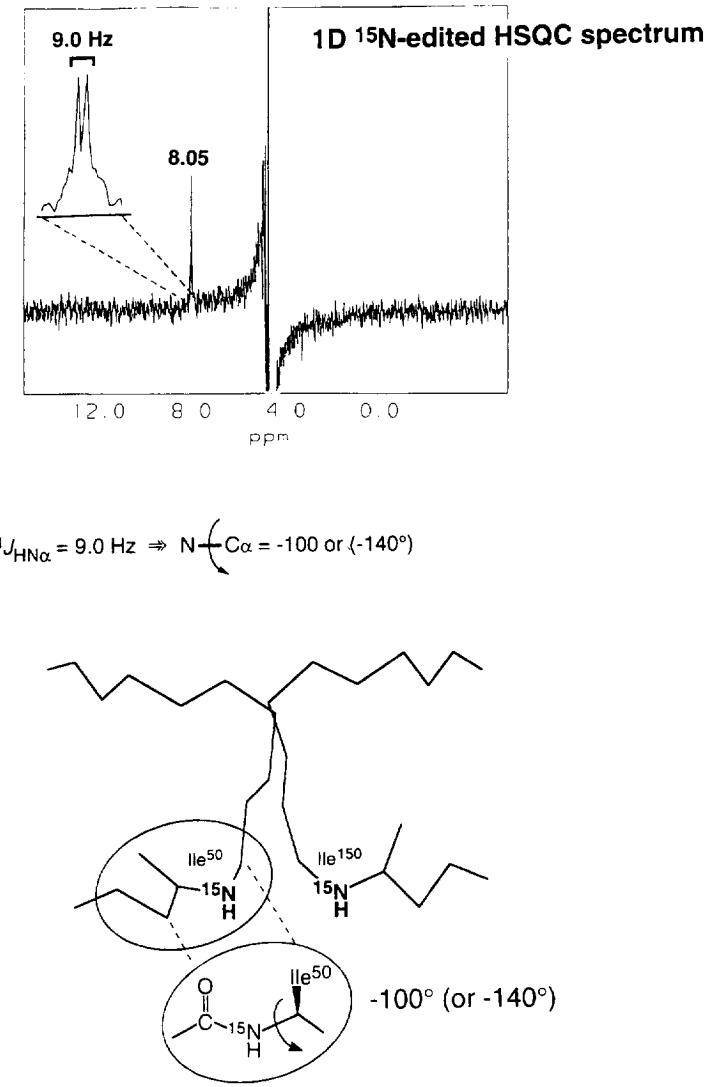

Fig. 6. ^{15}N NMR measurements, (for details see [36]): $^{1}D^{15}N$-edited ^{1}H 600 MHz HSQC spectrum of (^{15}N-ILE^{50}[COS]$^{51-52}$)HIV-1 proteinase homodimer. The single resonance at 8.05 ppm indicates that only one residue (i.e. Ile^{50} in each subunit) of the HIV-1 PR analogue is labeled with ^{15}N.

number (k_{cat}). This decrease in the catalytic activity corresponds to a 5 kcal mol^{-1} increase in the activation energy for the rate limiting step in catalysis and is consistent with the effect of deleting two hydrogen bonds.

The large decrease in activity resulting from the single atom substitution not only suggests an important role for flap–substrate hydrogen bonds, but also indicates that these critical interactions cannot be substituted by any neighboring groups in the flap regions of enzyme molecule. The results obtained with ([COS]$^{49-50}$)$_2$HIV-1 PR were consistent with both flaps contributing equally to the enzymatic activity. Nonetheless, we wondered whether, by analogy with the cell-encoded aspartyl proteinases, hydrogen bonding from the tip of only one flap was required for normal enzymatic activity. Although this is not what one would infer from the large body of

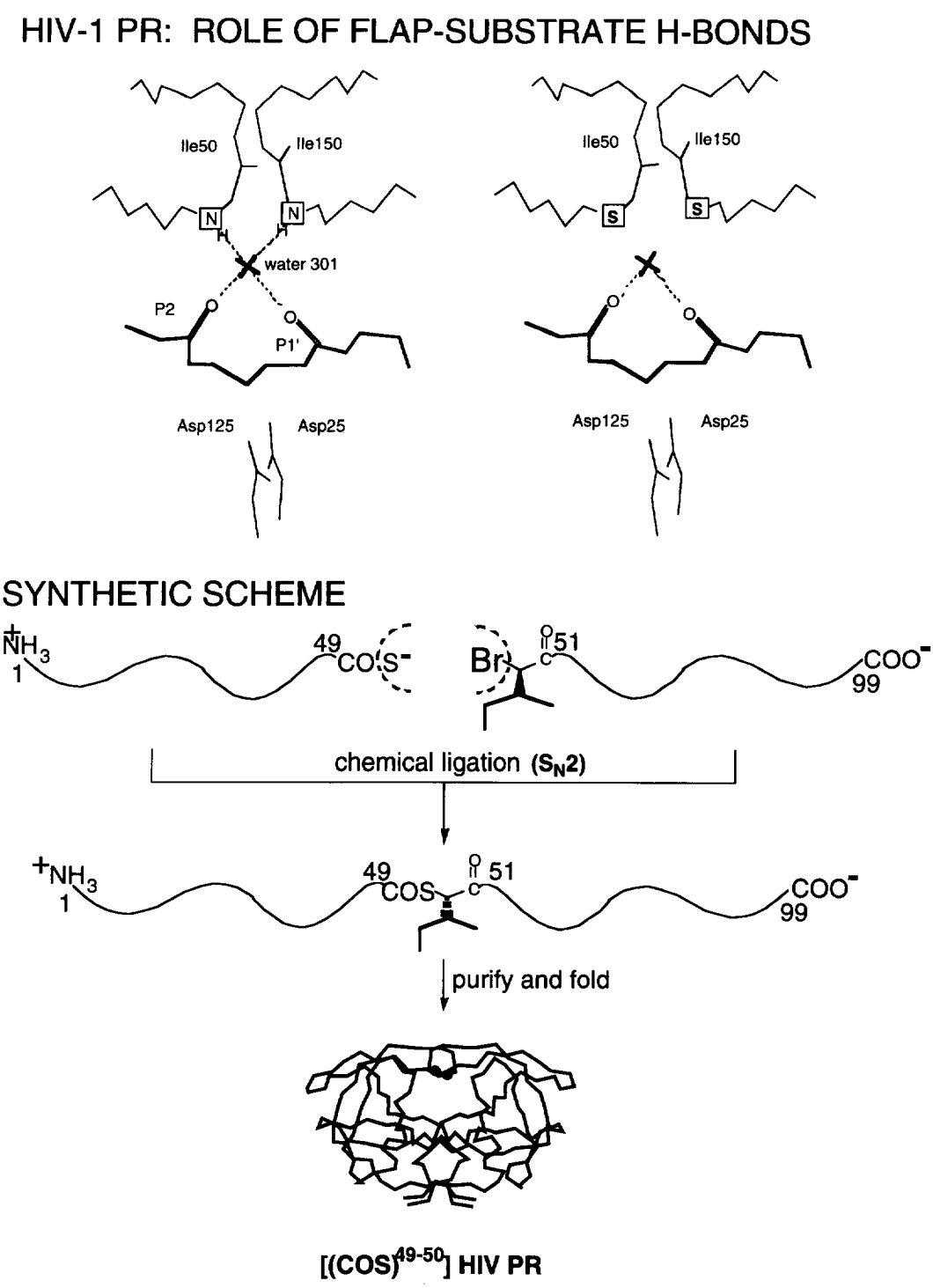

Fig. 7. Design/synthesis of ([COS]$^{49-50}$)2HIV-1 PR [37].

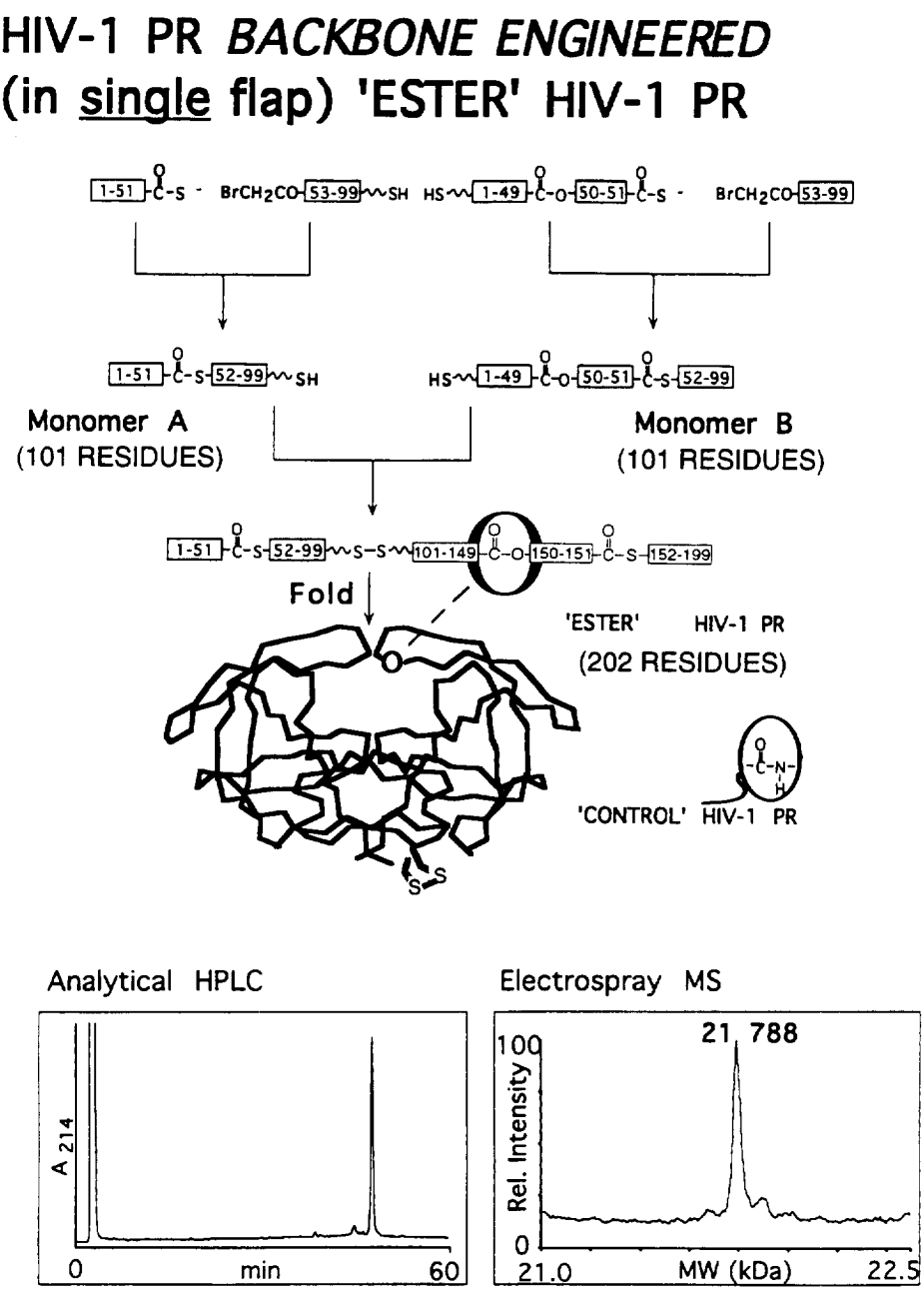

Fig. 8. Design/synthesis of ([COO]$^{49-50}$,[CONH]$^{149-150}$)HIV-1 PR [38].

HIV-1 PR inhibitor structures [17], it is a scenario worth considering when one recalls that the pepsin-like proteinases possess only a single functional flap. We considered this an avenue worth pursuing and accordingly set out to further explore this idea.

2.3. ([COO]$^{49-50}$,[CONH]$^{149-150}$)HIV-1 PR

Based on the need to further investigate the role of specific flap–substrate hydrogen bonds in the catalytic mechanism of HIV-1 PR, we designed an analogue of

HIV-1 PR in which hydrogen bond donor was deleted from the tip of one flap only. A covalent dimeric form of HIV-1 PR was prepared by total chemical synthesis in which the Gly^{49}–Ile^{50} peptide bond –NH– atom was specifically replaced, in one flap only, by an ester –O– atom (Fig. 8). This single atom substitution removes the hydrogen bond donating ability of the Gly^{49}–Ile^{50} backbone linkage. The normal peptide bond was maintained in the second subunit of the enzyme (numbered Gly^{149}–Ile^{150}). As a control molecule, a second covalent dimer analogue was simultaneously prepared [38] which differed from the single flap ester analogue only in possessing the native amide [CONH] linking Gly^{49}–Ile^{50} in both flaps. The control molecule was fully active, with kinetic parameters (k_{cat}, K_m) similar to those of either recombinant or chemically synthesized HIV-1 PR. Surprisingly, the analogue containing a backbone ester bond at Gly^{49}–Ile^{50} in one flap was also highly active, and kinetic analysis indicated that k_{cat} was reduced only by a factor of 2 relative to the control enzyme molecule. This was a remarkable result. Based on a 5 kcal mol^{-1} effect for deletion of both flap–substrate hydrogen bonds, a logical assumption is that deletion of one of these bonds would lead to a 2.5 kcal mol^{-1} effect, or $\approx$ 50-fold reduction in the intrinsic catalytic activity. The observation of only a 2-fold effect in k_{cat} is not consistent with this expectation. We interpreted the 2-fold reduction of k_{cat} as consistent with the suggestion that hydrogen bonding from the tip of only *one flap* is important to catalytic activity.

Additional evidence which calls into question the significance of hydrogen bonding from both flaps came from the study of inhibitor binding. DMP-323 [28], an HIV-1 PR inhibitor specifically designed to displace water 301 and hydrogen bond with the tip of both flaps, is actually bound 3-fold tighter to the monoester enzyme analogue relative to the control enzyme. This is despite the inability of the monoester analogue to form one of these two enzyme–substrate hydrogen bonds. Thus even for inhibitor binding, hydrogen bonding from the tip of the second flap makes no net contribution to function.

3. Discussion

In this paper we have described a series of experiments performed on the HIV-1 PR molecule that were designed to elucidate specific aspects of the molecular basis of its action as a proteolytic enzyme. We have used the capabilities provided by total chemical synthesis to prepare unique variant forms of the enzyme molecule in order to explore the role of the flaps in HIV-1 PR catalysis and inhibitor binding. Full kinetic characterization (including studies with key inhibitors) was carried out on each analogue; in addition, structural studies were carried out on selected enzyme chemical variants. Taken together, the kinetic and structural data on these backbone engineered enzyme molecules present a strong case that something is amiss with the conventional two-flap model of HIV-1 PR action. The ($[COS]^{51-52}$)HIV-1 PR retains full catalytic activity, but does not seem to coordinate water 301 in the co-crystal structure with MVT101; the "double (backbone H-bond) deletion" variant, ($[COS]^{49-50}$)HIV-1 PR, displays a loss of catalytic activity that shows that the Ile^{50} –NH– makes an important contribution to enzyme action; yet, the "single flap (backbone H-bond) deletion" tethered dimer HIV-1 PR retains full catalytic activity!

Based on this combined structural and biochemical characterization of a number of HIV-1 PR analogues engineered in the flap region, we find no evidence to support a mechanistic role for water 301. Water 301 was seen by crystallography also in catalytically non-relevant complexes [39], while changes to the enzyme molecules which result in the absence of this water molecule caused no change in the catalytic properties. We have further observed that removing the flap–substrate hydrogen bonds apparently mediated by water 301 has drastically different consequences according to whether one or both of these hydrogen bonds is disrupted. Deleting both of these hydrogen bonds (one from each flap) has a major deleterious effect on enzymatic activity. Deletion of just one of these hydrogen bonds has essentially no effect. These results, surprising given the highly symmetrical nature of the HIV-1 PR homodimer observed crystallographically, contrast with the hitherto assumed relevance, both for inhibitor design [17,28] and catalysis [26], of the hydrogen bonds from both flaps to the P2 and P1′ substrate/inhibitor carbonyls. What we propose based on these results is that the two flaps of the HIV-1 PR homodimer do not contribute equally to the catalytic process.

While this difference may be confined to the level

of a single hydrogen bond, we wish to raise the possibility that HIV-1 PR may in fact function in a manner analogous to the pepsin-like proteinases. Even before the three dimensional structures of the retroviral proteinases were predicted [3] and elucidated [2,4,6], Tang et al. [40] put forward the hypothesis that (1) the sequence of the retroviral proteinase corresponds to a domain of an aspartic proteinase molecule, (2) the retroviral proteinases must exist as dimers, and, (3) the pepsin-like enzymes are fusion proteins arising out of an ancestral protein similar to the retroviral proteinase. It is still not clear whether retroviral proteinases, evolving from an ancestral gene, are direct precursors to the cellular enzymes or whether they are independently derived from the ancestral gene by deletion events. In order to carry out its enzymatic functions, the aspartic proteinase must comprise two domains or two monomers, and must have two characteristic Asp–Thr–Gly signature sequences at the active site. However, if the aspartic proteinases from higher organisms could function with only one flap, may one conclude that the retroviral proteinase or a similar archetypal one also uses only one flap? If the convergence of the catalytic mechanism of the native enzymes belonging to the two classes, viz., two adjacent Asp residues from different domains or monomers, is any indication, one can extrapolate it to the entire process of the catalysis and conclude that perhaps only one flap is necessary and sufficient for enzymatic activity for both classes of aspartyl proteinases.

Over all, the data discussed in this paper support this hypothesis. The experimental observations presented here provide evidence that interactions from one flap are sufficient for full enzymatic activity of HIV-1 PR and are consistent with a model in which the retroviral enzyme uses only a single flap closed down over the substrate in the catalytically productive complex (see Fig. 2) similar to that of pepsin-like enzymes. This would involve direct flap–substrate interactions, with a quite different (flat vs. edge on) orientation of the flap with respect to the substrate. While not in itself an outrageous suggestion (after all, the cell encoded enzymes work perfectly well with only one flap), at first glance, it is not easy to see how such a mode of action can be reconciled with the extensive crystal structure data and with NMR evidence that both HIV-1 PR flaps close down over substrate-derived inhibitors, and that both flaps are involved in specific interactions of potential importance for enzyme action.

The appearance of two fully symmetrical flaps in X-ray structures of catalytically non-relevant HIV-1 PR complexes with symmetrical inhibitors is not surprising and is not in disagreement with the single flap hypothesis. In the case of complexes with substrate-based inhibitors the same observation may be an artifact caused by the bidirectionality of the inhibitor in the crystal lattice. Unfortunately X-ray data for HIV-1 PR with bound inhibitors are available in most cases only at low to medium resolution. In the crystal structure of HIV-1 PR/MVT101 complex, MVT101 hexapeptide, initially reported as bound in one orientation [7], on refinement of 2 Å data in fact exhibits a two-fold static disorder [34]. However, no attempt to correlate the structure of the flap with the orientation of the peptide chain was made. The first indication of "nonequivalence" of the two flaps of the homodimer came from the crystal structure of the $([COS]^{51-52})_2$HIV-1 PR (solved as a the mirror image of D-enantiomer) described here. At 2.5 Å resolution MVT101 inhibitor was modeled in one orientation. If this indeed is a case in this crystal form, then the second flap is visibly disordered (see Fig. 5(b)). The other possibility is that the poorly defined electron density corresponds to the flap which closes over the inhibitor bound in the alternative orientation with lower occupancy. The "nonfunctional" flap would be then in this crystal lattice completely disordered and undetectable, as were both flaps in the crystal structure of the unliganded form of RSV PR [4,41].

We have recently collected X-ray data extending to 2 Å resolution on co-crystals of D-ligated HIV-1 PR complexed with D-MVT101 inhibitor and we will try to verify these possibilities with carefully designed refinement procedures. The chemical synthesis of other flap-region analogues of the HIV-1 PR is also being undertaken to test the role of the flaps in catalysis.

4. Conclusions

The conventional "two-flap" model of HIV-1 PR catalysis has been subjected to a critical test, and on the face of it has been put in doubt by the structural and kinetic properties of the variant forms of the

enzyme prepared by total chemical synthesis. The "single flap" mode of catalytic action of the HIV-1 PR has to be subjected to more stringent experimental investigation, in order to clarify if it is the normal mode of action or an alternative mechanism in the event of a mutation in one of the flaps of the PR dimer. In either case, the results reported here have significant implications for understanding the molecular origins of substrate specificity in the HIV-1 PR and the problem of escape mutants, and will contribute to the design of improved inhibitors. More work remains to be done to explore these important questions.

Acknowledgements

We thank Drs. Jane Dyson and Afshin Karimi for their assistance in the design and execution of the NMR experiment. This research is sponsored in part by the National Cancer Institute (DHHS) under contract with ABL and by NIH grants R01 48897 and P01 GM 48870 (SBHK). The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

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