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Amprenavir Complexes with HIV-1 Protease and Its Drug Resistant Mutants Altering Hydrophobic Clusters

ChenHsiang Shen¹, YuanFang Wang¹, Andrey Y. Kovalevsky^{1,#}, Robert W. Harrison^{3,1}, and Irene T. Weber^{1,2,*}

¹ Department of Biology, Molecular Basis of Disease Program, Georgia State University, Atlanta, GA 30303, USA

² Department of Chemistry, Molecular Basis of Disease Program, Georgia State University, Atlanta, GA 30303, USA

³ Department of Computer Science, Molecular Basis of Disease Program, Georgia State University, Atlanta, GA 30303, USA

Summary

The structural and kinetic effects of amprenavir (APV), a clinical HIV protease (PR) inhibitor, were analyzed with wild type enzyme and mutants with single substitutions of V32I, I50V, I54V, I54M, I84V and L90M that are common in drug resistance. Crystal structures of the APV complexes at resolutions of 1.02 to 1.85 Å reveal the structural changes due to the mutations. Substitution of the larger side chains in PR_{V32I}, PR_{I54M} and PR_{L90M} resulted in formation of new hydrophobic contacts with flap residues, residues 79 and 80, and Asp25, respectively. Mutation to smaller side chains eliminated hydrophobic interactions in the PR_{I50V} and PR_{I54V} structures. The PR_{I84V}-APV complex had lost hydrophobic contacts with APV, the PR_{V32I}-APV complex showed increased hydrophobic contacts within the hydrophobic cluster, and the PR_{I50V} complex had weaker polar and hydrophobic interactions with APV. The observed structural changes in PR_{I84V}-APV, PR_{V32I}-APV and PR_{I50V}-APV were related to their reduced inhibition by APV of 6-, 10- and 30-fold, respectively, relative to wild type PR. The APV complexes were compared with the corresponding saquinavir (SQV) complexes. The PR dimers had distinct rearrangements of the flaps and 80's loops that adapt to the different P1' groups of the inhibitors while maintaining contacts within the hydrophobic cluster. These small changes in the loops and weak internal interactions produce the different patterns of resistant mutations for the two drugs.

Keywords

X-ray crystallography; enzyme inhibition; aspartic protease; HIV/AIDS; conformational change

INTRODUCTION

Currently, about 33 million people worldwide are estimated to be infected with human immunodeficiency virus (HIV) in the AIDS pandemic [1]. The virus cannot be fully eradicated

*Corresponding author: Irene T. Weber, Department of Biology, Georgia State University, P.O. Box 4010, Atlanta, GA 30302-4010, USA, Phone 404 413-5411, FAX 404 413-5300, iweber@gsu.edu.

#current address: Bioscience Division, MS M888, Los Alamos National Laboratory, Los Alamos NM 87545, USA

Database Accession Codes: The atomic coordinates and structure factors are available in the Protein Data Bank with accession code 3NU3 for wild-type HIV-1 PR-APV, 3NU4 for PR_{V32I}-APV, 3NU5 for PR_{I50V}-APV, 3NU6 for PR_{I54M}-APV, 3NUJ for PR_{I54V}-APV, 3NU9 for PR_{I84V}-APV, and 3NUO for PR_{L90M}-APV.

despite the effectiveness of highly active anti-retroviral therapy (HAART) [2]. Furthermore, development of vaccines has been extremely challenging [3]. HAART uses more than 20 different drugs, including inhibitors of the HIV-1 enzymes, reverse transcriptase (RT), protease (PR) and integrase, as well as inhibitors of cell entry and fusion. The major challenge limiting current therapy is the rapid evolution of drug resistance due to the high mutation rate caused by the absence of a proof-reading function in HIV RT [4].

HIV-1 PR is the enzyme responsible for the cleavage of the viral Gag and Gag-Pol polyproteins into mature, functional proteins. PR is a valuable drug target since inhibition of PR activity results in immature noninfectious virions [5–6]. PR is a dimeric aspartic protease composed of residues 1-99 and 1'-99'. The conserved catalytic triplets, Asp25-Thr26-Gly27, from both subunits provide the key elements for formation of the enzyme active site. Inhibitors and substrates bind in the active site cavity between the catalytic residues and the flexible flaps comprising residues 45-55 and 45'-55' [7].

Amprenavir (APV) was the first HIV-1 PR inhibitor (PI) to include a sulfonamide group (Fig 1A). Similar to other PIs, APV contains a hydroxyethylamine core that mimics the transition state of the enzyme. Unlike the first generation PIs, such as saquinavir (SQV), APV was designed to maximize hydrophilic interactions with PR [8]. The sulfonamide group increases the water solubility of APV (60 µg/mL) compared to SQV (36 µg/mL) [9]. The crystal structures of PR complexes with APV [8,10] and SQV [11–12] demonstrated the critical PR-PI interactions.

HIV-1 resistance to PIs arises mainly from accumulation of PR mutations. Conservative mutations of hydrophobic residues are common in PI resistance, including V32I, I50V, I54V/M, I84V and L90M that are the focus of this study [13]. The location of these mutations in the PR dimer structure is shown in Figure 1B. Multi-drug-resistant mutation V32I, which alters a residue in the active site cavity, appears in about 20% of patients treated with APV [14] and is associated with high levels of drug resistance to lopinavir (LPV)/ritonavir [13]. Ile50 and Ile54 are located in the flap region, which is important for catalysis and binding of substrates or inhibitors [8,15]. Mutations of flap residues can alter the protein stability or binding of inhibitors [15–18]. PR with mutation I50V shows 9-fold worse inhibition by DRV relative to wild type enzyme [19], and 50- and 20- fold decreased inhibition by indinavir (IDV) and SQV [17–18]. Unlike Ile50, Ile54 does not directly interact with APV, but mutations of Ile54 are frequent in APV resistance and the I54M mutation causes 6-fold increased IC₅₀ [20]. Mutation I54V appears in resistance to IDV, LPV, nelfinavir (NFV) and SQV [13]. I54V in combination with other mutations, especially V82A [21–22], decreases the susceptibility to PI therapy [18]. I84V, which is located in the active site cavity, significantly reduces drug susceptibility to APV [23]. L90M is commonly found during PI treatment [14] and is resistant to all currently used PIs, with major effects on NFV and SQV [13].

Mutations of hydrophobic residues are found in more than half of drug resistant mutants [13, 24] and several of these mutations show altered PR stability [17,25]. Hydrophobic interactions play an important role in protein stability. Aliphatic groups reportedly contribute about 70% of the hydrophobic interactions in proteins [26]. Removing a methyl group in the protein hydrophobic core affects protein folding and decreases the protein stability in mutant proteins [27]. In HIV PR, two clusters of methyl groups have been identified; one inner cluster surrounding the active site cavity and the second cluster in an outer hydrophobic core, as shown in Figure 1B [24]. Drug resistant mutations V32I, I50V, I54V/M, and I84V belong to the inner cluster around the active site, while L90M is in the outer cluster.

In order to establish a better understanding of the mechanism of resistance to APV, atomic and high resolution crystal structures have been determined of APV complexes with wild type PR

and its mutants containing single substitutions of Val32, Ile50, Ile54, Ile84 and Leu90. HIV-1 PR mutations can have distinct effects on the binding of different inhibitors. Therefore, the structural effects of APV and SQV were compared for wild type PR and mutants PR_{I50V}, PR_{I54M}, and PR_{I54V} complexes, using previously reported SQV complexes [12,18]. Exploring the changes in PR due to binding of two different inhibitors will give insight into the mechanisms of resistance and help in the design of new inhibitors.

RESULTS

APV Inhibition of HIV-1 PR and Mutants

The kinetic parameters and inhibition constants of APV for wild-type PR and the drug-resistant mutants PR_{V32I}, PR_{I50V}, PR_{I54M}, PR_{I54V}, PR_{I84V} and PR_{L90M} are shown in Table I. The lowest catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$) values were seen for PR_{V32I} and PR_{I50V} with 30% and 10% of the wild-type PR value, respectively. The PR_{L90M} showed a surprisingly high 11-fold increase in the catalytic efficiency, while the other mutants were similar to the wild type. The $k_{\text{cat}}/K_{\text{m}}$ values for PR_{L90M} appear to depend on the substrate, however, since only a modest 3-fold increase relative to wild type PR was observed using a different substrate with sequence derived from the MA/CA rather than the p2/NC cleavage site [19]. The six mutants and wild-type PR were assayed for inhibition by APV (Table I). APV showed sub-nanomolar inhibition with K_{i} of 0.16 nM for wild-type PR and PR_{L90M}. PR_{I54M} and PR_{I54V} showed modestly increased (3-fold) relative K_{i} values. The largest increases in K_{i} of 6-, 10- and 30-fold were observed for PR_{I84V}, PR_{V32I} and PR_{I50V}, respectively, relative to wild-type PR. The substantially decreased inhibition of PR_{V32I} and PR_{I50V} suggested the loss of interactions with APV.

Crystal Structures of APV Complexes

The crystal structures of PR and drug resistant mutants PR_{V32I}, PR_{I50V}, PR_{I54M}, PR_{I54V}, PR_{I84V} and PR_{L90M} were determined in their complexes with APV at resolutions of 1.02 to 1.85 Å to investigate the structural changes. The crystallographic data are summarized in Table II. All structures were determined in space group P2₁2₁2. The asymmetric unit contains one PR dimer of residues 1-99 and 1'-99' as well as APV. The lowest resolution structure of PR_{I84V} was refined to an R-factor of 0.20 with isotropic B-factors and solvent molecules. The other structures were refined at 1.50–1.02 Å resolution to R-factors of 0.12–0.16, including anisotropic B-factors, hydrogen atoms and solvent molecules. Wild-type PR had the highest resolution and lowest R-factor, concomitant with the lowest average B-factors for the protein and inhibitor atoms.

Because of the high resolution of the diffraction data, all structures except for PR_{I84V}-APV, were modeled with more than 150 water molecules, ions, and other small molecules from the crystallization solutions, including many with partial occupancy (Table II). The solvent molecules were identified by the shape and intensity of the electron density and the potential for interactions with other molecules. The non-water solvent molecules were: a single sodium ion, three chloride ions, two partial glycerol molecules in PR_{WT}-APV; one sodium ion, three chloride ions in PR_{V32I}-APV; three sodium ions, seven chloride ions, two partial acetate ions in PR_{I50V}-APV; one sodium ion, three chloride, two partial acetate ions in PR_{I54M}-APV; 19 iodide ions in PR_{I54V}-APV; 33 iodide ions in PR_{I84V}-APV; and 19 iodide ions in PR_{L90M}-APV. Many iodide ions have partial occupancy, however, they were identified by the high peaks in electron density maps, abnormal B factors, and contact distances of 3.4 to 3.8 Å to nitrogen atoms.

Alternative conformations were modeled for residues in all crystal structures. Alternate conformations were modeled for a total of 48, 13, 28, 11, 1, 8 residues in the PR_{WT}-APV, PR_{V32I}-APV, PR_{I50V}-APV, PR_{I54M}-APV, PR_{I54V}-APV, PR_{I84V}-APV, and PR_{L90M}-APV

structures, respectively. APV was observed in two alternate orientations related by a rotation of 180° in the complexes with PR_{WT} and PR_{I50V} with relative occupancies of 0.7/0.3 and 0.6/0.4, respectively. The highest resolution structure, PR_{WT}-APV, showed the most alternate conformations for main chain and side chain residues. Several residues in the active site cavity showed two alternate conformations and were refined with the same relative occupancies as for APV. Surface residues with longer flexible side chains, such as Trp6, Arg8, Glu21, Glu34, Ser37, Lys45, Met46, Lys55, Arg57, Gln61, and Glu65 were refined with alternate conformations. Also, some internal hydrophobic residues, such as Ile64, Leu97, showed a second conformation for the side chain. At the other extreme, the lowest resolution structure of PR_{I84V}-APV showed only one residue, Leu97, with an alternate side chain conformation. In all the structures, the two catalytic Asp25 residues showed negative difference density around the carboxylate oxygens. This phenomenon might be caused by radiation damage in the carboxylate side chains, especially due to their location at the active site, as described in [28].

The accuracy in the atomic positions was evaluated by the diffraction-data precision indicator (DPI), which is calculated in SFCHECK from the resolution, R-factor, completeness and observed data [29]. The highest resolution structure of PR_{WT}-APV had the lowest DPI value of 0.02 Å, while the lowest resolution structure of PR_{I84V}-APV had the highest DPI value of 0.13 Å (Table II). We estimate that significant differences in interatomic distances should be at least 3-fold larger than the DPI value [7]. Hence, structural changes of >0.06 Å are significant for PR_{WT}-APV and >0.4 Å for PR_{I84V}-APV at the two extremes of resolution. The quality of the crystal structures is illustrated by the 2Fo-Fc electron density maps for the mutated residues (Fig. S1). The mutated residues had single conformations, except for the side chains of Met54, Val54 and Met90 in one subunit that were refined with relative occupancies of 0.6/0.4, 0.7/0.3 and 0.5/0.5, respectively. Overall, the mutants and wild type enzyme had very similar structures, probably because they shared the same crystallographic unit cell. The PR_{I54M}, PR_{I54V}, and PR_{I84V} complexes had RMS deviations for the C α atoms ranging from 0.26 Å to 0.38 Å compared to the wild type structure. The structures of PR_{V32I}, PR_{I50V}, and PR_{L90M} were more similar to the wild-type PR with RMS deviations of 0.15–0.19 Å for the main chain atoms.

HIV-1 Protease Interactions with APV and Influence of Alternate Conformations

The atomic resolution crystal structure of PR_{WT}-APV was refined with two differently populated conformations for the inhibitor and several residues forming the binding site with relative occupancies of 0.7/0.3 (Fig 2A). Residues Arg8, Asp30, Val32, Lys45, Gly48, Ile50, and Pro81 showed alternate conformations in both subunits, and Asp25' had two alternate conformations for the side chain. Alternate conformations were also refined for the main chain of residues 24', 29', 30, 30', 31, 31', 48, 48', 79' and 80' around the inhibitor binding site. Moreover, the conserved water molecule between the flaps and inhibitor showed two alternate positions. Similar, although less extensive, disorder in the inhibitor binding site has been observed in other atomic resolution crystal structures of this enzyme [12,30]. In fact, the highest resolution structure reported to date (0.84 Å) of PR_{V32I} with DRV comprised two distinct populations for the entire dimer with inhibitor and one conformer contained an unusual second binding site for DRV [30]. Moreover, a similar asymmetric arrangement of Asp25/25' with a single conformation for Asp25 and two conformations for Asp25' was observed in the crystal structure of PR_{WT}-GRL0255A [31]. Only single conformations were apparent for APV in the mutant protease structures with the exception of PR_{I50V}. However, the mutant structures were refined with lower resolution data where alternate conformations may be less clearly resolved than for the PR_{WT}-APV structure.

APV interactions with PR_{WT} were analyzed in terms of the hydrogen bond, C-H...O and H₂O... π interactions, as described for the PR_{V32I} complex with DRV [30]. The polar interactions of the major conformation of APV with PR_{WT} are illustrated in Figure 2B. The central hydroxyl group of APV forms strong hydrogen bond interactions with the carboxylate oxygens of the catalytic residues Asp25 and Asp25'. APV formed four direct hydrogen bonds with the main chain amide of Asp30', the carbonyl oxygen of Gly27', and the amide and carbonyl oxygen of Asp30. Water molecules make important contributions to the binding site. The flap water molecule (H₂O^A in Fig. 2B), which is conserved in almost all PR-inhibitor complexes, formed a tetrahedral arrangement of hydrogen bonds connecting the amide nitrogen atoms of Ile50/50' in the flap region with the sulfonamide oxygen and the carbamate carbonyl oxygen of APV. The second conserved water (H₂O^B) bridged APV and the PR main chain by hydrogen bonds to the carbonyl of Gly27 and the amide of Asp29 and a H₂O... π interaction with the aniline group of APV. The interactions of H₂O^B are conserved in PR complexes with darunavir and antiviral inhibitors based on the same chemical scaffold [31–32]. The third water, H₂O^C, which is conserved in these APV complexes and in DRV complexes, mediated hydrogen bond interactions between the carboxylate of Asp30 and aniline NH₂ of APV. Also, several C-H...O interactions link the PR main chain to APV: the carbonyl oxygens of Gly48' and Asp30' interact with the tetrahydrofuran (THF) moiety, Gly27 carbonyl oxygen with isopropyl group, Gly48 carbonyl oxygen with aniline ring, the sulfonamide oxygens of APV to Gly49 and Ile50', APV carbonyl oxygen with Gly49' and APV oxygen to Gly27' (Fig. 2). The C-H...O interactions formed by the PR amides and carbonyl oxygens mimic the conserved hydrogen bond interactions observed in PR complexes with peptide analogs [33–34].

The minor APV conformation refined with 0.3 relative occupancy lies in the opposite orientation to the major conformation and interacts with the opposite subunits of the PR dimer. The minor conformation of APV retained almost identical hydrogen bond, C-H...O and H₂O... π interactions to major conformation, with the following exceptions (Table S1). The hydrogen bond between the aniline nitrogen of APV and the carbonyl oxygen of Asp30 was lost in the minor APV conformation (distance increased to 3.6 Å). The water mediated interaction between the APV aniline nitrogen and the carboxylate group of Asp30 was replaced by a weak direct hydrogen bond (distance of 3.4 Å). The hydrogen bond of the THF oxygen with the amide of Asp30' was lost in the minor APV conformation (distance increased to 3.7 Å). Instead, the THF oxygen of APV formed a new interaction with the carboxylate group in the minor conformation of Asp30. The water interaction with the amide of Ile50' was weakened (distance of 3.5 Å). The C-H...O interaction between the carbonyl oxygen of APV and the C $_{\alpha}$ of Gly49 was lost in the minor conformation of APV. Some of these differences are likely to reflect the lower occupancy and greater positional error in the minor conformation. Variability in the interactions of Asp30/30' due to flexibility of the side chains has been observed in other PR complexes [19]. Overall, the minor conformation of APV showed one less hydrogen bond, one less C-H...O interaction, and weaker interactions than the major conformation had with PR.

Effects of Mutations on PR Structure and Interactions with APV

The structures of the mutants and wild type PR complexes with APV were compared in order to identify any significant changes. Overall, the polar interactions between APV and PR were well maintained in the mutant complexes. In these seven complexes the distances between non-hydrogen atoms were observed to be in the range of 2.3 to 3.3 Å for hydrogen bonds and 3.2 to 3.8 Å for C-H...O interactions (Tables S1, S2). The estimated error in atomic position is about 0.05 Å in structures at 1.0–1.2 Å resolution compared to the higher estimated errors of 0.10–0.15 Å in structures at 1.5–1.8 Å resolution [7], such as the complexes of PR_{I54V}-APV and PR_{I84V}-APV. Structural changes are detailed in the next sections for the mutant complexes with respect to the major conformation in PR_{WT}-APV. Generally, the changes in the mutants

involved hydrophobic C-H...H-C contacts or C-H...O polar interactions, although shifts of main chain atoms were observed in some cases. The ideal distances between non-hydrogen atoms are considered to be 3.0–3.7 Å for C-H...O interactions and 3.8–4.2 Å for van der Waals interactions as described in [30]. The structural differences are described separately for each mutant.

Val32 is an important part of the S2 pocket in the active site cavity and forms van der Waals interactions with inhibitors. In the PR_{WT}-APV structure Val32 forms hydrophobic contacts with Ile47, Ile56, Thr80 and Ile84, while Val32' interacts with Thr80' and Ile84'. Mutation of Val to Ile, which adds one methyl group, can reduce the volume of the active site cavity and alter the hydrophobic interactions in the cluster. The mutant with Ile32 did not show significant alterations in the main chain conformation or the interactions with APV, however, the C δ 1 methyl of the Ile side chain provided new van der Waals contacts with other hydrophobic side chains. Ile32 formed new hydrophobic contacts with the side chains of Val56, Leu76, and the main chain atoms of residues 77-78, and Ile32' showed new interactions with the side chains of Ile47', Ile50 and Val56' in the flaps (Fig. 3A). The flaps can exist in an open conformation in the absence of inhibitor and a closed conformation when inhibitor is bound. The interactions of residue 32 differ in the closed and open conformations; Val32 has no hydrophobic contacts with flap residues in the PR-APV structure, while Val32 forms hydrophobic contacts with Ile47 in the open conformation structure [35]. The flexibility of the flaps is likely to be altered in PR_{V32I} by the new hydrophobic contacts of Ile32/32', which is expected to contribute to the 3-fold reduced catalytic activity and 10-fold decreased APV inhibition of the PR_{V32I} mutant relative to wild type enzyme (Table I).

Ile50 is located at the tip of the flap on each PR monomer where its side chain forms hydrophobic interactions with inhibitors. In the wild type enzyme, Ile50/Ile50' interacts with Pro81'/Pro81 and Thr80'/Thr80 in the 80's loop as well as Ile47'/47 and Ile54'/54 in the flaps. The C δ 1 methyl of Ile50 side chain formed C-H...O interactions with the hydroxyl oxygen of Thr80' and carbonyl oxygen of Pro79', and the C δ 1 of Ile50' interacts with the hydroxyl of Thr80. Mutation from Ile50 to Val shortens the side chain by a methyl group, which eliminates the C-H...O interaction with the hydroxyl oxygen of Thr80' and van der Waals contact with Ile54' (Fig. 3B). In the other subunit, mutation to Val50' eliminates the C-H...O interaction with the hydroxyl of Thr80 and a hydrophobic contact with Pro81. The APV in PR_{I50V} complex had two alternate conformations with 0.6/0.4 relative occupancy. The APV showed an elongated hydrogen bond than seen in the PR_{WT} complex between the aniline group and the carbonyl oxygen of Asp30, with interatomic distance of 3.4 Å for the major conformation and 3.5 Å for the minor APV conformation. Val50' has also lost hydrophobic interactions with the THF group of APV. The minor conformation of APV showed similar changes in interactions with Asp30/30' as described for the minor APV conformation in the PR_{WT} complex. Overall, the observed structural changes in PR_{I50V}-APV are loss of two C-H...O interactions and van der Waals contacts, the elongated hydrogen bond and reduced hydrophobic contacts with APV. PR_{I50V} shows a large decrease in sensitivity to APV shown by the 30-fold drop in the relative inhibition coupled with 10-fold decreased catalytic efficiency, which suggests the importance of Ile50. Loss of the C-H...O interaction of Val50 with Thr80' has not been described before. Thr80 is a conserved residue in the PR sequences and its hydroxyl forms a hydrogen bond with the carbonyl oxygen of Val82, which contributes hydrophobic interactions with the inhibitors. Moreover, the hydroxyl group of Thr80 was shown to be important for PR activity using site directed mutagenesis where only mutation to Ser retaining the hydroxyl group, and not to Val or Asn, maintained enzymatic activity [36]. These lines of evidence, taken together, strengthen the suggestion that loss of the C-H...O interaction of residue 50 with the hydroxyl of Thr80, as well as loss of hydrophobic contacts with inhibitor, are important for the decreased catalytic activity and APV inhibition of PR_{I50V}.

Ile54 is another flap residue that forms hydrophobic interactions with Ile50' and residues 79-80, although it has no direct contact with inhibitor. Mutation I54M introduces a longer side chain, and the nearby main chain atoms have shifted relative to their positions in the wild type PR (Fig. 3C). Compared to PR_{WT}, the C_α of Met54 moved by 0.7 Å, and the longer Met side chain pushed residues 79, 80 and 81 away by 0.7–1.4 Å. In the other subunit the C_α of Met54' moved by 0.5 Å toward Pro79', and there was a correlated motion of Pro79' of 0.9 Å relative to its position in PR-APV. The longer Met54/54' side chains formed more hydrophobic contacts with Pro79/79' and Thr80/80' in PR_{I54M} relative to those of wild type PR. Overall, the Ile54 to Met mutation improved contacts within the hydrophobic cluster, although the interatomic distances to residues 79-80/79'-80' were increased. Similar structural changes were observed in the PR_{I54M} complexes with darunavir and saquinavir [18]. Despite these correlated changes between the main chain atoms of the flaps and 80' loops, this mutant was similar to the wild type PR in catalytic efficiency and had only 3-fold reduced inhibition by APV.

In contrast to PR_{I54M}, mutation I54V substitutes the shorter Val in PR_{I54V}. In PR_{WT}, the C_{δ1} of Ile54 interacts with Ile50', Val56, Pro79 and Thr80, while the C_{δ1} of Ile54' shows van der Waals interactions with Ile47', Ile50', Val56', and Pro79 and one C-H...O interaction with the carbonyl oxygen of Pro79'. The shorter Val side chain in the mutant results in loss of several van der Waals contacts with the adjacent residues, thus decreasing the stability of the hydrophobic cluster formed by flap residues 47, 54 and 50' (Fig. 3D). No C-H...O interaction was possible with Pro79', which was associated with a shift of ~0.5 Å in Pro79' increasing the separation of the flap and 80's loop. The mutation I54V decreased the hydrophobic interactions within the flaps and with Pro79, however, PR_{I54V} showed similar *K_i* value and only 3-fold reduced activity relative to the wild type enzyme.

Ile84 forms part of the S1/S1' subsites of PR, and mutation to Val84 removes a methylene moiety, which can reduce interactions with substrates and inhibitors. In wild type PR, van der Waals contacts were found between C_{δ1} of Ile84 and the benzyl and aniline moieties of APV and from C_{δ1} of Ile84' to the isopropyl group of APV. These interactions were lost in the PR_{I84V} mutant structure since the interatomic distances increased to >4.3 Å (Fig. 3E). The loss of hydrophobic contacts with APV is consistent with the modest change of 6-fold in *K_i* value for PR_{I84V}.

Leu90 is located in the short alpha helix outside of the active site cavity, although it extends close to the main chain of the catalytic Asp25. Mutation of Leu90 to Met substituted a longer side chain and introduced new van der Waals contacts with residues Asp25-Thr26. Moreover, the long Met90/90' side chains formed close C-H...O interactions with the carbonyl oxygen of the catalytic Asp25 and Asp25' (Fig. 3F). The alternate conformations of the Met90 side chain were arranged as described previously [25]. The new interactions of Met90/90' with the catalytic aspartates and adjacent residues are presumed to play an important role in the observed 11-fold increase in catalytic activity, as described previously [19,25]. The increased catalytic efficiency of the PR_{L90M} mutant is mainly due to almost 5-fold higher *k_{cat}*. On the other hand, the *K_M* of the mutant is only about half that of the wild type enzyme. Therefore, the new interactions of Met90 with the catalytic residues that are absent in the wild type structure may minimally affect the binding of substrate, but at the same time dramatically lower the activation barrier for substrate hydrolysis leading to substantial improvement of the PR_{L90M} catalytic activity. No change, however, was detected in the APV inhibition of PR_{L90M}.

Comparison of the mutant complexes with APV and SQV

The structures of HIV-1 protease complexes with APV or SQV were analyzed in order to understand their distinct drug resistance profiles. PR_{WT}-APV was compared with PR_{WT}-SQV (2NMW) solved at 1.16 Å resolution in a different unit cell and space group P2₁2₁2₁ [12]. The mutant APV complexes reported here were compared with the published SQV complexes of

PR_{I50V}-SQV (3CYX), PR_{I54M}-SQV (3D1X), PR_{I54V}-SQV (3D1Y) and PR_{I84V}-SQV (2NNK) refined at resolutions of 1.05 to 1.25 Å in the isomorphous unit cell and identical space group P2₁2₁2 as for all the APV complexes [12,18]. No SQV complexes have been reported for mutants PR_{V32I} and PR_{L90M}. A lower resolution (2.6 Å) crystal structure has been reported for the SQV complex with the double mutant PR_{G48V/L90M}[37], in which Met90 showed interactions similar to those seen in the structure of PR_{L90M}-APV. To analyze how the PR conformation alters to fit the different inhibitors, all structures were superimposed on the PR_{WT}-APV structure. The superposition was tested for both possible arrangements of the two subunits in the asymmetric dimer of HIV PR, i.e., superimposing residues 1-99 and 1'-99' with 1-99 and 1'-99', as well as with the opposite subunit arrangement of 1'-99' and 1-99. The arrangement with the lowest RMS deviation was used in further comparison. Interestingly, the major conformation of SQV has the opposite orientation to that of APV for the superimposed dimers with the lowest RMS values. PR_{WT}-SQV had the highest RMS deviation value of 0.87 Å on C_α atoms due to the different space groups, while the RMS deviations for the mutant complexes with SQV were lower, ranging from 0.29 to 0.36 Å, as usual for two structures in the same space group (Table II).

The corresponding pairs of wild type and mutant complexes with the two inhibitors were compared (Fig. 4A). The structures of PR_{WT}-SQV and PR_{WT}-APV showed larger RMS deviations of >1.0 Å for residues in surface loops, probably arising from altered lattice contacts due to the different space groups as reported previously [25]. Moreover, the two subunits in the dimer show asymmetric deviations due to non-identical lattice contacts as well as the presence of different asymmetric inhibitors. Changes in residues 52'-56', 79'-81' in the active site cavity are assumed to reflect variation in the interactions with the two inhibitors, while the lower deviations of catalytic triplet residues 25-27/25'-27' reflect their important function. The pairs of mutant complexes were determined in isomorphous unit cells with less overall variation so that changes are more likely to arise from different interactions with APV and SQV. PR_{I50V} had the fewest RMS differences between the two inhibitor complexes with a peak of 1.3 Å for Phe53'. PR_{I54V} and PR_{I84V} showed the largest change for Pro81' of 1.9 and 1.6 Å, respectively, while PR_{I54M} showed the maximum RMS deviation of 1.2 Å at residue 54'. Three regions were analyzed in more detail due to their flexibility and proximity to inhibitors and mutations: the flaps, the 80's loops, and the hydrophobic clusters formed by residues Ile47, Ile54, Thr80, Ile84 and Ile50' from the opposite subunit.

The conformation of the flaps segregated into two categories corresponding to the APV complexes and the SQV complexes (Fig. 4B). The coordinated changes in the flaps were most obvious for residues 50-51 and 50'-51' at the tips of the flaps. The flap residues 50 and 51 showed differences in C_α position of 0.5–0.9 Å between the complexes with APV or SQV. Differences of 0.6–0.8 Å at Gly51' and 0.1–0.4 Å at Ile/Val50' were seen in the flap from the other subunit. The different flap conformations are probably related to the larger chemical groups at P2 and P1' in SQV compared to those in APV (Fig. 1).

Changes in the 80's loops, which have been described as intrinsically flexible [12,25,38] and function in substrate recognition [39–40], were assessed using the distance between the C_α atoms of Pro81 and Pro81' to reflect alterations in the S1/S1' subsites. Pro81 and Pro81' were separated by 17.6–19.4 Å in the APV complexes, while these residues were 0.7–2.5 Å further apart in the SQV complexes (separations of 18.5–20.5 Å). The comparison of wild type complexes is shown in Figure 4C. PR_{I50V} complexes had the smallest distance between Pro81 and Pro81', while the greatest separation was observed for PR_{I54M} complexes probably due to close contacts of the longer Met54/54' side chains with the 80's loops in both inhibitor complexes. The distance between Pro81 and Pro81' in the other structures was about 2.5 Å longer in the SQV complexes compared to the APV complexes, which corresponds to the increment of the width across the S1/S1' pockets caused by binding of the big

decahydroisoquinoline P1' group of SQV instead of the smaller P1' group in APV (Fig. 4C) [12].

The more rigid region of Asp30/30' showed smaller shifts of ~ 0.5 Å for the C α atoms. The different P2 and P2' groups, THF in APV or Asn in SQV at P2 and aniline in APV or t-butyl group in SQV at P2', are accommodated by these shifts, as shown in Figure 4C.

The side chain interactions within the inner hydrophobic cluster were analyzed in PR_{WT}, PR_{I50V}, PR_{I54M}, and PR_{I54V} complexes with SQV and APV. Overall, the main chains of the flaps were shifted relative to the 80' loops in APV complexes compared to SQV complexes. The hydrophobic cluster around the active site is formed by Ile47, Ile54, Thr80, and Ile84 from one subunit and Ile50' from the opposite subunit, as well as Val32 in a more rigid region in PR_{WT}. Differences in the side chain interactions are described. In PR_{WT}-APV the C γ 1 of Ile50 made good van der Waals contacts with Ile54', but the side chains were further apart in PR_{WT}-SQV (Fig. 5A). One C-H...O interaction between C δ 1 of Ile50/Ile50' and the hydroxyl of Thr80'/Thr80 was conserved in PR_{WT}-APV, PR_{WT}-SQV, and PR_{I50V}-SQV. The structure of PR_{I50V}-APV, however, has lost this C-H...O interaction of residue 50 with the hydroxyl of Thr80', which reduces the interactions between the flap and the 80's loop (Fig. 5B). Mutation of Thr80 to Val80, which eliminates the C-H...O interaction with Ile50 C δ 1, significantly reduces the catalytic activity and binding affinity of SQV [36]. Besides the different interactions between PR and inhibitors, the loss of the C-H...O contact between residue 50 and Thr80 in PR_{I50V}-APV and not in PR_{I50V}-SQV, appears to correlate with the observed drug resistance. Mutation I50V is associated strongly with resistance to APV, but not to SQV.

In the PR_{I54M} and PR_{I54V} complexes with both inhibitors, however, similar interactions were observed among the side chains of residues 50, 54' and 80' (Fig. 5C-5D). Val84 in PR_{I84V}-SQV had fewer hydrophobic contacts than in PR_{I84V}-APV, but it had one C-H...O interaction between C γ 2 of Val84 and the carbonyl oxygen of Val82 (Fig. 5E). In contrast to the significant conformational shifts in main chain atoms, the side chains in this hydrophobic cluster generally have rearranged to maintain internal hydrophobic contacts in the complexes with both inhibitors.

DISCUSSION

We have described an atomic resolution crystal structure of PR_{WT} with APV, and analyzed structural changes in the APV complexes with mutants PR_{V32I}, PR_{I50V}, PR_{I54V}, PR_{V82A}, PR_{I84V}, and PR_{L90M}. The mutated residues contribute to an inner hydrophobic cluster around the substrate binding cavity, with the exception of Leu90 that is located near the backbone of the catalytic Asp25 in the outer hydrophobic cluster (Fig. 1B). Studies of the patterns of resistance mutations and molecular dynamics simulations have suggested the importance of these hydrophobic mutations in drug resistance [14,41]. Our analysis showed that interactions within the inner hydrophobic cluster containing residues 32, 47, 54 and 50 were frequently altered relative to those in the wild type enzyme. Mutations to larger side chains in PR_{V32I}, PR_{I54M} and PR_{L90M} resulted in formation of new hydrophobic contacts with flap residues, residues 79 and 80, and Asp25, respectively. Mutation to smaller side chains caused loss of internal hydrophobic interactions in the PR_{I50V} and PR_{I54V} structures. PR_{I84V}, PR_{V32I}, and PR_{I50V} showed reduced APV inhibition by 6-, 10- and 30-fold, respectively, relative to wild type PR, which is consistent with the observed structural changes. The PR_{I84V}-APV complex had lost hydrophobic contacts with APV, the PR_{V32I}-APV complex showed increased hydrophobic contacts with the flaps that are likely to restrict the flexibility needed for catalysis, and the PR_{I50V} complex had weaker interactions with APV. Ile54 has no direct contacts with APV, which is consistent with the relatively small changes in inhibition, catalytic properties, protease stability, and structure shown by the I54 mutants. No compensating changes were

identified elsewhere in the hydrophobic core. In PR_{L90M}, the longer side chain of Met90/90' lies close to the main chain of the catalytic aspartates forming new van der Waals contacts and a C-H...O interaction. These new contacts with Asp25/25' near the dimer interface correlate with the reduced stability and altered catalytic parameters of this mutant, as described in studies with IDV and DRV [19,25]. No evidence was found, however, that PR_{L90M}-APV had substantially altered the volume of the S1/S1' substrate binding pockets, unlike the PR_{G48V/L90M}-SQV structure, which showed reduced volume for the S1/S1' subsites relative to the wild type complex [37]. Also, the structure of PR_{G48V}-DRV showed reduced volume of the active site cavity relative to the wild type complex, consistent with a major effect of the G48V rather than the L90M mutation in reducing the S1/S1' volume in PR_{G48V/L90M}-SQV [18]. Reduced interactions with inhibitors and conformational adjustments of the flaps and 80's loops were observed in our previous studies of these mutants with other inhibitors [17–19, 25,30,42]. These structural changes are expected to contribute to drug resistance. However, crystal structures show only a static picture of the effect of mutations, while changes in protein dynamic and thermodynamic properties likely also contribute to resistance. Future studies using molecular dynamics simulations and calorimetric analysis will help to address the changes in these other properties.

Individual mutations have distinct effects on the protease structure and activity, however, drug resistant clinical isolates generally accumulate multiple mutations. Previous studies of double and single mutants suggested that the structural changes due to a single mutation were retained in the double mutant, although other properties did not combine in predictable ways [43]. A variety of structural and biochemical mechanisms have been reported for different combinations of mutations including: compensating structural changes, altered interactions with inhibitor, and substantial opening of the active site cavity [44–46]. Clearly, further studies are needed to assess the effects of the multiple protease mutations that are observed clinically.

Comparison of the APV complexes with the corresponding SQV complexes for PR_{WT}, PR_{I50V}, PR_{I54V}, and PR_{I84V} showed changes in the conformation of the flexible flaps and 80's loops. Despite the conformational changes in main chain atoms, the internal side chains generally have rearranged to preserve the internal hydrophobic contacts in the complexes with both inhibitors. The structural changes can be correlated with the type of inhibitor. In particular, the separation of Pro81 and Pro81' was significantly smaller in the complexes with APV compared to the equivalent SQV complexes, which reflects the smaller size of the P1' group in APV relative to that of SQV. Also, the flexible side chains of Asp30 and Asp30' accommodate diverse functional groups at P2 and P2' of SQV and APV at the surface of the PR active site cavity. The functional group can be critical for a tight binding inhibitor. For example, DRV was derived from APV by changing THF to bis-THF, which introduces more hydrogen bonds with PR main chain atoms and dramatically increases the potency on drug resistant HIV [47].

APV was designed to include several hydrophilic interactions with PR, and SQV optimizes hydrophobic interactions with PR [12]. Both of the inhibitors have been classified as peptidomimetic inhibitors, which mimic PR-substrate interactions and block enzyme activity, although, APV has only a single CO-NH peptide bond compared to three in SQV [48]. Resistant mutations within the hydrophobic clusters frequently involve small changes such as addition or deletion of a methylene group [13]. Mutations within the hydrophobic cluster have the potential to alter the flap dynamics or stability of PR as well as the binding of inhibitors, as shown in our structural analysis. The current drugs target the active site cavity and demonstrate strong binding to the catalytic Asps [49]. The hydrophobic pockets around the substrate binding site have been proposed as an alternate drug target [50–51]. However, our structural analysis shows that side chains in the hydrophobic cluster can rearrange readily to maintain the PR structure and activity, suggesting this region is a poor target for drugs.

The accuracy of high and atomic resolution crystal structures is critical for deciphering how mutations and inhibitors alter the HIV-1 PR structure. Here, we describe how HIV-1 PR recognizes the inhibitors APV and SQV by structural rearrangements of the two beta-hairpin flaps and two 80's loops, and how the same mutation results in different structural changes with the two drugs. Comparison of the structures provides insight into why I50V is a major drug resistant mutation observed on exposure to APV but appears less critical in resistance to SQV therapy. Besides the different interactions between PR and inhibitors, the absence of the C-H...O contact between flap residue 50 and Thr80 in PR_{I50V}-APV, and its presence in PR_{I50V}-SQV and PR_{WT} complexes, contributes to the drug resistance of this mutation. The conclusion is that small rearrangements of the PR loops enclosing the inhibitor combined with changes in weak internal interactions produce the distinct patterns of resistant mutations for the two drugs.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

The HIV-1 PR and mutants were constructed with five mutations Q7K, L33I, L63I, C67A, and C95A to prevent cysteine-thiol oxidation and diminish autoproteolysis [52]. The expression, purification and refolding methods are described in [52–53].

Kinetic Assays

The fluorogenic substrate Abz-Thr-Ile-Nle-p-nitro-Phe-Gln-Arg-NH₂, where Abz is anthranilic acid and Nle is norleucine, (Bachem) with sequence derived from the p2/NC cleavage site of the Gag polyprotein was used in kinetic assays. Proteases were diluted in reaction buffer (100 mM MES, pH 5.6, 400mM sodium chloride, 1 mM EDTA, and 5% glycerol). 10 μ L diluted enzyme was mixed with 98 μ L reaction buffer and 2 μ L Dimethyl Sulfoxide (DMSO) or APV (dissolved in DMSO) and incubated at 37 °C for 5 min. Then, the reaction was initialized with addition of 90 μ L substrate. The reaction was monitored over 5 min in the POLARstar OPTIMA microplate reader at wavelengths of 340 nm and 420 nm for excitation and emission. Data analysis used the program SigmaPlot 9.0 (SPSS Inc., Chicago, IL). K_m and k_{cat} values were obtained by standard data-fitting with the Michaelis-Menten equation. The K_i value was obtained from the IC50 values estimated from an inhibitor dose-response curve using the equation $K_i = (IC50 - [E]/2)/(1 + [S]/K_m)$, where [E] and [S] are the PR and substrate concentrations.

Crystallographic Analysis

Inhibitor APV (from AIDS reagent program) was dissolved in DMSO by vortex-mixing. The mixture was incubated on ice prior to centrifugation to remove any insoluble material. The inhibitor was mixed with 2.2 mg/ml protein in molar ratio of 5:1 in most cases. The two exceptions were: 3.5 mg/ml of PR_{I50V} was used with inhibitor-protein ratio of 10:1, and 3.7 mg/ml of PR_{L90M}. The crystallization trials employed the hanging drop method using equal volumes of enzyme-inhibitor and reservoir solution. PR_{WT}-APV was crystallized from 0.1M MES, pH 5.6, and 0.6–0.8 M sodium chloride. Crystals of PR_{V32I}-APV and PR_{I50V}-APV were grown from 0.1M sodium acetate, pH 5.4, 0.4 M and 1.2 M sodium chloride, respectively. PR_{I54M}-APV crystals were grown from 0.1M sodium acetate, pH 4.6, and 0.67 M sodium chloride. PR_{I54V}-APV and PR_{I84V}-APV crystals were grown from 0.1 M sodium acetate, pH 5.4 and 4.0, respectively, and 0.13 M sodium iodide, and PR_{L90M}-APV crystals from 0.1 M sodium acetate, pH 4.8 and 0.2 M sodium iodide. Single crystals were mounted on fiber loops with 20 to 30 % (v/v) glycerol as cryoprotectant in the reservoir solution. X-ray diffraction data were collected at the SER-CAT beamline of the Advanced Photon Source, Argonne National Laboratories. Diffraction data were integrated, scaled, and merged using the HKL2000 package [54]. PR_{WT}-APV, PR_{V32I}-APV and PR_{I50V}-APV were solved by molecular

replacement program Phaser [55] with the protein atoms of structure 2QCI[32] as the starting model. The other complexes were solved by MOLREP [56], using the protein atoms of 2F8G as the starting model [19]. The crystal structures were refined using SHELX-97 [57], except that the lower resolution structure of PR_{I84V}-APV was refined with REFMAC 5.2 [58]. The diffraction-data precision indicator (DPI) was used for determining the accuracy in the atomic positions [59]. The molecular graphics program COOT was used for map display and model building [60]. Structural figures were made by PyMol [61]. The structures were compared by superimposing their C α atoms and using HIVAGENT [62] to calculate the distance between two atoms. The cut-off distances for different interactions were as described in [30].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HIV-1	human immunodeficiency virus type 1
PR	HIV-1 protease (EC 3.4.23.16)
PR_{V32I}	PR with V32I mutation
PR_{I50V}	PR with I50V mutation
PR_{I54V}	PR with I54V mutation
PR_{I54M}	PR with I54M mutation
PR_{I84V}	PR with I84V mutation
PR_{L90M}	PR with L90M mutation
APV	amprenavir
SQV	saquinavir
RMS	root mean square

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Figure 1A

Figure 1B

Figure 1.

(a) The chemical structures of amprenavir (APV) and saquinavir (SQV).

(b) Structure of HIV-1 PR dimer with the sites of mutation Val32, Ile50, Ile54, Ile84 and Leu90 indicated by green sticks for side chain atoms in both subunits. Amino acids are labeled in one subunit only. APV is shown in magenta sticks. The amino acids in the inner hydrophobic cluster are indicated by numbered red spheres, and the amino acids in the outer hydrophobic cluster are shown as blue spheres.

Figure 2A

Figure 2B *PR_{WT}-APV*

Figure 2.
Inhibitor Binding Site in *PR_{WT}*-APV.

a) APV and PR residues in the binding site with alternate conformations. Omit maps for major (green) and minor (magenta) conformations of APV, interacting PR residues Asp25, Gly48 and Asp30 from both subunits, and the conserved flap water are contoured at a level of 3.5 σ .
b) Hydrogen bond, C-H...O and H₂O... π interactions between PR (gray) and APV (cyan). Hydrogen bond interactions are indicated by dashed lines. C-H...O and H₂O... π interactions are indicated by dotted lines.

Figure 3.

The interactions of mutated residues in (a) PR_{V32I}-APV, (b) PR_{I50V}-APV, (c) PR_{I54M}-APV, (d) PR_{I54V}-APV, (e) PR_{I84V}-APV, and (f) PR_{I90M}-APV. The grey color corresponds to wild type PR-APV and the cyan color indicates the mutant complex. Dashed lines indicate van der Waals interactions and dotted lines show C-H...O interactions. Interatomic distances are shown in Å with black lines indicating the PR_{WT} and red lines indicating the mutant. Interatomic distances of > 4.3 Å are shown in dash-dot lines to indicate the absence of favorable interaction.

Figure 4.

Structural differences between APV and SQV complexes. (a) The root mean square (RMS) difference (Å) per residue is plotted for C_α atoms of SQV complexes compared with the corresponding APV complexes: PR_{WT} (blue line), PR_{I50V} (red line) and PR_{I54V} (green line). (b) Comparison of the flap regions in the structures. The complexes with APV are in cyan, and the complexes with SQV are in grey. The arrow indicates the shifts between C_α atoms at the residues 50 and 51 in the PR complexes with the two inhibitors. (c) The width across the S1-S1' subsites increases in PR_{WT}-SQV relative to PR_{WT}-APV. Similar changes were seen for the mutant complexes, except for PR_{I50V}.

Figure 5.

Interactions of Ile50, Ile54' and Thr80'. (a) PR_{WT}-APV compared to PR_{WT}-SQV. (b) PR_{I50V}-APV compared to PR_{I50V}-SQV. (c) PR_{I54M}-APV compared to PR_{I54M}-SQV. (d) PR_{I54V}-APV compared to PR_{I54V}-SQV. (e) PR_{I84V}-APV compared to PR_{I84V}-SQV. Dashed lines indicate van der Waals contacts with interatomic distances in Å. Dotted lines indicate C-H...O interactions. Black lines indicate interactions in SQV complexes. Red lines indicate interactions in APV complexes.

Table 1

Kinetic parameters for substrate hydrolysis and inhibition of amprenavir.

	K_m (μ M)	k_{cat} (/min)	k_{cat}/K_m (μ M/min)	Relative k_{cat}/K_m	K_i (nM)	Relative K_i
WT*	30 $\pm$ 5	190 $\pm$ 20	6.5 $\pm$ 1.3	1.0	0.15 $\pm$ 0.04	1
V32I	65 $\pm$ 6	120 $\pm$ 10	1.8 $\pm$ 0.2	0.3	1.5 $\pm$ 0.2	10
I50V*	109 $\pm$ 8	68 $\pm$ 5	0.6 $\pm$ 0.03	0.1	4.5 $\pm$ 0.6	30
I54M*	41 $\pm$ 5	300 $\pm$ 40	7.3 $\pm$ 0.8	1.1	0.50 $\pm$ 0.06	3
I54V*	43 $\pm$ 6	130 $\pm$ 20	3.1 $\pm$ 0.9	0.5	0.41 $\pm$ 0.05	3
I84V	73 $\pm$ 6	320 $\pm$ 30	4.4 $\pm$ 0.5	0.7	0.9 $\pm$ 0.2	6
L90M	13 $\pm$ 2	950 $\pm$ 120	73 $\pm$ 13	11.2	0.16 $\pm$ 0.01	1

* K_m and k_{cat} values from [18]

Error in k_{cat}/K_m is calculated as $(A/B) + / - (1/B^2)(\text{square root}(B^2a^2 + A^2b^2))$, where A is k_{cat} , a is k_{cat} error, B is K_m , and b is error in K_m .

Table 2

Crystallographic Data Collection and Refinement Statistics

APV complex	PR	PR _{V32I}	PR _{I50V}	PR _{I54M}	PR _{I54V}	PR _{I84V} *	PR _{L90M}
Space group	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2
Unit cell dimensions: (Å)							
a	58.11	57.77	57.95	58.12	57.50	59.51	57.94
b	85.97	86.13	86.01	85.91	86.00	86.88	85.91
c	46.42	46.28	46.21	46.10	45.95	45.44	46.10
Resolution range (Å)	50–1.02	50–1.20	50–1.29	50–1.16	50–1.50	50–1.85	50–1.35
Unique reflections	113,227	66,626	55,569	73,638	37,010	18,138	50,443
<i>b</i> R _{merge} (%) overall ^a	5.7 (38.2)	8.1 (44.2)	7.0 (40.2)	7.2 (35.7)	6.0 (46.2)	9.7 (34.5)	5.5 (46.2)
<i>I</i> /σ(<i>I</i>) overall ^a	15.3 (2.6)	11.3 (2.5)	15.2 (2.3)	20.1 (2.1)	16.8 (2.4)	15.8 (5.8)	17.9 (2.5)
Completeness (%) overall ^a	95.8 (65.0)	91.6 (62.7)	93.9 (70.4)	91.8 (58.9)	99.7 (99.2)	93.2 (76.6)	97.8 (97.3)
Data range for refinement (Å)	10–1.02	10–1.20	10–1.29	10–1.16	10–1.50	10–1.85	10–1.35
<i>c</i> R (%)	12.4	16.4	15.5	15.4	14.9	19.9	14.3
<i>d</i> R _{free} (%)	14.2	20.1	19.3	18.8	19.7	23.6	19.9
No. of solvent atoms (total occupancies)	292 (207.3)	151 (129.8)	177 (143.6)	242 (221.5)	152 (128.5)	84 (84)	211 (202.5)
RMS deviation from ideality							
Bonds (Å)	0.017	0.013	0.012	0.016	0.010	0.014	0.012
Angle distance (Å)	0.036	0.031	0.030	0.033	0.029	1.546 (Degree)	0.030
Average B-factors (Å ²)							
Main-chain atoms	10.8	16.0	14.4	14.2	23.2	25.6	20.3
Side-chain atoms	14.8	21.4	20.7	20.8	28.8	28.3	23.6
Inhibitor	10.5	16.9	17.1	17.8	28.5	23.7	16.1
Solvent	20.8	25.6	24.3	36.1	47.0	49.5	39.9
DPI	0.02	0.04	0.05	0.04	0.07	0.13	0.05
Relative occupancy of APV	0.7/0.3	-	0.6/0.4	-	-	-	-
RMS deviation from PR (Å)	-	0.15	0.19	0.33	0.26	0.38	0.19

APV complex	PR	PR _{V32I}	PR _{I50V}	PR _{I54M}	PR _{I54V}	PR _{I84V} [*]	PR _{L90M}
RMS deviation from PR-SQV (Å)	0.87	-	0.29	0.36	0.32	0.36	-

* Refined using REFMAC 5.0, others were refined with SHELX-97

^a Values in parentheses are given for the highest resolution shell.

^b $R_{\text{merge}} = \Sigma |hkl| |I_{hkl} - \langle I_{hkl} \rangle| / \Sigma |hkl| I_{hkl}$.

^c $R = \Sigma |F_{\text{obs}} - F_{\text{cal}}| / \Sigma F_{\text{obs}}$.

^d $R_{\text{free}} = \Sigma |F_{\text{test}}(F_{\text{obs}} - |F_{\text{cal}}|)^2 / \Sigma |F_{\text{test}}(F_{\text{obs}})|^2$.