Articles

Effect of Point Mutations on the Kinetics and the Inhibition of Human Immunodeficiency Virus Type 1 Protease: Relationship to Drug Resistance†

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ABSTRACT: Mutations of human immunodeficiency virus type 1 (HIV-1) protease at four positions, Val^{82}, Asp^{30}, Gly^{48}, and Lys^{45} were analyzed for the resulting effects on kinetics and inhibition. In these mutants, Val^{82} was substituted separately by Asn, Glu, Ala, Ser, Asp, and Gln; Asp^{30} was individually substituted by Phe or Trp; Gly^{48} by His, Asp, and Tyr, respectively; and Lys^{45} by Glu. By examination of the inhibition of a single inhibitor, the differences in K_i values between the native and mutant enzymes can range from very large to insignificant even for the mutants with substitutions at the same position. By examination of a single mutant enzyme, the same broad range of K_i changes was observed for a group of inhibitors. Thus, how much the inhibition changes from the wild-type enzyme to a mutant is dependent on both the mutation and the inhibitor. The examination of K_i changes of inhibitors with closely related structures binding to Val^{82} mutants also reveals that the change of inhibition involves subsites in which Val^{82} is not in direct contact, indicating a considerable flexibility of the conformation of HIV protease. For the catalytic activities of the mutants, the k_{cat} and K_m values of many Val^{82} mutants and a Lys^{45} mutant are comparable to the native enzyme. Surprisingly, Gly^{48} mutations produce enzymes with catalytic efficiency superior to that of the wild-type enzyme by as much as 10-fold. Modeling of the structure of the mutants suggests that the high catalytic efficiency of some substrates is related to an increase of rigidity of the flap region of the mutants. The examination of the relative changes of inhibition and catalysis of mutants suggests that some of the Val^{82} and Gly^{48} mutants are potential resistance mutants. However, the resistance is specific with respect to individual inhibitors.

Human immunodeficiency virus (HIV)[1] contains a protease which is responsible for the processing of the *gag* and *gag-pol* polyprotein precursors to yield structural proteins and enzymes of the mature virus. Mutational experiments have shown that this protease is essential for the maturation and propagation of the virus (Kohl *et al.*, 1988). Since HIV is an etiological agent of the acquired immunodeficiency syndrome (AIDS), intense effort has been made in the understanding of its protease. It is now well established that HIV protease is a member of the aspartic protease family. From the crystal structures (Wlodawer *et al.*, 1989; Navia *et al.*, 1989; Lapatto *et al.*, 1989), it is clear that this enzyme is a homodimer and contains two active-site aspartic residues nearly identical in conformation to those of other members of the aspartic protease family (Davies, 1990). Also similar to other aspartic proteases is the substrate binding site which can accommodate about eight amino acid residues.

Since HIV protease is an obvious therapeutic target, considerable efforts have been directed toward the designing and testing of its inhibitors. There are now a large number of potent inhibitors, many of which have inhibition constants in the nanomolar range [for review, see DeBouck and Metcalf (1991), Tomasselli *et al.* (1991), Petteway *et al.* (1991), Huff (1991), and Wlodawer and Erickson (1993)]. Most of these inhibitors are peptide-based transition-state analogues, and some of these are being tested clinically as drugs for treating AIDS. A number of crystal structures of HIV-1 protease−inhibitor complexes have been solved (Miller *et al.*, 1989; Swain *et al.*, 1990; Fitzgerald *et al.*, 1990; Erickson *et al.*, 1990; Jaskolski *et al.*, 1991). This body of information is important for providing a basic framework of the structure−inhibition relationships which may be utilized to design new

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[1] Abbreviations: HIV, human immunodeficiency virus; HIV-1, human immunodeficiency virus type 1; Nph, *p*-nitrophenylalanine.

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generations of better inhibitor drugs.

Despite the wealth of structural information, only limited studies have been carried out using site-directed mutagenesis to probe for the functional roles of different parts of the enzyme. The substitution of the active-site Asp^{25} results in an inactive mutant enzyme (Kohl *et al.*, 1988; Seelmeier *etal.*, 1988; Le Grice *et al.*, 1988; Loeb *et al.*, 1989; Navia *et al.*, 1989). Loeb *et al.* (1989) mutated each of the 99 positions of HIV-1 protease and demonstrated the functional tolerance of the residue replacements. However, the analysis of functional consequences was essentially qualitative and not kinetically analyzed. In order to assess the importance of the active-site hydrogen bonds, we have compared the kinetics and the active-site pK_a's of HIV-1 protease and its mutant in which Ala^{28} is changed to Ser (Ido *et al.*, 1991). Rodrigues *et al.* (1993) have substituted Phe^{53} with Trp to probe the conformational changes of the enzyme. The combination of mutation of residues in the substrate side-chain binding pockets of HIV-1 protease with kinetic studies would be a logical way to explore the structural requirements for specificity and inhibition.

A serious problem in all therapeutic approaches against HIV is the development of drug resistance by the virus during the course of treatment. It is known that HIV develops resistance toward 3′-azido-3′-deoxythymidine (AZT, zidovudine, Retrovir), a suicide-substrate inactivator of the viral reverse transcriptase, by mutations in the active site of the enzyme (Larder & Kemp, 1989; Kellam *et al.*, 1992; Lacey *et al.*, 1992). Recently, HIV resistance to another inhibitor, 2′,3′-dideoxyinosine (ddI), has also been reported (St. Clair *et al.*, 1991). The high frequency of errors in copying of the HIV genome during propagation (approximately 1 in 8000 bases) makes drug resistance a serious problem for all AIDS drugs irrespective of the target. *In vitro* experiments by Otto *et al.* (1993), Dianzani *et al.* (1993), El-Farrash *et al.* (1994), Kaplan *et al.* (1994), and Ho *et al.* (1994) have established that HIV in cell culture can develop resistance against different types of HIV protease inhibitors.

One of the advantages of HIV-1 protease inhibitors as drugs against AIDS is the small size of the enzyme target which may provide only limited mutational possibilities for resistance to inhibition. Thus, it is important to understand the structure−function basis of resistance mutations of HIV-1 protease. Functional mutants resistant to protease inhibitors should be those which combine effective catalytic activity and reduced sensitivity to inhibition. Thus, to understand the structure−function relationships of "resistance", it is important to analyze enzyme kinetics and inhibition of potential resistant mutants. In the current study, we mutated positions, some of which have been shown to lead to the development of resistance *in vitro*, and compared the kinetics of catalysis and inhibition between the wild-type and mutant enzymes. The data suggest that the effect of mutations on inhibition is highly specific toward inhibitor structures. In addition, some mutant enzymes have a large change in inhibition potency but little change in catalytic efficiency. These are potentially mutations which may render HIV with ability to resist inhibitors.

EXPERIMENTAL PROCEDURES

Materials. Recombinant HIV-1 protease was prepared from *Escherichia coli* inclusion bodies and purified according to previously published procedures (Ido *et al.*, 1991). Synthetic peptide substrate A, Lys-Ala-Arg-Val-Leu-Nph-Glu-Ala-Met, was synthesized at the Molecular Biology Resource Center, University of Oklahoma Health Science Center, using an Applied Biosystems peptide synthesizer, 430A. Other synthetic substrates used in kinetic studies were synthesized in the peptide synthesis facility, University of Florida, Gainesville. Pepstatin A was purchased from the Peptide Institute, Inc., Osaka, Japan. Other inhibitors (Figure 1) were synthesized at The Upjohn Co. All other reagents were of the highest grade available commercially and were used without further purification.

Kinetic Measurements. Inhibition constants of HIV-1 protease were measured using chromogenic substrate A. Enzyme activity was assayed spectrophotometrically according to the procedure described previously by Richards *et al.* (1990). A typical reaction mixture consisted of known concentrations of substrate and HIV-1 protease or its mutants in either 100 mM sodium acetate/sodium monochloroacetate buffer of appropriate pH with 0.1 M NaCl, incubated at 37 °C. The initial rate of hydrolysis was monitored at A_{300nm} in a HP8452A diode array spectrophotometer using the HP89531A UV/vis operating software. An extinction coefficient of 1000 M^{-1} was used in the calculation. The kinetic constants were calculated from the rate data using a nonlinear least-squares algorithm (Yamaoka *et al.*, 1981).

Enzyme inhibition was assayed using 0.24 mM chromogenic substrate and enzyme concentrations as follows: wild type, 18.4 nM; V82N, 172 nM; V82E, 26.2 nM; V82A, 31.6 nM; V82S, 59.1 nM; V82D, 144 nM; V82Q, 63 nM; D30F, 365 nM; K45E, 28 nM; G48H, 12 nM; G48Y, 26 nM; G48D, 40 nM; and D30W, 6.3 mM. Inhibitors were dissolved in 50% acetic acid, from which a small aliquot was taken to mix with the assay buffer. The inhibition constants, K_i, were determined for the tight-binding inhibitors from the slopes of the plots of $I_o/(1 - a)$ vs $1/a$ according to Beith (1974), where I_o is inhibitor concentrations and a is the ratio between the initial rates of substrate hydrolysis in the presence and absence of inhibitors. Steady-state kinetics were used when the inhibition constants are greater than 10^{-7} M.

Preparation of HIV-1 Protease Mutants. The mutants at position 82 of HIV-1 protease were constructed by cassette replacement of the *KpnI*/*Xma*III fragment of the designed gene described previously (Ido *et al.*, 1991). Two primers, 5′-TATCGGTACCGTTCTGGTTGGTCCGACTCC-3′ and 5′-GTTACGGCCGATGATGTTXNNCGGAGTCGGACC-AACC-3′, where X is a mixture of G and C and N is the mixture of four bases, were annealed and mutually extended using the Klenow reaction. After the resulting DNA was digested with *Kpn*I and *Xma*III, the 40-bp fragment was isolated by agarose gel electrophoresis and cloned into the *KpnI*/*Xma*III sites of expression vector pET-3b-HIVP (Ido *et al.*, 1991). After transfection of the resulting plasmid into *E. coli* strain BL21(DE3)pLysS (Studier *et al.*, 1990), individual colonies were selected, and the plasmids isolated from them were sequenced to determine the residue replacements.

Genes of HIV-1 protease with mutations at Asp^{30} were constructed as follows. Two primers, 5′-GGTCAGCT-GAAAGAAGCTCTGCTGGACACTGGCGCTGACNNX-ACTGTTCTGG-3′, where X is a mixture of G and C and N is the mixture of four bases, and 5′-ACGAAGCTTA-GAAGTTCAAAGTGCAACCGATCT-3′, were used to am-

plify a 260-bp fragment of the designed HIV-1 protease gene (Ido *et al.*, 1991) using the polymerase chain reaction. Vector pET-3b-HIVP (Ido *et al.*, 1991) was used as template. The resulting fragment, which covers from the *Pvu*II site to the end of the gene (Ido *et al.*, 1991), was digested with *Pvu*II and *Kpn*I, and the 167-bp *PVu*III/*Kpn*I fragment was recovered following agarose gel electrophoresis and cloned into the corresponding sites of expression vector pET-3b-HIVP. Since there is another *Pvu*II site in the expression vector (at position 2342), plasmid pET-3b-HIVP was partially digested with *Pvu*II and fully digested with *Kpn*I. The 4809-bp fragment, which represents the pET-3b-HIVP with the 167-bp *Pvu*II/*Kpn*I fragment removed, was purified from the agarose gel and used for the ligation. The mutants were again selected from the plate and sequenced to determine the substitutions.

The construction of plasmids expressing Gly^{48} and Lys^{45} mutants will be described elsewhere.[2]

The preparation of mutant HIV-1 proteases was carried out using a procedure similar to that described previously (Ido *et al.*, 1991) with modifications as follows: the dissolved inclusion bodies, in 8 M urea at 0.5 mg/mL, were dialyzed at 4 °C for 4−10 h against 10 mM sodium acetate, pH 3.5 (pH 4.0 for D30W and pH 4.5 for V82N), containing 1 mM dithiothreitol and 1% glycerol. After centrifugation at 10000*g* for 20 min, the supernatant (about 100 mL) was ultrafiltered in an Amicon cell (molecular weight cutoff: 10 000) to reduce the total volume to about 5 mL. The pH of the protein solution was adjusted to 4.4 by the addition of about 0.5 mL of 100 mM sodium acetate buffer, pH 4.4, containing 1 M NaCl. After 10 min at room temperature, the solution was again centrifuged at 10000*g* for 10 min to remove minor insoluble (probably unfolded) material. The supernatant was dialyzed against 10 mM sodium acetate, pH 3.5, for 2 h. The protease solution was concentrated by ultrafiltration in an Amicon Centricon-30 and stored at 4 °C for kinetic experiments.

Mutant proteases D30F and G48H did not refold efficiently by the above procedure and required a different refolding protocol. Inclusion bodies (10 mg) were dissolved in 100 mL of 67% acetic acid. This solution was dialyzed at 4 °C first against 4 L of distilled water for 12 h and then against 4 L of 10 mM sodium acetate and 1 mM dithiothrietol, pH 5.5 for D30F and pH 5.0 for G48H, for 4 h. After centrifugation at 10000*g* to remove any insoluble material, the protease solution was ultrafiltered in an Amicon Centricon-30 to reduce the volume to about 10 mL. For all protease preparations, the concentration of the protein was determined by A_{280nm} ($E^{0.1\%} = 1.0$) prior to kinetic experiments. Before use, the active protease concentrations were determined by active-site titration using Upjohn inhibitor U75875 for the wild-type enzyme and mutant enzymes G48D, V82N, V82S, V82A, V82E, G48Y, V82Q, and V82D (Ashorn *et al.*, 1990). For mutant enzymes K45E, G48H, and D30W, inhibitors U80773, U75320, and U76088 were used respectively. The wild-type protease is usually fully active, and the mutants contain about 50% of viable active sites.

[2] Y. Lin, X. Lin, J. A. Hartsuck, T. M., Laue, and J. Tang, Effect of Ionic Strength on the Activity and Inhibition of Human Immunodeficiency Virus Type 1 Protease, submitted for publication.

Modeling of HIV-1 Protease Mutant G48Y. The model of mutant G48Y was constructed from one selected coordinate data set of a high-resolution structure of an HIV-1 protease−inhibitor complex (Fitzgerald *et al.*, 1990). Several other structures (Miller *et al.*, 1989; Swain *et al.*, 1990; Erickson *et al.*, 1990; Jaskolski *et al.*, 1991; Thanki *et al.*, 1992) were least-squares fitted to this coordinate data set so that all possible variations in conformations for side-chain rotomers and main-chain geometry could be visualized together. The algorithm used to fit these crystallographic structures together was ALIGN (Satow *et al.*, 1986). The largest rms difference in these fitted structures calculated by least-squares superimposition was 0.56 Å for all main-chain atoms in these structures.

A model of the protease mutant Tyr^{48} was constructed using FRODO (Jones, 1978, 1985) with LORE (Finzel *et al.*, 1990) working on a Evans and Sutherland PS390, supported by a VAX computer. LORE was used to anneal new side-chain rotomers into the mutant HIV-1 model and to search for related fragments from other highly resolved crystallographic structures from the Brookhaven Protein Data Bank (Berstein *et al.*, 1977) in order to check the validity of the new side-chain orientations and main-chain geometry in our models.

To accommodate a Tyr at Gly^{48}, it was necessary to alter the conformations of other side chains at positions Met^{46} and Phe^{53}. Replacement conformations were chosen from the library of side-chain rotomers as defined by ROTOMER in LORE. We added the constraints that the replacement side chain could not introduce clashes into the model with any other parts of the protease or the inhibitors which occupy the active-site binding cleft. The residues Tyr^{48}, Met^{46}, and Phe^{53} were modeled to form a tightly clustered group of hydrophobic residues. The model was refined using the REFI option in FRODO (Hermans & McQueen, 1974) to regularize the geometry around the substitutions, particularly where we replaced the longer side-chain Tyr for Gly at position 48.

In order to further assess the validity of the modeled region, the conformations for all substitutions made in the model were searched in LORE using a database of 186 nonredundant medium-high and high resolution structures from the PDB data bank. Pentapeptide and heptapeptide fragments which spanned these substituted positions were searched, in order to confirm that the resultant model has some semblance of déjà vu from structures in the protein data bank. We discovered that numerous fragments visually confirmed our ideas that position G48 in the flap, which is part of the extended β-strand of the flap, could theoretically be replaced with a wide variety of amino acid substitutions. The model of the single site-directed protease mutant G48Y resulted in a model that introduced minimal perturbations into the starting structure and would provide a valuable tool with which to describe the changes we were observing in kinetic parameters of this particular mutant enzyme.

RESULTS

The k_{cat} and K_m parameters were determined for the cleavage of a synthetic substrate by wild-type HIV-1 protease and 12 mutant enzymes (Table 1). The inhibition constants (Table 2) against 7 potent transition-state inhibitors (Figure 1) were also established for the same 12 mutant enzymes.

Table 1: Kinetic Constants of Wild-Type and Mutant HIV-1 Protease[a]

	pH	wild type	V82E	V82A	V82D	V82N	V82Q	V82S	D30F	D30W	G48H	G48D	G48Y	K45E
k_{cat} (s^{-1})	3.5	9.5	9.7	8.3			9.1							
	5.0	11.0	13.0	13.7	13	3.3	8.9	7.2	2.2	0.12	30	10	12	13
K_m	3.5	48	240	170			100							
(μM)	5.0	40	115	190	132	300	130	157	730	760	65	57	30	60
k_{cat}/K_m	3.5	0.198	0.040	0.049			0.07							
(μM^{-1} s^{-1})	5.0	0.275	0.113	0.072	0.01	0.01	0.068	0.046	0.003	0.0002	0.46	0.18	0.40	0.22

[a] Substrate = Lys-Ala-Arg-Val-Leu-Nph-Glu-Ala-Met. Assay condition: 0.1 M NaOAc, 0.1 M NaCl, pH 5.0, at 37 °C.

Table 2: Inhibition Constants [K_i (nM)] of Wild-Type and Mutant HIV-1 Protease[a]

	pH	wild type	V82E	V82A	V82D	V82N	V82Q	V82S	D30F	D30W	G48H	G48D	G48Y	K45E
U71038	5.0	1.6	20.0	1.0	4100	1400	56	51.0	41.3		88	31	45	7.7
U93840	3.5	2.1	120	26										
	5.0	1.1			6100	370	11	14	65		ND	ND	8	5.6
U93965	3.5	1.0	28	6										
	5.0	1.6			189	250	39	2.3	140		4	44	14	10
U89360	3.5	120	620	155										
	5.0	20			560	2100	100	91	10000	28000	119	109	56	67
U85548	5.0	3	8.0	5.2	16	27.6		3	75	15000	135	69	96	31
U88566	5.0	11	260	15	51	420	26	16	40	<10	126	317	16	ND
U76088	5.0	0.3	3.7	3.4	56	27		1	0.9	83	36	18	ND	<1

[a] The inhibition constants, K_i, were determined for the tight-binding inhibitors from the slopes of the plots of $I_o/(1 - a)$ vs $1/a$ according to Beith (1974).

In 6 mutants, Val^{82} was changed to Glu, Ala, Asp, Asn, Gln, and Ser, respectively. In other mutants, Asp^{30} was changed to Phe and Trp, Gly^{48} was changed to His, Asp, and Tyr, and Lys^{45} was replaced by Glu. The same substrate was used in kinetic and inhibition studies in Tables 1 and 2. It is clear that k_{cat} values of Val^{82}, Lys^{45}, and most of the Gly^{48} mutants in Table 2 are close to that of the wild-type enzyme. Particularly interesting is mutant enzyme G48H, whose k_{cat} is 3 times higher than that of the wild-type enzyme. The k_{cat} values for 2 Asp^{30} mutants, on the contrary, are as low as 1% of the wild-type enzyme. With the exception of V82D, the K_m values of Val^{82} mutants are severalfold higher than that of the wild-type enzyme. In addition, the changes in K_m for Asp^{30} mutants are somewhat larger, just under 20-fold relative to the wild-type enzyme. The K_m values of Gly^{48} and Lys^{45} mutants are near that for the wild-type enzyme.

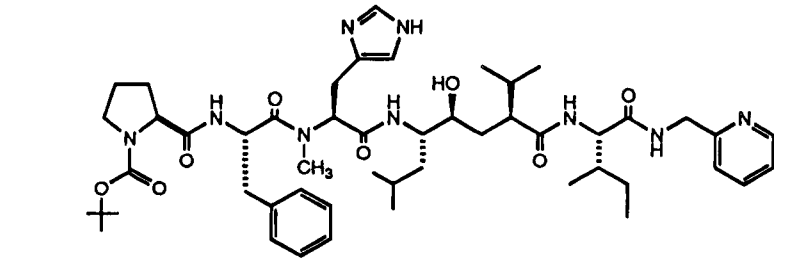
In contrast to the small changes of k_{cat} and K_m values, the change in K_i values is much less uniform within the groups of the same residue replacements (Table 2). For example, within the Val^{82} mutants, the K_i increase for inhibitor U71038 ranges from nearly the same (by a factor of 0.63 for V82A) to an extremely large change (by a factor of 2563 for V82D). This type of disparity is also observed for Asp^{30} mutants. The increase of K_i values due to a mutation also varies greatly for different inhibitors with a specific mutant enzyme. For example, the K_i increase with mutant enzyme V82D was 4.6- and 5545-fold for inhibitors U88566 and U93840, respectively.

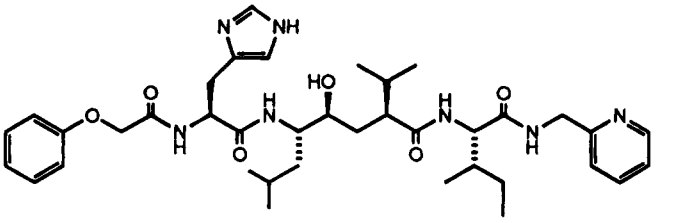
It is clear from the results in Table 2 that the inhibition constants of mutant enzymes are greatly influenced by the inhibitor structures. It was logical to ask then how does the substrate structure influence the kinetic parameters? To study this, the kinetic parameters, k_{cat} and K_m, were determined for HIV-1 protease and 10 mutants (out of the 12 mutants show in Table 1) using 12 different synthetic substrates (Table 3). These substrates contain Nph at P_1' position for the convenience of spectrophotometric assay but contain structure changes in three positions: P_1, P_2, or P_2' (see boldface residues in Table 3). Six mutants of Val^{82} and an Asp^{30} mutant produce mostly lower k_{cat} and higher K_m values as compared to that of the wild-type enzyme. The significance of some of these data will be further discussed.

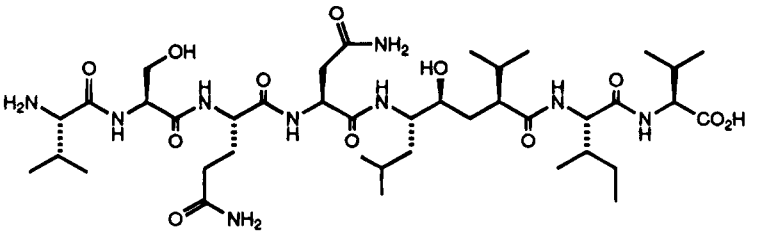
DISCUSSION

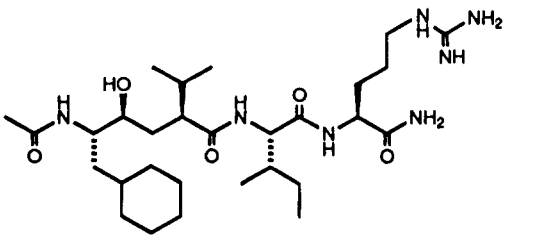
We chose Val^{82}, Asp^{30}, Gly^{48}, and Lys^{45} for mutations because these residues are located in the side-chain binding pockets of HIV-1 protease, but they have only peripheral contact with different inhibitors, as judged from the structures of enzyme−inhibitor complexes (Miller *et al.*, 1989; Swain *et al.*, 1990; Fitzgerald *et al.*, 1990; Erickson *et al.*, 1990; Jaskolski *et al.*, 1991). We consider the replacement of these residues as more likely to produce resistant mutants since the effective catalytic activity can be retained while the inhibition activity may be modulated. The replacements at major side-chain pocket residues would likely result in a drastic reduction of catalytic efficiency; thus these mutants may not be able to sustain the HIV life cycle. The mutation of Val^{82}, Asp^{30}, Gly^{48}, and Lys^{45} has also been shown to produce full or partial activity (Loeb *et al.*, 1989) and would therefore be suitable for kinetic analysis. In addition, the replacements of Val^{82} has been observed in several *in vitro* resistant HIV mutants (Otto *et al.*, 1993; Ho *et al.*, 1994; Kaplan *et al.*, 1994).

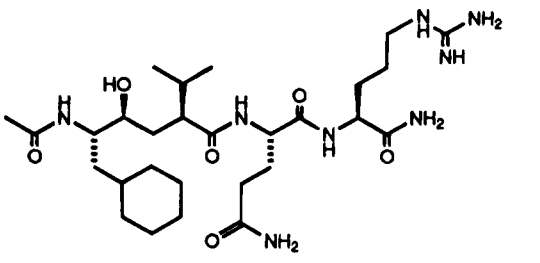
Effects of Residue Replacements on Inhibition Constants. From the inhibition data in Table 2 it is clear that the changes in K_i values due to mutations can range from insignificant to very large. Not surprisingly, this wide range was found for the inhibition of mutants with residue replacements at the same position by a given inhibitor. For example, the K_i of V82 mutants for U71038 can differ by a factor of 4000. This obviously reflects the ability of the replaced residue to form suitable side-chain pockets. More strikingly, however, is the comparison of K_i values of different mutant enzymes toward a set of inhibitors. For example, inhibitor U71038 is equally potent toward the wild-type enzyme and V82A. The same inhibitor is roughly 2500 and 870 times less potent

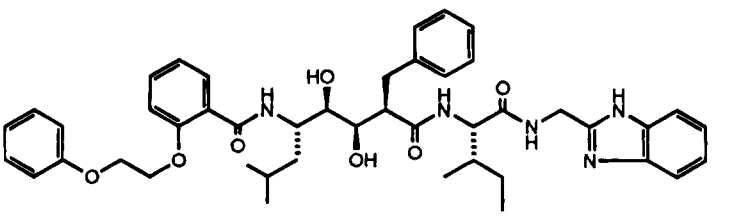












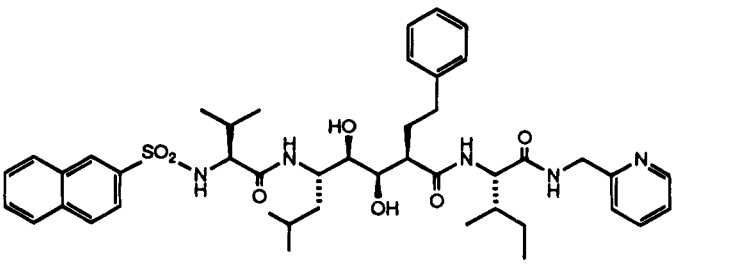


FIGURE 1: Structures of seven inhibitors used in this study.

for V82D and V82N mutants respectively. A similar disparity can be found for inhibitor U89360 toward V82A and V82N. These and other examples in Table 1 illustrate that the change in inhibition from the wild-type enzyme to

Table 3: Kinetic Parameters of the HIV-1 Wild-Type and Mutant Proteases for Peptides with Amino Acid Substitutions in P1, P2, Wild Type, and P2′

	substrates[a]										wild type			V82E			V82A			V82D			V82N			V82Q		
	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
1	K	A	R	V	**L**	X	E	A	Z	G	14	13	1.4	6.6	63	0.10	5.1	31	0.2	12	39	0.3	4.1	119	0.03	3.6	56	0.06
2	K	A	R	V	**Z**	X	E	A	Z	G	30	81	0.4	5.0	53	0.09	4.5	29	0.2	8	46	0.18	3.4	97	0.03	4.2	91	0.05
3	K	A	R	V	**F**	X	E	A	Z	G	31	40	0.8	13	74	0.18	10	57	0.2	6.4	26	0.26	5.6	96	0.06	8.9	83	0.1
4	K	A	R	**A**	Z	X	E	A	Z		23	38	0.3	4.1	151	0.03	6.3	97	0.07	4.8	180	0.03	1.4	176	0.01	2.3	157	0.01
5	K	A	R	**L**	Z	X	E	A	Z		3.3	48	0.1	PC[b]	PC	PC	2.9	109	0.03	1.6	75	0.02	PC	PC	PC	1.4	219	0.01
6	K	A	R	**I**	Z	X	E	A	Z		46	38	1.2	12	69	0.17	7.6	11	0.7	13	25	0.05	7.9	180	0.04	7.2	90	0.08
7	K	A	R	**P**	Z	X	E	A	Z		PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC
8	K	A	R	**D**	Z	X	E	A	Z		3.2	31	0.1	1.3	74	0.02	2.4	51	0.05	2.8	106	0.03	PC	PC	PC	1.1	95	0.01
9	K	A	R	**G**	Z	X	E	A	Z		PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC
10	K	A	R	V	Z	X	**Q**	A	Z		10	203	0.05	PC	PC	PC	4.0	225	0.02	PC	PC	PC	PC	PC	PC	PC	PC	PC
11	K	A	R	V	Z	X	**N**	A	Z		PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC
12	K	A	R	V	Z	X	**T**	A	Z		PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC

	substrates[a]										V82S			D30F			G48H			G48D			G48Y			K45E		
	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
1	K	A	R	V	**L**	X	E	A	Z	G	15	149	0.1	3.3	240	0.01	9.8	26	.038	0.86	17	0.05	12	18	0.65	1.1	5.9	0.18
2	K	A	R	V	**Z**	X	E	A	Z	G	18	219	0.1	4.4	300	0.01	11	34	0.31	0.98	20	0.05	23	5.0	4.5	5.4	4.5	1.2
3	K	A	R	V	**F**	X	E	A	Z	G	32	197	0.2	2.7	128	0.02	23	36	0.64	1.66	32	0.05	26	8.5	3.0	1.4	6.4	0.21
4	K	A	R	**A**	Z	X	E	A	Z		12	972	0.01	5.1	129	0.04	10	60	0.18	1.08	46	0.02	23	10	2.3	1.4	6.6	0.21
5	K	A	R	**L**	Z	X	E	A	Z		PC	PC	PC	PC	PC	PC	8.7	35	0.25	0.75	81	0.01	22	15	1.5	2.2	12	0.18
6	K	A	R	**I**	Z	X	E	A	Z		29	222	0.1	4.1	171	0.02	18	23	0.77	1.53	19	0.08	51	12	4.4	2.1	3.5	0.60
7	K	A	R	**P**	Z	X	E	A	Z		PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC
8	K	A	R	**D**	Z	X	E	A	Z		PC	PC	PC	PC	PC	PC	2.6	31	0.08	PC	PC	PC	3.0	7.0	0.43	0.35	5.0	0.07
9	K	A	R	**G**	Z	X	E	A	Z		PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	0.55	14	0.04
10	K	A	R	V	Z	X	**Q**	A	Z		PC	PC	PC	2.9	311	0.01	7.3	245	0.03	PC	PC	PC	18	34	0.54	5.2	35	0.15
11	K	A	R	V	Z	X	**N**	A	Z		PC	PC	PC	5.4	137	0.04	PC	PC	PC	PC	PC	PC	PC	PC	PC	0.59	32	0.02
12	K	A	R	V	Z	X	**T**	A	Z		PC	PC	PC	7.6	115	0.07	PC	PC	PC	PC	PC	PC	33	52	0.64	1.1	30	0.04

[a] X = *p*-nitrophenylalanine; Z = norleucine. [b] PC = poorly cleaved.

a mutant is not uniform across the board with all inhibitors. This change of inhibition is specific with respect to the inhibitor.

The above conclusion suggests a careful examination of the relationship of inhibitor structures with the mutation-induced changes in inhibition constants. From the current data, this examination can be best done with Val^{82} mutants with respect to their inhibition by two pairs of inhibitors. Inhibitors U71038 and U76088 are similar in structure, having identical S_1 and S_1' side chains and differing only at S_3 and S_4 sites (Figure 1). The second pair of inhibitors, U89360 and U88566, differs only at S_2' side chains. The ratio of K_i values of U71038 and U76088 is 5.3 for the wild-type enzyme (Table 1). This ratio is unchanged for V83E, lower (by a factor of 0.29) for V82A, and larger (by factors of 73, 51, and 51) for V82D, V82N, and V82S, respectively. A similar, but less dramatic, diversity in the K_i changes due to mutation is also seen for the other inhibitor pair mentioned above (Table 1). Val^{82} is known to be in contact with S_1 and S_1' side chains in the majority of HIV-1 protease−inhibitor complexes whose crystal structures are available (Wlodawer & Erickson, 1993). It may also be in contact with S_3 and S_3' at lower frequencies. The large change of K_i ratio by the second pair of inhibitors in mutants V82A and V82D, and to a lesser extent in mutants V82N, V82Q, and V82S, cannot be explained on the direct contact of the residue at position 82 with the S_2' side chains of the pair of inhibitors. Similarly, the side-chain differences between the first pair of inhibitors are in the outside sites and are not likely to be responsible entirely for the change of K_i ratios (5.3 for the wild-type enzyme, 0.29 for V82A, 51 for V82N and V82S, and 73 for V82D). The most plausible explanation to these observations is that, due to the small size of HIV-1 protease, the conformations of the side-chain pockets are flexible. Thus, the change of Val^{82} to another side chain not only affects the conformation of S_1 and S_1' subsites where there are direct contacts with inhibitors, but the flexibility may induce conformation changes in other subsites, such as S_2' in the case of the second pair of inhibitors. In addition to the mutation of the enzyme, the structure of the inhibitor may also play a role in the conformational change of distant pockets. Some conformation changes of the protease crystal structures associated with the binding of inhibitors (Wlodawer & Erickson, 1993) are consistent with the explanation given here. Interestingly, the mutation of Gly^{48} and Lys^{45} produces much less changes in K_i, and less K_i variation among mutants, than those observed for Val^{82} mutations. Positions 45 and 48 are located in the flap region which are more mobile and presumably more adaptable to different inhibitor structures. This may account for the lower sensitivity in inhibition to mutations in the enzyme. Two mutants of position 30, D30 and D30W, produced the most dramatic K_i increases, especially U89360 (Table 2). However, these mutants also produced smaller k_{cat} and higher K_m values, which may indicate a general structural perturbation associated with these mutations.

Modeling of specific mutations studied here has been attempted in order to explain the inhibition changes. The resulting models have permitted us to evaluate some possible conformations for the inhibitor−protease complex. With these visualization tools we have developed the premise of increased flap rigidity for the G48Y mutant enzyme in addition to other hypotheses that describe changes in binding affinity with mutant protease toward inhibitors. Recognizing the limitation in precision of modeling, we hope to validate these conclusions by X-ray structural analyses of complexes formed between U89360 and the wild-type enzyme and two mutant enzymes.[3] We have successfully crystallized and collected diffraction data for these complexes. The solutions to these structures will hopefully provide additional insights in the inhibition changes induced by enzyme mutations.

Effects of Residue Replacements on Catalytic Efficiency. Using substrate A, the k_{cat} values of six Val^{82} mutants are close to that of the wild-type enzyme (Table 1). However, the K_m values of these six mutants are severalfold higher than that of the wild-type enzyme (with the exception of V82D). Thus, the catalytic efficiencies of four of these six mutant enzymes, V82E, V82A, B82Q, and V82S, range from about $^1/_{10}$ to $^1/_2$ of the native enzyme values. The loss of catalytic efficiency appears primarily due to the increased K_m. In the processing of *gag* and *gag-pol* precursors, the substrate concentration would be high inside the immature virions, which may be able to partially compensate for the increase of K_m. This argument makes k_{cat} the main parameter to determine the processing efficiency and makes all six Val^{82} potentially effective in HIV virion processing. Two mutants of Asp^{30} are poor in catalytic activity in all parameters. It is doubtful that these mutants can sustain the life cycle of the virus. Surprisingly, three Gly^{48} mutant enzymes and a Lys^{45} mutant enzyme catalyzed substrate cleavage efficiently even though most of the side-chain replacements are dramatic. In the cases of G48H and G48Y, the k_{cat}/K_m values exceed that of the native enzyme (Table 1). It seems clear that these mutants could be effective in the virions.

The use of 12 substrates in Table 3 permits a comparison of kinetic parameters of mutants due to amino acid changes in the substrates. Several conclusions can be drawn. (a) There is a general correlation in the catalytic efficiency of mutants toward all substrates, including substrate A (Table 1) and 12 substrates in Table 3. Mutants V82S, G48H, and G48Y have comparable k_{cat} but larger K_m to the native enzyme. Other mutants all have smaller k_{cat} and larger K_m than that of the wild-type enzyme. (b) For the wild-type enzyme, the preference for P_1 residues is in the order L > F > Z based on k_{cat}/K_m values of substrates 1−3 in Table 3. The substitutions of Val^{82} resulted in the change of preference in the majority of mutants to F > L > Z. Since Val^{82} is involved in P_1 binding, the change of preference is not surprising. However, it is somewhat surprising that the change to Phe preference seems to accompany many different substitutions. (c) The change of P_2 and P_2' residues in the substrates (Table 3, substrates 4−12) does not result in significant changes of the substrate preference of the Val^{82} mutants from the wild-type enzyme. This is not unexpected since Val^{82} is not directly involved in side-chain binding at these pockets. (d) As observed in substrate A (Table 1), G48Y is a superior enzyme with nearly all the substrates in Table 3. The higher efficiency resulted from both the increase of k_{cat} (for substrates 5, 6, 10, and 12) and the decrease of K_m (substrates 2, 3, 4, 5, 6, 8, and 10). The increase of catalytic efficiency is as high as 10-fold for substrate 10. It is also surprising that the high catalytic efficiency is insensitive to the residue replacements in the

[3] L. Hong, J. A. Hartsuck, J. Tang, and S. Foundling, unpublished results.

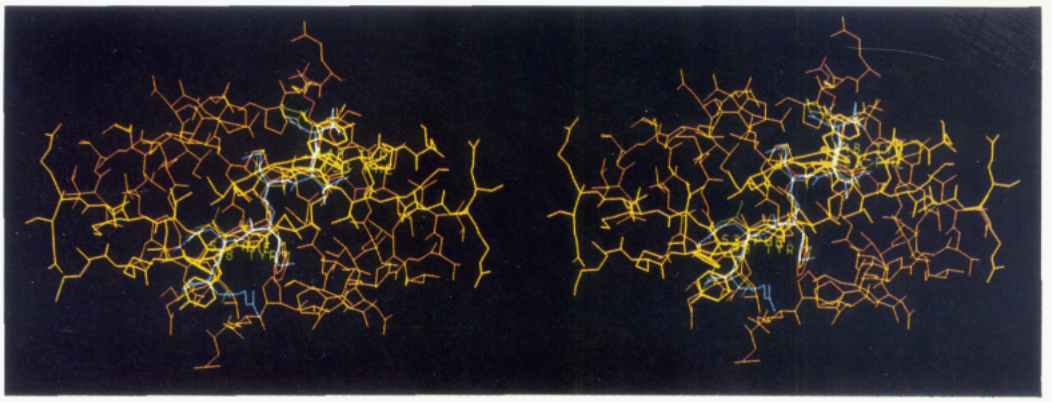


FIGURE 2: Stereo pair illustrating the modeled structure of HIV-1 protease G48Y. The structure shown is the region of the mutant enzyme in homodimer form. The view is from the top of the two flaps which run diagonally from southwest and northeast, respectively. The mutated residues are marked for α-carbon positions (CA 48 and CA 148). Phe^{53} and Phe^{153} nearby appear as lines in their end-on views. The structures of three inhibitors are also shown to be bound under the flap. The colors for the inhibitors are as follows: green, MVT101A (Miller *et al.*, 1989); blue, MVT101B (Miller *et al.*, 1989); red, U89360E (A. Wlodawer, personal communication).

substrates. As in the case of substrate A, G48H is catalytically comparable to the native HIV protease. G48D has relatively low activity with the substrates in Table 3.

Since position 48 is located in the flap region which is known to be mobile and must be flexible to allow the binding of inhibitors to the active site cleft, the effect of the substitution Tyr^{48} on catalysis is of interest. The model of the flap region with the Gly^{48} to Tyr^{48} replacement reveals that the Tyr side chain would be located on the surface of the flap (Figure 2). In order to accommodate this larger new side chain, the native side chains of Phe^{53} (shown only as a line from the end-on view in Figure 2.), which is located on the other strand of the β-hairpin structure of the flap, and Met^{46}, needed to be changed to different rotomers during the modeling. These changes also eliminated the close van der Waals contacts which existed between Phe^{53} and Met^{46} in the wild-type structure. Figure 2 shows that these three side chains are all on the outside of the flap structure and form a tightly packed cluster of hydrophobic residues. With glycines at positions 48 and 49 in the native enzyme, this part of the flap is relatively mobile. Substitutions with a larger side chain at position 48 and the potential interactions discussed above could result in less conformational freedom and greater rigidity of the flap. Thus, this model suggests that the flap rigidity contributes toward the higher catalytic efficiency for some substrates. This explanation is supported by the fact that Tyr, His, and Asp at position 48 all exhibit higher catalytic efficiency for some substrates.

Another possible effect may come from the added intermolecular contacts between the bulky Tyr^{48} residue and the side chain at position P_3 in the inhibitors and substrate. Both the phenolic ring atoms of Tyr^{48} and Tyr^{148} render additional contacts with side chains at P_3 and P_3'. This interaction would be most prominent when the P_3 and P_3' side chains are long or bulky. These potential interactions, which are illustrated in Figure 2 by the superimposition of four inhibitors, may also affect the catalytic efficiency of the mutants in a substrate-dependent manner.

An interesting question is why the wild-type HIV-1 does not select for a higher catalytic activity with a larger side chain at position 48. Since the processing of *gag-pol* polyprotein in the virion requires cleavage of at least eight different sequences, the flexible flat may be necessary to accommodate all of them. A larger side chain at position 48 may not hydrolyze all these sites at a higher rate than does the native enzyme and thus may not represent an advantage for virion maturation.

Relative Changes of K_i vs k_{cat}/K_m Due to Residue Replacements. The relative changes of inhibition and catalysis are of interest since a resistant mutant must maintain proteolytic efficiency in the virions while decreasing the sensitivity to inhibition. As discussed above, the kinetic parameters of some of the mutants of Val^{82}, Gly^{48}, and Lys^{45} are near that of the wild-type HIV-1 protease against specific substrates. These same mutants also exhibit a large increase of inhibition constants against specific inhibitors. Thus, we have observed kinetic parameters which are characteristics of resistance mutations. However, it should be emphasized again that the characteristic of resistance is observed with specific combinations of mutant, inhibitor, and substrate. A mutant can exhibit resistance against one inhibitor but lack resistance against a different inhibitor. Likewise, an inhibitor may be resisted by one mutant but not by another. For example, G48D and G48Y catalyze nearly as well as the wild-type enzyme against substrate A (Table 1), and G48Y catalyzes effectively against many substrates in Table 3. Compared to the inhibition constant of the wild-type enzyme, the K_i of G48D increases from 6- to 60-fold against six inhibitors while G48Y has K_i increases of 28- and 32-fold against U71038 and U85548, respectively (Table 2). Thus, the resistance potential is high for G48Y against U71038 and U85548 and for G48D against several inhibitors in Table 2. On the other hand, the resistance potential is low for G48Y against U88566 because the K_i is near that of the wild-type enzyme. Similar comparisons can also be made for Val^{82} mutants. For example, V82E, which catalyzes substrate cleavage with up to 40% of the efficiency of the native enzyme, but has a K_i increase of 55-fold against U93840, appears to have potential for resistance to this inhibitor. But against inhibitor U85548, the K_i of V82E is less than 3-fold higher than that of the wild-type enzyme. Sardana *et al.* (1994) have mutated Val^{82} to Ile in HIV-1 protease and observed only small changes in catalytic efficiency as well as in inhibition potencies of three tight-binding inhibitors. The absence of a significant change in this case may be due to a very conservative replacement of Val by Ile. Alternatively, the inhibitors used may be less sensitive to this mutation since larger changes in K_i were observed for

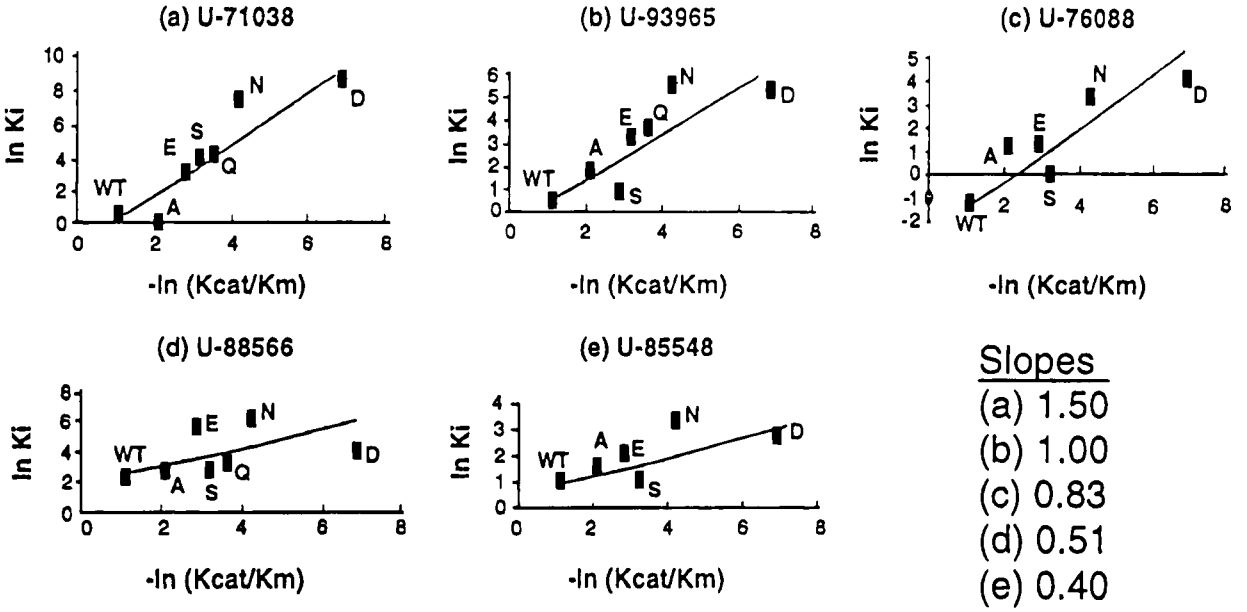


FIGURE 3: Plots of K_i of the individual inhibitor vs averaged values of $-\ln k_{cat}/K_m$ for all substrates. The inhibitors are shown in each panel. Only the data of Val^{82} mutants are plotted. The single amino acid code denotes the residues which replace Val at position 82.

mutants at other positions. Val^{82} to Ala mutation has been observed in two separate *in vitro* studies to resistance against different inhibitors (Otto *et al.*, 1993; Ho *et al.*, 1994), thus deserving special attention. At pH 5, the catalytic efficiency (k_{cat}/K_m) of V82A is lower than that of the wild-type enzyme, by a factor ranging from 5 to 10 dependent on the substrate (Tables 1 and 3). The most severe reduction of K_i of about 10-fold was observed for inhibitors U93840 and U76088 (Table 2). Other inhibitors are much less sensitive to this mutation.

Resistance to drug treatment is one of the major problems associated with the clinical management of AIDS, and it appears likely that it will also be a problem for drugs targeted against HIV protease. For this reason, it would be important to correlate the kinetic data of HIV protease mutants with clinical resistance. However, it is not possible at present to know the thresholds for changes of kinetic and inhibition parameters that constitute clinically significant drug resistance because none of the inhibitors used in this study have been used in a clinical trial. Resistant mutations which have been observed for HIV protease so far were done by selecting for resistance HIV strains grown in cell culture (Otto *et al.*, 1993; Rodriguez *et al.*, 1993; Ho *et al.*, 1994; Kaplan *et al.*, 1994). From these and other studies, resistant mutations have been observed for several positions. Whether the resistance of HIV protease inhibitor by the virus in cell culture is an accurate model for the clinical resistance is not clear at the present.

In the absence of information on clinical threshold, it may be appropriate to compare the relative changes of catalytic efficiency and inhibition for different mutants and inhibitors. A plot of $\ln K_i$ against average $-\ln k_{cat}/K_m$ (for all substrates in Tables 1 and 3) was made for individual inhibitors. Figure 3 shows such plots on Val^{82} mutants for five individual inhibitors. Two points become clear when these plots are compared. First, different inhibitors manifest different slopes. For example, inhibitor U71038 (Figure 3, panel a) has a slope more than 3 times that for U85548 (panel e). A steeper slope means that the loss of inhibition potency relative to the loss of catalytic activity is more severe for U71038 than for U85548. In other words, Val^{82} mutants are more likely to produce resistance against U71038 than against U85548. Second, individual mutants can sometimes be assessed for the ability to resist inhibitors. For example, mutant V82A loses catalytic activity much more than it loses inhibition potency against inhibitor U71038 (as indicated by its position below the line in Figure 3, panel a); thus, it is not likely to be a resistant mutant against this inhibitor. On the other hand, the same mutant V82A loses much more inhibition potency against U76088 (as indicated by its position above the trend line), indicating that this is a potential resistant mutation against this inhibitor. This type of analysis may be useful to assess the vulnerability of inhibitors to HIV protease mutations. The substrates used in the plots of Figure 3 are, however, artificial substrates. The true potential of this plot must be analyzed using as substrates the sequences of the natural cleavage sites in HIV *gag-pol* proteins.

Even though a large number of mutants have been prepared and studied (Table 2), 11 other substitutions for Val^{82} and 10 additional substitutions for Asp^{30} failed to provide information because these mutants either did not express in *E. coli* cells or did not refold properly. The former may represent a technical limitation in our current approach to study the influence of mutation on enzyme activity and inhibition. The latter group, however, would probably be unimportant because they cannot be correctly folded as resistant mutant enzyme.

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