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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)[1] provirus 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 ~3 kcal/mol in energetics.[2] 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 *Kpn*I/*Ava*I 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; Me_2SO, 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).

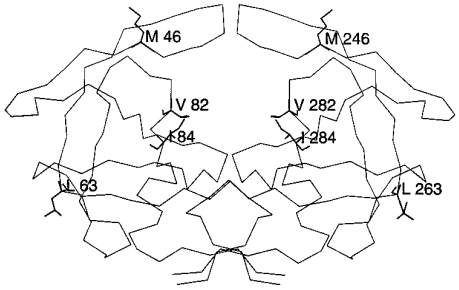

FIG. 1. **C_α structure of the homodimeric 4X mutant of the HIV-1 protease.** The four substitutions are labeled for both subunits of the protease. Residues Thr-82 and Val-84 are located in the active site of the enzyme within direct bonding distances from substrates and inhibitors. Residues Met-46 and Leu-63 are situated in the flap and hinge domains of the protease, respectively. See Ref. 2 for structural details.

3b-HIV1Pr *Kpn*I/*Ava*I fragment containing the wild-type protease sequence into the corresponding sites of the pET-3b-HIV1Pr-4X plasmid. Sequence-verified clones were transformed as described (2). Host cells were grown to an optical density of 0.4 to 0.6 at 600 nm in Luria-Burtani broth containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol and induced with 400 μM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Cells were pelleted and stored at −70 °C until use.

Enzyme Purification—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, 0.02% Triton X-100, 0.001% lysozyme, 5 mM $MgCl_2$, 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 3 h. The protease-bound resin was poured into an Econo-Column (Bio-Rad) and washed with 50 mM sodium acetate at pH 5.5, 1 mM DTT, 1 mM EDTA, 2 M NaCl, 10% glycerol, 5% ethylene glycol. The protease was then eluted with the same buffer without NaCl. Fractions containing active protease were pooled and adjusted to <70 mM NaCl by dilution into a solution of 10% glycerol, 5% ethylene glycol, 1 mM DTT, 1 mM EDTA. The diluted sample was filtered prior to loading onto a polysulfonethyl strong cation exchange (SCX) column (The Nest Group). Protease was eluted from the SCX column in 50 mM MES, pH 6.0, 1 mM DTT, 1 mM EDTA, 10% glycerol, 5% ethylene glycol with a gradient to 650 mM NaCl. Fractions containing the enzyme were concentrated in a CentriPrep device (Amicon) to a final volume of 8 ml and loaded on an affinity column of Pepstatin Agarose Resin (Sigma). The protease was eluted at pH 5.5. The purest fractions were pooled and concentrated with a final yield of 7 mg of protease per liter of induction culture. Both concentration and homogeneity of the purified enzyme were confirmed by amino acid analysis and N-terminal sequencing.

The M46I/L63P mutant was expressed as described above and extracted from inclusion bodies in 8 M urea, 10 mM Tris-HCl, pH 7.5, 10 mM DTT and refolded at room temperature by a 1–20-fold dilution into refold buffer (50 mM sodium acetate, 1 mM DTT, 1 mM EDTA, 10% glycerol, 5% ethylene glycol) at pH 3.7. After 3 h, the pH of the refold sample was adjusted to 5.5 prior to its chromatography on an SCX column and a pepstatin-agarose column as described above. The M46I/L63P mutant protease was eluted from the pepstatin column at pH 3.5. The purified protease was concentrated for a final yield of 0.4 mg of protease per liter of induction culture; its homogeneity and concentration were determined by amino acid analysis and N-terminal sequencing.

The V82T/I84V mutant was expressed in ratios of 5% mature protease to 95% miniprecursor. The protease was dissolved and chromatographed on a Sephacryl S-200 HR column as described. The fractions containing protease, as determined by SDS-polyacrylamide electrophoresis, were pooled and diluted 100-fold in refold buffer at pH 4.5 and

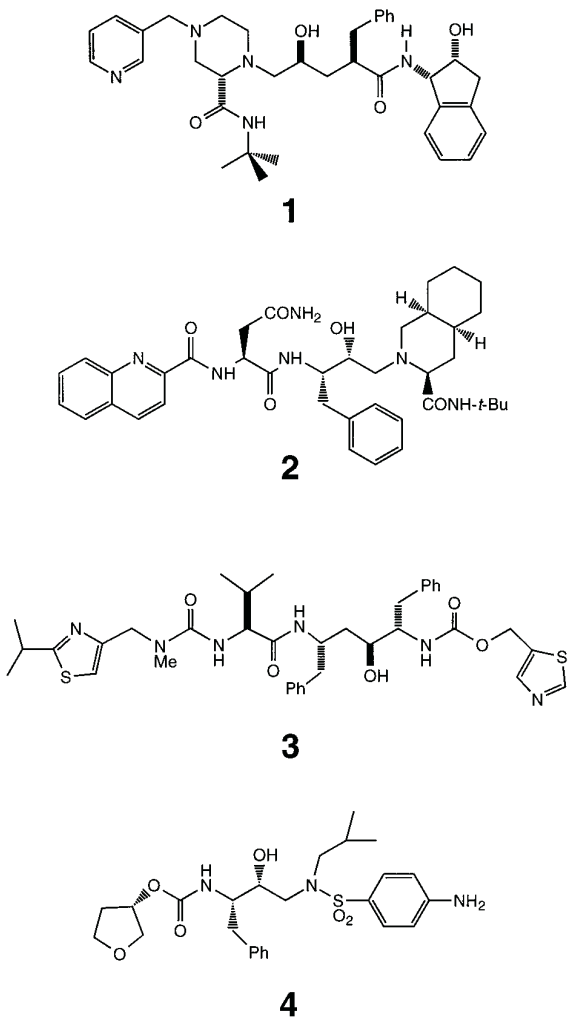

FIG. 2. **Chemical structures of: *1*, Indinavir (*MK-639*); *2*, Saquinavir (*Ro 31–8959*); *3*, Ritonavir (*ABT-538*); and *4*, Vertex (*VX-478*).**

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 (K_i ~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-HCl, 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-478 (7) were obtained from the Medicinal Chemistry Department of Merck Research Laboratories and dissolved in dimethyl sulfoxide (Me_2SO) 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 with standard Merrifield solid state methodologies (9). Each peptide was synthesized as a 13-mer containing 7 and 6 residues on the amino

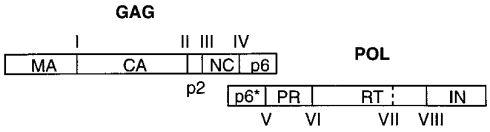

FIG. 3. **A schematic view of the HIV-1 GAG-POL polyproteins depicting the eight processing sites.**

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[3] as follows with their scissile bond marked by asterisks: *I* (MA/CA), NQVSQNY*PIVQNI; *II* (CA/p2), GHKARVL*AEAMSQ; *III* (p2/NC), TNSATIM*MQRGNF; *IV* (NC/p6), KGRPGNF*LQSRPE; *V* (p6*/PR), GTVSFNF*PQVTLW; *VI* (PR/RT), IGCTLNF*PISPIE; *VII* (RT-internal), IVGAETF*YVDGAA; *VIII* (RT/IN), AGIRKVL*FLDGID.

Substrates *I–VI* were solubilized in Me_2SO to a concentration of 25 mg/ml (12.53–15.65 mM). Substrates *VII* and *VIII* were initially solubilized in Me_2SO 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 Me_2SO concentration in the enzymic 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*, 313 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 Me_2SO (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 k_{cat} and K_M 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 k_{cat}/K_M was obtained from least-squares fits of the linear slope of plots of initial velocity as a function of substrate concentration at $[S] \ll K_M$.

The values of K_i 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 $[S] \ll K_M$. Reactions were initiated in the presence of inhibitor by addition of a protease, at a concentration at least less than one-half the K_i 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_i with use of Equation 1:

$$K_i = [I]_f \cdot \left\{ \frac{1}{(k_{cat}/K_M)_{-i} \div (k_{cat}/K_M)_{+i} - 1} \right\}, \qquad \text{(Eq. 1)}$$

where $[I]_f$ is the free inhibitor concentration in equilibrium with enzyme, $(k_{cat}/K_M)_{-i}$ and $(k_{cat}/K_M)_{+i}$ 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 nM 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.[4]

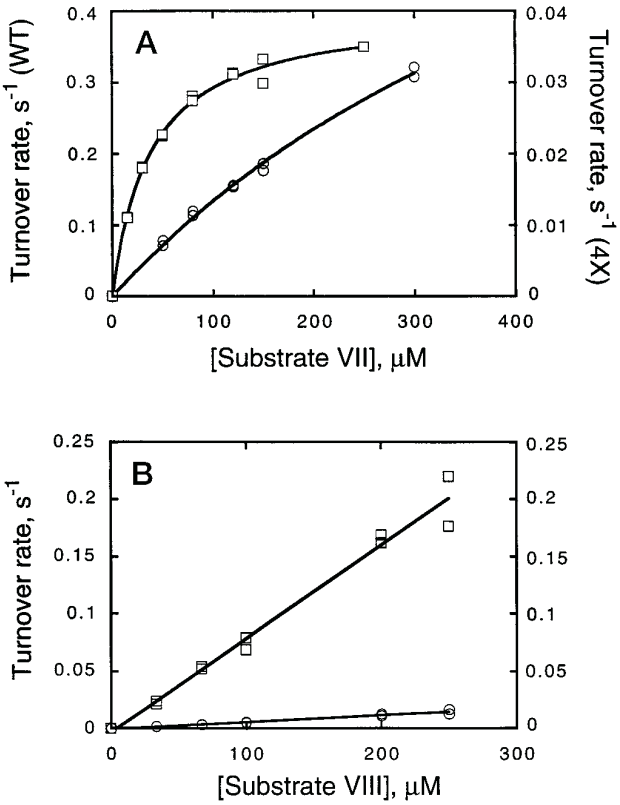

FIG. 4. **Saturation curves of the wild-type (*open squares*) HIV-1 protease and its resistance-conferring 4X point mutant (*open circles*) as obtained from steady-state turnover of *substrates VII* (*A*) and *VIII* (*B*) at 30 °C and pH 5.5 in 50 mM sodium acetate.** The saturation curves for the *substrates I–VI* are similar to those shown here. Bound by substrate solubility, only *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.

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 peptides 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% Me_2SO, 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 protease. For *III* and *VII*, the K_M values are 0.25 and 0.065 mM for the wild-type protease, 0.94 and 0.33 mM for the 4X mutant, and 0.05 and 0.043 mM for the M46I/L63P mutant, respectively. For the other substrates, K_M is likely to be in the very high micromolar range for the majority of the peptides (based on

[3] Amino acid residues are designated with the single-letter code.

[4] We note that for some point mutants of the HIV-1 protease an increase in the equilibrium constant has been observed (H. Schock and L. Kuo, unpublished data) for dissociation of the homodimer. For these mutants, dissociation of the protease subunits needs be accounted for (11) in the calculations of kinetic and equilibrium constants. Hence, analyses of the kinetic behavior of a mutant HIV-1 protease should begin with an analysis of its dimer stability.

TABLE I

The pseudo second-order rate constants (k_{cat}/K_M) for the hydrolysis of eight peptides representing the GAG-POL polyproteins 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 M46I/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 M46I/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 M46I/L63P protease in a 4-min reaction; *IV,* 150 nM wild-type, 100 nM M46I/L63P protease, 300 nM 4X, or 300 nM V82T/I84V protease in a 16-min reaction; *V,* 2 nM wild-type or 5 nM M46I/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 M46I/L63P protease in a 5.5-min reaction, 150 nM 4X or V82T/I84V protease in a 12-min reaction; *VII,* 25 nM wild-type or M46I/L63P protease, 250 nM 4X or V82T/I84V protease, all in an 8-min reaction; *VIII,* a 10- and 9-min reaction, respectively, with 75 nM wild-type and 50 nM M46I/L63P proteases, and a 30-min reaction with the 4X or V82T/I84V protease at 300 nM.

Cleavage site	Peptide sequence	Wild-type	4X	M46I/L63P	V82T/I84V
		$M^{-1}\ s^{-1}$	$M^{-1}\ s^{-1}$	$M^{-1}\ s^{-1}$	$M^{-1}\ s^{-1}$
I. MA(p17)/CA(p24)	NQVSQNY*PIVQNI	17,980	940	65,100	830
II. CA/p2	GHKARVL*AEAMSQ	720	310	790	220
III. p2/NC(p7)	TNSATIM*MQRGNF	27,970	3,440	76,300	1,770
IV. NC/p6	KGRPGNF*LQSRPE	370	140	490	43
V. p6*/PR	GTVSFNF*PQVTLW	113,400	6,810	221,400	3,760
VI. PR/RT	IGCTLNF*PISPIE	8,930	430	14,600	300
VII. RT (internal)	IVGAETF*YVDGAA	11,400	227	14,160	130
VIII. RT/IN	AGIRKVL*FLDGID	845	58	1,850	38

estimates with use of the double-reciprocal plots) so that accurate values are determinable only for k_{cat}/K_M measured from the linear increase of initial velocity with substrate concentration at [S] $\ll K_M$.

Catalytic Efficiency of HIV-1 Protease and Its Mutants—Table I compares the values of k_{cat}/K_M observed for the hydrolysis of the eight cleavage site peptides catalyzed by the HIV-1 protease and its three variants. It can be seen that k_{cat}/K_M varies drastically for these substrates. For the wild-type enzyme, the k_{cat}/K_M values range from <400 (*IV*) to ≥100,000 M^{-1} s^{-1} (*V*).[5] For the 4X mutant, the k_{cat}/K_M values are significantly smaller ranging from ~60 (*VIII*) to ~6,800 M^{-1} s^{-1} (*V*). These values are further diminished for all of the substrates by an additional 12–70% when mutational substitutions are restricted to V82T and I84V. In contrast, the catalytic efficiency of the M46I/L63P mutant is unexpectedly found to be greater than that of the wild-type enzyme for every substrate by 110–360%.

Binding Affinities of HIV-1 Protease and Its Mutants—Table II lists the binding constants (K_i) of the four potent HIV protease inhibitors (Fig. 2) for the wild-type and each of the mutant HIV proteases. Each of these inhibitors is currently being evaluated in various phases of clinical studies for antiviral effect. For the wild-type protease, all of these inhibitors display subnanomolar binding affinity in our low-salt (50 mM sodium acetate) reaction buffer. For the mutants, the K_i values are increased by the 4X and the V82T/I84V but not the M46I/L63P substitutions.

DISCUSSION

Characteristics of the Peptide Substrates—There is no clear classification of the sequences representing the eight cleavage sites of the GAG-POL polyproteins 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.[6] Under our assay conditions, peptide *V* is the fastest substrate (~10^5 M^{-1} s^{-1}), and peptides *II*, *IV*, and *VIII* are the slowest (<10^3 M^{-1} s^{-1}). Interestingly, all three slow 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.[7] Reviews of HIV protease substrates with natural and substituted sequences of the GAG-POL polyproteins have been presented by Dunn *et al.* (19) and by Tomasselli and Heinrikson (20).

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

[5] Our observed specificity (k_{cat}/K_M) is similar in magnitude to those reported, 10^3 to 10^5 M^{-1} s^{-1}, by Tozser *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).

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

[7] 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.

TABLE II

Dissociation constants (K_i) of four potent protease inhibitors as obtained from their inhibition of the hydrolytic reactions catalyzed by the HIV-1 protease and its resistance-conferring mutants.

The K_i 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
	nM	*nM*	*nM*	*nM*
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 mutations lead to relatively large catalytic defects without apparently being rate-limiting to the replication of the HIV (21–23). Table I also reveals that none of the peptides maintains an unvarying k_{cat}/K_M value when the double or quadruple 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 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 revealing that the M46I/L63P mutations ameliorate the deleterious effect of the V82T/I84V mutations on catalysis.[8] Taken together, the paired V82T/I84V and M46I/L63P mutations are apparently countervailing in the catalytic action of the 4X protease when viewed with a spectrum of substrates.

A more rigorous examination of the functional role of the two paired mutations can be made in terms of reaction energetics. The activation energy ($\Delta G_T^{\ddagger}$) for substrate-to-product turnover catalyzed by the protease may be calculated (24) with use of Equation 2 and the pseudo second-order rate constant:

$$\Delta G_T^{\ddagger} = -\left\{RT \cdot \ln[k_{cat}/K_M] + RT \cdot \ln\left[\frac{k_B T}{h}\right]\right\}, \qquad \text{(Eq. 2)}$$

where R is the gas constant, T is the temperature of the reaction in Kelvin, k_B is the Boltzmann's constant, and h is the Planck's constant. For the action of the two paired mutations to be compensatory, the $\Delta G_T^{\ddagger}$ of the reaction catalyzed by the 4X protease should be dictated by those of the reactions catalyzed separately by the double mutants. In other words, the change in activation energy ($\Delta\Delta G_T^{\ddagger}$) for substrate turnover upon alterations of the wild-type protease to the 4X variant should be equal to the sum of the $\Delta\Delta G_T^{\ddagger}$ for the same reaction catalyzed by the V82T/I84V and M46I/L63P mutants. Thus,

$$[\Delta\Delta G_T^{\ddagger}]^{4X} = [\Delta\Delta G_T^{\ddagger}]^{46/63} + [\Delta\Delta G_T^{\ddagger}]^{82/84}, \qquad \text{(Eq. 3)}$$

or

$$\{[\Delta G_T^{\ddagger}]^{4X} - [\Delta G_T^{\ddagger}]^{WT}\} = \{[\Delta G_T^{\ddagger}]^{46/63} - [\Delta G_T^{\ddagger}]^{WT}\} + \{[\Delta G_T^{\ddagger}]^{82/84} - [\Delta G_T^{\ddagger}]^{WT}\}, \qquad \text{(Eq. 4)}$$

or

$$[\Delta G_T^{\ddagger}]^{4X} = [\Delta G_T^{\ddagger}]^{46/63} + [\Delta G_T^{\ddagger}]^{82/84} - [\Delta G_T^{\ddagger}]^{WT}, \qquad \text{(Eq. 5)}$$

where $[\Delta G_T^{\ddagger}]^{4X}$, $[\Delta G_T^{\ddagger}]^{46/63}$, $[\Delta G_T^{\ddagger}]^{82/84}$, and $[\Delta G_T^{\ddagger}]^{WT}$ denote the activation energies of peptide hydrolysis as catalyzed by the 4X, the M46I/L63P, the V82T/I84V, and the wild-type (WT) proteases, respectively. The $[\Delta G_T^{\ddagger}]^{4X}$ values for the eight peptide reactions as measured directly and as predicted by Equation 5 can be calculated using the data listed in Table I. They are presented in Fig. 5 in the form of a linear free energy plot. It is seen that the data points fit reasonably well to a straight line with no obvious outliers. A least-squares regression calculation yields a slope of 1.1 with a correlation value of 0.95. The result reveals that $\Delta G_T^{\ddagger}$ for peptide hydrolysis catalyzed by the 4X mutant can be predicted with the $\Delta G_T^{\ddagger}$ for hydrolysis catalyzed by the double mutants. The actions of the V82T/I84V and M46I/L63P substitutions are quantitatively coupled.

Effect of Point Mutations of HIV-1 Protease on Binding—The action of the two pairs of mutants are easily discernible for inhibitor binding. For all four inhibitors examined (Fig. 2 and Table II), the resistance against binding for the 4X protease can be attributed to mutations in the active site of the enzyme (V82T and I84V). Within experimental errors, the K_i values of these inhibitors for the M46I/L63P mutant are the same as those obtained for the wild-type enzyme, whereas those for the 4X mutant are essentially identical to those for the V82T/I84V mutant.[9] The M46I and L63P substitutions bear no apparent effect on inhibitor binding, in agreement with our interpretations of the x-ray crystallographic data (2) on the 4X protease complexed with Indinavir. Clearly, the change in equilibrium binding energy (ΔG_b) for the protease due to the 4X mutations, limited to the data in Table II, can also be predicted from the changes due to the two double mutations.

A Molecular Mechanism for Resistance Mutations—The additive effect of $\Delta G_T^{\ddagger}$ and ΔG_b for point mutations of an enzyme has been seen previously for other enzymes (*e.g.* Refs. 25–27). Mutations of active site residues is an obvious means for an enzyme (E) to affect binding, which is expected to alter the affinity of inhibitors (I) as well as substrates (S) in the binary complexes (*i.e.* EI and ES in the ground state). In the case of the 4X protease, what is most intriguing is that point mutations away from and in the active site of the enzyme are functionally amendatory for catalysis but not for equilibrium binding, thus providing a mechanism to engender "resistance" against inhi-

[8] Among the eight substrates, only peptide *I* can be considered to be statistically unchanged (~12%).

[9] For the Vertex compound (VX-478), there is a change in binding energetic of ~0.33 kcal/mol between the 4X and the V82T/I84V mutants; this represents a 24% difference, a value ~2-fold higher than expected from the S.E. of the mean determined for K_i (see Table II).

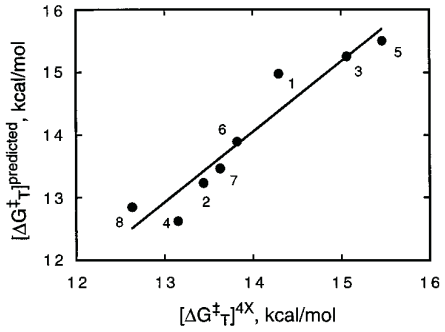

FIG. 5. **Correlation of the activation energies ($\Delta G_T^{\ddagger}$) for the hydrolysis of the eight GAG-POL polyprotein substrates (*I–VIII*) as measured for the 4X HIV-1 mutant protease and as predicted from those measured for the two double mutants (M46I/L63P and V82T/I84V substitutions).** The data can be fit to a straight line with a least-squares slope of 1.1 (r = 0.95).

bition. 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.* $ES^{\ddagger}$ 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_{95} 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_α 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).[10] 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] It is thus possible that, in the course of emergence of resistance, transient point-mutations appear in the protease sequence for reasons that may not be apparent in, or related to,

[10] Frequency of genetic variants has been attributed to the lack of a proofreading function of the HIV reverse transcriptase thus permitting the incorporation of DNA mismatches into the viral genome.

[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.

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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