Human Immunodeficiency Virus Type 1 Protease Inhibitors: Evaluation of Resistance Engendered by Amino Acid Substitutions in the Enzyme's Substrate Binding Site

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ABSTRACT: The human immunodeficiency virus type 1 (HIV-1) protease is a homodimeric aspartyl endopeptidase that is required for virus replication. A number of specific, active-site inhibitors for this enzyme have been described. Many of the inhibitors exhibit significant differences in activity against the HIV-1 and HIV type 2 (HIV-2) enzymes. An initial study was conducted to ascertain the HIV-1 protease's potential to lose sensitivity to several test inhibitors while retaining full enzymatic activity. The substrate binding sites of the HIV-1 and HIV-2 enzymes are almost fully conserved, except for four amino acid residues at positions 32, 47, 76, and 82. Accordingly, recombinant mutant type 1 proteases were constructed that contained the cognate type 2 residue at each of these four positions. The substitution at position 32 resulted in a significant adverse effect on inhibitor potency. However, this substitution also mediated a noted increase in the K_m of the substrate. Individual substitutions at the remaining three positions, as well as a combination of all four substitutions, had very little effect on enzyme activity or inhibitor susceptibility. Hence, the four studied active site residues are insufficient to be responsible for differences in inhibitor sensitivity between the HIV-1 and HIV-2 proteases and are unlikely to contribute to the generation of inhibitor-resistant mutant HIV-1 protease.

The human immunodeficiency virus type 1 (HIV-1),[1] a member of the *Lentiviridae* subfamily of animal viruses, is the etiologic agent of the acquired immunodeficiency syndrome (Barre-Sinoussi, 1983; Popovic, 1984; Gallo, 1984). The viral genome encodes a specific protease that acts to cleave polyprotein precursors into mature viral structural and nonstructural proteins (Ratner et al., 1985; Kramer et al., 1986; Debouck et al., 1987; Vernose et al., 1987). The enzyme participates primarily in a late stage of the viral replicative cycle. Its activity is essential for formation of the infectious viral particle. Thus mutant viruses that express inactive protease give rise to immature virions that are incapable of initiating new cycles of virus infection (Kohl et al., 1988). As a result, this viral enzyme has been a target for the development of anti-HIV-1 chemotherapeutic agents.

The protease is a homodimeric endopeptidase belonging to the aspartyl protease family (Pearl & Taylor, 1987; Meek et al., 1989; Wlodawer et al., 1989). Each monomer contributes one of the two catalytic Asp-Thr-Gly triads. Loeb et al. (1989) have identified three regions of the enzyme that are sensitive to amino acid substitution. High-resolution crystal structures of the protease and of protease–inhibitor complexes have demonstrated that these regions include residues involved in substrate binding (Navia et al., 1989; Wlodawer et al., 1989; Lapatto, 1989; Miller, 1989; Fitzgerald, 1990; Erickson, 1990; Swain, 1990). In addition, comparative modeling studies of the HIV-1 protease and the related HIV type 2 (HIV-2) enzyme have shown that, in spite of only 47.5% amino acid sequence conservation (Sanchez-Pescador et al., 1985; Guyader et al., 1987), the active site residues are almost totally conserved between the two enzymes.

Recently, several structurally distinct and potent inhibitors of the HIV-1 protease were described. These compounds (Figure 1), designated L-689,502 (Thompson et al., 1992), L-731,723 (Hungate et al., 1993), and RO 31-8959 (Roberts et al., 1990; Craig et al., 1991), bind to the enzyme's active site and are more potent against the HIV-1 than against the HIV-2 protease. An assessment of the compounds' clinical possibilities requires an understanding of the potential for the target enzyme's ability to become less susceptible to the inhibitors. Modeling studies were, therefore, performed to identify the amino acid residues which participate in the enzyme's interaction with the inhibitors and differ between the HIV-1 and HIV-2 proteases. A series of mutant HIV-1 enzymes, altered at the identified residues, were then constructed and expressed in *Escherichia coli*. These mutants were likely to retain activity and, yet, display altered affinity for inhibitors to the enzyme. This paper describes the kinetic analysis of the mutant proteases and the effects of the individual amino acid substitutions on the enzyme's sensitivity to each inhibitor.

MATERIALS AND METHODS

Reagents. Protease inhibitors L-689,502, L-731,723, and RO 31-8959, as well as the enzyme's peptide substrate (Heimbach et al., 1989), were synthesized and characterized

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[1] Abbreviations: DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIV-1, human immunodeficiency virus type 1; HIV-2, human immunodeficiency virus type 2; kDa, kilodalton(s); MES, 2-(*N*-morpholino)ethanesulfonic acid, PMSF, phenylmethanesulfonyl fluoride; RP-HPLC, reversed-phase high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

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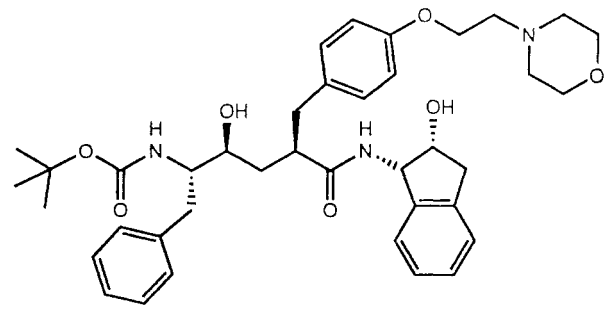
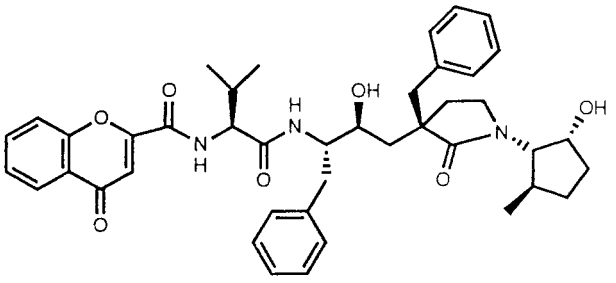
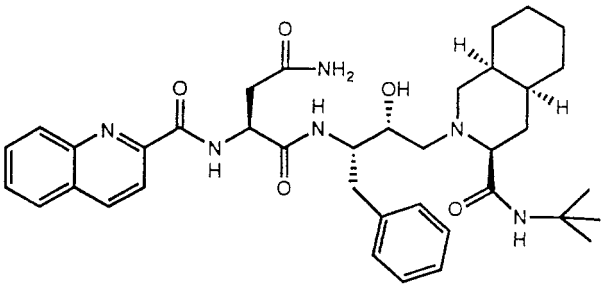


L-689,502



L-731,723



RO 31-8959

FIGURE 1: Three protease inhibitors based on hydroxyethylene isostere: L-689,502, *N*-[2(*R*)-hydroxy-1(*S*)-indanyl]-5(*S*)-[(*tert*-butyloxycarbonyl)amino]-4(*S*)-hydroxy-6-phenyl-2(*R*)-[[4-[2-(4-morpholinyl)ethoxy]phenyl]methyl]hexanamide; L-731,723, *N'*-[1(*R*)-hydroxy-3(*R*)-methyl-2(*S*)-cyclopentyl]-3(*S*)-[3(*S*)-[(*N*-(4-oxo-4*H*-1-benzopyran-2-carbonyl)-L-valinyl)amino]-2(*S*)-hydroxy-4-phenylbutyl]-3(*S*)-(phenylmethyl)pyrrolidin-2-one; and RO 31-8959, *N*-*tert*-butyldecahydro-2-[2(*R*)-hydroxy-4-phenyl-3(*S*)-[[*N*-(2-quinolylcarbonyl)-L-asparaginyl]amino]butyl]-(4a*S*,8a*S*)-isoquinoline-3(*S*)-carboxamide.

as described (Thompson et al., 1992; Hungate, 1993; Roberts et al., 1990). Pepstatin agarose and 3β-indoleacrylic acid were purchased from Sigma (St. Louis, MO); the Mono-S column was from Pharmacia (Piscataway, NJ). HPLC solvents were obtained from Burdick and Jackson (Muskegon, MI). All other chemicals were of the highest purity grade and were from standard suppliers.

Cloning and Mutagenesis of the Synthetic HIV-1 Protease Gene. A synthetic gene coding for protease from the NY5 strain of HIV-1 was assembled from six oligonucleotides ranging in length from 105 to 125 bases. The gene contained, from the 5'-end, a *Cla*I site, 33 base pairs with an *E. coli* ribosome binding site, a unique *Nde*I site overlapping the translational initiation codon (ATG), 297 base pairs encoding the 99 amino acids of the HIV-1 protease, a translational termination codon (TAA), and a *Hin*dIII site. Some of the codons used by the virus were changed, without altering the amino acid sequence of the encoded protein so as to introduce unique restriction sites into the gene. Oligonucleotides were synthesized by the solid-phase method on an Applied Biosystems 381A DNA synthesizer using phosphoramidate chemistry. Oligonucleotides were purified by electrophoresis through a 12% denaturing polyacrylamide gel and visualized by UV shadowing (Applied Biosystems User Bulletin No. 13). Following excision of the bands containing the full-length products, oligonucleotides were recovered from the acrylamide by soaking and desalted by dialysis against water (Applied Biosystems User Bulletin No. 13). Oligonucleotides were phosphorylated by polynucleotide kinase, and complementary fragments were annealed and ligated in two consecutive reactions to pUC19, which had been digested with restriction enzymes compatible with the cohesive ends of the annealed fragments. The *Cla*I/*Hin*dIII fragment containing the synthetic gene was isolated from the resulting plasmid and cloned into *Cla*I/*Hin*dIII-digested pTRP (Darke et al., 1989). The sequence of the entire synthetic gene was confirmed by dideoxy sequencing. The resulting plasmid, called syn 7, expressed amino acids 1–99 of the protease, preceded only by the initiator Met residue under the control of the *E. coli* trp promoter. Specific mutations were introduced by cloning synthetic double-stranded oligonucleotide cassettes between unique restriction sites of the protease gene. These mutations were verified by DNA sequencing.

Expression of the HIV-1 Protease. Cultures (500 mL) of LB (Maniatis et al., 1982), containing 100 μg/mL ampicillin, were inoculated with fresh transformants of expression plasmids in *E. coli* HB101 and shaken at 37 °C until A_{600} = 0.8–1.0. Cells were then harvested by centrifugation and resuspended in 500 mL of M9CA medium (Maniatis et al., 1982) containing 100 μg/mL ampicillin and 20 μg/mL 3β-indoleacrylic acid. Cultures were induced by shaking 2.5 h at 37 °C. Cells were subsequently recovered by centrifugation and stored at −70 °C prior to protease purification.

Purification of Expressed Protease. Wild-type and mutant enzymes were purified by the protocol described by Rittenhouse et al. (1990) with minor modifications. Bacterial cells (0.5–1.0 g) were thawed on ice, resuspended in 10 mL of lysis buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 5 mM EDTA, 5 mM DTT, 2 mM PMSF, 5 μg/mL aprotinin, 5 μg/mL soybean trypsin inhibitor, 10 μg/mL DNase I), and lysed by five passes through a Stansted press. The lysate (25 mL) was centrifuged at 28000*g* for 30 min at 4 °C. The resulting supernatant was then loaded on a 5-mL DEAE Sephadex A-25 column, previously equilibrated with 10 column volumes of 50 mM Tris-HCl, pH 7.8, 5 mM EDTA, and 1 mM DTT at 4 °C. The column was eluted with approximately 6 column volumes of equilibration buffer. The eluant was loaded onto a 1.25-mL bed volume pepstatin agarose column (1.5-cm diameter), equilibrated with 18 mL of 50 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM PMSF, and 1 mM DTT. The enzyme was eluted with 250 mM sodium ϵ-aminocaproate (pH 10.5), essentially as described earlier. The fractions expressing the protease activity (see below) were pooled and chromatographed on a cation-exchange FPLC Mono-S column (Pharmacia HR5/5). The active fractions were further analyzed by SDS–PAGE (Laemmli, 1970). Fractions with >90% purity were used for all the studies. No attempts were made to quantitate the recoveries of each mutant enzyme. Purified enzymes were stored at a concentration of 0.5–5.0 μM in 50 mM MES (pH 6.5), 1 mM DTT, 1 mM EDTA, 10% glycerol, 10% ethylene glycol, and 0.5–0.6 M NaCl at −80 °C. Protein concentrations were determined by quantitative amino acid analysis with norleucine as an internal standard. A molecular mass of 22 kDa for the dimeric form of the protease was used to calculate

the concentration.

Enzyme Activity Assay. Protease activity was determined by HPLC peptidolytic assay as described by Heimbach et al. (1989) using H_2N-Val-Ser-Gln-Asn-(β-naphthylalaninyl)-Pro-Val-Ile-Glu-OH peptide as substrate in 50 mM sodium acetate, pH 5.5. The reactions (100-μL final volume) were initiated by the addition of enzyme and, following incubation at 30 °C for 60 min, were quenched with phosphoric acid. Peptide substrate and products were separated and analyzed by RP-HPLC. Kinetic parameters were determined by fitting the velocity (initial rates at <5% of total substrate hydrolysis) versus substrate concentration data to the Michaelis–Menten equation (hyperbolic). The initial velocity and steady-state conditions for the enzymic reaction with each mutant enzyme were determined. k_{cat} was calculated from V_{max} by assuming 100% activity of the added enzyme and one active site per 22 kDa of dimeric protease. We demonstrated that the enzyme activity remained constant within the 60-min assay period and that this invariance in enzyme activity held within the protein concentration range of 0.1–2.0 nM (data not shown). Therefore, there was no further dissociation of the protease dimer into its monomeric (inactive) form during the course of our assays for all the mutant and wild-type enzyme kinetic analyses.[2]

Enzyme Inhibition Studies. The 50% inhibitory concentration (IC_{50}) values for inhibitor binding were determined using several (12–15) concentrations of the test inhibitors, varying from 0.01 nM to 0.1 mM, and a constant concentration of substrate (440 μM). Inhibitors were initially dissolved in DMSO, and dilutions in DMSO were performed to give a final concentration of 2% DMSO in every assay sample. The concentration of inhibitor that yielded 50% inhibition was obtained by fitting the data to a four-parameter sigmoidal equation (DeLean et al., 1978). A one-way analysis of variance was followed by a Student–Newman Keuls test to determine pairwise difference among pairs. The K_i values were determined by applying the equation

$$I_t/(1 - V_1/V_0) = e + K_i((S + K_m)/K_m)V_0/V_1$$

for tight-binding competitive inhibitors (Dixon & Webb, 1979). The equation was solved with Sigma plot (Jandel Scientific, San Rafael, CA 94901). K_i values were also independently calculated by nonlinear regression analysis using the equation

$$v = V_{max}S/(K_s/(1 + (I/K_i) + S)$$

for HIV-1, V32I, V32I/I47V, and V32I/I47V/M76L/V82I mutants. The assays were performed at five substrate concentrations (50–1000 μM) using at least five inhibitor concentrations. For competitive binding analysis, four concentrations of inhibitor were assayed with variable concentrations of substrate as described earlier. The data were analyzed by a Lineweaver–Burk plot.

Molecular Modeling. The high-resolution crystal structures of inhibited protease complexes were examined (Fitzgerald et al., 1990). Four amino acid residues that differed between the HIV-1 and HIV-2 proteases were identified in each subunit as important for defining substrate binding interactions. A model of the binding site for the HIV-2 protease was constructed by altering these four residues in each subunit using, as a starting point, the crystal structure of the HIV-1 enzyme complexed with inhibitor L-689,502 (Fitzgerald, personal communication; Thompson et al., 1992). The backbone and β-carbon coordinates were not altered because the relatively bulky indanal P2′ moiety of L-689,502 provided a good definition of possible interactions in the P2′ (P2) pockets. Plausible side-chain conformations were selected by visual modeling of steric interactions using Quanta version 3.2 (Molecular Simulations Inc., Waltham, MA) while consulting the preferred side-chain rotamer conformations tabulated by Ponder and Richards (1987). This procedure gives little weight to the side-chain conformations of the starting crystal structure. Almost identical folding of the main chain in several other inhibited complexes, i.e., acyl-pepstatin (Fitzgerald et al., 1990), L-365,862 (Fitzgerald, personal communication), and L-700,417 (Bone et al., 1992), was observed. Therefore, highly similar results would have been obtained by starting with any of these structures. In this model, no attempts were made to refine the structure by energy minimization. All ligand structures were constructed and energy minimized using the procedure described previously (Thompson et al., 1992). Graphics visualization and surface calculations were performed with a general purpose molecular viewing program (Culberson, unpublished).

RESULTS

Molecular Modeling of Inhibitors with HIV-1 Protease. Molecular models of each inhibitor with the HIV-1 protease identified four amino acid residues, at positions 32, 47, 76, and 82, that differ between the type 1 and type 2 proteases and that seem to participate in inhibitor binding. The amino acids at these positions for the HIV-1 and HIV-2 enzymes, respectively, are 32 (Val and Ile), 47 (Ile and Val), 76 (Leu and Met), and 82 (Val and Ile). The inhibitors were modeled in an HIV-1 protease active site containing the type 2 residues at each identified position. Figure 2 presents the composite view of the bound conformations of the three inhibitors. The inhibitors appear to be similarly accommodated in the fully altered enzyme. For reference, Figures 3 and 4 show the position of each substituted amino acid residue in the protease complexed with L-689,502 and L-731,723, respectively.

Kinetic Analyses of Mutant and Wild-Type Proteases. Seven mutant HIV-1 enzymes were recombinantly expressed and purified. The enzymes contained individual amino acid substitutions, as well as multiple substitutions (Table 1). Comparative kinetic analyses were performed with these enzymes and with recombinantly expressed, purified HIV-1 and HIV-2 wild-type enzymes. The activity and kinetic parameters of each enzyme with respect to substrate affinities (K_m), turnover values (k_{cat}), and specificity constants (k_{cat}/K_m) were determined with a peptide substrate (see Materials and Methods) and are summarized in Table 1. Significant differences were observed in the K_m values with these mutants. Specifically, the substitution of Val at position 32 by Ile resulted in a marked increase in the K_m of the substrate. The substitution of Ile by Val at residue 47 seemed to partially compensate for this effect. Additional alterations at residues 76 (Leu to Met) and 82 (Val to Ile) provided further compensation. The mutant enzyme containing all four substitutions had a lower K_m than the wild-type HIV-1 protease. Similar changes in K_m were noted with the enzymes containing individual and combined substitutions at positions 76 and 82. There were no significant differences among the catalytic activities of the enzymes.

[2] We note that Kuzmic (1993) describes the dissociation constant for the wild-type enzyme to be 440 nM. However, there are other reports (Zordan et al., 1992; Zhang et al., 1991; Cheng et al., 1990) describing much lower dissociation constants. We do not observe any change in the extent of dissociation under our assay conditions.

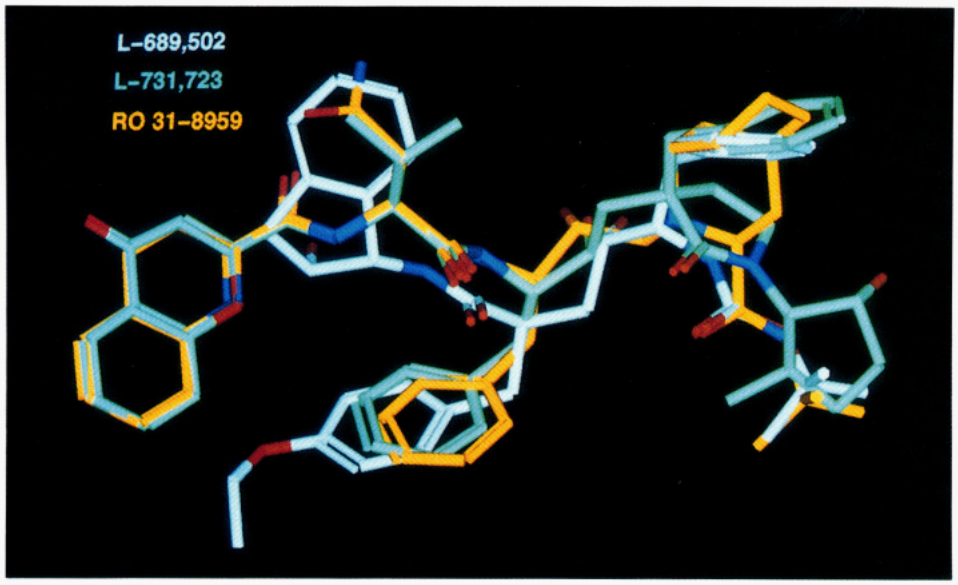


FIGURE 2: Modeled structures of the three inhibitors in the mutated HIV-1 active site. The nitrogens and oxygens of each inhibitor are in blue and red, respectively. The carbons of L-689,502 are in gray, the carbons of L-731,723 are in green, and the RO 31-8959 carbons are in orange.

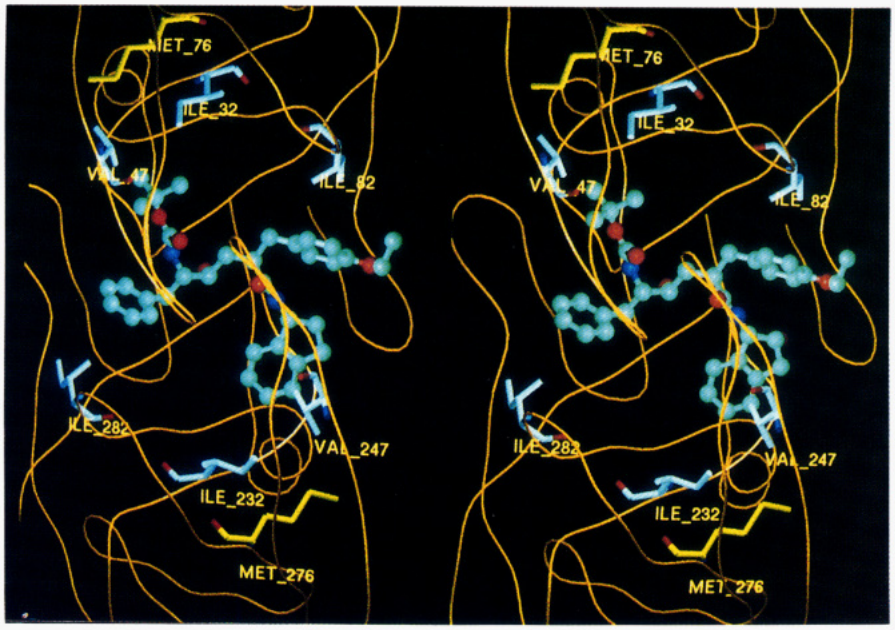


FIGURE 3: Modeled structure of L-689,502 in the modeled HIV-2 protease active site. The flaps of the enzyme are oriented toward the viewer. The mutated residues are shown in thick bonds, while the inhibitor is displayed as a ball and stick figure. One should note the proximity of the *tert*-butyl and indanyl side chains in the inhibitor to the mutated residues in the enzyme, particularly at residue positions 32 and 47.

The specificity constant (k_{cat}/K_m) of the type 1 enzyme was also adversely affected by the Ile for Val substitution at residue 32. This effect also was reversed by incorporation of the remaining substitutions. The catalytic efficiency of these mutants may be a reflection of the substrate binding affinity.

Effect of Inhibitors against Mutant and Wild-Type Proteases. Inhibition of the wild-type HIV-1 protease by L-689,502, L-731,723, and RO 31-8959 had been previously found to be linearly competitive by peptidolytic assay (Thompson et al., 1992; Roberts et al., 1990). Several representative mutant enzymes were similarly analyzed with L-689,502 and L-731,732. Both inhibitors were shown, by Lineweaver–Burk plots, to remain linear competitive inhibitors of the altered proteases (data not shown). Accordingly, linear competition was assumed for the subsequent inhibition assays.

The IC_{50} values for each of the three inhibitors with the type 1 and type 2 wild-type enzymes and with the type 1 mutant enzymes were determined at a constant concentration of substrate and are presented in Table 2. The K_i values for the inhibitors were calculated (as described in Materials and Methods) for tight-binding inhibitors using the measured K_m values and are also listed in Table 2. Independent K_i determinations with type 1 wild-type and with V32I, V32I/I47V, and V32I/I47V/L76M/V82I mutant enzymes, using varying substrate and inhibitor concentrations (as described in Materials and Methods), were in good agreement (data not shown). The three compounds were potent inhibitors of the HIV-1 protease. RO 31-8959 also exhibited good activity against the type 2 enzyme, as previously reported (Roberts et al., 1990). The substitution of Ile for Val at position 32 of the type 1 protease mediated a significant loss of enzyme sensitivity to each inhibitor. However, much of the enzyme's sensitivity to RO 31-8959 was regained by addition of a second substitution (Ile to Val) at position 47. The doubly altered

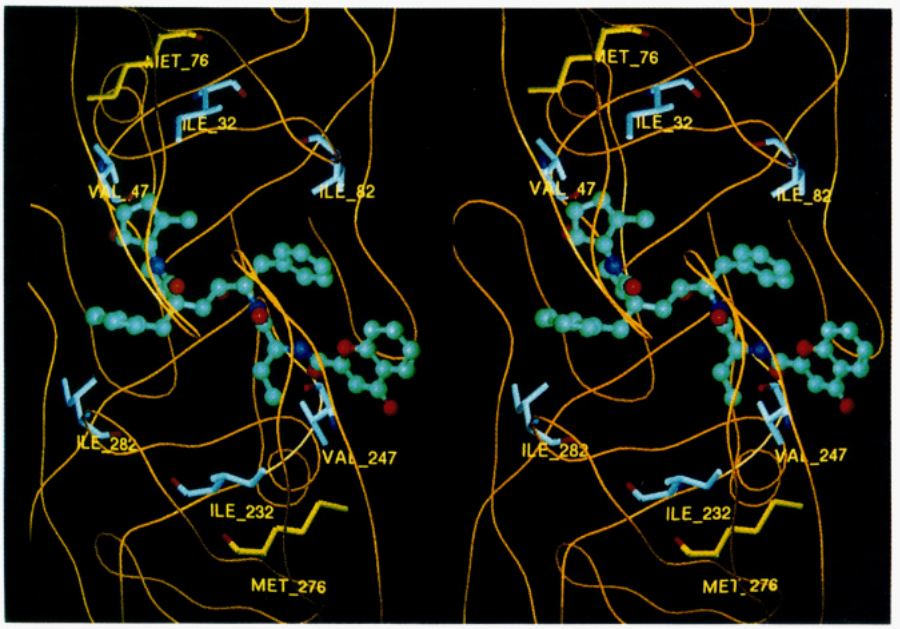


FIGURE 4: Modeled structure of L-731,723 in the modeled HIV-2 protease active site. The flaps of the enzyme are oriented toward the viewer. The mutated residues are shown in thick bonds, while the inhibitor is displayed as a ball and stick figure. Mutations in the protease at positions 32 and 47 are in close proximity to the isopropyl and cyclopentanone side chains.

Table 1: Kinetic Parameters of Mutant and Wild-Type Proteins[a]

enzyme[b]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
type 1 wild type	200 ± 17	13.2 ± 0.8	66.00 ± 7.00
type 2 wild type	1425 ± 180	0.6 ± 0.2	0.42 ± 0.14
32 (Val → Ile)	2357 ± 572	8.1 ± 0.3	4.22 ± 1.02
47 (Ile → Val)	432 ± 92	5.2 ± 1.2	12.04 ± 3.77
76 (Leu → Met)	91 ± 8	5.0 ± 0.2	54.95 ± 5.30
82 (Val → Ile)	68 ± 7	9.4 ± 1.1	138.24 ± 21.53
32 (Val → Ile)/47 (Ile → Val)	1227 ± 212	6.0 ± 1.7	5.32 ± 1.66
76 (Leu → Met)/82 (Val → Ile)	50 ± 1	5.1 ± 0.2	102.00 ± 4.49
32 (Val → Ile)/47 (Ile → Val)/76 (Leu → Met)/82 (Val → Ile)	97 ± 7	5.5 ± 0.8	56.70 ± 9.20

[a] Kinetic parameters were calculated from Michaelis–Menten plots of the initial velocity versus substrate concentration. Activity was determined as described in Materials and Methods. All values represent two or more independent experiments. [b] All mutant enzymes are type 1 (see text).

Table 2: Inhibition of Mutant and Wild-Type Proteases by Test Inhibitors

	L-689,502		L-731,723		RO 31-8959	
enzyme[a]	IC_{50} (nM)[b]	K_i (nM)[b]	IC_{50} (nM)	K_i (nM)	IC_{50} (nM)	K_i (nM)
type 1 wild type	0.85 ± 0.06	0.27 ± 0.03	0.34 ± 0.03	0.08 ± 0.01	0.33 ± 0.02[c]	0.09 ± 0.00
type 2 wild type	12.24 ± 0.46	7.81 ± 0.22	12.63 ± 0.92	8.73 ± 0.81	0.64 ± 0.04[c]	0.61 ± 0.23
32 (Val → Ile)	4.04 ± 0.16	2.96 ± 0.06	1.64 ± 0.21	1.03 ± 0.26	1.22 ± 0.01	0.66 ± 0.04
47 (Ile → Val)	1.34 ± 0.14	0.61 ± 0.07	0.25 ± 0.04	0.07 ± 0.01	0.22 ± 0.01	0.06 ± 0.01
76 (Leu → Met)	1.32 ± 0.12	0.18 ± 0.00	0.76 ± 0.12	0.05 ± 0.02	0.34 ± 0.00	0.04 ± 0.01
82 (Val → Ile)	0.50 ± 0.01	0.06 ± 0.00	0.59 ± 0.11	0.07 ± 0.02	0.49 ± 0.00	0.06 ± 0.01
32 (Val → Ile)/47 (Ile → Val)	5.43 ± 0.49	3.60 ± 0.22	1.36 ± 0.02	0.73 ± 0.02	0.39 ± 0.02	0.12 ± 0.03
76 (Leu → Met)/82 (Val → Ile)	0.97 ± 0.08	0.10 ± 0.02	0.92 ± 0.11	0.09 ± 0.03	0.54 ± 0.02	0.05 ± 0.01
32 (Val → Ile)/47 (Ile → Val)/76 (Leu → Met)/82 (Val → Ile)	5.46 ± 0.40	1.06 ± 0.05	2.64 ± 0.04	0.72 ± 0.11	0.52 ± 0.03	0.08 ± 0.00

[a] All mutant enzymes are type 1 (see text). [b] IC_{50} and K_i values were calculated as described in Materials and Methods and are the geometric means (±geometric standard errors) of multiple determinations. [c] The reported IC_{50} values are <0.4 and <0.8 for HIV-1 and HIV-2, respectively (Roberts et al., 1990).

enzyme remained significantly resistant to L-689,502 and L-731,723. The substitution at position 47, by itself, had no effect on sensitivity to any of the test compounds. Similarly, single and combined amino acid alterations at residues 76 and 82 did not influence sensitivity. The type 1 mutant enzyme that contained substitutions at all four positions was either fully or only slightly less sensitive to each of the three compounds as compared to the wild-type protease.

DISCUSSION

The HIV-1 protease is an essential enzyme in the replicative cycle of the virus. As such, specific inhibitors of this enzyme hold significant promise as chemotherapeutic agents for the treatment of persistent HIV-1 infection. However, the clinical potential of protease inhibitors may be limited if, during therapy, mutant forms of the enzyme can arise under selective pressure that are fully active and are less sensitive to inhibition. This study represents an assessment of the effects of specific amino acid alterations in the active site of the type 1 protease. Most of the amino acid residues that contribute to the substrate binding site are fully conserved between the HIV-1 and HIV-2 proteases. These residues are not likely to be amenable to change during mutant selection. There are, however, four active site residues that differ between the type 1 and type 2

enzymes and that, by molecular modeling, contribute to the enzyme's interaction with each of the studied inhibitors. It was possible that single amino acid alterations at any of these residues, located at positions 32, 47, 76, and 82, would alter the enzyme's ability to bind the inhibitors and not significantly affect substrate binding. Accordingly, mutant HIV-1 proteases, containing single and multiple substitutions at each of the four positions, were expressed in recombinant bacteria and were assayed for kinetic properties and for sensitivity to inhibition by the test compounds.

Alterations at residues 47, 76, and 82, by substitution of the wild-type amino acid with the cognate amino acid from the type 2 enzyme, had relatively little effect on protease inhibition by each of the compounds. In contrast, the conservative substitution of Val to Ile at position 32 rendered the enzyme significantly less sensitive to inhibition. However, the loss of sensitivity exhibited by this mutant was paralleled by lower K_m values and by a notably decreased enzymatic efficiency. The efficiency (k_{cat}/K_m) of the mutant enzyme was only 6% of the wild-type protease. The effects of the alteration at position 32 were partially overcome by introduction of the substitution at position 47, and the activity was almost fully restored by the further introductions of the substitutions at positions 76 and 82.

A possible explanation for these observations may be obtained by additional molecular model analysis. Residues 32 and 47 form the S2′ (and S2) active site pockets (see Figures 3 and 4). The side chains of both residues are directed approximately toward each other. Substitution of Val by Ile at position 32 would project the added methyl (CD1 atom) of the Ile into the S2′ pocket. This would make the pocket slightly smaller, resulting in stearic hindrance for the inhibitors modeled in this study. On the other hand, removal of the Ile CD1 carbon by introduction of a Val residue at position 47 increases the effective volume of the S2 pocket. Accordingly, the combination of the two substitutions at positions 32 and 47 results in an S2 pocket whose volume is unchanged. The amino acid residue at position 76 may participate indirectly through contact with residue 32. The role of residue 82, located at the periphery of the P1′ (P3) binding region, is less clear. It may contact large substituents at the distal ends of the inhibitor P1 and P1′ groups.

Our studies suggest that none of the four HIV-1 protease substrate binding site amino acid residues analyzed here are likely to contribute, as single substitutions, to the generation of an inhibitor-resistant, fully viable HIV-1 variant. The loss of susceptibility engendered by the conservative alteration at residue 32 was accompanied by a significant loss of substrate binding as well as enzyme efficiency. Of course, the studies presented here are limited in scope and represent an initial attempt to explore possible resistance to HIV protease inhibitors. The effects of alterations at other amino acid residues remain to be determined. For instance, the data presented here suggest that the observed disparities between the HIV-1 and HIV-2 wild-type enzymes with respect to inhibitor susceptibility are not due to the four studied binding site residue differences alone. Substitutions at residues outside of the catalytic site probably influence both enzyme structure and function. Further investigations are needed to identify these residues and define their contributions to the protease's activity and susceptibility to active site inhibitors.

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