Analysis of HIV-1 CRF_01 A/E Protease Inhibitor Resistance: Structural Determinants for Maintaining Sensitivity and Developing Resistance to Atazanavir†

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ABSTRACT: A series of HIV-1 protease mutants has been designed in an effort to analyze the contribution to drug resistance provided by natural polymorphisms as well as therapy-selective (active and non-active site) mutations in the HIV-1 CRF_01 A/E (AE) protease when compared to that of the subtype B (B) protease. Kinetic analysis of these variants using chromogenic substrates showed differences in substrate specificity between pretherapy B and AE proteases. Inhibition analysis with ritonavir, indinavir, nelfinavir, amprenavir, saquinavir, lopinavir, and atazanavir revealed that the natural polymorphisms found in A/E can influence inhibitor resistance. It was also apparent that a high level of resistance in the A/E protease, as with B protease, is due to it aquiring a combination of active site and non-active site mutations. Structural analysis of atazanavir bound to a pretherapy B protease showed that the ability of atazanavir to maintain its binding affinity for variants containing some resistance mutations is due to its unique interactions with flap residues. This structure also explains why the I50L and I84V mutations are important in decreasing the binding affinity of atazanavir.

The genetic variability encountered in the human immunodeficiency virus (HIV) poses a major challenge for the world health care community. There are two main types of HIV, type 1 (HIV-1) and type 2 (HIV-2), with HIV-1 being the most prevalent in the worldwide pandemic. HIV-1 has been classified into three groups, M, N, and O. Viruses in group M are further subdivided into subtypes, sub-subtypes, and recombinant forms (CRFs), which are prevalent in specific geographical regions. The subtype B virus is common to the Americas, Europe, and Australia. Non-subtype B viruses dominate the growing epidemic in the developing world (World Health Organization).

The nucleotide diversity between protease subtypes and CRFs maps to all regions of the viral genome, including the genes encoding the therapeutic targets protease and reverse transcriptase. Many of these polymorphisms are manifested as amino acid mutations in the protease at positions that are common variants in subtype B viruses in response to protease inhibitor (PI)[1] therapy. The currently available therapeutic protease inhibitors were developed and tested only against the subtype B (B) protease. There are limited data confirming that different HIV-1 subtypes and CRFs are more or less susceptible to the various protease inhibitors (*1*). It would be expected that the worldwide use of protease inhibitors would impart further selective pressure driving the evolution of HIV-1. The residue polymorphisms in the protease have raised concerns that the therapy for non-B viruses may be less effective than that for subtype B viruses (*2*−*5*).

In this study, we aimed to analyze the effects on biochemical properties, specifically substrate specificity and inhibitor binding, of natural polymorphisms in the CRF_01 A/E (AE) protease and therapy-selected active and non-active site mutations when compared to B protease. We determined the Michaelis−Menten constants for three substrates and K_i values for seven available therapeutic PIs against a pretherapy B protease, a pretherapy isolated AE protease containing the natural V3I, I13V, E35D, M36I, S37N, R41K, H69K, and L89M polymorphisms, and the post-therapy AE (AE-P)

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[1] Abbreviations: HAART, highly active antiretroviral therapy; IPTG, isopropyl thio-D-galactopyranoside; A_{600}, absorbance at 600 nm; dTT, dithiothreitol; Nph, *p*-NO_2-L-phenylalanine; nL, L-norleucine; PI, protease inhibitor; RTV, ritonavir; IDV, indinavir; NFV, nelfinavir; SQV, saquinavir; APV, amprenavir; LPV, lopinavir; ATV, atazanavir; H-bonds, hydrogen bonds; B, pretherapy HIV-1 subtype B protease; AE, pretherapy HIV-1 CRF_01 A/E protease; B^{V82F}, B protease with the V82F active site mutation; AE^{V82F}, AE protease with the V82F active site mutation; AE-P, post-therapy HIV-1 CRF_01 A/E protease; AE-P^{F82V}, AE-P protease with the F82V back mutation; Wat, water.

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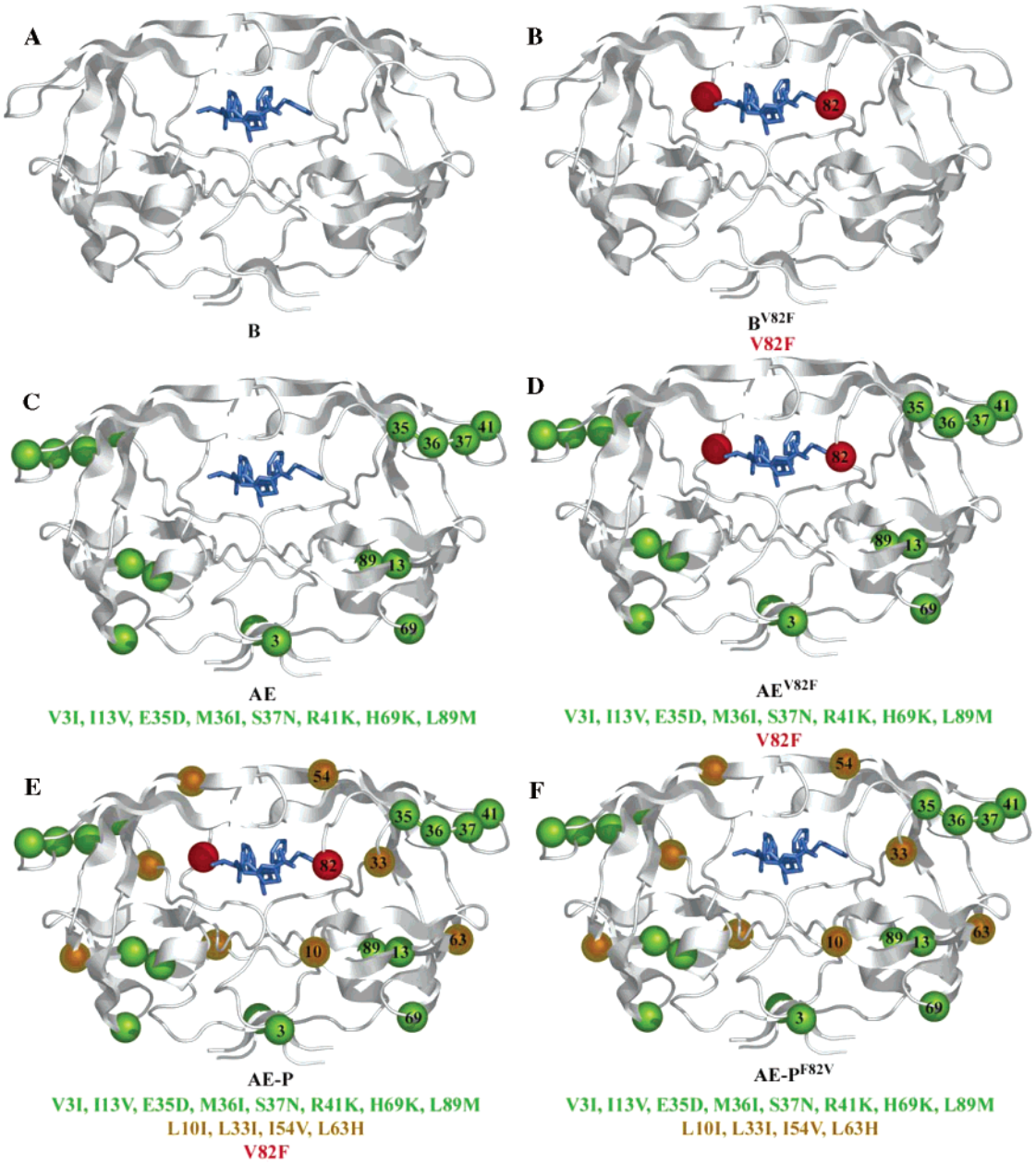

FIGURE 1: Ribbon diagrams of HIV-1 proteases: (A) pretherapy HIV-1 subtype B (B) protease, (B) B protease with the V82F active site mutation (B^{V82F}), (C) pretherapy HIV-1 CRF_01 A/E (AE) protease, (D) AE protease with the V82F active site mutation (AE^{V82F}), (E) post-therapy A/E protease (AE-P), and (F) AE-P protease with the F82V back mutation (AE-P^{F82V}). The red sphere marks the V82F active site mutation. The green spheres mark the natural polymorphisms found in AE protease when compared to the B protease. The brown spheres mark the therapy-acquired non-active site mutations in the AE-P protease.

protease isolated from a patient treated with saquinavir (SQV), ritonavir (RTV), and indinavir (IDV) PI therapy (Figure 1) (*6*).

To determine the effects of the active site V82F mutation and the natural polymorphisms in AE protease on substrate specificity and inhibitor susceptibility, the V82F mutation was added to B (B^{V82F}) and AE (AE^{V82F}) proteases. To assess the effects of the therapy-selected non-active site mutations, the V82F mutation in AE-P was back mutated to pretherapy (AE-P^{F82V}) protease. During the course of our study, it was evident that atazanavir (ATV) was able to maintain its binding affinity for all mutants. To understand the mechanism utilized by ATV to maintain its binding affinity for resistant proteases, we have determined the X-ray crystal structure of ATV bound to the pretherapy B protease.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of Protease. A complete description of the cloning, expression, and purification procedures can be found in Ido et al. (*7*) and Goodenow et al. (*8*). In brief, the HIV-1 protease DNA for all the HIV B and AE variants was subcloned into the pET23a expression vector (Novagen) (*9*). The construct was transformed into *Escherichia coli* strain BL21 Star DE3 PlysS from Invitrogen. The introduction of all mutations onto the B and AE background was done using the QuikChange mutagenesis kit from Stratagene. Protease expression in bacteria was initiated when the OD_{600} reached 0.6 by addition of 1 mM IPTG to a culture grown at 37 °C in M9 medium (6.8 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, and 1 g of NH_4SO_4); 5 g of casamino acids was autoclaved together in 987

mL of H_2O, and then 1 mL of 0.1 M $CaCl_2$, 2 mL of 1.0 M $MgSO_4$, 10 mL of 20% glucose, and 50 μg/L ampicillin were added. After 3 h, cells were harvested by centrifugation at 16000*g* for 5 min and resuspended in TN buffer [0.05 M Tris, 0.15 M NaCl, and 0.001 M $MgCl_2$ (pH 7.4)]. Inclusion bodies containing the protease were isolated by centrifugation through a 27% sucrose cushion. The inclusion bodies were solubilized in 8 M urea, and the protease was refolded by dialysis against 0.05 M sodium phosphate buffer [0.05 M Na_2HPO_4, 0.005 M EDTA, 0.3 M NaCl, and 0.001 M dTT (pH 7.3)]. The protease was purified through ammonium sulfate precipitation and gel filtration chromatography using a Superdex 75 16/60 column from Amersham Pharmacia attached to an FPLC LCC 500 Plus system, also from Pharmacia. The protease was eluted using potassium phosphate buffer [50 mM K_2HPO_4, 2 mM EDTA, 150 mM NaCl, 2 mM dTT, 5% glycerol, and 5% 2-propanol (pH 7.3)].

Protease Activity and Inhibitor Constants. The Michaelis−Menten constants k_{cat}, K_m, and k_{cat}/K_m and K_i values were determined for each variant as previously described (*10*). The chromogenic substrates K-A-R-V-*L**Nph-E-A-nL-G (S1), K-A-R-V-*nL**Nph-E-A-nL-G (S2), and K-A-R-V-*F**Nph-E-A-nL-G (S3), which mimic the CA/p2 cleavage site, were used to determine the catalytic activity of each variant at 37 °C in sodium acetate buffer [0.05 M NaOAc, 0.15 M NaCl, 0.002 M EDTA, and 0.001 M DTT (pH 4.7)]. The P1 residue was substituted with leucine (Leu), norleucine (nLeu), and phenylalanine (Phe). These three residues were analyzed because they are commonly found in the P1 position of the Gag-Pol cleavage sites. Norleucine is used to mimic the volume occupied and hydrophobicity of methionine (Met) while preventing any problems due to oxidation of the sulfur. K_i values for all inhibitors were measured under the same conditions. Cleavage of the substrate was monitored using a Hewlett-Packard 8452A spectrophotometer equipped with a seven-cell sample handling system as described by Dunn et al. (*11*). The inhibition constants K_i were determined by monitoring the inhibition of hydrolysis of the chromogenic substrate as described by Bhatt et al. (*12*).

Relative Vitality. To better describe the effect resistance mutations have on virus viability in the presence of a specific inhibitor, Gulnik et al. (*13*) introduced the vitality parameter that is described by the following equation:

$$\text{vitality} = \frac{(K_i \times k_{cat}/K_m)_{mutant}}{(K_i \times k_{cat}/K_m)_{wild-type}}$$

Because some second-generation inhibitors have low picomolar binding constants, a 10-fold drop in affinity will still leave an inhibitor with a sub-nanomolar binding constant. This inhibitor would still be able to arrest viral maturation since it would be able to outcompete the substrate. To better describe the effects of these inhibitors, Velazquez-Campoy (*1*) introduced a modified vitality value, which is normalized to a reference inhibitor, termed relative vitality described by the following equation:

$$\text{relative vitality} = \frac{(K_i \times k_{cat}/K_m)_{mutant}}{[(K_i)_{reference\ inhibitor} \times k_{cat}/K_m]_{wild-type}}$$

Crystallization of a Protein−Inhibitor Complex. After protease B was purified, the enzyme purification buffer was exchanged for 50 mM sodium acetate, 1 mM EDTA, and 1 mM DTT at pH 4.7 during concentration to 2 mg/mL. Atazanavir was dissolved at a concentration of 20 mM in 100% DMSO and mixed with B protease in a molar ratio of 3:1. The inhibitor and enzyme were allowed to equilibrate for 1 h at 4 °C, after which precipitated material was removed by centrifugation at 10000*g* at 4 °C. The enzyme−inhibitor complex was mixed with reservoir solution (Hampton Research Cryo Crystallization Kit 18) in a 1:1 (v/v) ratio to set up 4 μL hanging drops at 25 °C. Rod-shaped crystals grew in 2 days.

Table 1: Michaelis−Menten Constants

variant	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
	K-A-R-V-L*nF-E-A-nL-G (S1)		
B	18 ± 2	20 ± 1	1.2 ± 0.2
B^{V82F}	18 ± 2	7.3 ± 0.8	0.40 ± 0.05
AE	15 ± 2	8.2 ± 0.5	0.54 ± 0.07
AE^{V82F}	62 ± 8	11 ± 1	0.17 ± 0.02
AE-P	29 ± 3	11 ± 1	0.38 ± 0.04
$AE\text{-}P^{F82V}$	20 ± 3	8.9 ± 0.7	0.44 ± 0.07
	K-A-R-V-nL*nF-E-A-nL-G (S2)		
B	10 ± 1	9.0 ± 0.6	0.9 ± 0.1
B^{V82F}	29 ± 5	9 ± 1	0.31 ± 0.05
AE	15 ± 3	16 ± 1	1.1 ± 0.2
AE^{V82F}	32 ± 3	6.7 ± 0.6	0.21 ± 0.02
AE-P	31 ± 3	10 ± 1	0.32 ± 0.03
$AE\text{-}P^{F82V}$	67 ± 7	16 ± 1	0.24 ± 0.03
	K-A-R-V-F*nF-E-A-nL-G (S3)		
B	6.2 ± 0.6	9.0 ± 0.3	1.5 ± 0.1
B^{V82F}	17 ± 2	6.4 ± 0.7	0.38 ± 0.04
AE	9 ± 1	15 ± 1	1.7 ± 0.2
AE^{V82F}	41 ± 3	14 ± 1	0.33 ± 0.02
AE-P	12 ± 2	13 ± 1	1.0 ± 0.2
$AE\text{-}P^{F82V}$	20 ± 3	11 ± 1	0.5 ± 0.1

Data Collection, Structure Determination, and Refinement. X-ray diffraction images were collected at Brookhaven National Laboratory beamline X29 on an ADSC quantum 315 CCD detector. The crystal was dipped in cryoprotectant solution (30% glycerol in reservoir solution) prior to data collection at 100 K. A complete data set, from 65 images taken with 1.0° oscillation steps with 5 s exposures, was collected from a single crystal. The data were indexed, scaled, and reduced using DENZO and SCALEPACK (*14*). The complex crystallized in the $P6_1$ space group with the following unit cell dimensions: $a = 62.4$ Å and $c = 82.6$ Å.

Initial phases were calculated using the coordinates of B protease (Protein Data Bank entry 1SGU) after removal of the inhibitor and solvent molecule to prevent model phase bias. Standard methods of structure refinement were then employed using programs in the CNS suite (*15*). Electron density maps with $2F_o - F_c$ and $F_o - F_c$ coefficients were used to guide manual fitting of the protease and bound inhibitor followed by real space refinement using the molecular graphics program WinCoot (*16*). The inhibitor and water molecules were added into $F_o - F_c$ density at 3σ. During model building and refinement, 5% of the data was reserved for cross validation of the refinement progress. The atomic coordinates have been deposited in the Protein Data Bank (entry 2AQU).

RESULTS AND DISCUSSION

The aim of this study was to analyze the contributions to catalytic efficiency and inhibitor resistance by natural

Table 2: K_i Values (nanomolar)[a]

	RTV	IDV	NFV	APV	SQV	LPV	ATV
B	0.7 ± 0.1	3.1 ± 0.1	1.2 ± 0.2	0.17 ± 0.01	1.3 ± 0.3	0.05 ± 0.02	0.48 ± 0.06
B^{V82F}	4 ± 1 (6)	12 ± 1 (4)	1.2 ± 0.3 (1)	2.6 ± 0.4 (15)	0.52 ± 0.13 (−3)	0.2 ± 0.05 (5)	0.56 ± 0.03 (1)
AE	0.2 ± 0.1 (−4)	6.9 ± 0.3 (2)	2 ± 0.3 (2)	0.4 ± 0.1 (2)	1.8 ± 0.3 (0)	0.019 ± 0.004 (−2)	0.24 ± 0.07 (−2)
AE^{V82F}	3.1 ± 0.6 (4)	108 ± 12 (35)	3.7 ± 0.5 (3)	1.2 ± 0.2 (7)	0.43 ± 0.07 (−3)	0.48 ± 0.1 (12)	0.57 ± 0.19 (1)
AE-P	32 ± 3 (46)	1616 ± 119 (521)	146 ± 12 (122)	23 ± 3 (135)	24 ± 3 (18)	9 ± 1 (180)	0.7 ± 0.2 (1)
AE-P^{F82V}	1.2 ± 0.4 (2)	42 ± 6 (14)	17 ± 3 (14)	1.4 ± 0.4 (8)	48 ± 8 (38)	0.5 ± 0.1 (10)	1.1 ± 0.3 (2)

[a] The *x*-fold changes in K_i from that of B are shown in parentheses.

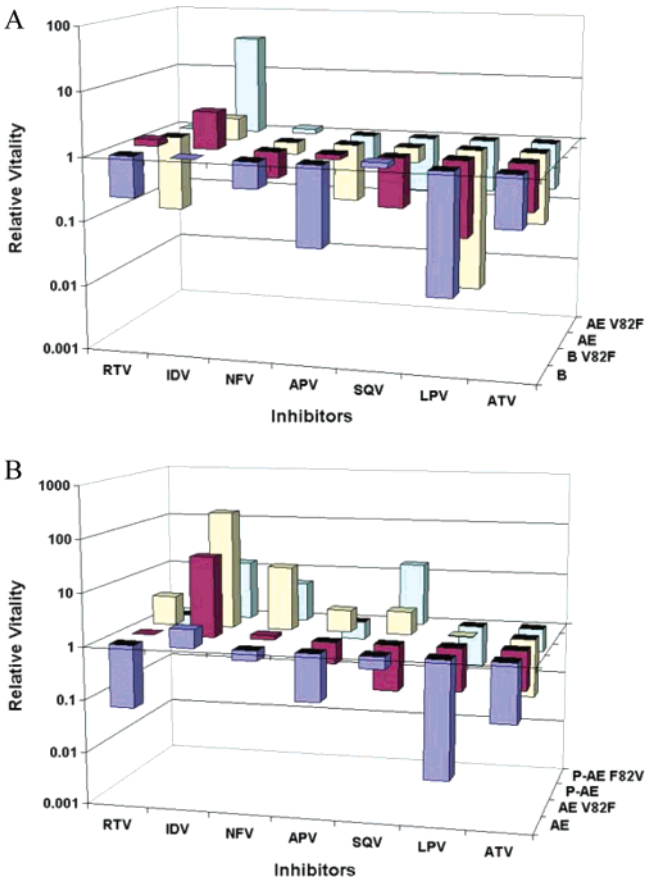

FIGURE 2: (A) Relative vitality values for B, B^{V82F}, AE, and AE^{V82F} using B and the K_i of IDV as a reference. (B) Relative vitality values for AE, AE^{V82F}, AE-P, and AE-P^{F82V} using AE and the K_i of IDV as a reference.

background polymorphisms and therapy-selected, active and non-active site, residue changes in AE compared to B protease (Figure 1). Though the natural polymorphisms found in AE when compared to the B protease have arisen in the absence of PI therapy, residue changes in AE at positions 35−37, 41, 69, and 89 have been associated with PI resistance in vivo and in vitro in B proteases (*17*−*20*). Thus, these polymorphisms can potentially influence substrate processing and the binding of currently available PIs and/or facilitate the development of resistance.

Michaelis−*Menten Constants*. The Michaelis−Menten constants were determined for three substrates with each variant to assess the effects of natural polymorphisms and drug-selected mutations on substrate specificity and are listed in Table 1. Of the substrates that were tested, the B and AE proteases preferred phenylalanine at the P1 position (S3 substrate). Phenylalanine is the most common residue found at the P1 position, occurring in 40% of cleavage sites in GagPol. Methionine and leucine occur in 10 and 20% of

Table 3: X-ray Data Collection and Refinement Statistics

wavelength (Å)	1.1
resolution range (Å)	20−2.0
space group	$P6_1$
unit cell parameters *a*, *c* (Å)	62.4, 82.6
no. of reflections	49001
no. of unique reflections	12351
overall completeness (%)	99.8 (100)
average I/σ	8.1
R_{sym}[a] (%)	8.4 (47.2)
refinement statistics	
R_{work}[b] (%)	22.7 (29.6)
R_{free}[c] (%)	23.8 (30.2)
rmsd for bond lengths (Å)	0.02
rmsd for bond angles (deg)	2.3
average *B*-factor (Å^2)	
Wilson plot	35.3
protein main/side chains	29.0/32.7
inhibitor atoms	28.3
water molecules	37.1
Ramachandran plot quality	
most favored (%)	94.9
additionally allowed (%)	5.1
generously allowed (%)	0
disallowed (%)	0

[a] $R_{sym} = \sum_{i(hkl)}|I(hkl) - \langle I_i(hkl)\rangle|_{i,hkl}/I_i(hkl) \times 100$, where $I_i(hkl)$ is the *i*th observation of the intensity of a reflection with indices *h*, *k*, and *l* and $\langle I(hkl)\rangle$ is the average intensity of all symmetry equivalent measurements of that reflection. [b] $R_{work} = \sum_{i(hkl)}|F_{obs(h)} - F_{calc(h)}|_{hkl}|F_{obs(h)}| \times 100$, where $F_{obs(h)}$ and $F_{calc(h)}$ are the observed and calculated structure factor amplitudes, respectively. [c] $R_{free} = \sum_{i(hkl)}|F_{obs} - F_{calc}|_{hkl}|F_{obs}| \times 100$. R_{free} is calculated using 5% of data excluded during refinement process. Statistics for the highest-resolution shell are given in parentheses.

cleavage sites, respectively. Though there is a preference by both proteases for phenylalanine, the differences seen in the Michaelis−Menten constants for all substrates suggest that the natural polymorphisms in AE can influence active site specificity and/or catalytic efficiency. In contrast to the data reported earlier for subtype C (C) and A (A) proteases, the AE protease does not show any clear catalytic advantage over B protease (*21*).

Additional evidence for the effects of the natural polymorphisms and active and non-active site therapy-selected mutations affecting substrate specificity can be seen when comparing the kinetic values for the variants B^{V82F}, AE^{V82F}, AE-P, and AE-P^{F82V} (Table 1). These variants exhibited a decrease in k_{cat}/K_m for all substrates when compared to those of the pretherapy proteases. A clear example of the effects on substrate binding and catalytic efficiency by the natural polymorphisms in AE can be seen when comparing the Michaelis−Menten parameters for the S3 substrate and the AE^{V82F} and B^{V82F} variants. Both proteases exhibited similar k_{cat}/K_m values for S3 (for AE^{V82F}, $k_{cat}/K_m = 0.33$; for B^{V82F}, $k_{cat}/K_m = 0.38$). While AE^{V82F} was able to maintain a k_{cat} value (14 s^{-1}) similar to that of AE (15 s^{-1}), the B^{V82F} protease exhibited a decrease in k_{cat} of 30% (from 9.0 to 6.4

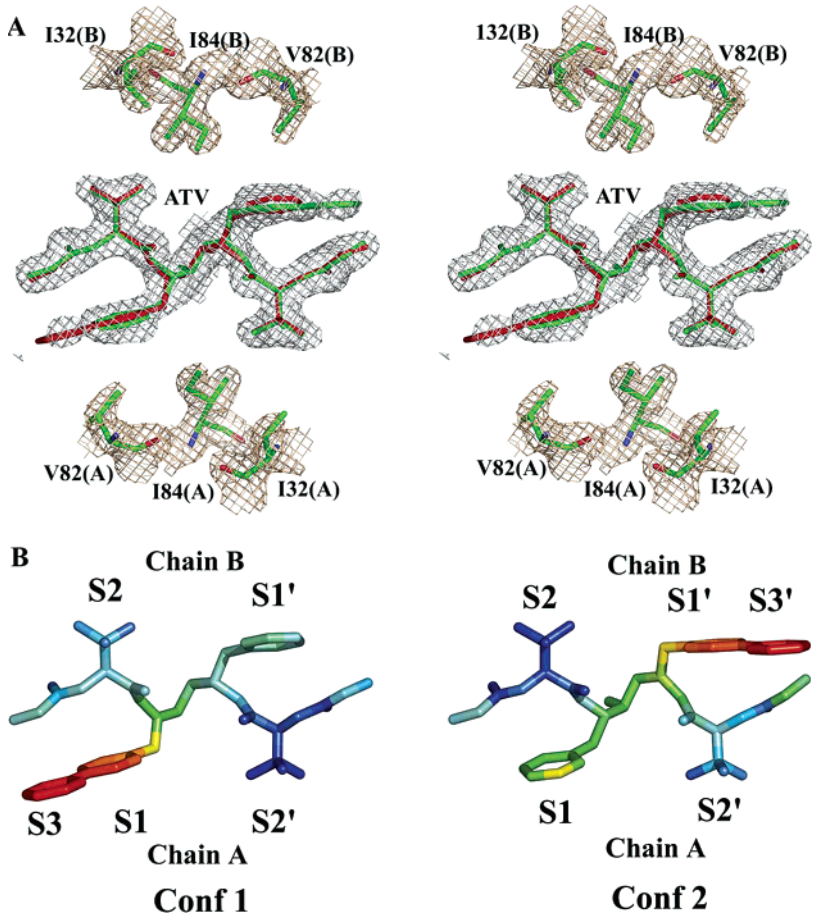

FIGURE 3: (A) Stereoview of ATV in the active site. Conformation 1 (Conf1) (red) and conformation 2 (Conf2) (green) are shown as sticks. Val32(A), Val82(A), and Ile84(A) in chain A and Val32(B), Val82(B), and Ile84(B) in chain B are shown as green sticks. The $2F_o - F_c$ electron density is drawn as a beige mesh at the 1σ level. (B) Stick drawing of ATV Conf1 and Conf2 colored by *B*-values with red the highest and blue the lowest.

s^{-1}) compared to that of B. Similar differences between the parameters contributing to k_{cat}/K_m were seen for all the substrates with B^{V82F} and AE^{V82F}.

It is also apparent that the natural polymorphisms in AE alone do not provide an advantage for substrate catalysis in combination with the primary active site mutation V82F. Comparing the AE, AE^{V82F}, AE-P, and AE-P^{F82V} proteases revealed that addition of the therapy-selected non-active site mutations (for AE-P, $k_{cat}/K_m = 1.0\ \mu M^{-1}\ s^{-1}$) improved the catalytic efficiency when compared to that of AE^{V82F} ($k_{cat}/K_m = 0.33\ \mu M^{-1}\ s^{-1}$), but removal of the active site mutation (for AE-P^{F82V}, $k_{cat}/K_m = 0.5\ \mu M^{-1}\ s^{-1}$) did not restore wild-type activity (for AE, $k_{cat}/K_m = 1.7\ \mu M^{-1}\ s^{-1}$). These data clearly displayed cooperativity between the active site and post-therapy non-active site mutations in improving or maintaining catalytic efficiency. As shown by other studies on B proteases, these cooperative effects between active site and non-active site mutations to improve catalytic efficiency are clearly not independent (*10*, *22*, *23*).

Inhibitor Dissociation Constants. The K_i values for all variants against seven clinically used inhibitors are listed in Table 2, and all fold changes are a comparison to the B protease. Fold changes to other proteases are specifically stated.

Velazquez-Campoy et al. (2003) showed that C and A proteases were 2−7-fold less susceptible to inhibition by clinically used inhibitors than B protease (*24*). The AE protease exhibited an increase in K_i of no greater than 2-fold compared to those of any of the inhibitors tested (Table 2). The effect of the V82F mutation on inhibitor binding on the wild-type background of B and AE varies from none to significant (>10-fold) and varies between subtypes. B^{V82F} exhibited a 15-fold increase in K_i for APV, while AE^{V82F} exhibited 12- and 35-fold increases in K_i for lopinavir (LPV) and IDV, respectively. The decrease in the sensitivity of AE^{V82F} to IDV and LPV and B^{V82F} to amprenavir (APV) inhibition compared to that of B predicts a predisposition to developing resistance to these three inhibitors by the AE or B proteases. Although LPV, IDV, and APV exhibited a decrease in activity against AE^{V82F} or B^{V82F}, it is not a clear marker of resistance, since LPV and APV maintained a binding strength sufficient for effective inhibition of the protease when using IDV against the pretherapy proteases as a gauge. To better determine the viability of viruses containing these mutations, we analyzed the variants using relative vitality as described in Experimental Procedures. The results for the S1 substrate are shown in panels A and B of Figure 2. The results for S2 and S3 mimic that of S1; therefore, they have been excluded. When using relative vitality values as a comparison, only IDV is predicted to have a decreased potency against a virus carrying the AE^{V82F} variant when compared to AE and B (Figure 2A). These data predict that the natural polymorphisms found in the AE protease can select for faster development of IDV resistance upon the acquisition of the V82F mutation.

The post-therapy AE-P protease exhibited an up to ~500-fold increase in K_i for the inhibitors tested. Analysis of relative vitality values for these inhibitors with AE-P also predicts a large degree of cross resistance (Figure 2B). Atazanavir showed no decrease in potency, though the AE-P protease contains mutations at five positions (10, 33, 36, 54, and 82) associated with resistance to this inhibitor (*25*). It is of interest that ATV has remained highly potent against an enzyme that exhibited an ~20−500-fold decrease in binding affinity for the other six inhibitors tested, and this will be discussed further below.

Back mutating the active site V82F mutation in AE-P (AE-P^{F82V}) removed a significant level of cross resistance to the inhibitors RTV, IDV, nelfinavir (NFV), APV, and LPV when compared to that of AE-P (Table 2). The AE-P^{F82V} and AE^{V82F} proteases exhibit similar susceptibility to RTV, APV, and LPV. The AE-P^{F82V} variant exhibited a 5- and 100-fold greater susceptibility to NFV and SQV, respectively, than did AE^{V82F}. AE^{V82F} exhibited a 3-fold greater susceptibility to IDV than did AE-P^{F82V}. As with substrate binding and processing, it is evident that both active and non-active site mutations have the ability to alter the binding of inhibitors to the active site. It is apparent that independently active or non-active site mutations (AE^{V82F} and AE-P^{F82V}) do not provide for a high level of cross resistance when compared to the AE-P protease. As seen with B proteases, comparison of the three variants, AE-P, AE-P^{F82V}, and AE^{V82F}, shows cooperativity between therapy-selected active and non-active site mutations, leading to a high level of resistance and cross resistance (*10*, *22*, *23*, *26*, *27*). It is also apparent that the cooperative contributions to resistance by active and non-active site mutations are not independent and are not additive but multiplicative.

Structural Analysis of ATV Binding. To analyze the possible interactions that permit ATV to be the only PI that is able to maintain its binding affinity for all proteases that have been studied (Table 2), the crystal structure of ATV bound to the B protease (B−ATV) was determined. Attempts to crystallize the AE variants were unsuccessful. The B−ATV complex X-ray crystallographic data and refinement statistics are given in Table 3. Density for two binding modes of the inhibitor was observed in the active site of the protease (Figure 3A). This is indicative of a 2-fold (180°) bimodal binding of the inhibitor and has previously been observed for other inhibitors (*28*). Weak density was observed for the pyridine ring occupying the S3 or S3′ pockets in the two conformations (Conf1 and Conf2) of the inhibitor, which is indicative of disorder in this region consistent with the high *B*-factors for the pyridine atoms (Figure 3B). Both inhibitor conformations displayed an average *B*-factor of 28 Å^2.

Hydrophobic interactions between ATV and the protease were analyzed using Ligplot (Figure 4) (*29*). Due to the fact that the HIV-1 protease is a homodimer, the naming of chains A and B is arbitrary for each monomer in the case where two inhibitor conformations are seen. In this study, we have assigned enzyme subsites S3, S1, and S2′ to be formed by the residues in chain A (Figure 4B) and subsites S2, S1′, and S3′ to be formed by residues in chain B. Given this structural assignment, the inhibitor group 4-(pyridin-2-yl)-phenyl occupies the S1 and S3 pockets in Conf1 and the S1′ and S3′ pockets in Conf2. Ile47 was found in two

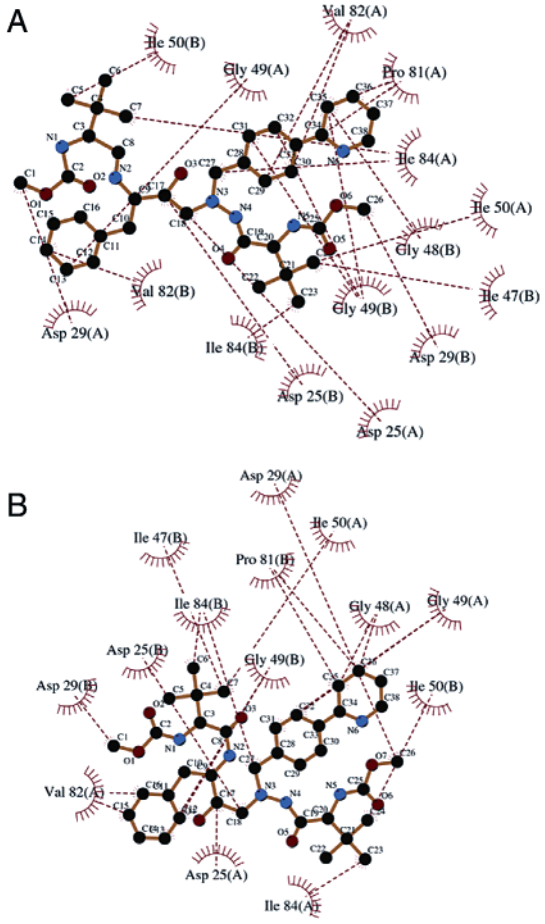

FIGURE 4: Ligplot two-dimensional representation of hydrophobic interactions of ATV with active site residues for ATV in (A) Conf1 and (B) Conf2. Hydrophobic interactions with residues in the first monomer [e.g., Gly49(A)] and the second monomer [e.g., Ile50-(B)] are shown as red dashed lines.

conformations. Only the conformation of Ile47 with atoms within 4 Å of the inhibitor was analyzed.

None of the proteases that were analyzed contain the I50L or I84V mutation, each of which is associated with a high level of ATV resistance in vivo and in vitro (*30*−*32*). Ile50 forms various interactions to lock down the flaps, and mutations at this residue may function to destabilize the protease closed conformation (*23*). Ile84 is located in the center of the active site, and we previously suggested that the I84V mutation may function to destabilize the interactions of the core of the inhibitor and its interactions with the flaps (*23*). Previous studies have also shown the importance of mutations in the flaps in providing a high level of resistance (*10*, *22*, *23*, *26*), and that the addition of non-active site mutations may decrease binding affinity by destabilizing the flaps in the bound conformation (*23*). Recent studies by Yanchunas and colleagues, utilizing thermodynamic and modeling techniques, have suggested the importance of interactions of ATV P2 and P2′ groups with Ile50 in maintaining binding affinity (*33*). We can see from the analysis of *B*-factor values of the bound ATV that the most stable regions are the P2 and P2′ *N*-methoxycarbonyl-L-*tert*-leucine groups (Figure 4B). The P2 and P2′ groups make hydrophobic contacts with Ile47and Ileu50 (flaps) and Ile84 (active site). Knowing the correlation between drug resistance and flap function, we find that the strong interactions that ATV P2 and P2′ groups make with the flap residues may

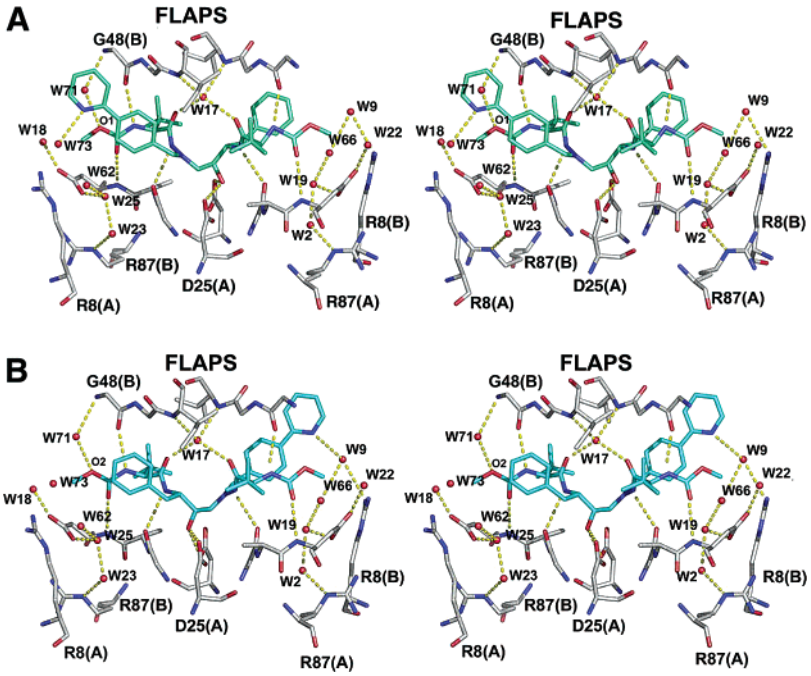

FIGURE 5: Stereoview of hydrogen bonding interactions of ATV for (A) Conf1 (green) and (B) Conf2 (aqua). Flap and active site residues are drawn as CPK sticks. Key residues described in the text are labeled. Hydrogen bonds are depicted with yellow dashes. Water molecules are drawn as red spheres.

provide for its ability to bind to resistant proteases containing numerous mutations. Given the key resistance mutations found in response to ATV, it appears that resistance to ATV is associated with weakening of the interactions of the *N*-methoxycarbonyl-L-*tert*-leucine group and destabilizing the flap interactions.

Atazanavir makes a total of 11 (Conf1) and 12 (Conf2) hydrogen bonds with the enzyme. Two of these H-bonds are with flap residue Gly48 (Figure 5A,B). The G48V mutation is found in proteases isolated from therapy-experienced patients after treatment with ATV. The side chain of Val48 points away from the inhibitor and does not make contact with ATV (*34*, *35*). No other residue with which ATV makes a direct H-bond is associated with resistance to any clinical inhibitor. Hong et al. showed that mutations at position 48 provide inhibitor resistance by decreasing the flexibility of the flaps. In the case of ATV, the G48V mutation may function to destabilize the H-bonds and cooperatively increase resistance with additional mutations. This further supports the prediction that the destabilization of the interactions of the flaps and ATV is important in developing resistance.

When compared to previously reported wild type−inhibitor complex structures of the HIV-1 protease, ATV is unique in that it binds in two conformations with each conformation making unique H-bonding and hydrophobic interactions (*28*, *36*−*39*). Analysis of the structure showed a substantial amount of hydrophobic and H-bonding interactions; thus, it is apparent why ATV binds with a highly favorable enthalpy (*33*, *40*). A large part of the binding affinity of ATV for the wild-type protease comes from the large number of H-bonds it makes with the enzyme. The two inhibitor conformations make equivalent direct H-bonds involving backbone atoms but differ in their water-mediated H-bonds (Figure 5A,B). The Conf1 and Conf2 inhibitors form three and four water-mediated H-bonds, respectively, bridging the enzyme and the inhibitor. The H-bond between the N6 atom of the 4-(pyridin-2-yl)phenyl group and Wat73 in Conf1 is mediated by Wat9 in Conf2. Wat9 is also within hydrogen bonding distance of NH1 of Arg8(B). Additional differences in H-bonding between the two inhibitor conformations can be seen in the ability of Wat9 to H-bond to Wat66, forming a H-bonding water chain involving two additional waters and ending at the NE atom of Arg87(A).

The disorder in Ile47(B) coincides with the Wat71-mediated H-bonding between O1 and O2 of the methoxycarbonyl group in Conf1 and Conf2, respectively, and Gly48(B) (Figure 5A,B). Given the symmetry of ATV, it is surprising that this H-bonding interaction is not observed with Gly48(A) at the other end of the inhibitor. It is difficult to determine if the H-bonding interaction mediated by Wat71 with Gly48(B) is in both inhibitor conformations or in only one. If it is found in both, then the interactions are distinctly different. In Conf1, the H-bond involves the methoxycarbonyl-L-*tert*-leucine opposite the 4-(pyridin-2-yl)phenyl group, while in Conf2, it is opposite the phenyl group. Given that juxtaposed groups will influence each other's interaction with the enzyme, the disorder of Ile47(B) may be due to differences in its interactions with each inhibitor conformation. It is possible that this water-mediated H-bonding interaction is only possible in one inhibitor conformation (*41*). If this is so, then the interactions of Conf1 and Conf2 with the enzyme can also be said to be different.

As with hydrogen bonding interactions, the hydrophobic interactions are unique between the two inhibitors. Atazanavir in Conf1 and Conf2 makes a total of 27 (14 with chain A and 13 with chain B) and 28 (14 with chain A and 14 with chain B) hydrophobic contacts, respectively (Figure 4A). The two inhibitor conformations interact with similar residues but in the opposite chain. The main difference is in the interactions of the 4-(pyridin-2-yl)phenyl group. The 4-(pyridin-2-yl)phenyl group of Conf1 make hydrophobic contacts

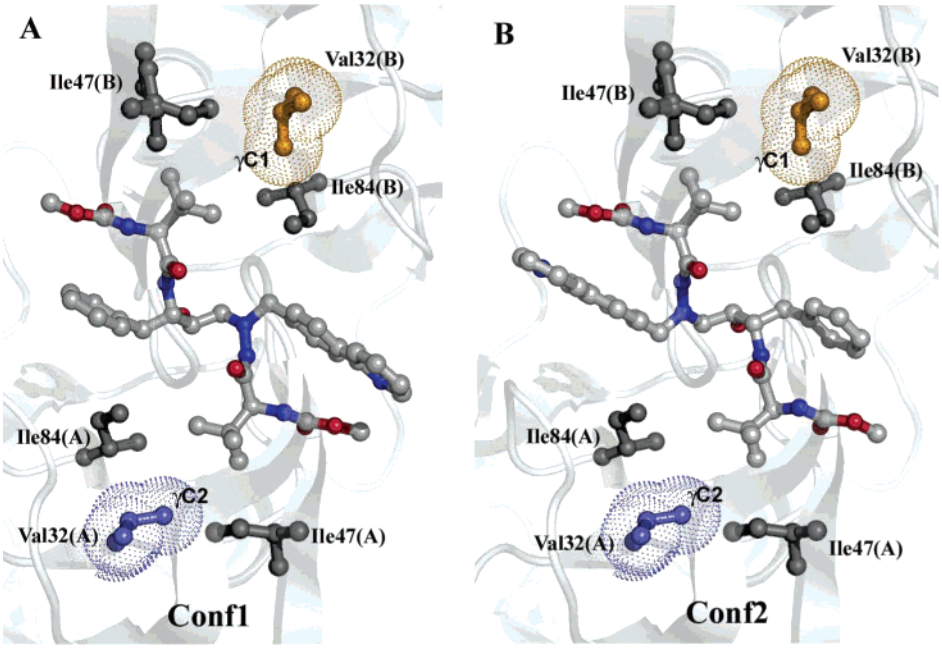

FIGURE 6: Interactions of residues Val32, Ile47, and Ile84 in inhibitor Conf1 (A) and Conf2 (B). In both panels, Ile84 and Ile47 (gray), Ile32(A) (blue), Val32(B) (orange), and inhibitor are modeled as ball-and-stick diagrams.

with Pro81(A), Val82(A), Ile84(A), Gly48(B), and Gly49-(B) for a total of nine hydrophobic contacts. The 4-(pyridin-2-yl)phenyl group of Conf2 makes hydrophobic contacts with Gly48(A), Gly49(A), and Pro81(B), for a total of five hydrophobic contacts. Unlike Conf1, this side chain group in Conf2 does not make contact with residues Val82(B) and Val84(B).

Analysis of residue Val32 interactions revealed additional flexibility in the binding of ATV. Residues Val32(A) (S2 pocket) and Val32(B) (S2′ pocket) in the active site occupy different conformations due to a 180° rotation around the Cα−Cβ bond (Figure 6). The average distances from L-*tert*-leucine atoms to γC2 of Val32(A) and γC1 of Val32(B) for Conf1 are 4.9 and 4.4 Å, respectively. The average distances from L-*tert*-leucine atoms to γC2 of Val32(A) and γC1 of Val32(B) for Conf2 are 4.8 and 4.2 Å, respectively. The difference in the orientation of residue 32 coincides with the alternate conformations of residue Ile47(B) described above. γC2 of Val32(A) makes van der Waals contacts with δC of Ile47(A). In chain B, this interaction is lost. In chain B, γC1 of Val32(B) makes van der Waals contacts with Ile84(B). It would be expected that the structural differences in chains A and B surrounding Val32 affecting inhibitor−enzyme hydrophobic interactions are different for the two inhibitor conformations. These differences are due to the distinct P1 and P1′ groups found opposite the S2 and S2′ pockets for each inhibitor conformation, and the alternate conformations of Ile47(B). It is possible that the alternate conformations of Ile47(B) are due to the conformation of Val32(B). Val32 in chain A and B also exhibited alternate levels of interactions with Ile84, which forms the juncture between the S1 and S2′ pockets and between the S1′ and S2 pockets. Differences in the interactions of Val32 and Ile84 will also affect the interactions between Ile84 and the inhibitors.

Conclusions. The current pattern of resistance development by HIV to protease inhibitors is the acquisition of protease active site mutation followed by several non-active site accessory mutations. It was originally believed that non-active site mutations were acquired to regain the loss of catalytic activity due to active site mutations. Recent studies from our laboratory and others have shown that many non-active site mutations in HIV-1 subtype B proteases will contribute to decreasing the binding affinity of inhibitors, while maintaining the catalytic efficiency (*10*, *22*−*24*, *26*, *27*). In this study, we provide insights into the effects of natural polymorphisms and the contributions to resistance made by therapy-selected active site and non-active site mutations found in the CRF_01 AE protease. We have also established the structural determinants for the unique ATV resistance-associated mutation I50L, and the ability of ATV to maintain its binding affinity for cross-resistant proteases.

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