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Review

# Structural and biochemical studies of retroviral proteases

Alexander Wlodawer *, Alla Gustchina

*Macromolecular Crystallography Laboratory, Program in Structural Biology, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702, USA*

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**Abstract**

Retroviral proteases form a unique subclass of the family of aspartic proteases. These homodimeric enzymes from a number of viral sources have by now been extensively characterized, both structurally and biochemically. The importance of such knowledge to the development of new drugs against AIDS has been, to a large extent, the driving force behind this progress. High-resolution structures are now available for enzymes from human immunodeficiency virus types 1 and 2, simian immunodeficiency virus, feline immunodeficiency virus, Rous sarcoma virus, and equine infectious anemia virus. In this review, structural and biochemical data for retroviral proteases are compared in order to analyze the similarities and differences between the enzymes from different sources and to enhance our understanding of their properties. 

*Keywords:* Retrovirus; Aspartic protease; AIDS; Protein structure; Enzyme kinetics

## 1. Introduction

In the past ten years, retroviral proteases (PRs) have gained the distinction of becoming the group of proteins most widely studied by crystallographic techniques. The structures of more than 180 complexes of PRs from human immunodeficiency virus type 1 (HIV-1), type 2 (HIV-2), and simian immunodeficiency virus (SIV), originating from more than 25 laboratories, have already been collected into an internet-accessible database [1] (http://www.ncifcrf.gov/HIVdb), and they most likely represent only a small fraction of the total. In addition, the structures of related PRs from Rous sarcoma virus (RSV), feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV) have also been reported. Because this interest in retroviral PRs has been largely sustained by their critical role as targets for a new generation of AIDS drugs [2–6], a number of reviews have already been published summarizing their properties, especially those important in the design of the five drugs that are now approved for clinical use [7] and of several more that are in trials or preclinical development [8–11]. However, other features of these proteins, such as those responsible for most of their structural and enzymatic properties, have not been compared in similar detail. In this review, we summarize the current state of knowledge of the structure, function, and activity of all retroviral PRs for which structural data are available.

* Corresponding author. Fax: +1-301-846-6128; E-mail: wlodawer@ncifcrf.gov

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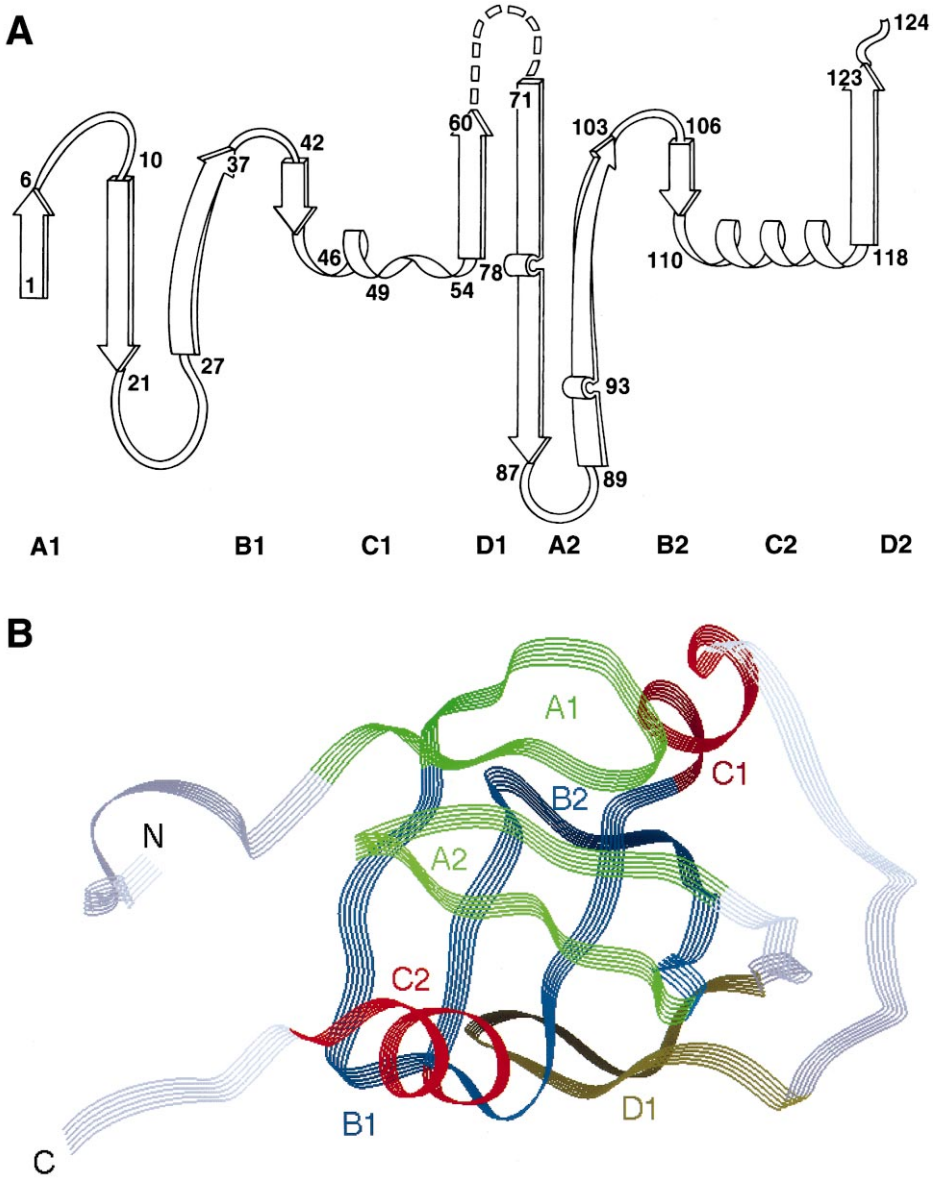


Fig. 1. Secondary-structure representation of retroviral PRs. (A) Diagram of the secondary structure, with residue numbers corresponding to the structural elements observed in RSV PR. Adapted from Jaskólski et al. [31]. (B) Three-dimensional structure of a monomer of EIAV PR, with secondary-structure elements marked. Adapted from Gustchina et al. [45].

## 2. Structures of retroviral PRs

Based on their amino acid sequences, retroviral PRs have been identified as belonging to a new class in the family of aspartic PRs almost as soon as the first sequences of the retroviral genomes became available [12,13]. These enzymes exhibit characteristics typical of aspartic proteases, such as inhibition by pepstatin [14–16] and inactivation by mutating the putative active site aspartates [14,17–19]. However, unlike cellular aspartic proteases such as pepsin or renin, a single molecule which contains two topologically similar domains and more than 300 amino acids [20], retroviral PRs are much smaller. They are active as symmetric dimers with a single active site, formed by the residues originating from two identical monomers. This type of molecular architecture is unique among enzymes, with no other known examples of active sites formed in a similar manner. Nevertheless, despite the uniqueness of retroviral

PRs, early modeling studies based on cellular PRs yielded models that were largely correct [21,22], due to the high similarity in the topology of a single domain of nonviral aspartic PRs and a monomeric molecule of retroviral PRs. The secondary structures of all retroviral PRs follow a structural template that was previously used to describe nonviral aspartic PRs [23]. According to the template, the monomeric molecule of a retroviral PR is formed by duplication of four structural elements: a hairpin (containing loops A1 and A2), a wide loop (B1, containing the catalytic aspartic acid, and B2), an α-helix (C1 and C2), and a second hairpin (D1 and D2) [24] (Fig. 1A). The hairpin D2 is substituted by a β-strand in all retroviral proteases for which structural information is available. The α-helix C1 is prominent only in EIAV PR (Fig. 1B), whereas it consists of a single helical turn in RSV and FIV PRs, and is replaced by a loop in HIV (both types 1 and 2) and SIV PRs. Also, the length of loops A1 and A2 is different in various retroviral PRs, as are the length and conformation of the connecting segments between these structural elements. These structural elements are present in all reported apoenzyme structures except for the original one of HIV-1 PR (PDB code 2hvp [25]), later shown to be partially incorrect [26]. The pseudodyad in the molecules of retroviral PRs was not considered to be of much evolutionary significance, but rather arising from the necessity of supporting the folding of the enzymes [27].

The most significant differences in the structures of viral and nonviral PRs are attributed to the symmetrical arrangement in the dimeric molecule of the retroviral PR. The two halves of the molecules are exactly or approximately the same, as opposed to the single chain molecule of nonviral aspartic protease, two domains of which, although topologically similar, are not identical. The flexible β-loop D1, known as a 'flap' in nonviral PRs, is functionally very important, since it changes orientation during the binding of the ligand and forms numerous interactions with it. Two such flaps are present in the symmetric dimers of retroviral PRs. The N- and C-termini in a dimer form a four-stranded β-sheet interface, as opposed to the six-stranded interdomain β-sheet in nonviral aspartic PRs. The amino acid sequences of retroviral PRs share considerable similarity, particularly in the locations of residues that are important

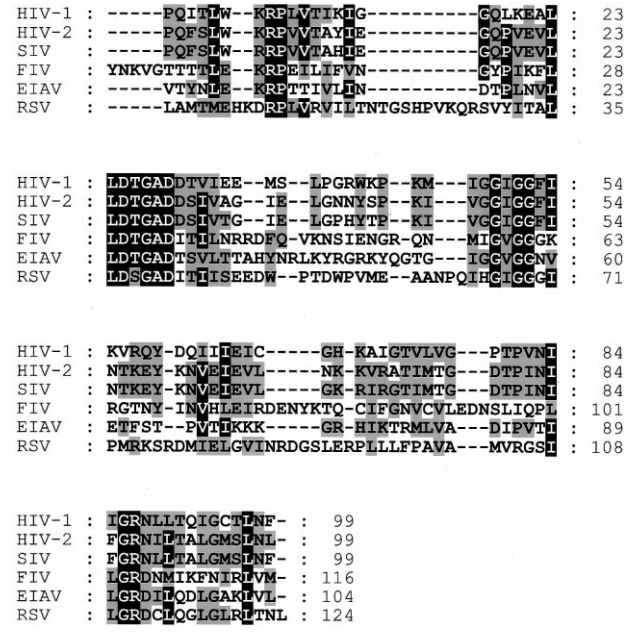


Fig. 2. Amino acid sequences of retroviral PRs, aligned based on their three-dimensional structures. Identical residues are shown in black boxes, while those conserved in the majority of the structures are shown in gray boxes.

in preserving both structure and function (Fig. 2). Structure-based alignments of their sequences have been already discussed in detail [28].

The structures of apoenzymes of four retroviral PRs have been solved and reported to date. Solving such structures was not without difficulties, since these enzymes tend to autodigest in the absence of inhibitors [29] and, thus, growing suitable crystals is not particularly easy. The resolution of the reported structures of apoenzymes is not very high. The best-refined structures are those of RSV PR [30,31] at 2.0 Å resolution and the nearly identical myeloblastosis associated virus (MAV) PR at 2.2 Å [32]. The structure of HIV-1 PR was solved independently in four different laboratories at 2.7–3.0 Å resolution, using recombinant or synthetic enzymes corresponding to several viral isolates [25,33–35], whereas the structure of HIV-2 PR was solved at 2.5 Å resolution [36]. Finally, the structure of the apoenzyme of SIV PR was reported at 2.3 Å resolution [37] with closed flaps, and at 2.0 Å with the flaps open and partially disordered [38].

Although the structures of the apoenzyme PRs superimpose very well throughout most of their sequences (Fig. 3), considerable differences are ob-

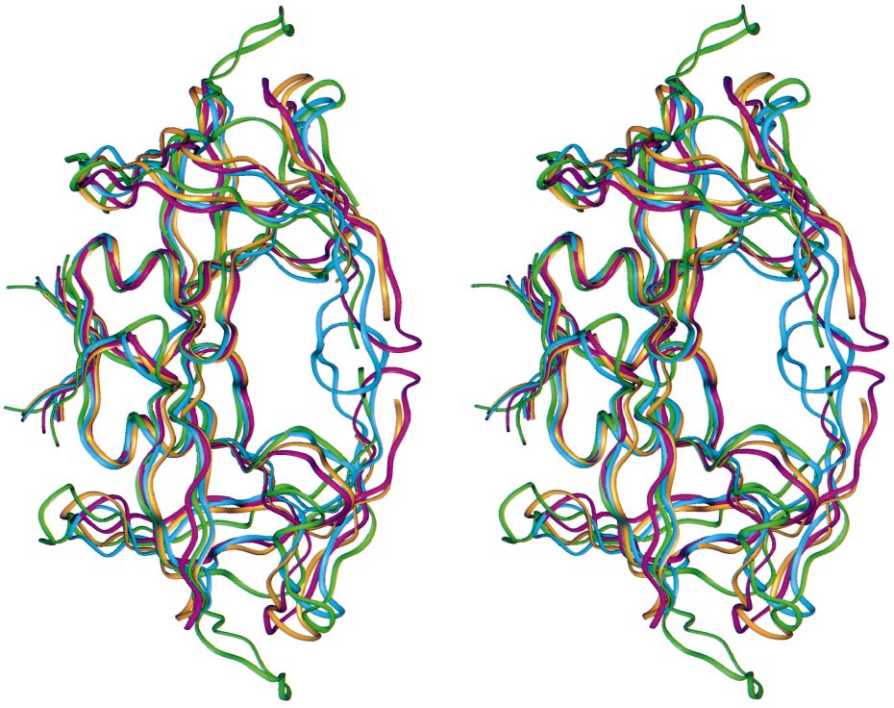

Fig. 3. Superposition of the Cα traces of the apoenzyme forms of RSV PR [31] (green) and HIV-1 PR [33] (magenta), as well as open [38] (gold) and closed [37] (cyan) forms of SIV PR. This figure, as well as Fig. 4, are prepared with the program InsightII (Molecular Simulations) and Photoshop (Adobe).

served in the area of the flap (defined above). This stretch is completely disordered in RSV PR, is found in an open conformation in all four structures of HIV-1 PR (it must be stressed, though, that all of the structures are isomorphous and thus subject to identical crystal contacts) and in HIV-2 PR, and either in a closed conformation or partially disordered in SIV PR. Clearly, the prominent flaps in retroviral PRs (which are much larger than the single flap in nonviral PRs) must open upon binding of the substrates [38,39]; therefore, their flexibility is an expected and necessary feature. The open structure of HIV-1 PR was postulated to be a potential crystallographic artefact, caused by the direct interactions between the flaps of two molecules in the crystal lattice [9,33]. However, although crystals of the open form of the apoenzyme HIV-2 PR are not isomorphous to those of HIV-1 PR [36], the open flaps are very similar (Fig. 3), while not involved in obvious crystal contacts. This similarity might indicate that the open conformation of the flaps is quite stable after all. Conversely, the closed flaps in SIV PR seem to be stabilized by crystal contacts [37]. The flaps in RSV PR [30] and in the open form of SIV PR [38] face open areas with no crystal contacts, so it is not surprising that they do not assume unique orientations that could be traced in the crystal structures. The closed flaps in SIV PR [37] make this structure more similar to the inhibitor complexes of HIV and SIV PRs than to the apoenzyme structure of HIV-1 PR (see below), whereas the partially disordered flaps in the other SIV PR structure [38] more closely resemble RSV PR. Recent NMR studies of the HIV-1 apoenzyme have indicated that an ensemble of open conformations might predominate in solution, being in slow dynamic equilibrium on ca. 100 μs time scale with the disordered flap conformations that permit access to the active site [40].

Direct comparison of the Cα tracings of HIV-1 PR (PDB code 3hvp [33]) to the open structure of SIV PR (PDB code 1az5 [38]) shows the deviation of 1.38 Å for 184 atom pairs; to the closed structure of SIV PR (PDB code 1sip [37]), 1.52 Å for 186 atom pairs; to RSV PR (PDB code 2rsp [31]), 1.78 Å for 174 pairs; and to HIV-2 PR (PDB code 1hsi [36]), 1.08 Å for 195 atom pairs. These differences exceed by

more than a factor of two those reported for the various independently determined structures of HIV-1 PR, so they are quite significant. It is not surprising that the deviations are larger for RSV PR than for SIV and HIV-2 PRs, which are of identical length to HIV-1 PR, and that the structure of SIV PR with partially disordered flaps would be more similar than the one with closed flaps. The open structures of HIV-1 and HIV-2 PRs are by far most similar.

Although the structures of HIV-1, HIV-2 and SIV PRs are quite similar, the polypeptide chains of the other three retroviral PRs are longer, thus allowing additional structural elements to be identified. FIV PR has an extension at the N-terminus that is not present in other PRs, but this part of the structure is disordered and, therefore, not visible [41,42]. Another feature specific to FIV PR is an insert Glu93-Asp94-Asn95 which follows the position equivalent to Gly78 in HIV-1 PR. This additional structural element might be involved in modulating the flap opening in FIV PR [41]. The much longer RSV PR has a two-residue extension of the loop after residue 6, a ten-residue insert after residue 18, a considerably different tracing of residues 48–54 (which interact with the loop 19–28), inserted residues 86–90, and a one-residue extension at the C-terminus. A unique feature of EIAV PR is the presence of helix C1 (residues 33–41), rather than the single helical turn seen in other retroviral PRs (Fig. 1B). The root mean square (rms) deviation between the dimers of EIAV and HIV-1 PRs is 1.35 Å (185 atom pairs); between EIAV and FIV PRs, 1.35 Å (187 atom pairs); and between EIAV and RSV PRs, 1.22 Å (171 atom pairs).

The active site of retroviral PRs contains a pair of aspartic acids (Asp25 and 25′ in HIV-1, HIV-2, SIV, and EIAV PRs, Asp30/30′ in FIV PR, and Asp37/37′ in RSV PR; in this review, we will follow the numbering of HIV-1 PR, unless explicitly noted otherwise). The conserved active site residues – Asp25, Thr26 (replaced by Ser38 in RSV PR), and Gly27 – are located in a loop whose structure is stabilized by a network of hydrogen bonds similar to that found in the eukaryotic PRs (for a review, see [20]). The carboxylate groups of Asp25 from both chains are nearly coplanar and show close contacts involving Oδ1 atoms. The network is quite rigid due to an interaction called the 'fireman's grip', in which each Thr26 Oγ1 accepts a hydrogen bond from the Thr26 main-chain NH of the opposing loop. Thr26 also donates a hydrogen bond to the carbonyl O atom of residue 24 on the opposite loop. Although the central features of the catalytic site are very similar between retroviral and cellular aspartic PRs, the residue following the triad differs, with Ala invariably present in retroviral PRs, and either Ser or Thr most common in the pepsins.

The carboxylate residues are bridged by a water molecule, located within hydrogen bonding distance to the oxygen atoms of the Asp25 carboxylates. Similar water molecules have also been reported in non-viral PRs [20] and might correspond to the catalytic water in this family of PRs. The distances between the inner O atoms of the coplanar carboxylates are 2.8–3 Å, indicating the presence of an acidic proton in the bridge.

## 3. Inhibitor complexes

Many crystal forms of the complexes of HIV-1, HIV-2, SIV, and FIV PRs with inhibitors have been reported [8,11,41,43,44]. The enzymes that were used for such studies were either wild-type (corresponding to a variety of viral strains) or mutants, particularly drug-resistant ones. For EIAV and RSV PRs, however, only the structures of mutant PR complexes have been described [45,46]. Some of these structures have been solved to quite high resolution; at least two of them have been refined at 1.7 Å with R-factors below 0.2 [47,48]. Most of the crystal forms of retroviral PRs contain a dimer of the protease and a single inhibitor molecule in the asymmetric unit, although in some cases only a monomer of the PR is present [49,50], while two dimers are present in other structures [48]. The availability of multiple crystal forms with significantly different packing makes it possible to differentiate between the intrinsic structural properties of the enzyme and the crystal-induced conformational adjustments. Although the inhibitors are clearly ordered in unique orientations in some structures (for example, see [51,52]), they are often present in two orientations occupying similar space; as a result, they can be separated only with difficulty [26,41,49,50,53].

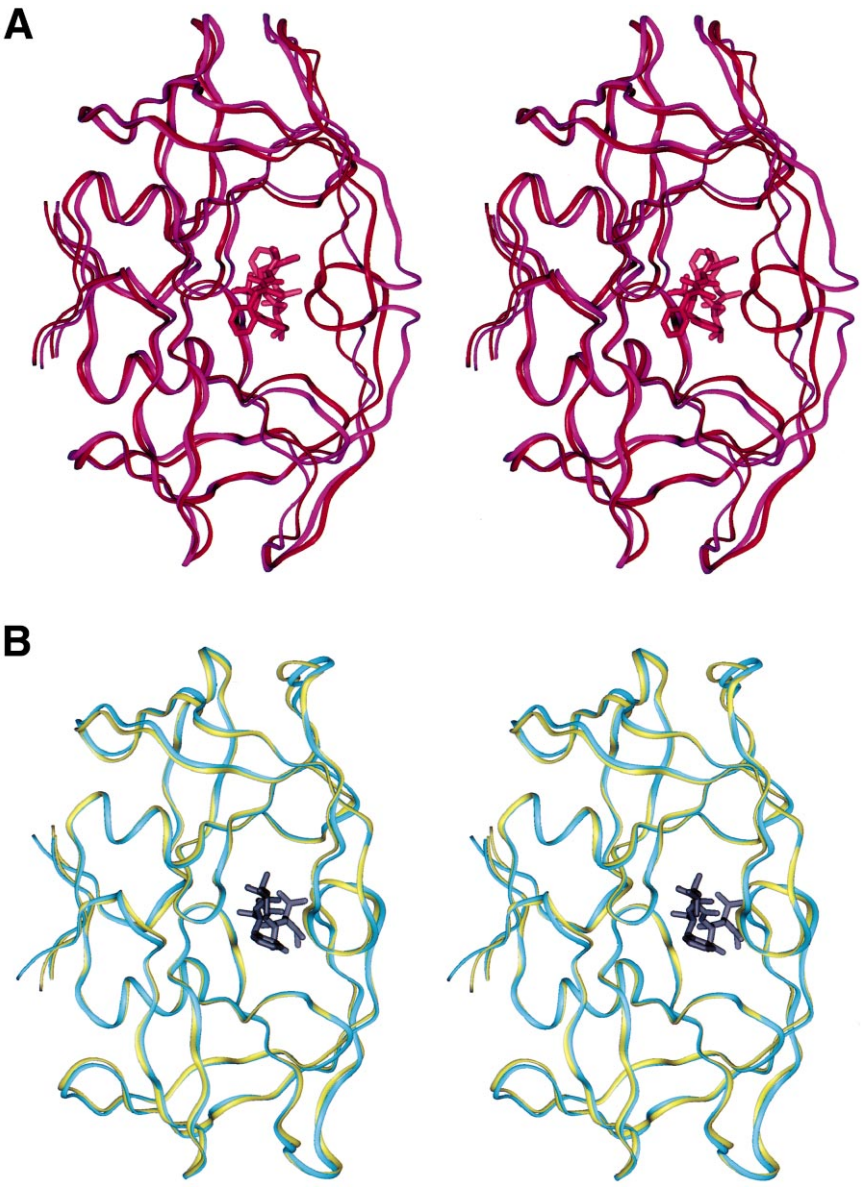


Fig. 4. Superposition of the Cα traces of the apoenzyme and inhibited forms of retroviral PRs. (A) Apoenzyme HIV-1 PR [33] (magenta) superimposed on the complex with a difluoroketone-containing inhibitor (A-79285, red), solved at 1.7 Å resolution, the highest reported to date [48]. (B) Apoenzyme SIV PR (closed form [37], cyan) superimposed on the complex with the hydroxyethylene isostere inhibitor SKF107457 [43] (yellow).

The structures of inhibitor complexes of retroviral PRs are available mostly for substrate-based, modified oligopeptide inhibitors, although some nonpeptidic inhibitors have also been studied crystallographically [44,54]. It was initially assumed that binding of an inhibitor introduces substantial conformational changes to the enzyme, exemplified by differences between the structures of the HIV-1 PR apoenzyme and its complex with an inhibitor (Fig. 4A). In this case, the overall movement of the subunits can be described as a rotation of up to 2° around a hinge axis located in the subunit β-sheet interface. This motion, which slightly tightens the cavity of the active site, is also accompanied by a very large motion of the flap region – as much as 7 Å for the tips of the flap [3]. As already mentioned, however, the differences between the free and inhibited forms of the enzyme could be modulated due to the influence of

crystal contacts on the structure of the apoenzyme. For example, considerably less profound differences can be seen when comparing the apoenzyme form of SIV PR with an inhibitor complex (Fig. 4B), but in this crystal form the flaps in the apoenzyme are involved in extensive interactions with the symmetry-related molecule.

The structures of complexes of any single PR with different inhibitors are well conserved, with rms deviations between the positions of Cα atoms seldom exceeding 0.6 Å [8]. Such differences are well within the agreement range for protein structures refined independently, or crystallized in different space groups [55].

Most of the inhibitors that have been cocrystallized with retroviral PRs, including all of the peptidomimetic inhibitors, are bound to the active site in extended conformations, such that when they are superimposed on each other, their functional elements align quite well [8]. The interactions between peptidomimetic inhibitors and retroviral PRs are very similar for all of the complexes. The hydrogen bonds are formed mostly between the main-chain atoms of both the enzyme and the inhibitor, and follow a similar pattern. These interactions are not sequence specific, and they dominate the total binding energy [56]. The nonhydrolyzable scissile bond analog of each inhibitor was found in close vicinity to the carboxyl groups of the active site aspartates Asp25/Asp25′. The hydroxyl group at the nonscissile junction, present in inhibitors other than those containing the reduced peptide bond isosteres, is positioned between the PR aspartate carboxyl groups, within hydrogen bonding distance to at least one carboxylate oxygen of each aspartate. A feature common to almost all complexes of retroviral PRs is a buried water molecule that bridges the CO groups at P2 and P1′ of the inhibitor and the Ile50/Ile50′ NH groups of the flaps. This water is approximately tetrahedrally coordinated and is completely inaccessible to the solvent [57]; furthermore, it is highly specific for retroviral PRs and has no equivalent in cellular aspartic proteases.

Although the structures of HIV PRs have been solved for a number of native and drug-induced variants of these proteins, for technical reasons it was necessary to use only mutated proteins to crystallize the complexes of EIAV and RSV PRs. The I54G mutant of EIAV PR [58] was used in crystallization studies, since the native protein was much more difficult to express and purify [45]. This mutation makes the enzyme more similar to its HIV-1 counterpart. Similarly, it has not been possible to grow crystals of the native RSV PR complexed with inhibitors; instead, a mutant with nine sites changed to their HIV-1 counterparts was utilized [46]. Several mutations in HIV-1 and SIV PRs were engineered in order to decrease the incidence of autolysis [29,44], and these forms of the enzyme were used for many structural investigations. Clearly, all of these differences have to be considered when analyzing the structures of retroviral PRs in detail.

## 4. Comparative structural investigations

In view of the wide range of binding constants for inhibitors interacting with retroviral PRs, comparative studies of the same inhibitor complexed with different enzymes have become very important. Unfortunately, it has not yet been possible to crystallize the same inhibitor with all PRs of known structure; therefore, the data must be extrapolated from several different inhibitors bound to multiple targets. However, some of the inhibitors that have been studied as complexes with multiple retroviral PRs are quite similar to each other, making the comparisons easier.

At least two different inhibitors have been studied bound to both HIV-1 and HIV-2 PRs. The clinical Merck compound L-735,524 (indinavir), with at least 7-fold difference of the inhibition constant against these enzymes, has been crystallized and analyzed bound to both PRs [36,59]. However, no explicit comparisons of its interactions have been presented. A more detailed comparative analysis of the structures of CGP-53820, a pseudosymmetric peptidic inhibitor, has shown that despite close overall structural similarities, small differences are evident in the architecture of the P1 and P1′ pockets [60]. The nine-site mutant of RSV PR [46] was crystallized with a reduced peptide bond inhibitor based on the CA-p2 target sequence (Fig. 5), and a structure with the same inhibitor was also solved for the complex with HIV-1 PR [61]. The inhibitor LP-130 was found to be universal for all retroviral PRs, and structures of its complexes with HIV-1, FIV, and EIAV PRs

| | |
|---|---|
| **HIV-1 PR** | |
| MA/CA | VSQNY*PIVQN |
| CA/CA* | KARVL*AEAMS |
| CA*/NC | SATIM*MQRGN |
| NC/p6 | RPGNF*LQSRP |
| p6/PR | VSPNF*PQITL |
| PR/RT | CTLNF*PISPI |
| RNaseH | GAETF*YVDG. |
| RT/IN | IRKVL*FLDGI |
| **HIV-2 PR** | |
| MA/CA | KGGNY*PVQHV |
| CA/CA* | KARLM*AEALK |
| CA*/NC | IPFAA*AQQRK |
| NC/p6 | KPRNF*PVAQV |
| p6/PR | RGLAA*PQFSL |
| PR/RT | MSLNL*PVAKV |
| RNaseH | GAETF*YTDGS |
| RT/IN | IRQVL*FLEKI |
| **RSV PR** | |
| MA/p2a | .TSCY*HCG.. |
| p2a/p2b | .IGCN*CAT.. |
| p2b/p10 | .PYVG*SGL.. |
| P10/CA | .VVAM*PVV.. |
| CA/SPa | .QGIA*AAM.. |
| SPa/SPb | .AAAM*SSA.. |
| SPb/NC | .PLIM*AVV.. |
| NC/PR | .PAVS*LAM.. |
| -RT | .ATVL*TVA.. |
| RT/IN | .FQAY*PLR.. |
| IN/p4 | .PLFA*GIS.. |
| **FIV PR** | |
| MA/CA | PPQAY*PIQTV |
| CA/NC#1 | YKMQL*LAEAL |
| CA/NC#2 | LTKVQ*VVQSK |
| NC(int) | PVNQM*QQAVM |
| NC/PR | IGFVN*YNKVG |
| PR/RT | IRLVM*AQISD |
| RNaseH | GAETW*YIDGG |
| RT/DU | LCQTM*MIIEG |
| DU/IN | STGVF*SSWVD |
| **EIAV PR** | |
| MA/CA | PSEEY*PIMID |
| CA/X | QKMML*LAKAL |
| X/NC | LAKAL*QTGLA |
| NC/p9 | QKQTF*PIQQK |
| TF/PR | GKFVG*VTYNL |
| PR/RT | AKLVL*AQLSK |
| RT/p15 | KEEIM*LAYQG |
| p15/IN | STGVF*WVENI |

Fig. 5. Cleavage sites in Gag-Pol retroviral polyproteins. Adapted from: EIAV PR [71], FIV PR [105], HIV-1 PR [72], RSV PR [106].

are available [62]. Another broad-specificity inhibitor is HBY-793, which has been cocrystallized with HIV-1 PR [63] and with EIAV PR [45]. Nevertheless, the structure of a single inhibitor bound to all retroviral PRs is still elusive. Some results of these comparisons will be discussed below.

## 5. Subsites of the inhibitor-binding pockets

A number of distinct subsites that accommodate the side chains of the inhibitors (or substrates) can be identified in retroviral PRs. These subsites are usually described using the nomenclature of Schechter and Berger [64], in which subsites on the N-terminal side of the scissile bond (or its nonscissile replacement) are unprimed and those on the C-terminal side are primed. The two residues that are immediately adjacent to the scissile bond are therefore labeled P1 and P1′; the next two P2 and P2′; and so on. Their corresponding binding pockets in the enzyme are labeled S1 and S1′, S2 and S2′, and so on. In retroviral PRs, the primed and unprimed sites are formed by identical residues, due to the symmetry of these enzymes. It must be stressed that some of the enzyme residues are part of more than a single subsite, since the pockets S4, S2, S1′ and S3′ are located on one side of the active site (Fig. 6), whereas the pockets S3, S1, S2′ and S4′ are located in a similar manner on the other side of the active site. Three subsites (S1–S3) are very well defined, whereas the more distant subsites are not as distinct (although sites S4 and S5 can also be delineated). Cameron et al. [65] compared in detail the subsites in HIV-1 and RSV PRs; those in FIV and EIAV PRs were described in the original structural papers [41,45,62].

The PR side chains comprising pockets S1 and S1′, with the exception of the active site aspartates, are mostly hydrophobic. The residues, which contact the P1 or P1′ side chains of the substrates (or inhibitors) in HIV-1 PR, include Arg8, Leu23, Asp25, Gly27, Gly48, Gly49, Ile50, Thr80, Pro81, and Val82. Residues Arg8, Leu23, Asp25, Gly27, and Gly49 are invariant in all retroviral PRs that are discussed here. Gly48 is substituted by Ile in FIV PR and by His in RSV PR. Ile50 is replaced by Val in FIV and EIAV PRs. Thr80 is conserved in HIV-1, HIV-2, and SIV PRs, and substituted by hy-

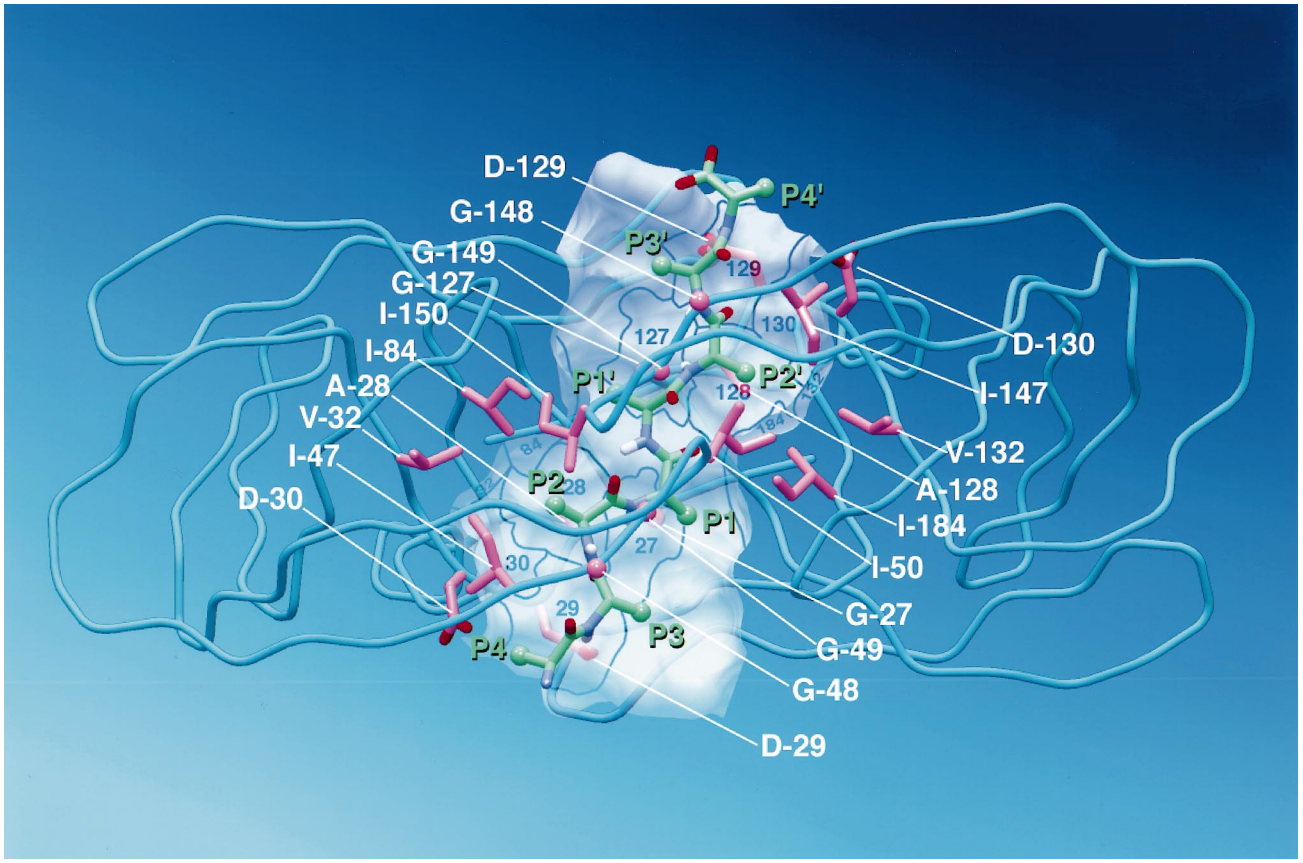


Fig. 6. Binding of an extended polypeptide chain to HIV-1 PR. The subsites and the residues forming them are marked. Figure prepared by Dr. Garrett Morris, using the programs AVS and Photoshop.

drophobic residues in the other enzymes: Leu in FIV PR, Ile in EIAV PR, and Val in RSV PR. Pro81 is present in four PRs, and replaced by Ile in FIV PR and Arg in RSV PR. Val82 is quite variable; it is present only in HIV-1 and EIAV PRs, and replaced by Ile in HIV-2 and SIV PRs, Gln in FIV PR, and Gly in RSV PR. The presence of two positively charged residues (Arg105 and His65) in these pockets in RSV PR correlates well with the preference of the parent type enzyme for the Glu at P1/P1′, in contrast to the R105P or H65G mutants of this enzyme [66].

The side chains of the active site aspartates and the main-chain hydroxyls of inhibitors that contain such a central group are involved in polar contacts mediated by hydrogen bonds. Almost all of the documented inhibitors have hydrophobic moieties at P1 and P1′ with the exception of the statine- and glycine-containing inhibitors, in which no groups occupy the PR subsite S1′.

Subsites S2 and S2′ are interior pockets in all retroviral PRs and are smaller than the S1/S1′ or S3/S3′ binding sites. They were also shown to be more specific, restricting the size and the type of the residue at P2/P2′ in the substrates or inhibitors relative to the other binding pockets in the PRs [67,68]. Such characteristics are also responsible for the lower tolerance in the drug-resistant mutants of the residues forming these binding sites [69]. In HIV-1 PR, S2 and S2′ are formed by Ala28, Asp29, Asp30, Val32, Ile47, Gly49, Ile50, Leu76, and Ile84. Conserved residues in all of the compared enzymes are limited to Ala28 and Asp29. Ile84 is mostly conserved with the exception of FIV PR, in which it is substituted by Leu. Ile50 is replaced by Val in FIV and EIAV PRs, while Ile47 is also substituted by Val in HIV-2 and SIV PRs, and by Met in FIV PR. Val32 is present in HIV-1 and EIAV PRs, and in the other four enzymes it is replaced by Ile. Leu76 is located on the periphery of the pocket and appears to be the most variable residue in the S2/S2′ subsites. It is present in HIV-1 and

EIAV PRs, and is substituted by Met in HIV-2 and SIV PRs, by Cys in FIV PR, and by Ala in RSV PR. Asp30 is present in HIV-1, HIV-2, and SIV PRs. It has been shown that mutations of this residue change the specificity of the enzymes (S. Gulnik, personal communication). Asp30 is replaced by Ile35 in FIV PR and Ile42 in RSV PR, making the S2/S2′ binding sites even more hydrophobic, and by Thr30 in EIAV PR. The change of this residue in RSV and EIAV PRs also affects the specificity of the pocket [45,70], while the 'inverse' replacement of Ile35 by Asp in FIV PR virtually inactivates this enzyme (Y.-C. Lin, personal communication).

Although it is clear that these pockets are at least partially hydrophobic, both hydrophilic and hydrophobic residues of substrates/inhibitors can occupy the individual sites. Hydrophobic side chains are observed at P2 and P2′ in various orientations for the different inhibitors, forming contacts with different groups in the binding pocket of the enzyme. For inhibitors containing Asn or Gln, the amide side chains are also stabilized by polar contacts with the carbonyl oxygens of the previous residues in the inhibitor. Some polar contacts are also observed between the P2/P2′ amide groups and polar side chains of the enzyme.

Subsites S3 and S3′ in retroviral proteases are known to have a rather broad specificity, being able to accept residues of different types and sizes [8,67]. This phenomenon is due to the variation of the residues forming them and to their ability to maintain aliphatic, polar and ionic interactions with different ligands. In addition, these subsites are quite large and exposed to the solvent. In HIV-1 PR, they are formed by Arg8, Leu23, Asp29, Gly48, Gly49, Pro81, and Val82. Conserved residues include Arg8, Leu 23, Asp29, and Gly49. Gly48, Pro81, and Val82 also form parts of the S1/S1′ pockets, and the variations of these residues in retroviral PRs have been described above. These subsites can be accommodated by a variety of side chains, some of which are very large (for example, naphthylalanine in inhibitors such as LP-130 [62], LP-149 [41], or HBY-793 [45]). Multiple conformations have been detected even for such bulky side chains, emphasizing the large size of these pockets.

The three PRs with chains of identical length – HIV-1, HIV-2, and SIV PRs – are most similar to each other. Of the other enzymes, EIAV PR exhibits much higher similarity to these three than do RSV and FIV PRs. For example, only three residues are of a different type between HIV-1 and EIAV PRs: Val56 (Ile50 in HIV-1 PR), Thr30 (Asp30), and Ile54 (Gly48). These residues participate in the formation of the S1/S1′, S2/S2′, and S3/S3′ binding sites, respectively. This similarity allows EIAV PR to successfully attack the cleavage junctions in the HIV-1 polyprotein with various degrees of efficiency and to mimic some characteristics of HIV-1 PR, such as the general substrate specificity and pH dependence [58,71]. The I54G mutant EIAV PR, however, for which a crystal structure has been reported [45,62], has impaired activity toward some, but not all, of the peptide substrates used in the characterization of native, mutant, and chimeric HIV-1 and HIV-2 PRs [72,73], as well as for the native EIAV PR [58]. Removal of the four carbons in the Ile54 side chain of EIAV PR was thus not without effect [58]. Nevertheless, the I54G and native EIAV PRs were: (i) equally susceptible to inhibition by HBY-793, (ii) equally resistant to the closely related A-76889; but (iii) not inhibited to any significant extent by a variety of other synthetic inhibitors that were developed as potent inhibitors of HIV-1 PR [58]. Thus, EIAV PR (either native or mutant) is almost identical to HIV-1 PR in the central features (subsites) of its active site and can function to process HIV-1 PR substrates, yet is resistant to all except one (HBY-793) of the potent HIV-1 PR inhibitors that have been tested.

The 'inverse' mutation G48V was found in the drug-resistant mutants of HIV-1 PR, which are about 30-fold more resistant than wild-type PR to an inhibitor Ro31-8959 (saquinavir, an FDA-licensed AIDS drug) [58]. Saquinavir is a potent inhibitor of HIV-1, HIV-2, and SIV PRs, all of which have Gly at position 48; in contrast, it does not significantly inhibit the proteases from human T-cell leukemia virus type 1 (HTLV-1) [74], FIV [75], or EIAV ([58] and references therein), all of which have an amino acid containing a side chain at the position structurally equivalent to Gly48 in HIV-1 PR (Fig. 2). However, when the I54G mutation was introduced into EIAV PR, the resultant single-mutant PR was no more susceptible than native EIAV PR to inhibition by saquinavir. Indeed, the

I54G mutation did not significantly affect the binding to EIAV PR of about 20 potent HIV-1 PR inhibitors [58]. Thus, despite having virtually identical residues in structurally equivalent locations, both the native EIAV PR and the I54G mutant are completely distinct from HIV-1 PR in their susceptibility to inhibition (with the exception of HBY-793).

As indicated above, the substrate-binding sites in RSV and FIV PRs have less similarity to those in EIAV PR. Each subsite has several residues of a different type in structurally equivalent positions. Three residues forming the subsites S1/S1′ in EIAV PR (Pro86, Val87, and Ile89) are substituted in FIV PR with Ile98, Gln99, and Leu101, respectively. The first two residues are also substituted with different types of residues in RSV PR (Arg105 and Gly106), whereas the third residue (Val56) is substituted with Ile67. These substitutions, particularly of Pro86 and Val87, are quite dramatic and would therefore explain the different selectivity that has been reported for RSV PR [76]. We can thus see that although the overall structures of retroviral PRs are quite similar, even in the vicinity of their substrate-binding areas, considerable differences that are ultimately responsible for their different activity and specificity can be clearly identified.

Subsites S4/S4′ and S5/S5′ have not been described in comparable detail, due to the paucity of available structures of retroviral PRs with ligands extending beyond P3/P3′ [1,77]. A comparison of those few for which structural data are available shows similar interactions between the residues at P4/P4′ positions and the respective enzyme pockets in HIV-1 PR (8hvp; [51]), RSV PR (1bai; [46]) and FIV PR complexed with an inhibitor TL-3 [107]. In HIV-1 PR complexes, only Asp30 is involved in the interactions with the ligand in this binding site, whereas in the FIV PR:TL-3 complex, in addition to Ile35 (which is a structural analog of Asp30 in HIV-1 PR), Gln54 interacts with the Cbz groups at the inhibitor's termini, which extend parallel to the flaps beyond S4 and S4′. Both flaps in this region in FIV PR are significantly shifted toward the active site in comparison with their location in HIV-1 PR. These differences may explain the conformational changes involving the Cbz groups of TL-3 when it is bound to HIV-1 and FIV PRs [107]. In HIV-1 PR, the Cbz groups follow the shift of the flaps away from the active site, possibly indicating the importance of β-sheet formation between the main chain of the substrate and the flaps during the early stages of substrate binding. Two insertions are present in RSV PR, consisting of His7 and Lys8 on one side of the active site, and residues 58–61 in the flap region on the other side; these residues form a bulge, narrowing the active site groove near this pocket. Some of these residues, such as Asn59, interact with the longer ligand, and their presence is likely to change the specificity of this pocket in comparison with the other PRs.

## 6. Complexes with substrates

Although the design of inhibitors of retroviral PRs was originally based on the assumption that their binding mode would resemble that of peptide and protein substrates, this hypothesis needed experimental verification. Because it was clear that a true substrate could not be cocrystallized with an active enzyme, as it would be processed before crystals could be grown and data collected, the enzyme had to be rendered inactive. This would, however, pose another question – namely, whether the structures of the active and inactive enzymes would be similar.

This problem was approached by expression of a mutant of FIV PR, designated FIV PR(D30N), in which the catalytic Asp30 was mutated to Asn, leading to an inactive PR [42]. To investigate the extent of perturbation of the enzyme's active site caused by this mutation, the crystal structure of FIV PR(D30N) was determined in a complex with LP-149, a statine-based inhibitor patterned after the junction between the CA and NC in the HIV-1 PR Gag polyprotein (Fig. 5). The structure was then compared with that of the parent enzyme, FIV PR(wt), complexed with the same inhibitor. This comparison revealed that the mode of binding of LP-149 to FIV PR(D30N) is similar to that of LP-149 to FIV PR(wt).

To provide a crystallizable substrate, a hexapeptide based on the primary structure of the inhibitor LP-149 was synthesized. This peptide (LP-149S) incorporated a Leu-Ala junction with an unmodified peptide bond in place of the statine, thus restoring the sequence of the CA-NC junction in HIV-1 PR

Gag. Crystals of the complex of FIV PR(D30N) with this substrate were grown, and the structure was solved [42]. The crystal structures of the complexes of FIV PR(D30N) with the inhibitor LP-149 and the substrate LP-149S showed that, as expected, the single active site mutation of the catalytic Asp30 to Asn did not affect the overall structure of the protein. The rms deviation between the Cα atoms of FIV PR(wt) and FIV PR(D30N), both complexed to LP-149, is 0.175 Å (234 pairs); between FIV PR(wt) complexed to LP-149 and FIV PR(D30N) complexed to LP-149S, 0.182 Å (229 pairs); and between the complexes of FIV PR(D30N) with LP-149S and LP-149, 0.179 Å (230 pairs). For the backbone atoms and all atoms, the respective values are 0.166 Å (919 pairs) and 0.188 Å (1603 pairs), 0.182 Å (906 pairs) and 0.200 Å (1514 pairs), and 0.175 Å (887 pairs) and 0.190 Å (1576 pairs).

The differences in the structures of the native and D30N FIV PRs with their ligand in the vicinity of the catalytic residues at the active site region are imposed by the symmetry of the crystals. Because the asymmetric unit of the crystals contains only one monomer, both halves of the active site, formed by the residues from monomers A and B, are related by the symmetry operation. In the structure of FIV PR(wt), the two catalytic aspartates are related by crystallographic symmetry and have only one conformation each, whereas in the structure of FIV PR(D30N):LP-149, the side chains of both asparagines, AsnA30 and AsnB30, have dual conformations due to the hydrogen bond formation between the N and O atoms of the side chain amides. Recent NMR studies of the ionization states of the catalytic aspartates in HIV-1 PR [78] have shown that, in the complexes of HIV-1 PR with pepstatin A, the catalytic aspartate that interacts with the statyl hydroxyl is protonated. These data are in good agreement with the interaction scheme deduced from the crystal structure of FIV PR(wt):LP-149.

The crystal structure of FIV PR(D30N):LP-149S revealed that the P1-P1′ peptide bond of the bound substrate does not deviate from planarity, indicating that the tetrahedral intermediate is not formed, and thus the asparagines most likely do not polarize a water molecule sufficiently to become a strong nucleophile. Another possible reason for the inactivation of the enzyme by the D30N mutation might be the different location of the nucleophilic water molecule with respect to the asparagines (vs. aspartates in the active enzyme) and the bound substrate, which is indicated by the different positions of the statyl hydroxyls in their active sites. These experiments have provided a solid basis for modeling enzyme-substrate complexes for active PRs as well as contributing to a better understanding of the mechanism of action. Similar work was also done independently by at least two groups using D25N mutants of HIV-1 PR (C. Schiffer and A. Silva, personal communication).

## 7. Tethered dimers of retroviral PRs

Because all naturally occurring retroviral PRs are noncovalent dimers of identical subunits, and since the C-terminus of one subunit is located very close to the N-terminus of the other, it is possible with the use of suitable spacers to create artificial monomeric enzymes, which are more analogous to pepsins, but which still preserve the structural features of the parent PRs. Such constructs were prepared for RSV PR [79], HIV-1 PR [73,80], and fusions of HIV-1/HIV-2 PRs [73]. The typical method of joining the two chains was by introducing potentially flexible links consisting of sequences such as GG [81], GGGG or GGD [79], and GGSSG [82]. Crystallographic investigations of the tethered dimer of HIV-1 PR at 1.8 Å resolution [82] have shown that although the covalent linkage could be well ordered and visible in the electron density maps, its presence has not led to significant modifications of the two subunits, nor to the introduction of asymmetry beyond what was expected based on earlier crystal structures of unmodified HIV-1 PR. The covalently dimeric molecule is organized in the crystal lattice in a unique manner, with distinct packing of the two monomers. The interactions with the symmetric inhibitor were also reported as being unique [82]. Enzymatic studies of the tethered dimer of HIV-1 PR have shown that the single-chain protein is more stable than the parent PR at pH 7.0, most likely by preventing dissociation of a noncovalent enzyme, for which a surprisingly high dissociation constant of $5\times10^{-8}$ M has been measured [80].

The presence of a covalent linker also enables the creation of truly asymmetric variants of retroviral

PRs, in which only one subunit of the dimer would be modified. An experiment in which only one Asp25 side chain was mutated to Gly resulted, not surprisingly, in a completely inactive enzyme [80]. In another experiment, mutants containing deletions or substitutions in only one flap of HIV-1 PR were constructed and analyzed [83]. These mutants showed a degree of self-processing, whereas their catalytic activity was significantly reduced. Surprisingly, the substrate cleavage was more efficient at low salt concentrations for a mutant containing a shortened hydrophilic flap than for the parent enzyme. The substrate specificity, however, was not affected by these mutations.

## 8. Protein substrates of retroviral PRs

Each retroviral PR is most specific for sequences corresponding to the cleavage sites in the polyproteins of its own virus (Fig. 5), but some degree of cleavage of other viral substrates can usually also be observed. Viral polyprotein substrates are processed in a well-defined order, with the initial cleavage occurring at the N-terminus of the PR itself [84], and with the first cleavage of the Gag polyprotein in HIV-1 occurring between p2 and NC [85]. Other cleavage reactions could be one to two orders of magnitude slower than the initial ones.

A number of nonviral protein substrates of retroviral PRs have also been identified. Such targets of opportunity include proteins as diverse as vimentin, desmin, and glial fibrillary acidic protein [86], ribonuclease A [87], lactate dehydrogenase [88], and calcium-free calmodulin [89], as well as actin, troponin C, Alzheimer amyloid precursor protein, and pro-interleukin 1β [90]. Based on these observations, a cumulative specificity model for HIV-1 and HIV-2 PRs has been proposed [91]. This model assumed independent contributions of the subsites to the specificity of the enzyme, an assumption that was later modified based on the proven influence of the adjacent sites on the specificity of the enzyme [92]. Nevertheless, the model has been generally quite successful in predicting new cleavage sites. Mathematical elaboration of the model led to a vectorized sequence-coupling technique for predicting HIV-1 PR cleavage sites in proteins [93].

## 9. Processing of peptide substrates and reengineering of retroviral PRs

Because the protein substrates of retroviral PRs need to be either unfolded or in an extended conformation for the cleavage to occur, and because only a limited number of subsites in the substrate need to interact with the PR, it is not surprising that peptide substrates have been extensively used to probe the activity and specificity of these enzymes [68,94]. These substrates are usually 8–12 amino acids in length and consist of common retroviral cleavage sequences (Fig. 5), sometimes augmented at their ends by the addition of hydrophilic amino acids such as Arg, in order to enhance their solubility [95]. Selected highlights of these studies will be summarized below.

Cleavage of the oligopeptides is best followed by techniques such as FPLC/HPLC, which enable direct identification of the extent of processing and of the identity of the products. The measurements are routinely conducted in rather high salt concentrations, typically 0.3 M for HIV PR and 1 M for RSV PR [68]. Incubation times range from 10 min to 24 h at 37°C. Another approach, useful only for the substrates normally containing a hydrophobic residue at P1′, involves their modification via substitution of P1′ residues by a chromophoric reporter group such as *p*-nitrophenylalanine, which, without modifying the kinetic properties of the peptides, makes their measurements much easier by allowing the use of spectrophotometric techniques [72,96]. Other substrates designed for easy monitoring of the reactions incorporate fluorogenic groups on both their termini, which lead to increased fluorescence upon cleavage [97].

Peptide substrates of retroviral PRs can be broadly divided into at least two classes, although there are no clear consensus sequences within each group (Fig. 5). One such division identifies cleavage sites containing either aromatic*Pro or hydrophobic*hydrophobic junctions [72] (where the asterisk denotes the cleavable bond). Although a few sites may not fall exactly into these broad categories, the vast majority do. It must be stressed that the aromatic*Pro sites are quite unique to this class of enzymes, since cleavage of peptide bonds on the N-terminal side of a Pro is very rare and few, if any, other PRs are specifically capable of such activity.

A detailed comparison of the processing of the peptide based on the MA-CA cleavage site of HIV-1 PR and its modifications, initially containing a Tyr*Pro junction, by HIV-1 and HIV-2 PRs has shown that both enzymes are capable of processing such substrates with good efficiency, although HIV-2 PR is slightly less efficient [67]. Various amino acids could be substituted at subsites P3 and P3′ with only a limited change in the catalytic efficiency of either enzyme, whereas P2 and P2′ were much more restrictive. Substitution of polar uncharged residues resulted in good binding at P3 and P2, whereas they resulted in high binding constants when substituted at P3′ and P2′, despite the overall similarity of the unprimed and primed binding pockets. Substitution of other hydrophobic residues at P1 resulted in cleavable peptides, but polar and β-branched amino acids prevented processing. Very importantly, any substitution of the P1′ Pro, even by residues found in other cleavage sites, prevented processing, suggesting a strong sequence context of the substrates [67]. Significant interactions between the subsites of HIV-1 and RSV PRs have also been shown in other studies [92].

Both classes of substrates defined above are best processed by HIV-1 PR if subsite P2′ contains Glu, while Gln is usually, but not always, a suitable replacement. Although Val in P2 provides an excellent substrate for the hydrophobic*hydrophobic substrates, it is very poor in the aromatic*Pro substrates, in which Asn is preferred in that location [72].

Analysis of the processing patterns of peptide substrates was useful in an engineering experiment in which RSV PR was mutated in order to cleave new substrate sequences [98]. A mutant of RSV PR with the substitutions S38T, I42D, I44V, M73V, A100L, V104T, R105P, G106V, and S107N had characteristics much closer to those of HIV-1 PR than to the parent enzyme. The mutant protein was active at lower salt concentrations, was inhibited by a specific HIV-1 PR inhibitor (KNI-272, which does not inhibit wild-type RSV PR), and was capable of processing HIV-1 PR cleavage sites such as RT-IN or CA-NCb (Fig. 5) with an efficiency approaching that of HIV-1 PR.

Another attempt to reengineer a retroviral PR involved FIV PR, which was mutated in order to make it more similar to HIV-1 PR. The mutations (V59I and Q99V) led to more efficient inhibition by TL-3, a specific inhibitor of FIV PR [99], which is still more active against HIV-1 PR [75]. Crystal structures of complexes of TL-3 with mutant PRs and with HIV-1 PR elucidated the differences in binding, especially at subsites P4 and P4′ [107].

## 10. Summary and conclusions

In just over a decade since the first structures of retroviral PRs were solved, this important class of enzymes has become one of the best characterized, both structurally and biochemically. Clearly, the importance of such knowledge to the development of new drugs has been the driving force behind this progress. The introduction of no fewer than five inhibitors of HIV-1 PR into clinical practice as anti-AIDS drugs has dramatically improved the life expectancy and quality of life for AIDS patients [6,7]. However, rapid development of resistance to PR inhibitors has been documented [100–102], with at least one-third of the sequence of HIV-1 PR shown by now to be subject to drug-induced mutations [103]. It has also been postulated that PRs from viruses other than HIV-1 could be excellent models for investigating the resistance phenomena, because, as shown above, their overall structures are quite similar, yet the specificities and details of the interactions with the substrates and inhibitors are often subtly different, yielding hints on the nature of the resistance phenomenon [41,45,58,99]. In addition, cats infected with FIV could provide a small-animal model for the studies of retroviral infection and drug resistance [104]. It is clear that the studies of retroviral PRs should continue with undiminished intensity in the future.

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