Articles

Molecular Basis of HIV-1 Protease Drug Resistance: Structural Analysis of Mutant Proteases Complexed with Cyclic Urea Inhibitors†

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ABSTRACT: In cell cultures, the key residues associated with HIV-1 resistance to cyclic urea-based HIV-1 protease (PR) inhibitors are Val82 and Ile84 of HIV-1 PR. To gain an understanding of how these two residues modulate inhibitor binding, we have measured the K_i values of three recombinant mutant proteases, I84V, V82F, and V82F/I84V, for DMP323 and DMP450, and determined the three-dimensional structures of their complexes to 2.1−1.9 Å resolution with *R* factors of 18.7−19.6%. The K_i values of these mutants increased by 25-, 0.5-, and 1000-fold compared to the wild-type values of 0.8 and 0.4 nM for DMP323 and DMP450, respectively. The wild-type and mutant complexes overall are very similar (rms deviations of 0.2−0.3 Å) except for differences in the patterns of their van der Waals (vdw) interactions, which appear to modulate the K_i values of the mutants. The loss of the CD1 atom of Ile84, in the I84V mutant complexes, creates a hole in the S1 subsite, reducing the number of vdw contacts and increasing the K_i values. The V82F mutant binds DMP323 more tightly than wild type because the side chain of Phe82 forms additional vdw and edge-to-face interactions with the P1 group of DMP323. The K_i values of the single mutants are not additive because the side chain of Phe82 rotates out of the S1 subsite in the double mutant (the χ^1 angles of Phe82 and -182 in the V82F and V82F/I84V mutants differ by 90 and 185°, respectively), further reducing the vdw interactions. Finally, compensatory shifts in the I84V and V82F/I84V complexes pick up a small number of new contacts, but too few to offset the initial loss of interactions caused by the mutations. Therefore, our data suggest that variants persist in the presence of DMP323 and DMP450 because of a decrease in vdw interactions between the mutant proteases and inhibitors.

The human immunodeficiency virus (HIV)[1] protease processes the polyprotein gene products of *gag* and *gag-pol* into active structural and replicative viral proteins (Henderson *et al.*, 1988; Loeb *et al.*, 1989a). Incomplete processing of the polyproteins results in the formation of immature (noninfectious) viral particles (Crawford & Goff, 1985; Katoh *et al.*, 1985; Kohl *et al.*, 1988; Göttlinger *et al.*, 1989; Peng *et al.*, 1989; McQuade *et al.*, 1990). Currently, many competitive inhibitors of HIV protease (PR) effectively block polyprotein processing (Appelt, 1993; Wlodawer & Erickson, 1993; Winslow & Otto, 1995); however, the recent discovery of drug-resistant variants of HIV has cast doubt on the long-term therapeutic effectiveness of these compounds. Many resistant variants have been isolated from patients and cell cultures exposed to these inhibitors (Otto *et al*., 1993; Ho *et al.*, 1994; Kaplan *et al.*, 1994; El-Farrash *et al.*, 1994; Condra *et al.*, 1995; Markowitz *et al.*, 1995). The ability of HIV to persist in the presence of potent inhibitors is based in part on its high rate of genetic evolution: a highly error prone reverse transcriptase (Williams & Loeb, 1992) and a rapid rate of replication (Wei *et al.*, 1995).

In clinical trials, variants resistant to HIV PR inhibitors possess point mutations within conserved regions of the protease (Winslow & Otto, 1995). Forty weeks of therapy with indinavir (crixivan or MK639) produces a 15-fold-resistant variant with five mutations (L10R/M46I/L63P/V82T/I84V), and a constructed proviral genome with the five mutations exhibits an 8-fold increase in the IC_{95} (Condra *et al.*, 1995). In vitro selection with ritonavir (Norvir or ABT538) produces a 10−25-fold-resistant variant with five substitutions (M46I/L63P/A71V/V82F/I84V) (Markowitz *et al.*, 1995). A 100-fold-resistant virus with two mutations (G48V/L90M) emerges in the presence of saquinavir (invirase or Ro31-8959), and a constructed proviral genome containing both mutations exhibits a 20-fold increase in the IC_{50} (Jacobsen *et al.*, 1995). Recombinant HIV-1 PR with three mutations (M46I/I47V/I50V) has a K_i value for VX478 that is ∼270-fold greater than the corresponding value for

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[1] Abbreviations: HIV, human immunodeficiency virus; PR, protease; K_i, inhibition constant; SA, simulated annealing; rms, root mean square; vdw, van der Waals; Ile184, symmetry-related residue of Ile84;I84V, mutant protease containing a valine at position 84; WT, wild-type.

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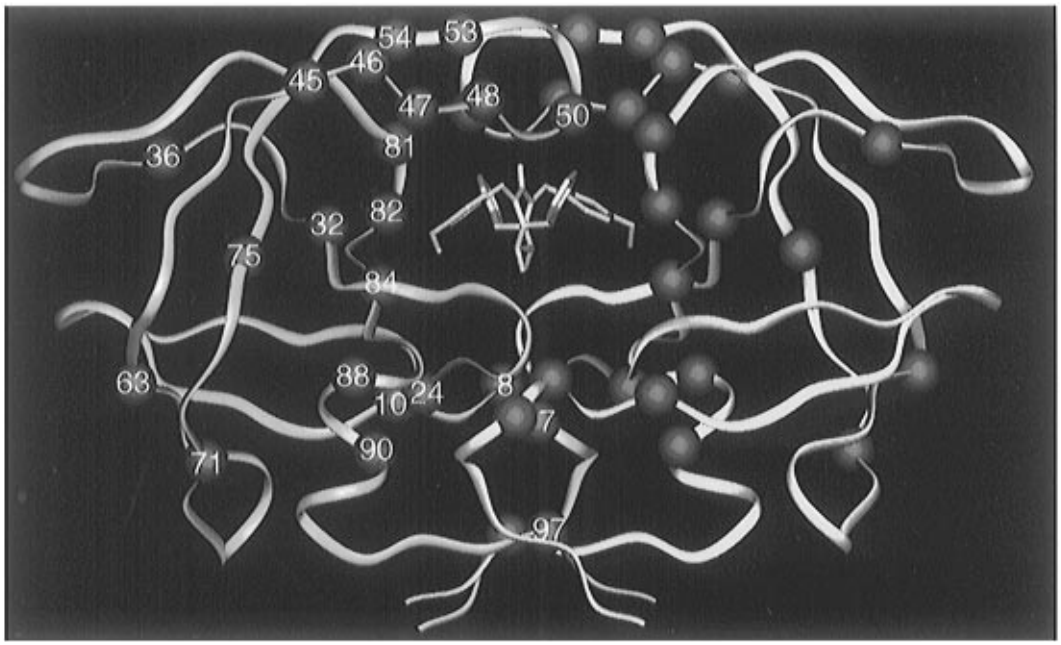


Inhibitor:	INDINAVIR	RITONAVIR	SAQUINAVIR	VX478	DMP323	DMP450
Mutation:	L10R	M46I	G48V	L10F	V82F	K45I
	M46I	L63P	L90M	M46I	I84V	M46L
	L63P	A71V		I47V		V82I
	V82T	V82F		50V		I84V
	I84V	I84V				L90M

FIGURE 1: Residues associated with HIV PR drug resistance. The red spheres indicate mutations observed in resistant variants isolated from cell cultures or patients. Note the overlap between resistance profiles of six protease inhibitors (bottom); C^{α} atoms of HIV PR (blue and yellow) and DMP323 (gray).

wild type (WT) (Partaledis *et al.*, 1995). Apparently, mutations that confer resistance can be located anywhere in the protease: the active site, flaps, dimer interface, and surface loops (Figure 1). As few as two substitutions within the active site are often sufficient to reduce the affinity of the protease for an inhibitor by several hundredfold. Substitutions outside of this region are thought to produce compensatory changes that affect the enzymatic activity of the protease by (1) directly altering the stability of the protein and (2) indirectly influencing substrate binding through long-range perturbations. The protease's tolerance for mutations suggests that all HIV PR inhibitors will, to some degree, select for variants that have a reduced affinity for inhibitor but retain the ability to process the polyprotein precursors.

To identify variants resistant to our cyclic urea inhibitors (Lam *et al.*, 1994), HIV-1 was passaged in the presence of increasing concentrations of DMP323 and DMP450 (King *et al.*, 1995; Hodge *et al.*, 1996). Variants resistant to DMP323 contain two mutations in the substrate binding pocket (S1 subsite): Val82 to Phe and Ile84 to Val. Recombinant 82F and 84V strains increase the IC_{90} by 8-fold and 50-fold, respectively. The most resistant variants encode proteases with both mutations and increase the IC_{90} by 100-fold. Variants isolated under the selective pressure of DMP450 encode proteases with five substitutions (K45I/M46L/V82I/I84V/L90M) and increase the IC_{90} by 45-fold. Furthermore, there is evidence for cross resistance between cyclic urea inhibitors since the IC_{90} of DMP450 for the DMP323-selected virus containing Phe82 and Val84 increased by 100-fold. The effectiveness of new cyclic urea inhibitors may therefore also be reduced by the emergence of these drug-resistant variants.

The future success of treating patients with HIV PR inhibitors may depend on understanding the molecular basis of resistance. So far, drug discovery groups have used the structural characteristics of only WT protease to design inhibitors. Perhaps inhibitors designed against both WT and the predicted mutants will reduce the rate of appearance of resistant variants. In this study, we attempt to identify the structural features of three recombinant HIV-1 protease mutants, V82F, I84V, and V82F/I84V, that confer resistance to DMP450 and DMP323.

MATERIALS AND METHODS

Inhibitor Preparation. The inhibitors DMP323 and DMP450 were synthesized at the DuPont Merck Pharmaceutical Co. (Lam, *et al.*, 1993). Stock solutions of the inhibitors were prepared in dimethyl sulfoxide.

Mutant Preparation, Purification, and Crystallization. The synthetic genes of three HIV-1 PR mutants (HxB2), V82F (Val82 → Phe), I84V (Ile84 → Val), and V82F/I84V (Val82 → Phe + Ile84 → Val), were constructed with mutagenic oligonucleotide primers (containing a unique *Eag*I site) using PCR. A 5′ coding strand oligonucleotide containing the WT sequence and a unique *Xma*I site was paired with each of the following mutagenic 3′ noncoding strand oligos: WT, 5′TCTCTCCCGGGCCGTTGGAAGCCGAAGATGATC; V82F, 5′CAGGTTACGGCCGATAATGTTAAACGGAGTCGG; I84V, 5′CAGGTTACGGCCGATAACGTTAACGTTAACCGGAGTCGG; and V82F/I84V, 5′CAGGTTACGGCCGATAACGTTAAACGGAGTCGG. Fragments of 155 base pairs were amplified, cleaved with *Xma*I and *Eag*I, and ligated into analogous sites of the WT gene. The mutant genes were then inserted into the

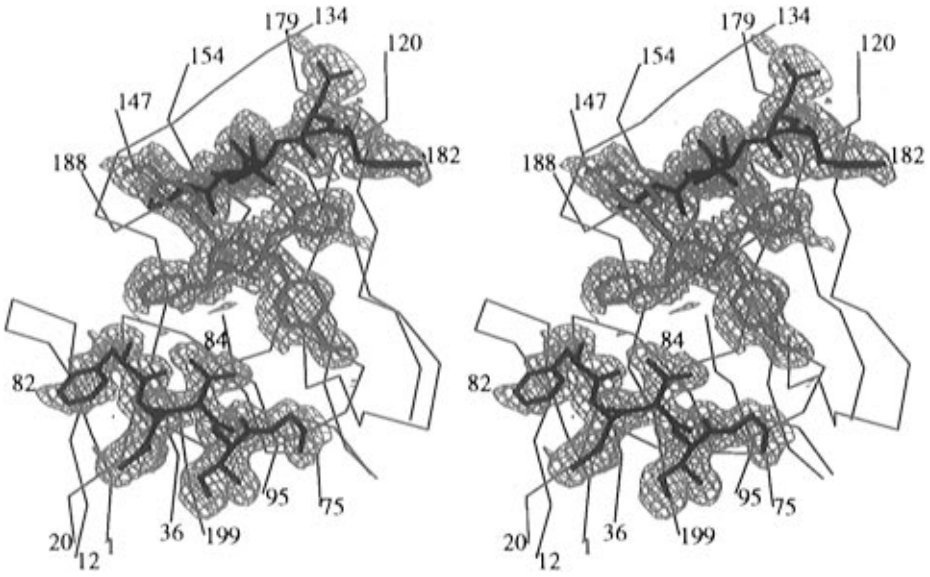


FIGURE 2: Stereodiagram of a simulated annealed omit map ($|2F_o - F_c|$) and atomic models of DMP323 (green) and neighbors of the mutated residues of V82F/I84V (blue). Residues displayed were omitted from the model prior to a cycle of simulated annealing and were not used in the calculation of phases. Electron density was contoured at 1.0σ; C^α atoms of the double mutant (red).

Table 1: Data Collection and Refinement Statistics

	HIV PR·DMP323			HIV PR·DMP450
	V82F	I84V	V82F/I84V	I84V
data collection statistics with all the data				
resolution (Å)	1.9	1.9	1.9	1.9
total reflections	58 279	65 630	41 114	41 830
unique reflections	15 034	14 779	14 386	14 673
completeness (%)	93.2	91.9	89.6	91.1
R_{sym} (%)[a]	9.2	7.5	8.8	9.3
refinement statistics with $F > 2\sigma(F)$				
R_{factor}[b]	19.5	18.9	19.3	18.7
no. of protein atoms	1850	1840	1848	1840
no. of inhibitor atoms	42	42	42	40
no. of solvent atoms	84	123	124	126
rms deviations, angle (deg)	3.03	2.92	3.12	2.88
rms deviations, bond (Å)	0.012	0.012	0.013	0.011

[a] $R_{sym} = \sum|\langle I\rangle - I|/\sum I$. [b] $R_{factor} = \sum||F_o| - k|F_c||/\sum|F_o|$.

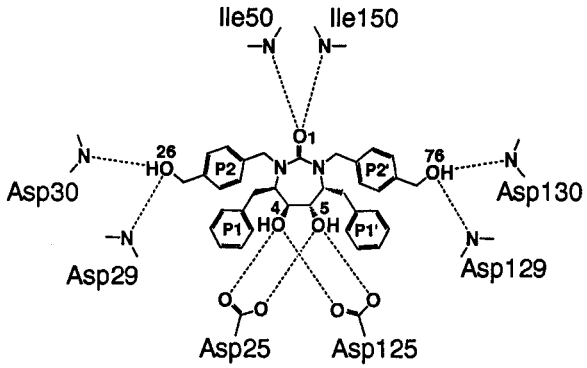
translation vector pET11C and expressed in *Escherichia coli* BL21(DE-3). The protein was purified from inclusion bodies and refolded using a hydrophobic interaction column (Geng & Chang, 1992).

The WT and mutant proteases were crystallized at 18 °C in hanging drops by vapor diffusion in the presence of inhibitor at concentrations of a 1000−5000-fold molar excess over their K_i values (Erickson *et al.*, 1990). Hexagonal rods ($0.08 \times 0.08 \times 1.4$ mm) appeared within 5 days from 4 μL drops containing 1 mg/mL protease, 250 mM acetate buffer (pH 5.0−5.6), and 80−240 mM ammonium sulfate. Diffraction data were collected at room temperature on a RAXIS IIc imaging plate mounted on a RU-H2R Rigaku rotating anode (Cu Kα) generator operating at 50 kV and 100 mA using a 0.3 mm cathode and Yale double-focusing mirrors. The unit cell parameters were determined from four still frames, at 15° intervals, using the RAXIS processing software. Full data sets were obtained from a single crystal by collecting 35−60 oscillation images (2° intervals) with an exposure time of 60−90 min at a detector distance of 70 mm. All protease−inhibitor complexes crystallized in the space group $P6_1$ with a dimer in the asymmetric unit and the following cell dimensions: $a = b = 62.8$ Å and $c = 83.5$ Å.

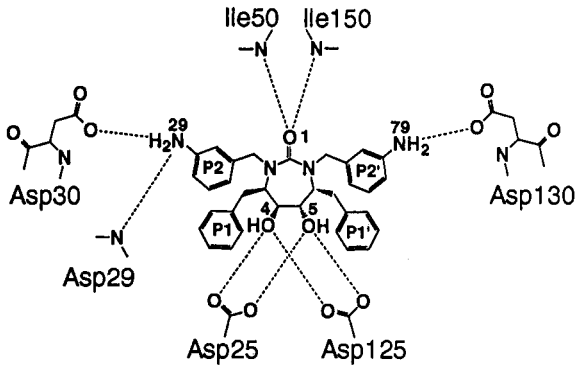
Structure Refinement. The atomic coordinates of the protein in the HIV PR·XK216 complex (XK216 is a symmetric cyclic urea inhibitor with benzyl and allyl groups at P1 and P2, respectively; 1HVR, in the Brookhaven Protein Data Bank) were used as the starting model for refinement except that alanine was substituted for the mutant residues. The data were refined by performing several cycles of simulated annealing (SA) followed by positional and restrained *B*-factor refinement (Brünger *et al.*, 1987). The conformations of the mutant residues and inhibitors were clearly defined in the electron density, as indicated by the typical omit map shown in Figure 2. Refinement reduced the starting *R* factors of ~40% to final values of 19−20%. The data collection and refinement statistics are tabulated in Table 1.

Kinetics Measurements. All K_i measurements were performed as reported (Erickson-Viitanen *et al.*, 1994). Briefly, HIV PR was incubated with DMP323 or DMP450 and then incubated with the fluorescent cationic peptide substrate (2-aminobenzyl)-ATHQVYF(NO_2)VRKA for 60 min at 20 °C in an assay buffer containing 1.0 M NaCl, 50 mM morpholineethanesulfinic acid (pH 5.5), 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol. The enzymatic hydrolysis of the substrate yielded the fluorescent anionic product, (2-

Table 2: Hydrogen Bonds between HIV PR and Inhibitors



HIV PR-DMP323



HIV PR-DMP450

	distance between heavy atoms (Å)							
HIV PR residue	DMP323 group	WT	V82F	I84V	V82F/I84V	DMP450 group	WT	I84V
D25−OD	O4	2.9/3.1	2.9/2.9	2.8/3.1	3.0/3.1	O4	2.9/2.7	2.9/2.9
D125−OD	O5	3.1/3.1	2.9/3.1	2.9/3.0	3.2/3.2	O5	3.1/3.2	3.5/3.5
D29−N	O26	3.2	3.4	3.5	3.3	N29	3.5	
D129−N	O76	3.2	3.4	3.3	3.5			
D30−N	O26	2.9	3.0	3.1	2.9			
D130−N	O76	2.9	3.0	3.0	3.0			
D30−OD						N29	2.9	2.9
D130−OD						N79	2.8	3.5
I50−N		3.3	3.4	3.2	3.1		3.3	3.3
I150−N	O1	3.2	3.0	3.3	3.4	O1	3.4	3.3
total no. of H bonds		10	10	10	10		9	8

aminobenzoyl)-ATHQVY, that was purified by anion exchange HPLC on a mono-Q column (Pharmacia) while monitoring at an emission wavelength of 430 nm with an excitation wavelength of 330 nm. The K_i values for inhibitor binding were estimated by use of the rearranged Michaelis−Menten equation: $K_i = I/[[(K_m + S - faS)/(faK_m)] - 1]$. In this equation, I = inhibitor concentration, S = substrate concentration, fa = fractional activity, and K_m = Michaelis constant. The K_i values reported are averages of two or three measurements.

RESULTS

The K_i values of WT HIV PR for DMP323 and DMP450 are 0.8 and 0.4 nM, respectively. These values increase by 25-fold for the I84V mutant but decrease by 2-fold for V82F. When the two mutations are combined, however, the K_i values increase by 1000-fold, indicating that the changes induced by the single mutations are not additive.

Correlation between K_i Values and Inhibitor Structures. DMP323 and DMP450 are symmetric molecules that contain a seven-membered cyclic urea ring, a phenyl group at P1, and a *p*-hydroxymethylbenzyl and *m*-aminobenzyl, respectively, at P2 (Table 2). When bound to HIV PR, their P1 and P2 substituents are related to P1′ and P2′ by a pseudo-2-fold symmetry axis coincident with the carbonyl bond of urea. Both inhibitors induce similar structural changes in the protease except in the S2 subsite where the hydroxymethylbenzyl group of DMP323 displaces Asp29 and Asp30 by 0.5 Å (Figure 3). The carbonyl oxygens of these two residues form a helix cap at the N-terminal end of the C-terminal helix by hydrogen bonding to Arg87 (NH) and Asn88 (NH). When DMP323 binds to the protease, it pushes the helix cap which in turn pushes the C-terminal helix away from the active site by ∼0.4 Å. Cyclic urea inhibitors that do not have a *p*-hydroxymethylbenzyl group at P2 do not induce these changes in the protease. This induced structural perturbation may explain why DMP323 is less potent than DMP450 and reveals a potential pathway for modulating protease activity from the surface of the protein.

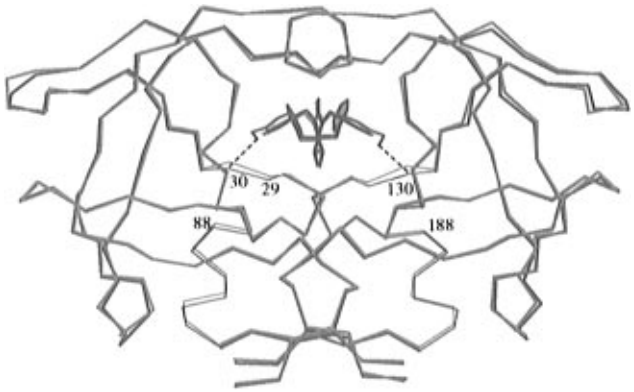


FIGURE 3: Overlay of C^α atoms of I84V complexed with DMP450 (blue) and DMP323 (red). Note the movement of helix caps (Asp29 and -30, thin bonds) and C-terminal helices (thin bonds) away from the active site when protease is complexed with DMP323. Dashed lines indicate hydrogen bonds between P2 (P2′) (OH) and Asp30 (130) (NH), respectively.

Effects of Mutations on Inhibitor Binding. The WT and mutant complexes overall are very similar (rms deviations of 0.2−0.3 Å). The substitutions produce local perturbations that do not disrupt the WT hydrogen bonding patterns between the protease and inhibitors (Table 2). Instead, the mutations alter the patterns of vdw contacts (Table 3). In the WT complexes, contacts are distributed over three regions of the protein: (1) residues 23−28 at the base of the substrate binding pocket, (2) residues 47−50 of the flap, and (3)

Table 3: van der Waals Interactions between HIV PR and Inhibitors[a]

	8	23	27 30	47 50	81 84	Total no. of Contacts (<4.1 Å)	H−bonds (<3.6 Å)
	R P..... L L	D T G A D D T V	L.....M I G G I	G.....T P V N I		
HIV PR•DMP323							
		10 5 4 3 10 2	3 3 5 7	2 7 3			
WT		11 5 5 4 11 2	2 2 5 5	2 4 3		125	10
		12 5 3 4 12 1	0 3 6 6	3 4 2			
V82F		12 3 4 4 12 3	1 2 7 10	3 11 3		136	10
	0	12 5 3 3 9 2	3 2 5 8	3 4 0			
I84V	2	12 5 5 4 10 1	0 2 4 8	2 6 1		121	10
		2 12 5 3 5 12 2	1 2 6 12	3 2 2			
V82F/I84V		1 11 4 4 4 8 2	2 3 3 5	2 0 1		119	10
HIV PR•DMP450							
WT	1	12 5 6 1 4 2	6 3 5 5	5 4 4			
	1	11 5 4 2 6 2	3 1 5 4	3 5 3		118	9
I84V	2	12 5 4 1 5 1	0 3 7 8	2 5 1			
	0	12 5 5 1 2 2	0 1 5 2	1 5 1		99	8

[a] Boxed letters identify WT residues in vdw contact with the inhibitors. Numbers correspond to the actual number of contacts between one residue and the inhibitor. The first and second rows of numbers represent the distribution of contacts in each monomer.

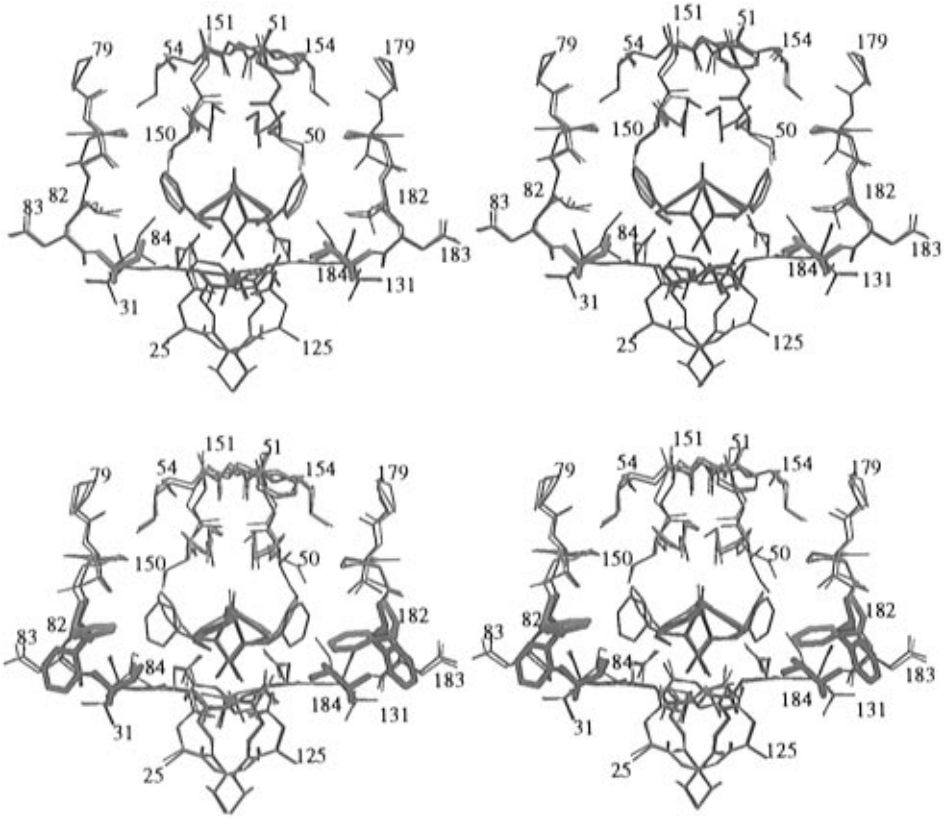


FIGURE 4: Stereodrawings of WT and mutant protease binding sites. Overlay of WT (red) and I84V (blue) complexed with DMP323 (top) and V82F (blue) and V82F/I84V (red) complexed with DMP323 (bottom); note the different rotamers of mutant side chains (thick bonds).

residues 29−32 and 81−84 at the side of the pocket (Table 3). Most important to this study is the fact that the CG1 and CG2 atoms of Val82 and the CD1 atom of Ile84 are involved in a total of 16 and 17 vdw contacts (<4.1 Å) with DMP450 and DMP323, respectively (Table 3). These interactions correspond to ∼14% of the total number of contacts to the inhibitors and appear to modulate the binding affinities of the mutants.

The substitution of Ile84 to Val shifts the backbone atoms of residues 181−185 toward the inhibitor by ∼0.3 Å (Figure 4, top). This compensatory shift, a displacement insufficient to fill the space created by the loss of the CD1 atom of Ile184, picks up only one new contact to the inhibitor. The total number of vdw contacts formed between the pair, Val84 and Val184, and the inhibitor is thus decreased by five (Table 3). The loss of these interactions is consistent with the observed increase in the K_i values of I84V for DMP323 and DMP450. In V82F·DMP323, the backbone atoms of Phe82 and Phe182 move away from the inhibitor by ∼0.5 Å to accommodate their bulky phenyl groups, which bind asymmetrically (their χ^1 angles differ by 110°) (Figure 4, bottom). The significance of the different

rotamers is not apparent since both residues are in vdw contact with the inhibitor and form weak edge-to-face interactions (Burley & Petsko, 1988) with the benzyl groups of P1 (P1′) and Arg8 (108), respectively. A total of 11 new contacts are formed between V82F and DMP323, and four of those contacts are between the benzyl groups of Phe82 (182) and P1 (P1′) (Table 3). The combined effect of additional electrostatic and vdw interactions may explain the observed decrease in the K_i values of V82F for inhibitors.

When both Val82 and Ile84 are mutated, ~70% of the contacts formed between these residues and DMP323 is lost. The most significant structural feature is the symmetric binding of Phe82 and Phe182 (their χ^1 angles differ by only 15°) in a hydrophobic pocket composed of Val10, Ala22, and Leu23 located outside of the active site. The χ^1 values of Phe82 and Phe182 differ from those in the V82F mutant by about 90 and 185°, respectively (Figure 4, bottom). In this orientation, the phenyl groups are not in vdw contact with the inhibitor but still form edge-to-face interactions with the guanidino groups of Arg8 and Arg108. Contacts are also lost between the pair, Val84 and Val184, and the inhibitor, as in the I84V complex. The combined loss of vdw interactions from Phe82 and Val84 to DMP323 may therefore account for the increase in the K_i value of this mutant.

Compensatory Shifts in Mutant Complexes. The conformations of the inhibitors are almost identical when bound to WT, V82F, and I84V, differing only by small changes in the torsion angles of their substituents; this was excepted because the cyclic urea system is preorganized for binding (Lam *et al.*, 1996). The only significant change in the conformation of DMP323 is a 0.7 Å shift of P1 (P1′) deeper into the S1 (S1′) subsite of the double mutant (Figure 4, bottom). This shift is important for inhibitor binding because it is responsible for picking up all five of the vdw contacts to Phe82 and Val84 (Table 3).

The mutations also cause the side chains of Ile50 and Ile47 to shift, increasing and decreasing the number of vdw contacts to DMP323, respectively (Table 3). Ile50 is located at the S1−S2 junction in vdw contact with Ile184 and Ile147 in the S1 and S2 subsites, respectively. When Ile184 is mutated to Val, Ile50 moves toward the hole created by the loss of the CD1 atom of Ile184 (a 0.3 Å shift) (Figure 4, bottom), and Ile147 in turn moves toward Ile50 but away from the inhibitor. These isoleucines appear to form a network that can communicate changes between the S1 and S2 subsites. These shifts are more subtle than those described above, and it is not clear what their net effect is on inhibitor binding.

DISCUSSION

The key residues associated with HIV-1 resistance to cyclic urea inhibitors are Val82 and Ile84 of HIV-1 protease. According to our data, the extent of vdw contacts formed between these two residues and the inhibitors modulates viral resistance. Variants encoding mutant proteases unable to reach a threshold of interactions with the inhibitor are more likely to emerge as resistant variants. The size, number, and location of the substituted residues and the flexibility of the inhibitor need to be considered to explain variations in the binding affinities of mutant proteases.

Mutant residues at position 82 retain some interactions with inhibitors because they reside in a flexible region of the protein (ψ-loop, 82−78). In V82F, the protease accommodates the bulky side chain of Phe82, within the binding pocket, without forming steric interactions with the inhibitor. Baldwin *et al.* (1995) observed even greater flexibility in the single mutant structure of V82A; the backbone moved 0.6−0.9 Å toward the inhibitor (A-77003) to partially fill the gap created by the loss of two methyl groups. In contrast, the reported isosteric substitution of Val82 to Thr in quadruple mutant M46I/L63P/V82T/I84V complexed with indinavir produces an unfavorable interaction between the OG1 atom of Thr82 and the pyridyl methyl piperidine group of the inhibitor; this interaction is believed to be in part responsible for the observed loss of affinity (Chen *et al.*, 1995). Residues replacing Ile84 are not expected to experience the same degree of reorganization because they are located within a β-sheet. A lack of backbone movement in I84V prevents Val84 from interacting with the inhibitor. The loss of two methyl-group contacts (one in each monomer) decreases the binding energy by ~1.8 kcal/mol, which is consistent with the observed 20-fold increase in the K_i values of I84V (Andrews *et al.*, 1984). A lack of flexibility is also apparent in the quadruple mutant complexed with indinavir since Val84 and surrounding residues do not fill the gap created by the loss of the CD1 atoms of Ile84 (Chen *et al.*, 1995). These examples suggest that mutations in restrained regions of the protease will produce the most resistant variants because the protein will not be able to compensate for changes in the binding pocket, and favorable interactions with the inhibitor will be lost.

In addition to protein flexibility, inhibitor mobility plays an important role in determining the binding affinities of mutants. When multiple mutations occur in the same subsite, a flexible inhibitor can move deeper into the enlarged subsite and regain lost interactions with the protein. This is exactly what occurs in the double mutant when the P1 group of DMP323 moves deeper into the S1 subsite. Without this shift, all vdw contacts between the pair, Phe82 and Val84, and the inhibitor would be lost. A more flexible inhibitor than DMP323 may even remain potent against the double mutant. Similar shifts in the inhibitor conformation were not observed in I84V because any movement of the P1 groups of DMP323 or DMP450, deeper into S1, is sterically hindered by the presence of Val82.

Nonadditive Effects on Inhibitor Binding. Another complicating feature of resistance is the possibility of nonadditive mutational effects on inhibitor binding. Mutations that affect substrate (or inhibitor) binding commonly exhibit simple additivity when the sites of mutation are spatially distinct (Wells, 1990; Wagner *et al.*, 1995). In other words, a mutation within one subsite does not alter the affinity of other subsites for substrate. Complex additivity occurs when mutated sites interact directly through steric, electrostatic, or hydrophobic interactions or indirectly through conformational changes. In HIV PR·DMP323, Val82 and Ile84 are in direct vdw contact with each other; the CG2 atom of Val82 and CG1 of Ile84 are separated by 3.8 Å, and the CG2 atom of Val182 interacts with the CG1 and CD1 atoms of Ile184 (Figure 5). Similar interactions are also present in the single mutant complexes but not in the double mutant complex. A lack of vdw interactions between Phe82 and Val84, in the double mutant, may have caused the rotamer of Phe82 to bind in an alternate conformation, outside of the S1 subsite. Alternatively, the substitution of Ile84 to Val may have

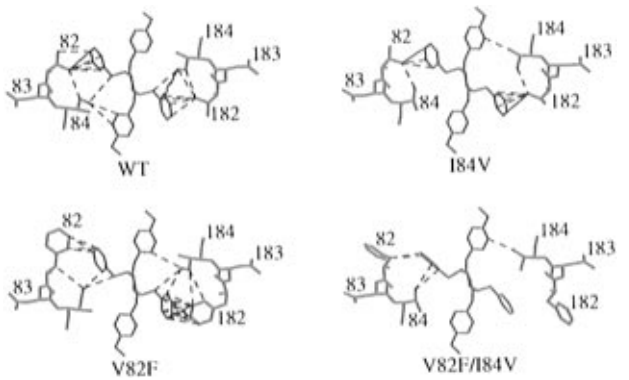


FIGURE 5: Contacts formed between active site residues (light blue) and DMP323 (red). Dashed lines identify distances less than 4.1 Å; note the lack of interactions between Phe82 (182) and Val84 (184) and the inhibitor in the double mutant.

enlarged the S1 pocket sufficiently to accommodate the bulky benzyl group of Phe82. In this scenario, the P1 of the inhibitor would have to push Phe82 out of the S1 subsite in order to bind to the protease. This could not occur in V82F because theCD1 atom of Ile84 sterically prevents the benzyl group of Phe82 from moving deeper into the S1 subsite. In either case, the structural changes induced by the mutations are not additive because different rotamers of Phe82 are stabilized in the single and double mutants. The K_i values of the double mutant have thus increased because Phe82 no longer interacts with the inhibitor. Structural studies of the uncomplexed mutants of HIV PR may help to elucidate whether Phe82 reorganizes in the absence or presence of the inhibitor.

Implications for Drug Design. Ideally, inhibitors should be designed to interact with residues that are essential for protease activity because resistant variants would produce inactive enzymes. Unfortunately, mutants often retain the ability to process viral precursors (Loeb *et al.*, 1989b; Winslow *et al.*, 1995). It is therefore likely that all reversible protease inhibitors designed against WT will select for resistant variants unless the dose of the drug is sufficiently high to compensate for the reduced affinities of the mutants. Consequently, we would like to design inhibitors that retain high affinity even against mutant proteases. In this study, we have shown that inhibitor binding is modulated by vdw interactions. In particular, the interactions lost between residues in the S1 subsite and the phenyl group of P1 are responsible for the decreased affinity of the double mutant. The therapeutic effectiveness of DMP323 and DMP450 may therefore be improved by increasing the size of the inhibitor or making it more flexible. In the first case, a larger inhibitor may be able to keep the number of interactions from dropping below the threshold required for complex formation, even in the double mutant, by forming additional interactions with the protease. In the second case, a more flexible inhibitor may be able to recruit new interactions by shifting in response to changes in the subsites. Finally, an asymmetric cyclic urea inhibitor may have higher efficacy than symmetric ones because resistant variants may emerge more quickly against symmetric compounds; a single substitution produces two symmetrically related changes, one in each monomer. All of these modifications are based on the assumption that the rate of emergence of resistant variants is inversely proportional to the complexity of the mutational pattern needed to reduce the protease's affinity for the inhibitor.

CONCLUSIONS

In this study, we have determined that vdw interactions modulate the binding affinities of the V82F, I84V, and V82F/I84V mutant proteases for DMP323 and DMP450. The loss of interactions between the mutants and inhibitors appears to be the major factor involved in the development of resistance, but it is not the only one. Mutations outside of the protease-coding region play a minor role in determining viral fitness (King *et al.*, 1995; Rose *et al.*, 1996). The fact that changes can occur anywhere within the full length genome of HIV (9000 nucleotides) makes it very difficult to predict and prevent the emergence of resistant variants during the treatment of AIDS. So far, success has been achieved by combining high doses of protease and reverse transcriptase inhibitors. Our aim is therefore to improve the effectiveness of monotherapy by developing a cyclic urea-based inhibitor that does not select for viable mutants, in light of our new structural information.

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