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# Capturing the Reaction Pathway in Near-Atomic-Resolution Crystal Structures of HIV-1 Protease

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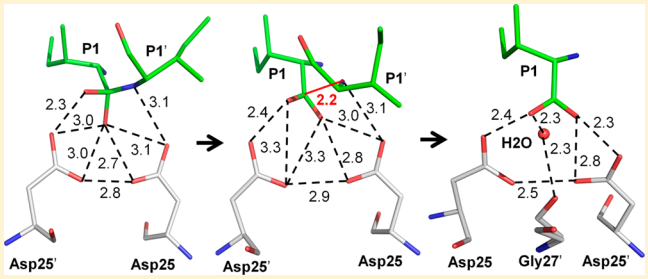
Supporting Information

**ABSTRACT:** Snapshots of three consecutive steps in the proteolytic reaction of HIV-1 protease (PR) were obtained in crystal structures at resolutions of 1.2−1.4 Å. Structures of wild-type protease and two mutants ($PR_{V32I}$ and $PR_{I47V}$) with V32I and I47V substitutions, which are common in drug resistance, reveal the gem-diol tetrahedral intermediate, the separating N- and C-terminal products, and the C-terminal product of an autoproteolytic peptide. These structures represent three stages in the reaction pathway and shed light on the reaction mechanism. The near-atomic-resolution geometric details include a short hydrogen bond between the intermediate and the outer carboxylate oxygen of one catalytic Asp25 that is conserved in all three structures. The two products in the complex with mutant $PR_{I47V}$ have a 2.2 Å separation of the amide and carboxyl carbon of the adjacent ends, suggesting partial cleavage prior to product release. The complex of mutant $PR_{V32I}$ with a single C-terminal product shows density for water molecules in the other half of the binding site, including a partial occupancy water molecule interacting with the product carboxylate end and the carbonyl oxygen of one conformation of Gly27, which suggests a potential role of Gly27 in recycling from the product complex to the ligand-free enzyme. These structural details at near-atomic resolution enhance our understanding of the reaction pathway and will assist in the design of mechanism-based inhibitors as antiviral agents.




The human immunodeficiency virus type 1 protease (HIV-1 PR) acts as a dimer of two identical 99-amino acid subunits to process the viral Gag and Gag-Pol polyproteins into functional proteins (Figure 1A).[1] The indispensable function of PR in replication of infectious virus makes it an important target for antiretroviral therapy. However, the efficacy of PR inhibitors decreases over time because of the evolution of drug resistance, primarily by mutations in the PR.[2] Even treatment with highly active antiretroviral therapy (HAART) does not completely eliminate resistant virus. Therefore, there is a continuing need for new PR inhibitors to combat drug resistance. Improved knowledge of the PR reaction intermediates will help in the design of novel mechanism-based inhibitors.

The aspartyl protease family is widely distributed in a variety of organisms, and its members participate in diverse biological functions; however, the detailed proteolytic mechanism is not fully understood.[3] Several experimental or theoretical studies have addressed the reaction mechanism of peptide cleavage by aspartyl proteases. $^{18}O$ exchange mass spectrometry experiments with HIV-1 PR suggest that the peptide hydrolysis reaction proceeds via the formation of a reversible and metastable gem-diol reaction intermediate.[4] Recent analysis by neutron crystallography has provided critical information about the location of hydrogen atoms in the active site of HIV-1 PR complexed with an inhibitor, which does not contain the gem-diol reaction intermediate.[5] X-ray crystallographic analysis of reaction intermediates trapped in the enzyme structure has proven to be a powerful tool for probing catalytic mechanisms.[6] Veerapandian et al. used the aspartic protease endothiapepsin complexed with a renin inhibitor mimicking both hydroxyls in the putative intermediate to study the proteolytic mechanism.[7,8] X-ray structures were reported for N-terminal and C-terminal peptide products bound to PRs from HIV-1 and the closely related simian immunodeficiency virus.[9] Other studies soaked tethered HIV-1 PR crystals with a substrate peptide to trap

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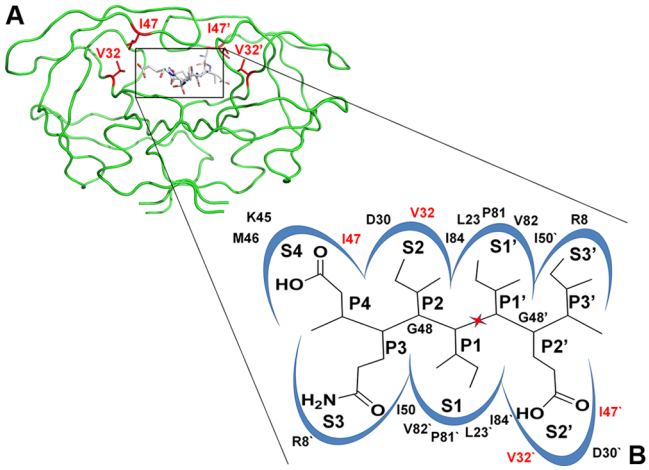


**Figure 1.** (A) Structure of the HIV-1 PR dimer in a green backbone representation. The sites of mutations Val32 and Ile47 are shown as red sticks for the side chain atoms in both subunits, with the prime indicating the "second" subunit. The tetrahedral intermediate (TI) peptide is shown as sticks colored by atom type. (B) Schematic illustration of the substrate binding site of HIV-1 PR. The peptide DQIIxIEI (P4−P3′) is shown in the S4−S3′ subsites of the PR dimer. The scissile peptide bond is indicated by the red star. PR residues contributing to the binding site are indicated.

different components of the reaction, including the tetrahedral intermediate.[10,11] Our group has reported the highest resolution of 1.5 Å for the structures of a tetrahedral reaction intermediate in the wild-type and mutant HIV-1 PR.[12] In these examples, however, the structural information is limited by the resolution and the disorder observed frequently for peptide intermediates.

More recently, our studies have focused on understanding the influence on the reaction intermediates of selected mutations found in drug resistance. Moreover, we identified a 1.2 Å resolution structure of the wild-type PR with a tetrahedral intermediate. PR recognizes peptide substrates of at least six residues binding in subsites S3−S3′ within the active site cavity (Figure 1B).[1] Mutations of PR residues Val32 and Ile47 were selected because they contribute hydrophobic interactions with substrates or inhibitors (Figure 1A), and mutations at these sites are common in drug resistance.[13] Multidrug resistant mutation V32I appears in ∼20% of patients treated with amprenavir[14] and is associated with high levels of drug resistance to lopinavir and ritonavir.[13] Drug resistant mutation I47V is located in the flexible flap and interacts with the inhibitor. Mutation I47V is associated with resistance to darunavir, lopinavir, tipranavir, and ritonavir in therapy.[13,15]

We describe crystal structures of PR and its mutants with different reaction intermediates and the implications for the proteolytic mechanism. The crystal structures of wild-type HIV-1 PR ($PR_{WT}$) and its mutants containing the single substitutions I47V ($PR_{I47V}$) and V32I ($PR_{V32I}$) were refined at near-atomic resolutions of 1.2−1.4 Å and, by serendipity, illustrate three different steps in the hydrolytic reaction. Peptide products and the reaction intermediate corresponding to an autoproteolytic cleavage site were observed in the structures, as described previously.[12] These near-atomic-resolution crystal structures provide more accurate information for the catalytic mechanism and the design of next-generation antiviral inhibitors.

## EXPERIMENTAL PROCEDURES

**Protein Preparation and Crystallization of HIV-1 $PR_{WT}$, $PR_{V32I}$, and $PR_{I47V}$.** The mutants were constructed and expressed in bacteria, and the protein was purified as described previously.[12] The crystallization trials employed the hanging drop method using equal volumes of enzyme/inhibitor and reservoir solution. PR and mutant proteins at 2.2 mg/mL were mixed with the inhibitor or peptide (dissolved in DMSO) at a molar ratio of 1:5 and incubated on ice for 30 min prior to centrifugation to remove any insoluble material. $PR_{WT}$ was crystallized from 0.1 M sodium acetate buffer (pH 4.8), 0.41 M potassium chloride, and an investigational inhibitor. $PR_{V32I}$ was crystallized from 0.06 M sodium acetate buffer (pH 5.6), 0.67 M sodium chloride, and a synthetic peptide. $PR_{I47V}$ crystals were grown from 0.05 M sodium acetate buffer (pH 5.0), 1.2 M sodium formate, and 2.5% PEG8000.

**X-ray Data Collection and Refinement.** Single crystals were mounted on fiber loops with 25% (v/v) glycerol as a cryoprotectant in the reservoir solution. X-ray diffraction data were collected at the SER-CAT beamline of the Advanced Photon Source, Argonne National Laboratories (Argonne, IL). Diffraction data were integrated, scaled, and merged using the HKL2000 package.[16] Structures of $PR_{WT}$, $PR_{V32I}$, and $PR_{I47V}$ were determined by the molecular replacement program Molrep[17] using structures 3B7V, 1FG6, and 2F8G as the respective starting models.[12,18,19] Refinement was conducted using SHELX-97.[20] No electron density was seen for the inhibitor used in the crystallization solution for $PR_{WT}$; however, the gem-diol intermediate of autoproteolysis of PR residues 59−67 (YDQIIxIEIA) fit the observed density. The synthetic peptide sequence did not fit the electron density in $PR_{V32I}$ and was replaced by the single C-terminal product of residues 59−63 (YDQII). Tyr59 at P5 was refined as Ala because of the poor electron density for its side chain atoms in $PR_{WT}$ and $PR_{V32I}$. $PR_{I47V}$ was refined with both N-terminal and C-terminal products extending in opposing directions (residues 60−63 with residues 64 and 65 in one orientation and residues 61−63 with residues 64−66 in the other). The structures were refined with anisotropic atomic displacement parameters (*B* factors). Hydrogen atoms were added at the final stages of the refinement. The molecular graphics program COOT was used for map display and model building.[21] Structural figures were made using PyMol.[22] The structures were compared by superimposing their C$\alpha$ atoms and using HIVAGENT[23] to calculate the distance between two atoms. The cutoff distances for different types of interactions were as described in ref 24.

## RESULTS AND DISCUSSION

**Crystallographic Analysis.** The crystal structures of $PR_{WT}$, $PR_{V32I}$, and $PR_{I47V}$ were determined in the same space group $P2_12_12$, and the crystallographic statistics are summarized in Table 1. The asymmetric units include one PR homodimer, and the residues in the two subunits are labeled 1−99 and 1′−99′. The diffraction data extend to a resolution of 1.2 Å for $PR_{WT}$, 1.3 Å for $PR_{I47V}$, and 1.4 Å for $PR_{V32I}$, and the structures were refined to *R* factors of 14.4−17.5%. The majority of protein residues and solvent molecules showed clear electron density in all the structures. The tetrahedral intermediate and cleavage products of PR residues 59−67 (YDQII*IEIA, where the asterisk indicates the cleavage site between P1 and P1′ residues) matched the electron density in the active site cavities of the $PR_{WT}$, $PR_{I47V}$, and $PR_{V32I}$ structures. These


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**Table 1. Crystallographic Data Collection and Refinement Statistics**

| | $PR_{WT}$ | $PR_{I47V}$ | $PR_{V32I}$ |
|---|---|---|---|
| space group | $P2_12_12$ | $P2_12_12$ | $P2_12_12$ |
| unit cell dimensions (Å) | | | |
| *a* | 58.41 | 58.08 | 58.06 |
| *b* | 86.20 | 86.30 | 86.14 |
| *c* | 46.36 | 46.35 | 46.30 |
| resolution range (Å) | 10−1.2 | 10−1.31 | 50−1.4 |
| no. of unique reflections | 66188 | 55879 | 45833 |
| overall $R_{merge}$ (%) (final shell) | 9.7 (39) | 6.3 (51) | 7.2 (43) |
| overall $I/\sigma(I)$ (final shell) | 15.03 (2.2) | 27.8 (2.1) | 18.3 (2.1) |
| overall completeness (%) (final shell) | 94.3 (56.8) | 98.8 (90.6) | 93.0 (98.7) |
| data range for refinement (Å) | 10−1.2 | 10−1.31 | 10−1.4 |
| *R* (%) | 0.14 | 0.15 | 0.17 |
| $R_{free}$ (%) | 0.18 | 0.18 | 0.23 |
| no. of solvent atoms (total occupancies) | 206 (191) | 181 (169.5) | 140 (134) |
| rmsd from ideality | | | |
| bonds (Å) | 0.015 | 0.012 | 0.010 |
| angle distances (Å) | 0.034 | 0.031 | 0.029 |
| average *B* factor (Å$^2$) | | | |
| main chain atoms | 19.2 | 17.1 | 16.8 |
| side chain atoms | 24.9 | 20.4 | 23.3 |
| peptide intermediate | 54.1 | 53.5 | 32.2 |
| solvent | 36.4 | 33.3 | 30.5 |
| intermediate peptide | (Y)[a]DQIIxIE | (D)(Q)II+IE | (Y)DQII |
| peptide relative occupancy | 0.5/0.4 | 0.5/0.4 | 1.0 |

[a]Parentheses indicate peptide residues refined as alanine because of poor electron density for the longer side chain.

peptides are thought to derive from very slow autoproteolysis, because the L63I substitution almost eliminates a site of autoproteolytic cleavage.[25] The $PR_{WT}$ dimer included two partial occupancy gem-diol intermediates: residues 59−65 (designated P5−P2′) were fit in one conformation and 61−67 (P3−P4′) for the conformation in the opposite orientation with relative occupancies of 0.4 and 0.5, respectively. The two mutants trapped the product peptides. A single C-terminal product containing residues 59−63 (P5−P1) was seen in $PR_{V32I}$. Ala was refined instead of Tyr59 at P5 because of weak electron density for the side chain in $PR_{WT}$ and $PR_{V32I}$. $PR_{I47V}$ was refined with both N- and C-terminal products in two alternate conformations comprising residues 60−63 (P4−P1) with residues 64 and 65 (P1′−P2′) at 0.5 occupancy and residues 61−63 (P3−P1) with residues 64−66 (P1′−P3′) at 0.4 occupancy. The side chains of P4 Asp and P3 Glu were not visible in the electron density. The solvent, consisting of water molecules, sodium ions, chloride ions, and glycerol, was modeled with 204, 181, and 140 molecules in $PR_{WT}$, $PR_{I47V}$, and $PR_{V32I}$, respectively. Alternate conformations were refined for 26 residues in $PR_{WT}$, 13 residues in $PR_{I47V}$, and 7 residues in $PR_{V32I}$. Generally, alternate conformations were seen for the longer side chains on surface residues. A few internal residues showed alternate conformations for the side chain or main chain: Ile84, Leu97, Ile33′, and Ile84′ of $PR_{WT}$, Gly27, Ile84, and Ile33′ of $PR_{V32I}$, and Val47, Ile64, Ile84, Ile33′, and Ile84′ of $PR_{I47V}$. The backbone structures were almost identical for the three dimers with low pairwise rmsd's of 0.15−0.17 Å for all Cα atoms.

**Structural Changes around the Mutated Residues.** The two drug resistant mutations are conservative substitutions of hydrophobic residues in the substrate binding cavity. The side chain of Val32 in the $PR_{WT}$ structure forms van der Waals contacts with the internal hydrophobic cluster comprising residues Ile50′, Ile47, Ile56, Leu76, Thr80, and Ile84, and similar interactions were seen in the other subunit. The longer Ile32 side chain in the $PR_{V32I}$ mutant has the potential to form new van der Waals contacts within the cluster, as described for the $PR_{V32I}$ complex with amprenavir.[26] In one subunit, the Cδ1 methyl of the Ile32 side chain is directed toward the hydrophobic cluster, providing new van der Waals contacts with several hydrophobic side chains. In the other subunit, however, the Cδ1 methyl of Ile32′ rotates to form new hydrophobic contacts only with Ile47′ and Ile50 (Figure 2A).

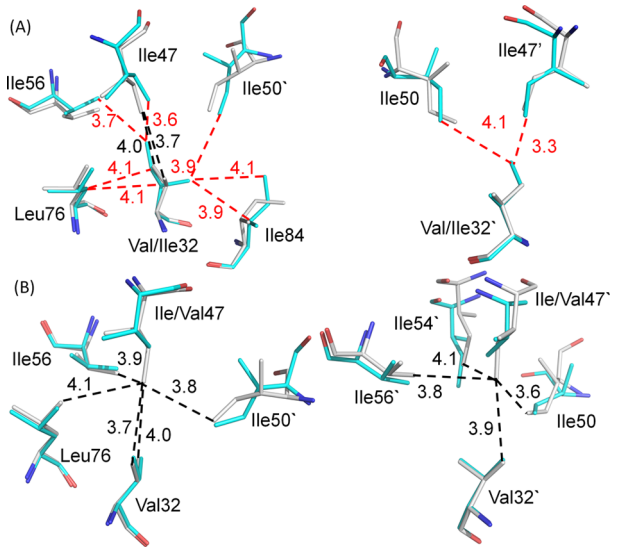


**Figure 2.** Mutations alter internal hydrophobic contacts. Residues are shown from superimposed structures of $PR_{WT}$ with (A) $PR_{V32I}$ and (B) $PR_{I47V}$. The two subunits are shown in the left and right panels, respectively. The $PR_{WT}$ residues are colored gray for carbon atoms, while the mutants are colored cyan. The van der Waals interactions are indicated by dashed lines in black for $PR_{WT}$ and red for the mutants with interatomic distances in angstroms. The mutation of I47 to a smaller side chain in $PR_{I47V}$ eliminates hydrophobic contacts seen for Ile47 in $PR_{WT}$. The opposite effect occurs with substitution of the large side chain in $PR_{V32I}$.

The 3.3 Å distance between the Cδ1 methyl groups of Ile32′ and Ile47′ is unusually short for a C−H···H−C interaction, which may indicate a destabilizing interaction.[27] Ile32 forms a van der Waals contact with Ile at the P2 position in the product peptide similar to the contact seen for Val32 in $PR_{WT}$, while no product is seen in the other subunit.

The hydrophobic side chain of Ile47 forms internal hydrophobic contacts and interacts with P2 and P2′ residues of the reaction intermediate in both subunits of the $PR_{WT}$ complex. These hydrophobic interactions are retained in the $PR_{I47V}$ mutant. In $PR_{WT}$, Cδ1 of Ile47 interacts with Ile50′, Val32, Val56, and Leu76, while Cδ1 of Ile47′ shows van der Waals interactions with the corresponding residues in the other subunit and with Ile54′. Mutation I47V substitutes the shorter Val side chain and eliminates van der Waals contacts with


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adjacent residues, thus probably decreasing the stability of the hydrophobic cluster in $PR_{I47V}$ (Figure 2B).

**$PR_{WT}$–TI Interaction.** The tetrahedral intermediate (TI) of residues $Y_{59}$DQII*IEIA$_{67}$ (where the asterisk indicates the gem-diol group) was observed in the omit $F_o - F_c$ electron density map in $PR_{WT}$ in two alternate conformations extending in opposite directions (Figure 3A and Figure S1A and S2A of the Supporting Information), as found frequently in PR complexes with peptide analogues.[19,28] Residues P3–P4′ were fit in one conformation and residues P5–P2′ in the opposite orientation, with occupancies refined to 0.5 and 0.4, respectively. The omit electron density ($F_o - F_c$) map for the gem-diol structure clearly indicates four hydroxyl oxygens (Figure 3B and Figure S1B of the Supporting Information). The two alternate conformations of the TI peptide formed essentially identical interactions with $PR_{WT}$. Interactions with the catalytic Asp25 and -25′ are described later. The main chain atoms of the TI peptide formed hydrogen bond interactions with residues Gly27, Asp29, Gly48, Gly27′, Asp29′, Asp30′, and Gly48′ (Figure 4A). The side chain of Glu at P2′ showed hydrogen bond interactions with Asp29 and Asp30. A shorter interaction of 2.4 Å seen between the carboxylate side chains of P2 Glu and Asp30 is consistent with protonation of P2 Glu, as described in other crystal structures of PR with peptide analogues.[19,28] Additional stabilizing interactions include water-mediated hydrogen bonds and hydrophobic contacts between $PR_{WT}$ and the peptide.

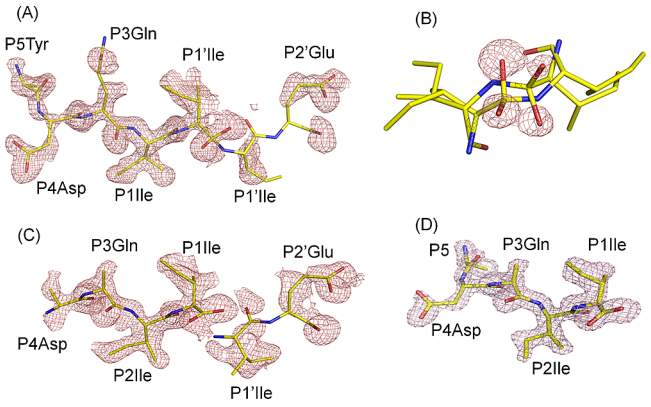


**Figure 3.** Electron density maps for the peptide intermediates. (A) Omit map ($F_o - F_c$) for the major conformation of TI in the $PR_{WT}$ structure. The contour level is 2.0$\sigma$. (B) $F_o - F_c$ omit map for the hydroxyl oxygen atoms of P1 Ile in the major and minor conformations contoured at 3.5$\sigma$. (C) Omit electron density map ($F_o - F_c$) for the major conformation of product peptides in the $PR_{I47V}$ structure. The contour level is 2.0$\sigma$. (D) $F_o - F_c$ omit map for the peptide C-terminal product of $PR_{V32I}$ contoured at 2.5$\sigma$. The corresponding stereofigures are given in Figure S1A–D of the Supporting Information.

**Interactions of $PR_{I47V}$ with Two Products.** The $PR_{I47V}$ structure revealed two alternate conformations of both the N- and C-terminal products extending in opposite directions (Figure 3C and Figures S1C and S2B of the Supporting Information). The occupancy of residues P4–P1 and P1′–P2′ in one conformation was 0.5, and residues P3–P1 and P1′–P2′ were refined with an occupancy of 0.4 for the opposite orientation. The alternate conformations maintained similar interactions with the two subunits of $PR_{I47V}$. Interactions with the catalytic Asp25 and -25′ are described below. Four hydrogen bond interactions connected the main chain atoms of product P4–P1 with residues Gly27, Asp29, and Gly48, and two hydrogen bond interactions linked main chain atoms of product P1′–P2′ with residues Gly27′ and Asp29′ (Figure 4B). The side chain carboxylate oxygen atoms of P2′ Glu interact with Asp29′ and Asp30′, as described for P2′ in the TI peptide.

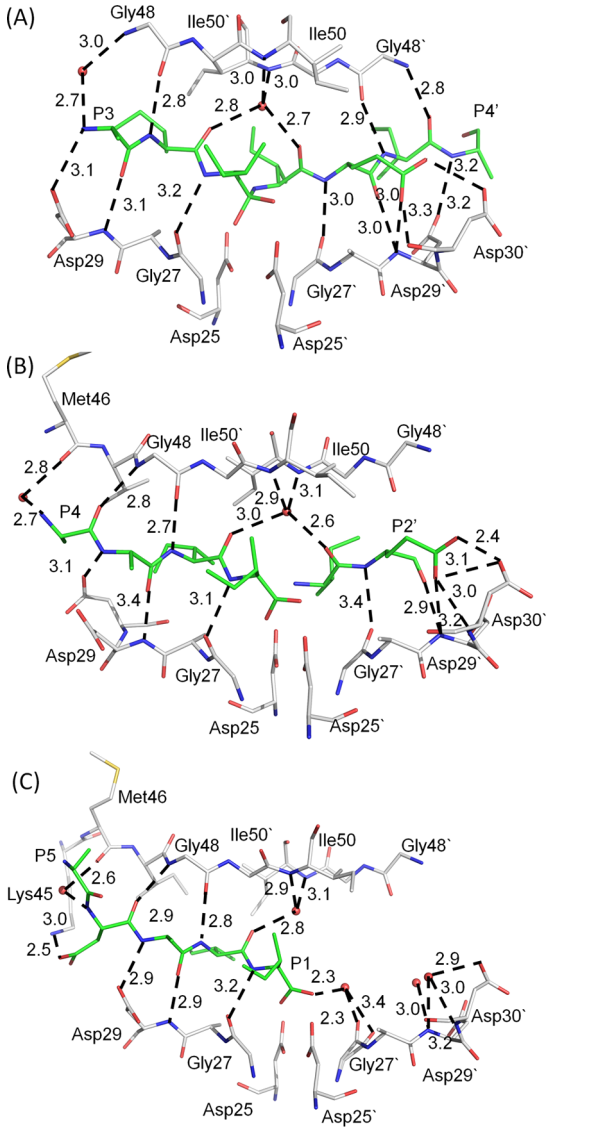


**Figure 4.** Hydrogen bond interactions: (A) the tetrahedral intermediate with $PR_{WT}$, (B) the two products with the $PR_{I47V}$ mutant, and (C) the single product with the $PR_{V32I}$ mutant. Only the major conformation is shown for the peptide intermediate or products in panels A and B. Hydrogen bond interactions are indicated by dashed lines with distances in angstroms. Interactions of Asp25 and -25′ have been omitted for the sake of clarity. PR is shown with gray carbons, and TI and product peptides are shown with green carbons. The water and carbonyl oxygen of Gly27′ in panel C were refined with a partial occupancy of 0.45.

**Interactions of $PR_{V32I}$ with the P5–P1 Product.** The $PR_{V32I}$ structure showed clear electron density for the single peptide product of residues P5–P1, except for the side chain of P5 (Figure 3D and Figure S1D of the Supporting Information). Individual water molecules were fit in the other subunit because no peptide product was visible in the electron density. The polar interactions of the single product with $PR_{V32I}$ are shown in Figure 4C. Two waters near the P2′ position formed


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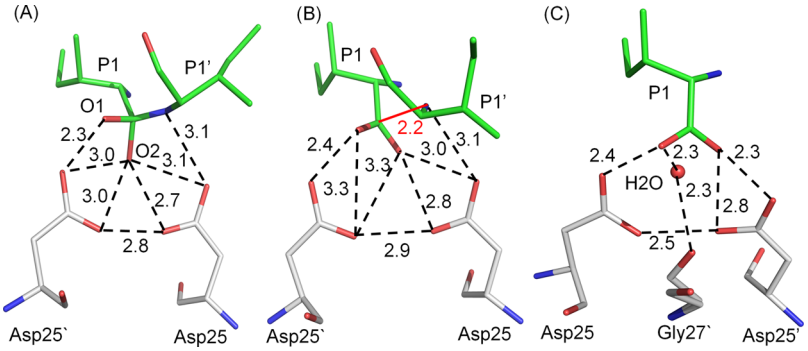


**Figure 5.** Hydrogen bond interactions with the catalytic residues Asp25 and -25′: (A) $PR_{WT}$−TI complex, (B) $PR_{I47V}$ with both products, and (C) $PR_{V32I}$ with a single product. Hydrogen bond interactions are indicated by dashed lines with distances in angstroms. The PR is colored gray, and TI and products are colored green. The short separation of 2.2 with distances in angstroms between the N- and C-terminal products is indicated by the red line in panel B. Only one conformation (occupancy of 0.5) of Gly27′ and interacting water is shown in panel C.

hydrogen bond interactions to the main chain of Asp29′ and main chain and side chain of Asp30′, resembling the interactions of the P2′ Glu in the other peptides. Another water molecule at an occupancy of 0.5 interacts with both alternate conformations of the carbonyl of Gly27′ and with the product carboxyl terminus. Hydrogen bond interactions connect the main chain of the product to $PR_{V32I}$ residues Gly27, Asp29, and Gly48, as observed in the other complexes. A new salt bridge was seen between the side chains of P4 Asp and Lys45, and the P4 main chain amide had a water-mediated interaction with the carbonyl oxygen of Met46. Hydrophobic interactions were also observed between the side chains of the product and side chains of $PR_{V32I}$.

**Interactions with Catalytic Residues.** The interactions with the catalytic residues are essentially identical in the major and minor conformations of the TI and two product peptides as shown by the superposition in Figure S3 of the Supporting Information. In the TI complex with $PR_{WT}$, the gem-diol structure lies between P1 Ile and P1′ Ile (Figure 5A). The O1 hydroxyl of P1 Ile forms a very short 2.3 Å hydrogen bond to the outer carboxylate Oδ2 atom of Asp25′, while the O2 hydroxyl interacted with all four carboxylate oxygens of Asp25 and -25′. The nitrogen of the gem-diol-amine moiety had a hydrogen bond interaction with Oδ2 of Asp25.

The $PR_{WT}$−TI interactions are comparable to those described in the 1.0 Å resolution crystal structure of endothiapepsin, a fungal aspartic proteinase, complexed with a gem-diol analogue.[8] In endothiapepsin, one short hydrogen bond interaction was seen between the O1 hydroxyl and Oδ2 of Asp35 with a distance of 2.5 Å; the O2 hydroxyl formed two hydrogen bond interactions with Oδ1 and Oδ2 of Asp219 with distances of 2.6 and 3.0 Å, respectively; and one hydrogen bond interaction with Oδ2 of Asp35. Unlike $PR_{WT}$, the hydrogen bond interaction between the amide nitrogen of the gem-diol analogue and the catalytic aspartate was absent in the endothiapepsin complex.

The inhibitor KNI-272 has been used as a transition state mimic in neutron diffraction studies to locate important hydrogen atoms at the catalytic site of HIV PR.[5] Although the hydroxymethylcarbonyl isostere of KNI-272 is not identical to the gem-diol of TI, the hydrogen bond interactions of O2 of KNI-272 are similar to those of O2 of P1 Ile with both catalytic aspartates. Also, O4 of KNI-272 resembles O1 of TI in forming a single hydrogen bond interaction to Oδ2 of Asp25, although the hydrogen bond is not particularly short.

The two product peptides bound in $PR_{I47V}$ are partially separated with a 2.2 Å distance between the P1′ amide nitrogen and the P1 carbonyl carbon (Figure 5B), whereas the standard C−N separation of 1.3−1.4 Å occurs in the TI complex. The 2.2 Å distance may indicate that there is still some bonding interaction present between the N atom of the amino product and the C atom of the carboxylate product. The N-terminus of P1′ Ile has one hydrogen bond interaction with Oδ2 of Asp25. One carboxylate oxygen of P1 Ile forms a short 2.4 Å hydrogen bond interaction with the outer Oδ2 atom of Asp25′, as seen for the tetrahedral intermediate. The other carboxylate oxygen of P1 has hydrogen bond interactions with the four aspartate carboxylate oxygens. This analysis suggests that the structure of $PR_{I47V}$ with two products represents the stage immediately after hydrolysis of the peptide bond.

In $PR_{V32I}$, a partial (0.5) occupancy water (Figure 5C) in the catalytic site forms hydrogen bond interactions linking the carboxylate oxygen of P1 and the alternate conformations of the carbonyl oxygen of Gly27′. One carboxylate oxygen of P1 forms a short hydrogen bond with Oδ2 of Asp25 with a distance of 2.4 Å, and the other carboxylate oxygen of the product forms two hydrogen bond interactions (2.3 and 2.8 Å) with Oδ1 and Oδ2 of Asp25′. These hydrogen bond interactions of the P1 carboxylate group are similar to those reported in other structures with synthetic peptide products.[9] This complex appears to represent the reaction step after release of the N-terminal product from the catalytic site. The alternate positions of the carbonyl oxygen of Gly27′ may reflect conformational changes occurring upon release of the N-terminal product, because this oxygen usually interacts with an amide of the bound substrate peptide, as shown in the $PR_{WT}$−TI complex and the $PR_{I47V}$ complex with two products.

**Implications for the Reaction Pathway.** Reaction intermediates have been described in a variety of enzyme crystal structures, as reviewed in ref 29. Several metastable intermediates can sometimes exist along a reaction path indicating the catalytic process proceeds through a series of energy barriers. In the case of HIV PR, a number of intermediates can be trapped by simply crystallizing the protein in the presence of a peptide substrate, which suggests that the energy barriers of the hydrolysis reaction may be of similar height. Thus, it is entirely possible that the reaction pathway of the peptide hydrolysis by PR does not include a single rate-limiting step. The three new crystal structures represent three consecutive steps in the proteolytic reaction of HIV PR and provide improved geometric details because of the near-atomic-


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resolution X-ray data. The majority of the interactions with the bound peptides are conserved in the wild-type enzyme and the mutants, in agreement with the report that mutants share similar transition states with wild-type PR.[30] The interactions observed in the new structures have been mapped on the scheme for the reaction (Figure 6). The hydrogen atoms

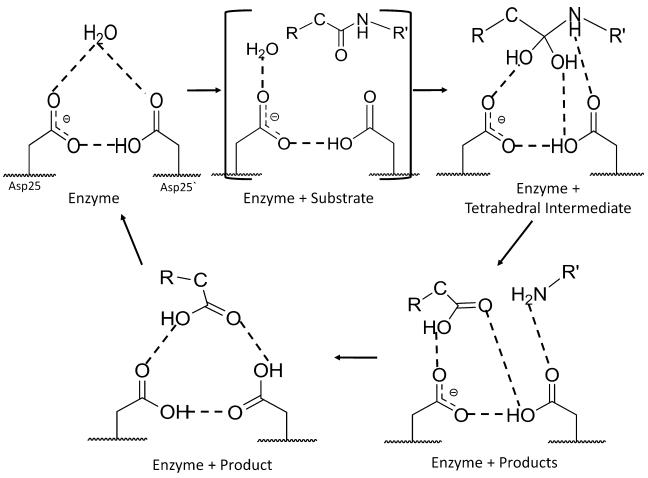


**Figure 6.** Scheme of the reaction pathway. The interactions of the peptide intermediates with the catalytic residues are illustrated on the basis of the new crystal structures. The minimal number of hydrogen atoms and hydrogen bonds is indicated based on the interaction distances in the crystal structures. Crystal structures have been described for four stages. Only the active enzyme with bound substrate has not been seen, as indicated by the large brackets.

around the catalytic sites cannot be identified in X-ray structures at this resolution; however, their locations were deduced from the short interactions. One Asp25 is protonated and the other deprotonated in most states, in agreement with neutron diffraction studies of an inhibitor complex.[5] In the absence of substrate, the active site cavity of PR generally contains water, or possibly a metal cation, interacting with the two catalytic aspartates.[31] This water reacts with the bound peptide to form the gem-diol intermediate, represented by the structure of the $PR_{WT}$–TI complex (Figure 5A). The tetrahedral intermediate dissociates into two products in the step shown in the $PR_{I47V}$ complex (Figure 5B). Then, the N-terminal product is released, in association with rotation of the carbonyl group of Gly27′, as shown in the $PR_{V32I}$ structure (Figure 5C). This state with a single product is shown with protonation of both Asp25 and -25′ to reflect the short interactions observed in the crystal structure, which suggests diprotonation in the simplest interpretation, as found in theoretical studies of PR with some inhibitors.[32] Finally, the C-terminal product is released, and the enzyme recycles to the first step. All three intermediate stages retain the short 2.3–2.4 Å hydrogen bond, which may be a low-barrier hydrogen bond, of the hydroxyl group of the peptide intermediate with one of the catalytic aspartates, as reported in lower-resolution crystal structures.[9−11] Moreover, the amide of the cleaved bond in the gem-diol intermediate and the freed amino terminus of the product also form hydrogen bond interactions with the carboxylate of Asp25.

The conserved catalytic Asp25-Thr26-Gly27 triplets are important for the activity and dimerization of PR. Previous X-ray and neutron diffraction studies have shown hydrogen bond interactions with the catalytic Asp25 and -25′ similar to those described in our new structures.[5,12] The position of hydrogen atoms, however, cannot be determined unambiguously in these X-ray structures, and further studies by neutron crystallography will be important in determining the protonation state of the catalytic residues in the reaction steps. Our structures suggest a new role for rotation of Gly27 to facilitate release of product as well as its known role in binding the main chain amides of peptides.[28] Analysis of these structures emphasizes the essential roles of residues 25–30 and flap residues 48–50 in binding peptide substrates and products and provides details of the geometry around the catalytic site at near-atomic resolution. The critical interactions can be targeted in the design of new antiviral inhibitors for resistant HIV.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Minor conformations of peptides and their comparison with major conformations. This material is available free of charge via the Internet at http://pubs.acs.org.

### Accession Codes

The structure coordinates and factors have been deposited in the Protein Data Bank as entries 4FL8 for $PR_{WT}$, 4FM6 for $PR_{V32I}$, and 4FLG for $PR_{I47V}$.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

HIV-1, human immunodeficiency virus type 1; $PR_{WT}$, wild-type HIV-1 protease; $PR_{V32I}$, protease with the V32I mutation; $PR_{I47V}$, protease with the I47V mutation; rmsd, root-mean-square deviation; TI, gem-diol-amine tetrahedral intermediate.

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