Kinetic Characterization of Human Immunodeficiency Virus Type-1 Protease-resistant Variants*

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Passage of human immunodeficiency virus type-1 (HIV-1) in T-lymphocyte cell lines in the presence of increasing concentrations of the hydroxyethylamino sulfonamide inhibitor VX-478 or VB-11328 results in sequential accumulation of mutations in HIV-1 protease. We have characterized recombinant HIV-1 proteases that contain these mutations either individually (L10F, M46I, I47V, I50V) or in combination (the double mutant L10F/I50V and the triple mutant M46I/I47V/I50V). The catalytic properties and affinities for sulfonamide inhibitors and other classes of inhibitors were determined. For the 150V mutant, the efficiency (kcat/Km) of processing peptides designed to mimic cleavage junctions in the HIV-1 gag-pol polypeptide was decreased up to 25-fold. The triple mutant had a 2-fold higher processing efficiency than the 150V single mutant for peptide substrates with Phe/Pro and Tyr/Pro cleavage sites, suggesting that the M46I and I47V mutations are compensatory. The effects of mutation on processing efficiency were used in conjunction with the inhibition constant (Ki) to evaluate the advantage of the mutation for viral replication in the presence of drug. These analyses support the virological observation that the addition of M46I and I47V mutations on the 150V mutant background enables increased survival of the HIV-1 virus as it replicates in the presence of VX-478. Crystal structures and molecular models of the active site of the HIV-1 protease mutants suggest that changes in the active site can selectively affect the binding energy of inhibitors with little corresponding change in substrate binding.

HIV protease is an attractive target for antiretroviral therapy (1-4). This enzyme is required for maturation of the viral capsid structural proteins and enzymes necessary for viral replication (5, 6). It is a homodimeric aspartic protease. Inhibition or impairment of HIV protease by mutation of the active site Asp to Asn or Ala results in noninfectious virus particles (5). This finding spurred efforts to design potent inhibitors for use in antiretroviral therapy. More than 200 crystal structures of co-complexes of small molecule inhibitors with HIV-1 protease have been solved (7). Compounds of diverse chemical structure have been designed to have high affinity for this enzyme, i.e. subnanomolar K, values (8, 9). HIV protease inhibitors have been shown to inhibit the replication of the virus in cell culture (10), and several protease inhibitors have advanced to clinical testing in AIDS patients and HIV-infected individuals (11-13). One of these compounds is VX-478, a potent inhibitor of HIV protease with high oral bioavailability in humans. The structure of the co-complex of VX-478 with HIV-1 protease has been solved at high atomic resolution (14).

In vitro selection of resistant virus in the presence of HIV protease inhibitors is useful in understanding resistance at the molecular level (15-22). For example, the HIV-1 strain G88 in CEM cells was grown at increasing concentrations of saquinavir (23). At passage 11, the IC50 increased about 40-fold over the wild type, and 9 out of 10 proviral clones examined contained both G48V and L90M mutations in the protease. In addition, earlier heterogeneity in the wild type sequences at other sequence positions had resolved into a homogeneous pattern after multiple passages. For indinavir, passage six of HIV-1HXB2-infected MT4 cells yielded a double (M46L/V82A) and a triple (V32I/M46L/V82A) mutant, whereas by passage eight a quadruple mutant (V32I/M46L/A71V/V82A) became the dominant protease mutant (22). Cross-resistance studies of the molecular clone with the quadruple mutation showed 0.2-, 6-, and 1-fold increases in IC50 for saquinavir, indinavir, and VX-478, respectively.

Partaledis et al. (24) reported that passage of HIV-1 virus in CEM-SS, a transformed T-lymphocyte line, in the presence of increasing concentrations of the sulfonamide inhibitors VB-11328 (K, < 0.1 nM) or VX-478 (K, = 0.6 nM) also led to the sequential accumulation of mutations in the protease gene. The identity and order in which the mutations appeared was identical for both inhibitors (Fig. 1). Each of these mutations observed is at or near the enzyme active site, except for L10F (Fig. 2). Ile80 and Ile84 are at the center of the active site and are located on the same plane as the carboxyl groups of the active site aspartate Asp25 and Asp259. Residues 46 and 47 are located on the flap region, which closes upon binding of the substrate or the inhibitor. Mutation of these residues, therefore, has the potential to affect flap dynamics and hence ligand binding. Since HIV is known for its high mutation frequency, it is important to determine which of the mutations observed in the in vitro passage experiment are due to selection pressure. It is also important to understand the implications for viral processing of gag-pol polypeptide during maturation of the virus. Hence, biochemical characterization of these mutations is necessary in understanding the contribution of each mutation to inhibitor binding and catalysis of polypeptide processing.

We have expressed and purified these mutant enzymes sin-

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‡ The abbreviations used are: HIV, human immunodeficiency virus; HPLC, high pressure liquid chromatography.

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Molecular Modeling—Analysis of the interactions of the four substrates used in this study with the HIV-1 protease active site was based on our earlier models of the natural substrates.\(^3\) Modeling of the natural substrate peptides spanning from P1 to the P4 region was carried out using the published crystal structures of peptidyl inhibitor complexes such as MVT-101 (PDB code 4hv) and G-365 (PDB code 7hv). The peptides with Phe-Pro and Tyr-Pro cleavage junctions were modeled using the enzyme coordinates described in the 7hv crystal structure.\(^27\) All other peptides were modeled using the 4hv enzyme structure.\(^28\) The carbonyl of the scissile peptide bond of these substrates was modeled to occupy the transition state carbonyl of the inhibitors. Initially the substrate models were built to mimic the bound conformations of the inhibitors G-365 or MVT-101. These models were energy-minimized (200 steps of steepest descent followed by 2000 steps of Adopted-Basis Newton Raphson method) using the CHARMM molecular modeling program.\(^29\) All enzyme atoms and the "flap water" were kept fixed for the first 1200 steps of minimization. The enzyme and flap water atoms were allowed to move during the last 1000 steps of minimization.

RESULTS

We investigated the catalytic parameters of HIV protease using four synthetic peptide substrates representing three natural cleavage sequences in the gag-pol polypeptide. Substrate \(1\) is a non-natural version of substrate \(2\) representing cleavage junction p24-p16 (p16 further gets cleaved into X[p2], p7, p1, and p6 proteins) in the gag polypeptide.\(^30\) The difference between substrate \(1\) and \(2\) is that Ala at P1\,' notation of Schechter and Berger; Ref. 36) of substrate \(2\) was substituted with \([\text{pNO2}]\text{Phe}\) in \(1\), and Met at P4 of \(2\) was changed to Nle in \(1\) for stability. Substrate \(1\) was designed to be used in the spectrophotometric assay of HIV-1 protease and has been used by other investigators to study effects of HIV protease mutations on catalysis.\(^33\) The change in extinction coefficient of this substrate (\(\Delta \lambda \sim 1000\) \(\text{m}^{-1}\) cm\(^{-1}\) at 300 nm) is not large enough to provide a sensitive assay for evaluating tight binding inhibitors using subnanomolar concentrations of enzyme. We chose, therefore, to use an HPLC assay that has the required sensitivity. Peptide substrate \(1\) has good aqueous solubility (\(\sim 6\) \(\text{m}\)) and is an excellent substrate for HIV protease. Hence, this was the substrate of choice for the evaluation of inhibition constants and for initial study of the catalytic parameters for the wild type and mutants of HIV-1 protease.

The catalytic parameters for processing the substrates by the wild type HIV-1 protease are presented in Table I. Among the four substrates, substrate \(1\) has the best catalytic parameters, with \(k_{cat}/K_m\) and \(k_{cat}/K_m\) parameters of 16.3 \(s^{-1}\), 17 \(\mu M\), and 9.4 \(\times 10^3\) \(s^{-1}\) \(\text{M}^{-1}\), respectively. An eight-amino acid peptide, generated from substrate \(1\) by deleting two amino acids from the N terminus and one from the C terminus and modifying \([\text{pNO2}]\text{Phe}\) to Phe at P1\,' was cleaved with a similar catalytic
efficiency ($k_{\text{cat}}/K_m$) (4, 2-fold decrease compared with substrate 1). On the other hand, substrate 2 is cleaved about 54-fold less efficiently than substrate 1. Most of this effect is attributable to the loss of hydrophobic interactions by changing from (pNO2)Phe to Ala at P1. Substrates 3 and 4 have poor affinity for the enzyme, as exemplified by their $K_m$ parameters. As reported by others (31, 32), HIV protease substrates with cleavage sites flanked by proline and/or aromatic amino acids (substrates 3 and 4) tend to have high $K_m$ values (mM). The $k_{\text{cat}}/K_m$ parameters for these substrates are also lower by a factor of 1340 and 1880, respectively, than for substrate 1.

The same set of substrates was used to evaluate the kinetic parameters for the mutants L10F, M46I, I47V, I50V, the double mutant L10F/I50V, and the triple mutant M46I/I47V/I50V. The catalytic parameters $k_{\text{cat}}$, $K_m$, and $k_{\text{cat}}/K_m$ were evaluated and are presented in Table I. For the L10F and M46I mutants, the kinetic parameters were evaluated for substrate 1 only. All other mutants were characterized against each of the four substrates. Without exception, the catalytic efficiency for these mutants is less than that of the wild type enzyme. For substrates 1 and 2, the reduction in catalytic efficiency is modest (≤5-fold). For ≤5-fold decreases in $k_{\text{cat}}/K_m$, the effects are due largely to altered $K_m$ values. For substrates 3 and 4, there is a 25-fold decrease in the $k_{\text{cat}}/K_m$ values against 150V, one of the largest effects we observed. Although there are experimental uncertainties associated with the evaluation of such high $K_m$ values for these substrates, it appears that both $K_m$ and $k_{\text{cat}}$ values are significantly affected to produce the observed 25-fold decreases.

**Table I**

| Cleavage site | Substrate | $K_m$ | $k_{\text{cat}}$ | $k_{\text{cat}}/K_m$ |
|--------------|-----------|------|--------------|----------------|
| p24-X        | 1         | 17   | 16.3         | 940            |
| p24-X        | 2         | 69   | 1.2          | 17.5           |
| p17-p24      | 3         | 740  | 8.6          | 11.6           |
| PR-RT        | 4         | 1465 | 0.7          | 0.5            |

*Substrate 1 is His-Lys-Arg-Val-Leu-[pNO2]Phe-Glu-Ala-Nle-Ser-NH2, substrate 2 is His-Lys-Arg-Val-Leu-Ala-Glu-Ala-Met-Ser-NH2, substrate 3 is Ser-Gln-Asn-Tyr-Pro-Ile-Val-OH, and substrate 4 is Ac-Thr-Leu-Asn-Phe-Pro-Ile-Ser-Pro-OH. Residues flanking the cleavage site are in boldface type.

4 S. Pazhanisamy, unpublished results.
decrease in $k_{cat}/K_m$. The double mutant L10F/I50V processes the substrates as efficiently as the 150V single mutant. The catalytic efficiency for the cleavage of substrates 2, 3, and 4 by the triple mutant is higher than that for the 150V mutant by a factor of 1.3–2.0.

The inhibition constants, $K_i$, for VX-478, indinavir, and saquinavir, were evaluated against all of the mutants (Table III). The single mutations L10F, M46I, and I47V have little effect on the binding of any of these inhibitors. On the other hand, the 150V mutation, either alone or in combination with the above mutations, weakens the binding of these inhibitors significantly. The largest increases in $K_i$ were observed for VX-478 binding to the single mutant 150V and the triple mutant M46I/I47V/I50V. Decreased affinities of 83- and 247-fold, respectively, were observed.

Vitality, as defined in Equation 1, is an index of survivability of the mutant virus and its ability to replicate in the presence of the inhibitor. The higher the vitality value, the greater the advantage offered by the mutation. Since vitality is a function of $k_{cat}/K_m$, it is dependent on the nature of the substrate. Vitality values were calculated for each inhibitor against each mutant using substrates 1–4. The 150V mutant showed significantly larger vitality values for VX-478. The vitality values obtained for 150V and the triple mutant against VX-478, saquinavir, and indinavir were plotted as a function of substrates as in Fig. 4. The mutant enzymes have much higher vitality values for VX-478 than for saquinavir or indinavir, against which these mutant enzymes remain sensitive.

**DISCUSSION**

HIV protease plays a vital role in the post-translational processing of gag and gag-pol polypeptides into functional structural proteins and enzymes. The effect of certain mutations on the catalytic efficiency of the protease has been evaluated by systematically modifying the residues on the substrate (32, 33). Although this type of structure-activity relationship is useful in understanding which substrate interactions are affected most by the mutations, it is less helpful if one wishes to determine to what extent the actual cleavage sites on the gag-pol sequence would be affected. Catalytic efficiency data for authentic cleavage sites, we believe, are useful in correlating biochemical data on in vitro selected mutations in the protease with the viability of the virus.

We chose four substrates, representing three cleavage sequences on the gag-pol polypeptide, to study the effects of mutation of the protease on processing polypeptides. It is clear from Table I that the efficiency of cleavage varies for each processing site of these mutations. Among the substrates we examined, substrate 4, which represents the PR/RT cleavage junction, is the most slowly processed substrate. It is likely that in vivo processing of PR/RT junction is one of the rate-limiting steps for the production of mature proteins from the gag-pol polypeptide. Any mutation that decreases the catalytic efficiency for processing the PR/RT cleavage site may have significant consequences for the replication kinetics of the virus.

**TABLE II**

| Enzyme    | Substrate 1 | Substrate 2 | Substrate 3 | Substrate 4 |
|-----------|-------------|-------------|-------------|-------------|
| Wild type | 940 ± 240   | 17.5 ± 1.9  | 11.6 ± 1.7  | 0.5 ± 0.1   |
| L10F      | 425 ± 41    | ND a        | ND          | ND          |
| M46I      | 530 ± 55    | ND          | ND          | ND          |
| I47V      | 320 ± 25    | 20.7 ± 3.1  | 7.0 ± 0.9   | 0.34 ± 0.06 |
| 150V      | 300 ± 37    | 3.0 ± 0.6   | 0.46 ± 0.09 | 0.02 ± 0.004|
| L10F/I50V | 230 ± 15    | 2.8 ± 0.8   | 0.35 ± 0.36 | 0.015 ± 0.02|
| M46I/I47V/I50V | 185 ± 42 | 3.05 ± 0.4 | 0.7 ± 0.14 | 0.037 ± 0.001|

**TABLE III**

| Enzyme    | VX-478 | Indinavir | Saquinavir |
|-----------|--------|-----------|------------|
| Wild type | 0.6 ± 0.05 (1) | 1.0 ± 0.13 (1) | 0.8 ± 0.3 (1) |
| L10F      | 0.5 ± 0.1 (1)  | 4.0 ± 0.4 (4)  | 1.4 ± 0.6 (2)  |
| M46I      | 0.3 ± 0.05 (1) | 4.0 ± 0.7 (4)  | 0.8 ± 0.03 (1) |
| I47V      | 0.4 ± 0.03 (1) | 3.3 ± 0.3 (3)  | 0.4 ± 0.03 (1) |
| 150V      | 50 ± 5 (83)    | 10 ± 1.1 (10)  | 17 ± 5 (21)    |
| L10F/I50V | 36 ± 4 (40)    | 35 ± 4 (35)    | 27 ± 5 (34)    |
| M46I/I47V/I50V | 160 ± 16 (267) | 29 ± 5 (29)    | 33 ± 12 (41)   |

a ND, not determined.

Effect of Mutations on Molecular Interactions with Inhibitors and Substrates—HIV-1 protease variants with Phe10 are naturally occurring (34), but the wild-type virus used in the past studies has Leu10 at this position. In the protease, Leu10 is located on the surface of the enzyme about 10 Å away from the active site aspartate residues. Its side chain is in contact with the side chains of Arg5, Glu21, Leu23, and Val82. When Phe10 was replaced with Leu and energy-minimized, it was found that the Phe side chain makes an aromatic-charge interaction with the Arg9 side chain.
in addition to the hydrophobic interactions with the side chains of Leu23 and Val52. These additional interactions are likely to shift the monomer-dimer equilibrium toward the catalytically inactive monomer. A moderate reduction of 2.5-fold in turnover number (k_cat) is in qualitative agreement with the model. For the L10F mutation to have any effect on binding, the ligand must have P1/P2 side chains that interact with Arg90/Arg98 side-chains. Even then, only a small effect is expected. The modest increases in K_i for saquinavir and indinavir, 2- and 4-fold, respectively, and the lack of effect on the K_i of VX-478 are consistent with the predicted interactions between the inhibitor and mutant. Met46 is located on the flap of the enzyme with the amino acid side chain exposed to solvent. It is not involved in any direct contact with the inhibitors bound at the active site. The M46I mutation causes no reduction in binding of VX-478 or saquinavir binding, but a modest 4-fold decrease is observed in indinavir binding. A recent molecular dynamics study by Collins et al. (35) showed that the M46I mutation stabilizes the closed form of the enzyme flap. It is not clear why such stabilization of the flap would have selective effects on binding of different inhibitors or substrates. We are investigating the effect of the M46I mutation on the dynamics of the flap in the closed form and also the interactions of the neighboring residues (Ile47 and Gly48) with the inhibitors and the substrates by molecular dynamics simulations. Ile47 is also located on the flap and is part of the constellation of residues forming the S2/S2' pocket. The phenyl ring of the indan group in indinavir at P2 buries deep into the S2 pocket, closer to Ile47 than the other inhibitors, and makes a number of contacts with the C-ethyl methyl group of this side chain compared with the smaller tetrahydrofuran and Asn side chains of VX-478 and saquinavir, respectively. It is understandable, therefore, that I47V affects indinavir binding and not VX-478 or saquinavir binding. There is a 3-fold decrease in k_cat/K_m for substrate 1, which may also result from the loss of interaction between Val at P2 of the substrate and the Val417 side chain of the mutant. Ile47 and Ile84 are at the heart of the active site and are capable of interacting with the peptide side chain of P2, through P2'. All three inhibitors show >10-fold increases in K_i against 150V (see Table II) and 184V (data not shown) single mutants. Therefore, it is not surprising that during in vitro selection against VX-478, the virus first generated an 184V mutation in the protease but at the end selected a 150V mutation, which offered more resistance to VX-478 binding (24- and 83-fold increase in K_i for 184V and 150V mutants, respectively). Molecular modeling and solvent-accessible surface area calculations suggest that the increase in K_i is due to the loss of hydrophobic interactions but not due to loss of any hydrogen bond interactions of the flap water. The C-ethyl methyl group of Ile47 makes hydrophobic interactions with all of the carbon atoms of the P2' phenyl ring of VX-478 (Fig. 5). On the other hand, in saquinavir and indinavir, only two of the three methyl groups of t-butyl at P2' are in direct contact with the C-ethyl methyl group of Ile47. On the nonprime side, the P2 indanol group of indinavir makes a direct hydrophobic interaction with the entire side chain of Ile47, not just with the terminal C-ethyl group. Therefore, absence of the C-ethyl methyl group of Ile47/Ile47 residues by the 150V mutation causes a greater loss in binding of VX-478 than for indinavir or saquinavir.

The 150V mutation also affects catalysis significantly. The catalytic efficiencies (k_cat/K_m) decrease by 3-5-fold for substrates 1 and 2 and by 25-fold for substrates 3 and 4. The substrate models show that Ile47 and Ile47' side chains are in contact with P2, P2', P1', and P2' side chains of the substrates, as in the case of inhibitors noted earlier. The degree of contact of the Ile47/Ile47 residues with these different substrates varies, however. Using a 5.0-A distance cut-off, we found that the C-ethyl methyl groups of Ile47 and Ile47' side chains make the largest number of contacts (four) with the Ile side chains at P2' of substrates 3 and 4 but only one contact with the P2' Glu side chain of substrates 1 and 2 (see Fig. 6). On the nonprime side, they make two contacts with the Val side chain of substrates 1 and 2 one with the Asn side-chain of substrates 3 and 4. The larger number of interactions of the P2' Ile is due to the fact that Ile side chain is buried deeper into the hydrophobic part of the S2' pocket, whereas the Glu side chain is swung away from the hydrophobic part but toward the solvent-exposed portion of the enzyme, making hydrogen bonds with the main chain and side chain of Asp40. Therefore, a larger effect observed on the catalytic efficiency of substrates 3 and 4 compared with substrates 1 and 2 is consistent with the predicted interactions of these substrates with the Ile47/Ile47' side chains. Effect of Mutations on Appearance of Resistance—During in vitro passaging of HIV-1, a number of mutations appear sequentially under the selection pressure of increasing concentrations of HIV protease inhibitors. Which mutation, if any, imparts a selective advantage to the virus? We have used the model by Gulnik et al. (25), which derives a predictive parameter for the viability of the virus from the K_i and k_cat/K_m values. Vitality is defined as the ratio of -fold increase in K_i for the mutant over -fold decrease in k_cat/K_m values for the same mutant with respect to the wild type enzyme values (Equation 1). A high vitality ratio signifies that this set of mutations will

![Fig. 4. Vitality values for inhibitors, calculated according to Equation 1, plotted against substrates mimicking the cleavage sites on the gag-pol polypeptide. For the peptide sequence of the substrates, see "Materials and Methods." Panels A and B are for the 150V single mutant and M46I/A47V/I50V triple mutant, respectively. The dotted, gray, and black bars represent the vitality values for VX-478, indinavir, and saquinavir, respectively.](image-url)
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activesite of HIV-1 protease (indicate close contacts (5-Å cut-off) between C- residues of these substrates (Glu and Ile for substrates 2 and 3, respectively) and residues of the substrates and details of modeling of substrates in the active site. See “Materials and Methods” for the structure and catalytic efficiency for substrates 3 and 4 by a factor of 1.5–2.0. Clearly, M46I and I47V are compensatory mutations. Addition of the M46I and I47V mutations therefore, to the existing I50V mutation increases the survival value for VX-478, but not in the presence of indinavir or saquinavir. 3) The vitality factors are consistently higher for all substrates for the triple mutant than for the I50V single mutant. There is no increase in K for VX-478 and saquinavir either for the M46I or I47V single mutants. Against the triple mutant, however, we observe a 3- and 2-fold increase in the K values for VX-478 and saquinavir, respectively, compared with I50V. Moreover, the addition of the M46I and I47V mutations results in an increased catalytic efficiency for substrates 3 and 4.

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