Mutational Anatomy of an HIV-1 Protease Variant Conferring Cross-resistance to Protease Inhibitors in Clinical Trials

COMPENSATORY MODULATIONS OF BINDING AND ACTIVITY*

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Site-specific substitutions of as few as four amino acids (M46I/L63P/V82T/I84V) of the human immunodeficiency virus type 1 (HIV-1) protease engenders cross-resistance to a panel of protease inhibitors that are either in clinical trials or have recently been approved for HIV therapy (Condra, J. H., Schleif, W. A., Blahy, O. M., Gadryelski, L. J., Graham, D. J., Quintero, J. C., Rhodes, A., Robbins, H. L., Roth, E., Shivaprakash, M., Titus, D., Yang, T., Teppler, H., Squires, K. E., Deutsch, P. J., and Emini, E. A. (1995) Nature 374, 569–571). These four substitutions are among the prominent mutations found in primary HIV isolates obtained from patients undergoing therapy with several protease inhibitors. Two of these mutations (V82T/I84V) are located in, while the other two (M46I/L63P) are away from, the binding cleft of the enzyme. The functional role of these mutations has now been delineated in terms of their influence on the binding affinity and catalytic efficiency of the protease. We have found that the double substitutions of M46I and L63P do not affect binding but instead endow the enzyme with a catalytic efficiency significantly exceeding (110–360%) that of the wild-type enzyme. In contrast, the double substitutions of V82T and I84V are detrimental to the ability of the protease to bind and, thereby, to catalyze. When combined, the four amino acid replacements institute in the protease resistance against inhibitors and a significantly higher catalytic activity than one containing only mutations in its active site. The results suggest that in raising drug resistance, these four site-specific mutations of the protease are compensatory in function; those in the active site diminish equilibrium binding (by increasing $K_i$), and those away from the active site enhance catalysis (by increasing $k_{cat}/K_M$). This conclusion is further supported by energy estimates in that the Gibbs free energies of binding and catalysis for the quadruple mutant are quantitatively dictated by those of the double mutants.

Analyses of mutational effects in the human immunodeficiency virus type-1 (HIV-1) propruvirus have revealed that as few as four amino acid side chain substitutions, M46I/L63P/V82T/I84V (4X), in the protease suffice to yield a viral variant cross-resistant to a panel of protease inhibitors in clinical studies (1). Three of these inhibitors, Ritonavir (Ro 31–8959), Saquinavir (ABT-538), and Indinavir (MK-639), have been approved by the U. S. Federal Drug Administration as therapeutic agents for the treatment of HIV infection and AIDS. The 4X mutant protease is a poor enzyme. Two of its mutations, V82T and I84V, are located in the active site of the protease (see Fig. 1), and their perturbations of binding have been deduced from the x-ray structure (2); the V82T substitution introduces an unfavorable hydrophilic moiety for binding in the active site, and the I84V substitution creates a void (unoccupied by water) that should lead to a decrease in van der Waals contacts with the inhibitor. The combined contribution accounts for a total loss of $\sim$3 kcal/mol in energetics. The other two mutations are in the flap (M46I) and hinge (L63P) domains of the protease, and their role in drug resistance is not apparent from the crystallographic data. These changes only induce minor perturbations in the flap domain, as well as in the hinge region, of the native enzyme. One possibility is that the M46I and L63P substitutions affect the stability and/or activity of the enzyme unrelated directly to but ameliorate for the deleterious effect on equilibrium binding by the V82T/I84V mutations.

The 4X mutant is derived from clinical studies of AIDS patient viral isolates and is not an intermediate found in the path of emergence of HIV resistance, but it provides a model system with which we may be able to gain some understanding of HIV drug resistance at the molecular level. With the aid of a panel of inhibitors and peptide substrates, the functional roles of these four mutations have now been evaluated in terms of the binding affinity and catalytic efficiency of the two double mutants (M46I/L63P and V82T/I84V) that comprise the 4X protease. The results reveal that contributions of the two pairs of mutations toward the energetics of binding and catalysis are quantitatively countervailing, with those in the active site of the enzyme (the V82T/I84V substitutions) diminishing affinity for inhibitors and those away from the active site (the M46I/L63P replacements) enhancing catalysis. The coupled action of these or similar mutations could possibly be necessary for the production of viable, resistant HIV variants.

MATERIALS AND METHODS

Cloning and Expression—The pET-3b-HIV1Pr-4X plasmid containing the synthetic gene for the 4X mutant of the HIV-1 protease was constructed as described previously (2). The pET-3b-HIV1Pr-M46I/L63P plasmid was constructed by subcloning the pET-3b-HIV1Pr-4X KpnI/Aval fragment containing the M46I/L63P mutations into the corresponding sites of the wild-type (3) pET-3b-HIV1Pr plasmid. The pET-3b-HIV1Pr-V82T/I84V plasmid was constructed by subcloning the pET-

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$^1$ The abbreviations used are: HIV-1, human immunodeficiency virus type 1; 4X, resistant mutant HIV-1 protease containing the M46I/L63P/V82T/I84V substitutions; MεSO$_2$, dimethyl sulfoxide; DTT, dithiothreitol; HPLC, high performance liquid chromatography; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; SCX, strong cation exchange; GAG, precursor protein containing HIV non-envelope structural proteins; POL, precursor protein containing HIV protease, reverse transcriptase, and integrase.

$^2$ This estimate is in agreement with the 70-fold drop in binding affinity obtained from kinetic measurements (see Ref. 2 for details).
The 4X protease was expressed in a ratio of ~90% mature protease (11 kDa) to ~10% miniprecursor protease (14 kDa), as demonstrated previously for the wild-type enzyme (3). Cells were lysed in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2% Triton X-100, 0.001% lysozyme, 5 mM MgCl₂, 0.0001% Dnase I on ice. The mutant protease was extracted from inclusion bodies with solubilizing buffer (6 M guanidine HCl, 50 mM Tris, pH 8.0, 5 mM DTT). Insoluble particulates were removed with ultracentrifugation, and the supernatant was applied to a Sephacryl S-200 HR (Pharmacia Biotech Inc.) sizing column pre-equilibrated with solubilizing buffer. Elution was performed at 1.25 ml/min, and fractions containing the active protease were pooled and re-folded for 5 h at 4 °C by a 1–10-fold dilution in 50 mM MES, pH 5.5, 1 mM EDTA, 1 mM DTT, 10% glycerol, 5% ethylene glycol. The diluted sample was adjusted to 2 M NaCl and added batch-wise to Bakerbond Wide-Pore Hi-Propyl*C₃ resin for 1 h. The protease-bound resin was poured into an Econo-Column (Bio-Rad) and washed with 50 mM Tris-HCl, pH 7.5, 10% glycerol, 5% ethylene glycol. The diluted sample was adjusted to 25 °C. The unprocessed miniprecursor protease was precipitated with 2 M NaCl, washed with refold buffer, solubilized in 3 M acetic acid, and diluted to 0.05 mg/ml. The sample was stirred gently at pH 4.5 for 2 h to allow for conversion to the mature protease prior to its pH being readjusted to 5.5. The supernatant containing the mature form of the V82T/I84V protease was applied at a rate of 2.5 ml/min to an affinity column (Affi-Gel 10 Resin (Bio-Rad) coupled to the protease inhibitor L-734,353 (Kᵣ = ~11 nm)); the column was preequilibrated in equilibration buffer (50 mM sodium acetate, 10% glycerol, 1 mM EDTA, pH 5.5). The column was washed with 5 volumes of equilibration buffer containing 0.1% Triton X-100 followed by 10 volumes of equilibration buffer, 2.5 volumes of 50 mM Tris-Cl, pH 7.5, 10% glycerol, 0.01% Triton X-100, 1 mM EDTA, 1 mM DTT, and 2 volumes of equilibration buffer. The mutant protease was eluted from the column with 50 mM CAPS, 20% ethylene glycol, 5% glycerol, 1 mM EDTA, and 1 mM DTT at pH 10.5. Fractions were immediately neutralized to pH 5.5 with 3 M acetic acid, and those containing pure protease were pooled and concentrated in a CentriPrep prior to diluting in 3 volumes of pre-neutralized elution buffer containing no ethylene glycol. The homogeneity of the V82T/I84V mutant protease was determined to be >95% by N-terminal sequencing.

**Inhibitors**—The HIV-1 protease inhibitors Indinavir (MK-639) (4), Ritonavir (ABT-538) (5), Saquinavir (Ro 31–8959) (6), and Vertex VX-476 (7) were obtained from the Medicinal Chemistry Department of Merck Research Laboratories and dissolved in dimethyl sulfoxide (Me₂SO) to a stock concentration of 10 mM until use. The structures of the inhibitors are shown in Fig. 2.

**Substrates**—Eight peptides, mimicking the natural protease cleavage sites within the HXB-2 viral strain (8) of HIV-1, were synthesized and used in the activity assays. The inhibitors were synthesized with standard Merrifield solid state methodologies (9). Each peptide was synthesized as a 13-mer containing 7 and 6 residues on the amino and carboxyl terminus.
and carboxyl side of the scissile bond, respectively.

The cleavage sites of the HIV-1 polyproteins are shown schematically in Fig. 3. The substrates are designated as follows with their scissile bond marked by asterisks: I (MA/CA), NQVSQNY.PIVQNI; II (CA/p2), GKHARVL.AEMSQL; III (p2/NC), TNSATIM.MQRGNSF; IV (NC/p6), KGRPGNLF.QSRRPE; V (p6/PR), GTVSFN.PQVTLL; VI (PR/RT), IGCTLNFL.PSPIE; VII (RT/IN), AGIRKVL.FLDGID.

Substrates I–VII were solubilized in MeSO to a concentration of 25 mg/ml (12.53–15.65 mM). Substrates VII and VIII were initially solubilized in MeSO at 12.5 mg/ml; the solubility of these peptides was then increased to 25 mg/ml with additional powder peptides and flash-heating in boiling water for 5 s and immediately returned to room temperature. To restrict the final MeSO concentration in the enzyme assays to ~4%, the maximum assay concentration of substrate was limited to the following: I, 352 μM; II, 322 μM; III and VI, 358 μM; IV, 913 μM; V, 367 μM; VII, 391 μM; VIII, 343 μM.

Enzymic Assays—The catalytic activities of the wild-type and mutant HIV-1 proteases were measured with peptide hydrolysis assays (10, 11). Assays were performed at 30 °C and pH 5.5 in 50 mM sodium acetate, 0.1% bovine serum albumin, and MeSO (at 3.75% for inhibition assays and at 2.5% for other assays). Reactions were initiated by the addition of enzyme following a 5-min pre-incubation at 30 °C and were quenched by the addition of phosphoric acid to 4% (v/v). The appearance of products and the corresponding loss of substrate were monitored with ultraviolet absorbance at 225 nm on a 5-cm C-18 reversed phase HPLC column (Vydac). Gradients employing 0.1% phosphoric acid and acetonitrile were developed and optimized for separation of each substrate and its corresponding products (see figure and table legends for details).

All substrates and their hydrolyzed products were stable in assay buffer and 4% phosphoric acid at 25 °C for 24 h with the exception of V. At room temperature, the hydrolyzed products of V precipitated in assay buffer and 4% phosphoric acid within 15 min, while at 4 °C, there was no detectable precipitation for ~24 h. Consequently, samples containing V and its products were placed at 4 °C immediately after quenching and were incubated at the same temperature in a chilled auto-sampler until just prior to HPLC injection.

Steady-state and initial velocity conditions were established and strictly observed in the reactions catalyzed by the wild-type and mutant enzymes for every peptide substrate. The conditions employed are given in the legend of Table I. The kinetic parameters of each competitive inhibitor in steady-state turnover assays of I, or the derivative of I (VSQN-(β-naphthylalanine)-PIV), catalyzed by each protease, under the condition that [S] ≪ [Km] were determined from nonlinear least-squares fits of initial velocity data to the Michaelis-Menten equation when substrate saturation of the enzyme was achieved. When saturation was not achieved; the value of kcat/Km was obtained from least-squares fits of the linear slope of plots of initial velocity as a function of substrate concentration at [S] ≪ [Km].

The values of K were obtained for each competitive inhibitor in steady-state turnover assays of I, or the derivative of I (VSQN-(β-naphthylalanine)-PIV), catalyzed by each protease, under the condition that [S] ≪ [Km]. Reactions were initiated in the presence of inhibitor by addition of a protease, at a concentration at least less than one-half the K value, and were allowed to proceed at 30 °C for 1 h. At least three different concentrations of each inhibitor were employed to determine K with use of Equation 1:

$$K = \left[\frac{1}{k_{cat}/K_m + k_{cat}/K_m - 1}\right]$$

(Eq. 1)

where [I] is the free inhibitor concentration in equilibrium with enzyme, (kcat/Km), and (kcat/Km), are the apparent substrate specificity of the enzyme in the absence and presence of a competitive inhibitor, respectively.

RESULTS

Stability of Mutant HIV-1 Proteases—All three purified enzymes, the 4X, the V82T/I84V, and the M46I/L63P proteases, are stable homodimers in the 5–375 mM concentration range, as is the wild-type protease (11). We have found that there is no detectable dissociation of these dimers into their monomeric subunits during catalysis, either in the presence or absence of inhibitors; the specificity of each protease is independent of enzyme concentration.

Hydrolysis of the Eight Peptide Substrates—Fig. 4 shows the saturation curves of the wild-type HIV-1 protease and its 4X mutant in their catalysis of steady-state hydrolysis of peptides representing two of the eight cleavage sites in the GAG-POL polyproteins. One (VII) is saturating while the other (VIII) is not. The saturation curves for the other six substrates are similar, with characteristics as described below, and are not shown separately here. Bound by solubility varying from 60 to 300 μM in a reaction buffer containing 3.75% MeSO, only substrates III and VII are saturating for the wild-type, the 4X, and the M46I/L63P proteases. None is saturating for the V82T/I84V enzyme. See “Results” and legend of Table I for details.

![Fig. 3. A schematic view of the HIV-1 GAG-POL polyproteins depicting the eight processing sites.](Image 82x683 to 264x729)

![Fig. 4. Saturation curves of the wild-type (open squares) HIV-1 protease and its resistance-conferring 4X point mutant (open circles) as obtained from steady-state turnover of substrates VII (A) and VIII (B) at 30 °C and pH 5.5 in 50 mM sodium acetate.](Image 315x429 to 547x729)
TABLE I

HIV Protease Resistance

The pseudo second-order rate constants (k_{cat}/K_{M}) for the hydrolysis of eight peptides representing the GAG-POL polypeptides processing sites as catalyzed by the HIV-1 protease and its resistance-conferring mutants

Each 13-residue peptide substrate contains a scissile bond as designated by the asterisk 7 amino acids from its N terminus. The values of k_{cat}/K_{M} are obtained from multiple runs (n > 3) of substrate saturation, for each peptide and enzyme, conducted under steady-state and initial velocity conditions. The overall standard error of the mean is 10%. For II, IV, and VI, where detector sensitivity and/or difficult product peak separation were limiting, the error is in the 12–14% range. The conditions employed to obtain steady-state and initial velocity kinetic data are as follows: I, 2 nm wild-type or M46L/L63P protease in a 10-min reaction, 300 nm 4X or V82T/I84V protease in a 2-min reaction; II, 100 nm wild-type or M46L/L63P protease, 250 nm 4X or V82T/I84V protease, all in a 6-min reaction; III, 150 nm wild-type or 300 nm 4X protease in 10-min reaction, 200 nm V82T/I84V or 10 nm M46L/L63P protease in a 4-min reaction; IV, 150 nm wild-type, 100 nm M46L/L63P protease, 300 nm 4X, or 300 nm V82T/I84V protease in a 16-min reaction; V, 2 nm wild-type or 5 nm M46L/L63P protease in a 2-min reaction, 150 nm 4X or 150 nm V82T/I84V protease in a 1-min reaction; VI, 25 nm wild-type or M46L/L63P protease, 250 nm 4X or V82T/I84V protease, all in an 8-min reaction; VII, a 10- and 9-min reaction, respectively, with 75 nm wild-type and 50 nm M46L/L63P proteases, and a 30-min reaction with the 4X or V82T/I84V protease at 300 nm.

Cleavage site  | Peptide sequence | Wild-type  | 4X | M46L/L63P | V82T/I84V
--- | --- | --- | --- | --- | ---
I | MA(p17)/CA(p24) | NQVSQNY+P1VQNI | 17,980 | 940 | 65,100 | 830
II | CA(p2) | GKHARVL+AEAMSQ | 750 | 310 | 790 | 220
III | gp2NC(p7) | TNSATM+MQRGNF | 27,970 | 3,440 | 76,300 | 1,770
IV | NC(p6) | KGPRGNF*+LQSRPE | 370 | 140 | 490 | 43
V | p6*PR | GTVSFNF*+PQVTWL | 113,400 | 6,810 | 221,400 | 3,760
VI | PRRT | ICGTINF*+P1S1PE | 8,930 | 430 | 14,600 | 300
VII | RT (internal) | IVGAEFT*+VDGAA | 11,400 | 227 | 14,160 | 130
VIII | RT/IN | AG15KVFL+P1DGD1 | 845 | 58 | 1,550 | 38

Our observed specificity (k_{cat}/K_{M}) is similar in magnitude to those reported, 10^{2} to 10^{3} m^{-1} s^{-1}, by Tölszer et al. (12) whose assay buffer contains 7.5% glycerol and 2 m NaCl. The notable difference is that their observed apparent K_{M} values are smaller than ours. One possible reason is that the high ionic strength of their buffer may increase the binding affinity of their substrates that are different in length (8–11 residues) from ours. A second possibility is that our longer substrates intrinsically possess higher K_{M} values. We have noted that the apparent affinity of HIV-1 protease inhibitors is substantially increased by buffer ionic strength (P. Darke and L. Kuo, unpublished data).

5 Similar observations have been reported previously for peptides of different lengths assayed under different conditions (12, 15, 16).

6 By determining the accessibility of the protease to the various cleavage sites, Petit et al. (18) have suggested that conformation of the polyprotein precursor is a limiting factor in the order of proteolytic processing.

DISCUSSION

Characteristics of the Peptide Substrates—There is no clear classification of the sequences representing the eight cleavage sites of the GAG-POL polypeptides of HIV. Most researchers consider these sites to be represented by two types of substrates, those containing Tyr/Phe-Pro and those containing other hydrophobic-hydrophobic residues at the P1–P1′ positions (13, 14). Bulky aromatic residues are not found in the P2 and P2′ positions. We have attempted to mimic better the natural protease substrates and to make comparison as direct and relevant as possible, by increasing the length of our peptides to 13 residues for all eight cleavage sites, with each peptide containing 7 residues on its N-terminal side (P-sites) of the scissile bond. Low solubility (<400 μm) is a severe limitation that discourages us from further increasing the length of these peptides.

The HIV-1 protease specificity for the eight substrates varies enormously and bears no obvious structure-activity relationship (Table I). In particular, there is no apparent specificity dependence on the identities of the immediate residues linked by the scissile bond. Under our assay conditions, peptide V is the fastest substrate (10^{6} m^{-1} s^{-1}), and peptides II, IV, and VIII are the slowest (<10^{5} m^{-1} s^{-1}). Interestingly, all three slowest substrates have a leucine at either the P1 or P1′ position. On the other hand, both the fastest (V) and the slowest (IV) peptides contain phenylalanine in the P1 and asparagine in the P2 sites. Also, both peptides V and VI contain phenylalanine, asparagine, and proline in the P2, P1, and P1′ sites, respectively, but the k_{cat}/K_{M} value for VI is only ~8% that of V. These results are consistent with the suggestion (13, 17) that specificity of the HIV protease substrates is dictated to a significant extent by sequence composition distal from the immediate surroundings of the scissile bond.

We note that although our substrates are of equal length and span well beyond the entire active site cleft of the protease, the k_{cat}/K_{M} values reported here do not necessarily reflect the precise in vivo specificity of polyprotein processing since short oligopeptides in general possess little or no definable secondary structure. Reviews of HIV protease substrates with natural and substitutional sequences of the GAG-POL polypeptides have been presented by Dunn et al. (19) and by Tomasselli and Heinrikson (20).

Effect of Point Mutations of HIV-1 Protease on Catalysis—

There is no clear classification of the sequences representing the eight cleavage sites of the GAG-POL proteolytic enzymes of HIV. Most researchers consider these sites to be represented by two types of substrates, those containing Tyr/Phe-Pro and those containing other hydrophobic-hydrophobic residues at the P1–P1′ positions (13, 14). Bulky aromatic residues are not found in the P2 and P2′ positions. We have attempted to mimic better the natural protease substrates and to make comparison as direct and relevant as possible, by increasing the length of our peptides to 13 residues for all eight cleavage sites, with each peptide containing 7 residues on its N-terminal side (P-sites) of the scissile bond. Low solubility (<400 μm) is a severe limitation that discourages us from further increasing the length of these peptides.

The HIV-1 protease specificity for the eight substrates varies enormously and bears no obvious structure-activity relationship (Table I). In particular, there is no apparent specificity dependence on the identities of the immediate residues linked by the scissile bond. Under our assay conditions, peptide V is the fastest substrate (~10^{6} m^{-1} s^{-1}), and peptides II, IV, and VIII are the slowest (~10^{5} m^{-1} s^{-1}). Interestingly, all three slowest substrates have a leucine at either the P1 or P1′ position. On the other hand, both the fastest (V) and the slowest (IV) peptides contain phenylalanine in the P1 and asparagine in the P2 sites. Also, both peptides V and VI contain phenylalanine, asparagine, and proline in the P2, P1, and P1′ sites, respectively, but the k_{cat}/K_{M} value for VI is only ~8% that of V. These results are consistent with the suggestion (13, 17) that specificity of the HIV protease substrates is dictated to a significant extent by sequence composition distal from the immediate surroundings of the scissile bond.

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Effect of Point Mutations of HIV-1 Protease on Catalysis—
The $K_v$ values are obtained with use of Equation 1 as described under “Materials and Methods” employing substrate turnover assays under steady-state condition as catalyzed by each protease at $[S] \ll K_M$ in the presence of an inhibitor. Each value is the average of three determinations with a standard error of the mean of 17%.

| Inhibitor | Wild-type | 4X | M46I/L63P | V82T/I84V |
|-----------|-----------|----|-----------|-----------|
| MK-639    | 0.40      | 21.6 | 0.47      | 23.7      |
| ABT-538   | 0.19      | 28.5 | 0.15      | 30.1      |
| Ro 31–8959 | 0.33     | 11.9 | 0.37      | 13.3      |
| VX-478    | 0.57      | 5.8  | 0.53      | 10.1      |

The values of $k_{cat}/K_M$ for the eight GAG-POL peptides are affected by the 4X mutations to an extent that reflects a substantial loss of enzymic efficiency. It is seen from Table I that the pseudo second-order cleavage rates for substrates I and V–VIII are reduced to <7% of the wild-type values, whereas those for II, III, and IV to 12–43%. Despite the drastic drop of protease efficiency, the HIV-1 virion containing the 4X protease is a viable virus (1) suggesting that the action of the wild-type protease is not rate-limiting in the replication of the wild-type virion. Similar observations have been made that certain protease is not rate-limiting in the replication of the wild-type HIV-1; however, peptides reveal that the M46I/L63P mutations ameliorate the deleterious effect of the V82T/I84V mutations on catalysis.8 Taken together, the paired V82T/I84V mutations are introduced; however, peptides I, III, and V are invariably the fastest substrates while II, IV, and VIII are the slowest.

The loss in catalytic prowess introduced by the quadruple mutations can be attributed to the V82T and I84V substitutions. Each of the pseudo second-order rate constants shown in Table I for the wild type is decreased when mutations are restricted to the Met-46 and Leu-63 sites. When compared head to head, the specificity of the V82T/I84V protease is 12–70% that of the 4X protease when mutations are restricted only to the active site. On the other hand, each of the rate constants is increased over that seen for the wild-type protease when mutations are restricted to the Met-46 and Leu-63 sites. When compared head to head, the specificity of the V82T/I84V protease is 12–70% that of the 4X protease when mutations are restricted only to the active site. On the other hand, each of the rate constants is increased over that seen for the wild-type protease when mutations are restricted to the Met-46 and Leu-63 sites. When compared head to head, the specificity of the V82T/I84V protease is 12–70% that of the 4X protease when mutations are restricted only to the active site. On the other hand, each of the rate constants is increased over that seen for the wild-type protease when mutations are restricted to the Met-46 and Leu-63 sites. When compared head to head, the specificity of the V82T/I84V protease is 12–70% that of the 4X protease when mutations are restricted only to the active site. On the other hand, each of the rate constants is increased over that seen for the wild-type protease when mutations are restricted to the Met-46 and Leu-63 sites. When compared head to head, the specificity of the V82T/I84V protease is 12–70% that of the 4X protease when mutations are restricted only to the active site. On the other hand, each of the rate constants is increased over that seen for the wild-type protease when mutations are restricted to the Met-46 and Leu-63 sites. When compared head to head, the specificity of the V82T/I84V protease is 12–70% that of the 4X protease when mutations are restricted only to the active site. On the other hand, each of the rate constants is increased over that seen for the wild-type protease when mutations are restricted to the Met-46 and Leu-63 sites. When compared head to head, the specificity of the V82T/I84V protease is 12–70% that of the 4X protease when mutations are restricted only to the active site.

The ad-
HIV-1 Protease Resistance

It would appear that to circumvent the undue detrimental effect of point mutations in the protease active site, the HIV-1 virus is capable of selecting point mutations elsewhere to specifically stabilize transition state binding (i.e. ES$^1$ in the excited state) in productive reactions but not inhibitor binding in nonproductive reactions. The combined effect should then be desirable for the emergence of viable viruses that are drug-resistant. The virus containing the 4X mutant is resistant to the protease inhibitors with CIC$_{50}$ values 8–15-fold increased over the wild-type values (1).

Although the functional role of the double M46I/L63P replacements is apparent from the data presented, the structural alterations due to these mutations are not. The root mean square deviation for all 198 C$_\alpha$ atoms between the 4X and the wild-type proteases is 0.5 Å in the native and 0.2 Å in the Indinavir-bound form (2). Therefore, the structural changes due to these two mutations are undetectable with x-ray crystallography, they are either too small (less than 0.2–0.5 Å in bond length) or they provide structural flexibility essential for chemistry but not for binding.

Other Biochemical Studies of HIV-1 Protease Mutants—Studies of an enzymic nature have been conducted on HIV resistance to the protease inhibitors. The majority of these studies document kinetic analysis of substrate turnover and inhibitor affinity for protease mutants containing either single, double, or multiple amino acid changes (21, 28–37). In general, impairments of catalysis and binding are found for mutations in the active site of the protease including the Val-82 and Ile-84 residues. Compensatory actions due to mutations of nonactive site residues of the protease have also been suggested but not demonstrated (21, 30, 32, 33, 36, 37). The M46I mutation, whether alone or combined with an active site mutation (21, 36, 37), generally exhibits a minimal effect on binding ($K_i$) and catalysis ($k_{cat}/K_m$) that is either compensatory or deleterious; however, in a few cases, a more significant effect has been observed. To our knowledge, the L63P mutation has not been analyzed.

HIV Drug Resistance and the Protease Inhibitor—The most distinguishing feature of the HIV-1 virus as an infectious agent lies in its ability to replicate at a remarkably high rate. It has been shown that the half-life for virus clearance from the plasma and corresponding elimination of virus-producing T cells is around 2 days, and the plasma population of virus is completely replaced within 2 weeks (38, 39). It is this high replication rate that allows the accumulation of a wide array of genetic variants in the virus population of a single individual (40). Selectivity is based not only upon resistance but also upon the viral replication rate. A variant that is highly resistant to a particular inhibitor yet is slow in its ability to process the precursor polyproteins to their corresponding mature and functional forms will inevitably show poor replication kinetics and be less likely to emerge as the dominant genotype than a variant that is capable of both rapid catalysis and significant resistance. It follows then that prior to drug treatment, a single individual’s virus population may contain numerous variants, whereas the basic wild-type genome is predominant. It has been shown in sequencing studies of clinical isolates that prior to drug treatment, the amino acid sequence of the protease is conserved and varies minimally from the sequences of known HIV-1 strains; variances at residues 37 and 63 appear to be common among isolates, but mutations to residues in the active site are not in the majority (41, 42).

When faced with selective pressure of an inhibitor, some 20 of the 99 amino acid residues of the protease undergo mutations (43, 44). The mutations that the virus selects in the protease, as anticipated, appear to be inhibitor-specific and dependent upon particular interactions of the inhibitor with the subsites of the enzyme. However, most of the combinations in the protease, containing a constellation of as many as 10 or more residues, encompass a set of mutations in the active site and another set of mutations located in other regions of the protease (1, 21, 30, 32, 45, 46). Common active site mutations include changes at residues 8, 50, 82, and 84. Mutations to the flap region include changes at 46, 48, 50, and 54. While changes to residues 48 and 50 have been shown to contribute to resistance (21, 45, 46), changes to residue 46 appear to play a compensatory role for other deleterious mutations in vitro (21, 30, 32, 46). Along with previous studies of site-specific mutants of the HIV-1 protease (21, 28–37), the action of the 4X protease in mounting resistance as delineated in this report provides a starting point toward our understanding of the molecular basis of HIV resistance against protease inhibitors.

The 4X mutations are but just one set of a minimal, base-line mutation profile found to confer cross-resistance to a panel of potent protease inhibitors. The resultant protease variant is not found to be an intermediate in the path of emergence of HIV resistance. It is thus not surprising that the 4X protease remains a poor enzyme and that the compensatory effect of the M46I/L63P mutations is inadequate to overcome entirely the deleterious effect of the V82T/I84V mutations. For the same reason, the clear-cut role of the point mutations in the quadruple mutant is not expected to be duplicated in clinical variants that are true intermediates in the path of the virus engendering resistance against the protease inhibitor. It is expected that mutations of the GAG-POL cleavage sites can also affect the rate and extent of HIV polyprotein processing (33, 47). Furthermore, mutations of a gene arise for viral fitness to replicate under selection pressure and are likely to have effects on both the properties of the gene product of the mutated gene itself (protein function, inhibitor binding, etc.) and the properties of viral genes/proteins unrelated to the mutated gene (RNA stability, codon usage, polyprotein folding/processing, etc.).

11 For example, Taddeo et al. (48) have shown that two point mutations in HIV-1 integrase (S81 and P109) block viral DNA integration and also lead to production of progeny viral particles that exhibit reduced reverse transcriptase activity.
HIV-1 Protease Resistance

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the action of the protease. Nevertheless, the general conclusion drawn from the data presented here is likely extensible to clinical resistant HIV proteases evolved from a sustained selection pressure of a protease inhibitor. Indeed, a comparison of several HIV-1 protease variants, identified (1) from viral isolates of patients participating in clinical studies of Indinavir and containing all or some of these substitutions, reveals that all are resistant and all are (from being mildly to drastically) more effective catalysts than the V82T/I84V protease which in and by itself may suffice as a resistant but perhaps nonviable variant.12

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