Drug-Resistant HIV-1 Proteases Identify Enzyme Residues Important for Substrate Selection and Catalytic Rate†

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Received March 17, 1998; Revised Manuscript Received June 17, 1998

ABSTRACT: A series of mutations, first identified in protease inhibitor-resistant HIV-1 viral isolates, were introduced into HIV-1 PR as individual substitutions. Mutants containing *R8*K, *V32*I, *V82*T, *I84*V, *G48*V/*L90*M, or *V82*T/*I84*V substitutions were analyzed for differences in substrate preference and catalytic efficiency using a set of single amino acid substituted HIV-1 CA-NCa cleavage site peptides. All mutants exhibited wild-type preference for large hydrophobic residues, especially Phe, in the P1′ substrate position. Only the *R8*K and *V32*I mutants showed significant differences in subsite selection compared to wild-type enzyme. In a parallel study, the individual mutations R10K, L12V, I44*V*, A60*M*, I71V, and I108V were introduced into RSV PR. These amino acid positions are structurally equivalent to *Arg8*, *Leu10*, *Val32*, *Met46*, *Ile54*, and *Ile84* in HIV-1 PR, respectively, which mutate in drug-resistance. The RSV R10K substitution significantly altered substrate specificity and catalytic rate, compared to wild-type, in a manner similar to that of the HIV-1 *R*8K mutant. Crystal structures of the RSV PR R10K, I44*V*, I71V, and Il08V mutant enzymes presented here indicate that each of these substitutions has little effect on the overall structure of the respective enzymes. Taken together, these data provide an explanation for the reported *in vivo* predilection for selection of large hydrophobic residues in the P1′ substrate position of second locus mutations in the Gag polyprotein PR cleavage sites. The data also suggest that the selection of resistant enzymes is not simply limited to loss of binding to inhibitor but affects other steps in proteolysis.

RSV[1] and HIV-1 PRs, both of which play an essential role in their respective viral replication pathways, share a common structural topology, but only about thirty percent amino acid sequence identity. Despite their structural similarities, these enzymes display significantly different substrate specificities. RSV PR has a narrow specificity, limited to specific cleavage sites in the RSV Gag and Gag-Pol polyproteins, while HIV-1 PR can cleave both its natural HIV-1 Gag and Gag-Pol polyproteins and most sites in the RSV polyproteins. Initial efforts to identify important specificity-determining amino acids used crystallographic information describing both RSV and HIV-1 enzymes (see ref 1 for a review) to identify residues positioned within 10 Å of the substrate. In RSV PR, these residues included Ser38, Ile42, Ile44, Gln63, His65, Met73, Alal00, Val104, Arg105, Gly106 and Ser107. The structurally analogous positions in HIV-1 PR are *Thr26*, *Asp30*, *Val32*, *Met46*, *Gly48*, *Val56*, *Leu76*, *Thr80*, *Pro8l*, *Val82*, and *Asn83*, respectively (see Figure 1). (Note that amino acids from wild-type HIV-1 PR will be *italicized* throughout the text.) RSV PR Gln63 has no HIV-1 PR structural equivalent. Site-directed mutagenesis was used to substitute the amino acids found in HIV-1 into their structurally equivalent positions of the RSV PR (*2*−*5*). The resultant RSV protease mutants, containing substitutions at one or more of these key-positions, displayed significant HIV-1-like substrate selection (*2*−*5*). Additionally, a mutant known as RSV S9 PR, which combined all of the above substitutions except at residues Gln63 and His65, and whose structure has been solved (*6*), became specifically susceptible to inhibition by HIV-1 protease inhibitors at concentrations too low to inhibit wild-type RSV protease (*7*).

† This work was supported in part by U.S. Public Health Service Grants CA52047 (to J.L.), CA58166 (to I.T.W.), and AI41380 (to I.T.W. and R.W.H.), and by the National Cancer Institute, DHHS Contract with ABL (to T.C. and A.W.) and SAIC (to J.E.).

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[1] Abbreviations: RSV, Rous sarcoma virus; HIV-1, human immunodeficiency virus, type 1; AMV, avian myeloblastosis virus; PR, retroviral protease; NC, retroviral nucleocapsid protein; CA, retroviral capsid protein.

S0006-2960(98)00612-6 CCC: $15.00
Published on Web 09/11/1998

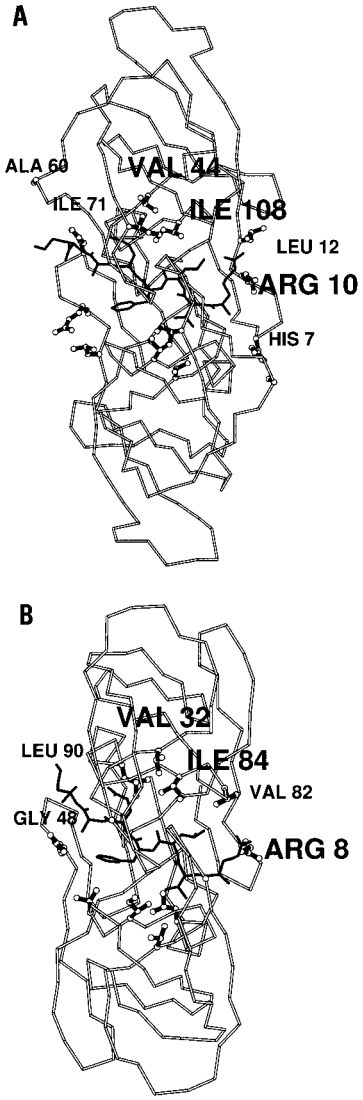

FIGURE 1: Comparison of the structures of the RSV S9 and HIV-1 proteases. (A) Structure of RSV S9 protease with inhibitor. The α-carbon backbone of the dimer is shown in the open lines, and the closed lines indicate the inhibitor, Arg-Val-Leu-r-Phe-Glu-Ala-Nle-NH_2, a reduced peptide analogue of the HIV-1 CA−p2 cleavage site. The sites of mutations His7, Arg10, Leu12, Val44, Ala60, Ile71, and Ile108 are shown in a ball-and-stick representation for the side chain atoms. Large letters indicate identical mutations placed in both enzymes. (B) Structure of HIV-1 protease with the same inhibitor. The view is similar to that of (A), with similar representations. Side chains are shown for residues Arg8, Val32, Gly48, Val82, Ile84, and Leu90.

Recent studies of HIV-1-infected patients have shown that inhibitors directed at the HIV-1 PR reduce blood virus levels. However, resistant PRs are selected by these drugs even during multi-drug therapy (*8*). Many of the nonconserved residues between HIV-1 and RSV PRs identified above were predicted to (*4*) and subsequently found to mutate during the development of drug resistance (*9*−*16*). To stem the development of resistance, the next generation of protease-directed inhibitory agents will have to target not only the wild-type HIV-1 PR but also its drug-resistant variants. To design effective future inhibitors against these drug-resistant mutants, the resistance of these enzymes to the protease inhibitors available today must be understood. In this manuscript, we have characterized selected mutations found in drug-resistant HIV-1 PR enzymes, and describe how these mutations influence the overall catalytic activity and substrate selection in both the HIV-1 and RSV PRs. The data obtained from this study suggest that the development of resistance is not solely due to reduced binding affinity for inhibitors but probably also involves other steps in proteolysis.

EXPERIMENTAL PROCEDURES

Protease Assay. HIV-1 and RSV PR activity was assayed using the standard fluorescamine assay described previously (*2*), except that reactions contained 4% DMSO to increase the solubility of the substrate peptide. Steady-state kinetic measurements were conducted as described previously by fitting the Michaelis−Menten equation to the activity data (*2*). All experiments were repeated in duplicate. Standard error for the reported kinetic constants was not greater than 20%.

Peptides. Peptide substrates, 10 and 12 amino acids in length, were synthesized based on the CA-NCa (PARVL-AEAMR) and NC-PR (PPAVS-LAMTMRR) cleavage sites of the HIV-1 and RSV Gag polyproteins, respectively. These peptides include the eight P4 to P4′ amino acids required for efficient and specific cleavage by the retroviral protease. Peptides were synthesized with an amino terminal Pro residue to prevent uncleaved substrate from reacting with fluorescamine. Arginine residues were added to the carboxyl termini to improve solubility. All peptides were dissolved in 1 mM dithioerythritol.

Identification of Cleavage Sites within the Peptide Substrate. Standard protease assay conditions were employed except that the reactions were allowed to go for 60 min. A 25 μL reaction volume was diluted by addition of 225 μL water and 20 μL of this dilution was used directly for N-terminal sequencing employing an automated ABI amino acid sequencer at the Molecular Biology Core Facility, Case Western Reserve University. The final concentration of salt in the sample did not interfere with the N-terminal analysis.

Purification of Wild Type and Mutant AMV Proteases. AMV PR was purified directly from viral particles obtained from Molecular Genetic Resources (Tampa, FL), as described previously (*17*). Recombinant wild-type and mutant RSV proteases were purified from the soluble fraction of *E.* coli extracts, as described previously (*7*). Briefly, the protease was expressed with an N-terminal polyhistidine tag and purified by Ni−NTA affinity chromatography. The His tag was subsequently removed by proteolytic cleavage with bovine Factor Xa. The recombinant wild-type RSV enzyme had a specific activity equal to that seen with the native AMV PR purified directly from virions. Both enzyme preparations were >95% pure and suitable for crystallization studies. Note that viral AMV and bacterially expressed RSV protease differ in their primary sequence by only two amino acids and are biochemically indistinguishable.

Construction of Mutant RSV Protease Clones. All mutants were constructed using the mega-primer PCR method described previously (*18*). Plasmids containing the mutant genes were transformed into *E.* coli M15 pDM 1.1 by electroporation. Clones containing the plasmid were selected in the presence of appropriate antibiotic. The presence of all mutations was confirmed by sequencing of individual clones.

Preparation of Wild-Type and Mutant HIV-1 Proteases. The construction, expression and purification of wild-type

Table 1: RSV Protease Amino Acids Predicted To Influence Substrate Selection

amino acid position in RSV PR	HIV-1 PR equivalent	subsite predicted from structural comparisons[a]	HIV-1 drug-resistant mutation[b]
His7	none	S3,S4,S3′,S4′	
Arg10	*Arg8*	S1,S3, S1′,S3′	yes
Leu12	*Leu10*	none	yes
Ser38	*Thr26*	S3,S3′	
Ile42	*Asp30*	S2,S2′,S4,S4′	yes
Ile44	*Val32*	S2,S2′	yes
Ala60	*Met46*	S3,S4,S3′,S4′	yes
Asn61	none	S4,S4′	
Gln63	none	S4,S4′	
His65	*Gly48*	S2,Sl′	yes
Ile71	*Ile54*	none	yes
Met73	*Val56*	S2,S2′,S4,S4′	
Ala100	*Leu76*	S2,S2′	
Val104	*Thr80*	S1,Sl′,S3,S3′	
Arg105	*Pro81*	S1,S1′	yes
Gly106	*Val 82*	S1,Sl′,S3,S3′	yes
Ser107	*Asn83*	S1,S1′	
Ile108	*Ile84*	S1,S1′,S2,S3,S3′	yes
Leu114	*Leu90*	none	yes

[a] Subsite designation is from analysis of the RSV S9 and HIV-1 PR crystal structures with the CA-p2 analogue inhibitor (*6*). [b] Amino acid residues that mutate in drug-resistant HIV-1 protease (*9*−*16*).

and mutant HIV PRs were as previously described (*19*). Efficiency of folding of the wild-type and mutant HIV-1 PRs varied from 60 to 90% as determined by active site titration with a nM inhibitor of the enzyme, as was previously described (*2*). The purity of enzymes was sufficient for crystallization.

Determination of Crystal Structures of RSV Protease Mutants. Crystals of the RSV protease mutants were grown by the vapor diffusion technique using a solution of 2−5 mg/mL of protein with a reservoir solution of 0.1 M citrate buffer, pH 5.0−5.3, 0.2−0.4 M ammonium sulfate and either 5% DMF or 5% PEG300. X-ray diffraction data were collected on an R-AXIS imaging plate detector mounted on an RU200 Rigaku rotating anode X-ray generator. The unit cell dimensions had the average value of $a = b = 88.8 \pm 0.4$ Å, and $c = 78.8 \pm 0.5$ Å in space group $P3_121$. The crystals of mutant RSV PRs were all isomorphous with the crystals of wild-type RSV protease (*20*). The structures were solved by molecular replacement and refined using the program XPLOR (*21*). The crystal structures were visualized on a Silicon Graphics workstation using CHAIN (*22*). Root-mean-square deviations (RMSD) on Cα atoms were calculated with ProFit (Martin, A. C. R., University College, London, U. K).

RESULTS

Rationale for Introduction of Specific Mutations into HIV-1 and RSV PRs. Many of the amino acids that contribute to differences in substrate selection by the HIV-1 and RSV PRs were identified previously by structural and biochemical comparisons of the two enzymes (*2*−*5*). (See Table 1 for a list of amino acids predicted by analysis of PR structure to influence substrate selection.) Moreover, several of these key residues were predicted (*4*) and later shown to change in drug resistant HIV-1 PRs. Of these HIV-1 PR amino acid residues, *Asp30*, *Val32*, *Gly48* and *Val82* (see Figure 1B) are among the first to mutate (*9*−*16*). Several other residues, such as *Arg8*, *Leu10*, *Met46*, *Ile54*, and *Ile84* subsequently and repeatedly mutate in drug resistant enzymes (*9*−*16*) suggesting that they, too, may be involved in substrate selection determination. We, therefore, investigated the effects of selected drug resistance mutations on substrate selection within both HIV-1 and RSV PRs.

Four HIV-1 PR constructs containing the single substitutions *R8*K, *V32*I, *V82*T or *I84*V, and two containing the double substitutions, *V82*T/*I84*V and *G48*V/*L90*M, were examined. The *G48*V, *L90*M and, more rarely, the *G48*V/*L90*M mutations arise predominantly in viral isolates recovered from patients resistant to the drug Saquinavir (*23*). The remaining five mutations arise in isolates resistant to other protease inhibitors, including Indinavir and Ritonavir (*9*−*16*). Analysis of crystal structure data has shown that the above residues are in direct contact with the substrate with the exception of *Leu90*. This residue is positioned away from the substrate binding site but close to the main chain atoms of *Asp25*/*Asp25*′. Thus, mutations to *Leu90* are predicted to influence the catalytic *Asp25*/*Asp25*′ residues.

Single substitutions of amino acids in HIV-1 PR that arise during drug selection were also examined in the context of RSV PR, including R10K, L12V, I71V, and I108V. These are analogous to HIV-1 positions *8*, *10*, *54*, and *84*, respectively (see Figure 1), and the amino acids at these positions are conserved between the two enzymes. Several other RSV mutants were examined including I44*V* and A60*M*, which substituted the amino acid found in HIV-1 PR into the structurally related position of RSV PR (see Table 1). These sites are also involved in drug resistance in HIV-1 PR. Changes at Ala60 were examined, particularly, since this residue is located in the flexible flap region. The main chain but not the side chain atoms of the equivalent HIV-1 PR residue, *Met46*, are involved in binding the substrate (*6*). Another RSV mutant, H7A, was analyzed since crystallographic data suggested that it forms part of the S4 and S4′ subsites in RSV PR (*6*). There is no HIV-1 equivalent to this residue, therefore, His 7 was changed to Ala so as to remove the large positively charged side chain while attempting to preserve the backbone conformation of the enzyme.

Activity of HIV-1 Proteases on the CA-NCa Peptides. The effects of PR mutations were analyzed using two peptide libraries containing substitutions of single residues from P4 to P4′. One library was based on the RSV NC-PR Gag junction sequence (PPAVS-LAM-TMRR) previously shown to distinguish changes in specificity in individual enzyme subsites (*2*−*5*). The other library was based on the HIV-1 CA-NCa cleavage junction (PARVL-AEAMR). HIV-1 PR was recently discovered to cleave the RSV NC-PR peptide at a second site between the Met and Thr residues (data not shown). Since cleavage at two sites would complicate interpretation of the kinetic data, all HIV-1 enzymes were analyzed using only the CA-NCa single amino acid substituted peptides; RSV enzymes were analyzed exclusively with the NC-PR peptide set. The wild type HIV-1 and six PR mutants cleaved this substrate set exclusively at the Leu-Ala junction, as determined by direct N-terminal amino acid sequencing of products (see Experimental Procedures). The HIV-1 PR *V32*I mutant was the least active on the wild-

type CA-NCa peptide, followed by the *R8*K and then the *V82*T/*I84*V mutants (see legend to Table 5 in appendix). The *I84*V substitution resulted in a more active protease on this substrate while the *V82*T and *G48*V/*L90*M enzymes had specific activities similar, but not identical, to the wild-type enzyme.

Activity of HIV-1 Mutant Proteases on Substituted CA-NCa Peptides. The relative activities of the six HIV-1 PR mutants on the substituted CA-NCa peptide set are presented in Table 5. For these data, a fixed substrate concentration of 20 μM was used for all peptides, with the exception of the P2 and P2′ substituted peptides which were at 100 μM. Enzyme/substrate combinations that displayed selection differences in this initial screen were subjected to steady-state kinetic analysis to determine specific k_{cat} and K_m values (Table 2). While most amino acid substitutions in the CA-NCa peptide resulted in decreased activity for the substrate by the wild-type HIV-1 PR, there were a few exceptions. Substitution of Arg in P3 with Phe, and Ala in P1′ with Met, Leu, Phe, Val, or Ile, all resulted in increased relative k_{cat}/K_m values for the respective peptides (Table 2). The increase in activity with Phe in P3 was due to a decrease in the K_m value, while the improved activity observed with substitutions in P1′ was due in each case to an increase in k_{cat} and, in some cases, a decrease in K_m.

Surprisingly, all six HIV-1 mutants displayed substrate specificities that were similar to the wild-type enzyme (Tables 2 and 5). HIV-1 protease residue *32* was identified previously as important in substrate selection, affecting the S2 and S2′ subsites, using the RSV NC-PR peptide library. However, neither the *V32*I mutant nor any of the other HIV-1 PR mutants tested here tolerated the majority of the substitutions in the P2 or P2′ positions of the CA-NCa peptide, with the exception of the P2 Ile substituted peptide (Table 5). This confirms the results of previous studies which found that β-branched amino acid residues (Ile and Val) optimally fit into the S2 subsite and Glu optimally fits into the S2′ subsite (*24*). In contrast, the *V32*I mutant displayed significantly enhanced activity for substrates with substitutions in the P1′ position of the modified CA-NCa peptides compared to wild type (Tables 2 and 5). For instance, wild-type HIV-1 PR was 6 times more efficient at cleaving a peptide with P1′ Phe compared to the unsubstituted peptide, while the *V32*I mutant was 56 times more efficient on the same substrate. This increase in activity was due to both an increase in k_{cat} and a decrease in K_m (Table 2). Comparable changes in P1 substituted peptides were not observed since the unmodified CA-NCa peptide already has an optimal residue in P1. The *R8*K mutant also displayed an enhanced activity over the wild-type enzyme for the CA-NCa peptides substituted with larger hydrophobic residues at the P1′ position, and with the P3 Phe substituted peptide. This increase in activity, however, was less than that observed for the *V32*I mutant.

Results with the *V82*T mutant were also somewhat surprising. Substitutions of amino acids at position 82 are predicted based on crystal structure analysis to influence substrate selection in the S1 and S1′ subsites. While changes in selection were observed with the HIV-1 *V82*T mutant with P1′ substituted CA-NCa peptides, these effects were comparable or less than those of wild type. Also, no stimulatory effects were detected with P1 substituted peptides for wild type or the *V*82T mutant (Table 5).

Table 2: Comparison of Steady-State Kinetic Activity by HIV-1 and Mutant HIV-1 Proteases on HIV-1 CA-NCa Peptides with Single Amino Acid Substitutions in Different Substrate Positions[a]

protease	substrate[b]	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})	$(k_{cat}/K_m)/(k_{cat}/K_m)_{WT}$
HIV-1	CA-NCa	16	33	0.5	1
	P3F	15	9	1.7	3.4
	P1′M	56	32	1.8	3.6
	P1′L	35	34	1.0	2
	P1′F	62	20	3.1	6.2
	P1′V	50	10	5.0	10
	P1′I	22	23	1.0	2
*G48*V/*L90*M	CA-NCa	12	27	0.4	1
	P3F	11	10	1.1	2.8
	P1′M	61	24	2.6	6.5
	P1′L	28	30	1.0	2.5
	P1′F	69	25	2.8	7
	P1′V	43	10	4.3	11
	P1′I	64	90	0.7	1.8
*V32*I	CA-NCa	4	105	0.04	1
	P3F	5	10	0.5	12.5
	P1′M	36	36	1.0	25
	P1′L	38	61	0.6	15.6
	P1′F	28	13	2.2	56
	P1′V	30	14	2.1	53
	P1′I	36	129	0.3	7.5
*V82*T	CA-NCa	32	46	0.7	1
	P3F	35	13	2.7	3.9
	P1′M	112	28	4.0	5.7
	P1′L	73	46	1.6	2.3
	P1′F	104	57	1.8	2.6
	P1′V	120	16	7.5	10.7
	P1′I	62	27	2.3	3.3
*I84*V	CA-NCa	45	51	0.9	1
	P3F	54	9	6.0	6.7
	P1′M	78	33	2.4	2.7
	P1′L	133	70	1.9	2.1
	P1′F	153	33	4.5	5
	P1′V	121	15	8.1	9
	P1′I	109	54	2.0	2.2
*V82*T/*I84*V	CA-NCa	20	72	0.3	1
	P3F	19	13	1.5	5
	P1′M	77	31	2.5	8.3
	P1′L	96	61	1.6	5.3
	P1′F	94	46	2.0	6.8
	P1′V	79	24	3.2	10.6
	P1′I	96	71	1.4	4.6
*R8*K	CA-NCa	8	56	0.1	1
	P3F	10	7	1.4	14
	P1′M	64	24	2.7	27
	P1′L	37	33	1.1	11
	P1′F	66	27	2.4	24
	P1′V	74	44	1.7	17
	P1′I	70	108	0.7	7

[a] Varying concentrations of the CA-NCa or CA-NCa substrates with amino acid substitutions in the P4 to P4′ positions (as indicated) were incubated with purified HIV-1 PR or HIV-1 PR mutants. The extent of cleavage was determined using the fluorescamine assay described under Experimental Procedures. [b] The wild-type CA-NCa peptide substrate is **PARVL-AEAM**R. The amino acid sequence is presented in one-letter notation with the amino terminus to the left. The specific PR cleavage site is indicated by a hyphen with the natural sequence in boldface letters. The nonboldface Arg residue was added to improve substrate solubility. Amino acid substitutions in the P3 to P1′ substrate positions are as indicated, also in one-letter notation.

Effect of Amino Acid Substitutions on the Specific Activity of Mutant RSV PRs. Seven RSV PR mutants were prepared

Table 3: Comparison of Steady-State Kinetic Activity by AMV and Mutant RSV Proteases on RSV NC-PR Peptides with Single Amino Acid Substitutions in Different Substrate Positions[a]

protease	substrate[b]	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1}\,\mu M^{-1}$)	$(k_{cat}/K_m)/(k_{cat}/K_m)_{NC-PR}$
AMV	NC-PR	18	75	0.2	1
	P1W	57	10	5.7	24
	P1F	85	9	9.4	39
	P3F	22	13	1.7	7
	P3R	32	92	0.4	2
	P4′H	60	304	0.2	1
	P1I	5	10	0.5	2
	P4V	3	24	0.13	0.7
R10K	NC-PR	10	154	0.06	1
	P1W	63	17	3.7	62
	P1F	278	38	7.3	122
	P3F	22	39	0.6	10
L12V	NC-PR	14	60	0.2	1
	P1W	33	12	2.8	12
	P1F	77	16	4.8	21
	P3F	18	12	1.5	7
H7A	NC-PR	17	39	0.4	1
	P1W	45	6	7.5	17
	P1F	72	6	12	27
	P4′H	77	157	0.5	1
I108V	NC-PR	15	254	0.06	1
	P3F	10	20	0.5	8
	P3R	38	422	0.1	1
I71V	NC-PR	18	70	0.3	1
	P1W	35	8	4.3	17
	P1F	62	7	8.9	34
	P1I	3	8	0.4	2
	P3F	28	19	1.5	6
A60*M*	NC-PR	1.4	59	0.02	1
	P3R	3.6	154	0.02	1
I44*V*	NC-PR	7.6	38	0.2	1
	P2L	5.4	244	0.02	0.1
	P2L,P2′L	1.1	5	0.2	1
	P2′L	7.5	17	0.4	2
	P1W	25	8	3.1	16
	P1F	37	8	4.7	24
	P4V	1.6	27	0.06	0.3

[a] Varying concentrations of the NC-PR or NC-PR substrates with amino acid substitutions in the P4 to P4′ positions as indicated were incubated with purified AMV PR or RSV PR with single amino acid substitutions as indicated. The extent of cleavage was determined using the fluorescamine assay described under Experimental Procedures.[b] The wild-type NC-PR peptide substrate is **PPAVS-LAM-TM**RR. The amino acid sequence is presented in one-letter notation with the amino terminus to the left. Specific PR cleavage sites are indicated by hyphens with the natural sequence in boldface letters. Nonboldface Arg residues were added to the substrate to improve solubility. Amino acid substitutions in the P4 to P4′ substrate positions are as indicated, also in one-letter notation.

from the soluble fraction of bacterial extracts as described in Experimental Procedures. The specific activity of each mutant with the unsubstituted RSV NC-PR peptide substrate is presented in the legend to Table 6 in the appendix, and steady state kinetic data are presented in Table 3. Amino acid substitutions at positions 7 and 71 produced enzymes with specific activities that were slightly greater than those observed with wild type RSV PR. This modest enhancement in activity resulted primarily from small decreases in K_m (Table 3). The L12V and I44*V* mutants displayed slightly decreased specific activities while the I108V, R10K, and A60*M* mutants all showed significantly decreased specific activities relative to wild-type RSV PR (Table 6). The A60*M* flap mutant was the least active enzyme.

Activity of Mutant RSV PRs on Substituted NC-PR Peptide Substrates. Each of the above-mentioned mutants was examined for changes in substrate preference using a library of synthetic RSV NC-PR based peptides with amino acid substitutions in the P4 to P4′ substrate positions. The data are presented in Table 6 and use a fixed substrate concentration of 40 μM for all peptides, except for the P2 substituted peptides, which were at 100 μM. As for the HIV-1 PRs, enzyme/substrate combinations that displayed selection differences in the initial screen were examined further by steady-state analysis to determine specific k_{cat} and K_m values (Table 3). The cleavage sites for the peptide substrates were confirmed by direct N-terminal amino acid sequencing; with the exception of the I44*V* mutant, cleavage of the NC-PR peptide by each of the mutants occurred between the Ser-Leu bond, as expected.

Most of the mutants had little or no detectable change in substrate preference relative to wild type (Table 6), with the exception of the R10K mutant. While both the wild type and R10K enzymes exhibited enhanced cleavage of peptides containing aromatic side chains in the P1 position, the enhancement seen with the R10K mutant was more dramatic. Wild-type RSV PR had relative k_{cat}/K_m values for the P1 Ser to Trp and P1 Ser to Phe substituted peptides that were approximately 24 and 39 times higher, respectively, than those seen for the unsubstituted NC-PR peptide (Table 3). For the same substrates, the k_{cat}/K_m values of the R10K mutant increased by factors of 62 and 122, respectively. This represents a substrate preference increase of 300−400% from that seen with wild-type RSV PR. Improved substrate cleavage appeared to be due to both an increase in k_{cat} and a decrease in K_m (Table 3). The enhanced cleavage by the R10K mutant for NC-PR peptides substituted with aromatic residues in the P1 position mirrors that observed for the structurally equivalent HIV-1 PR *R8*K mutant on CA-NCa peptides substituted with large hydrophobic residues in the P1′ position (Tables 5 and 6). This common effect suggests strongly that these residues, conserved between the two enzymes, play a role in defining substrate selection. The RSV PR mutants L12V, I71V, and I44*V* also showed an increased preference for aromatic substitutions in the P1 position similar to that of wild-type (Table 3).

The only additional selection change noted was a modest preference by the H7A enzyme for His in the P4 and P4′ substrate positions. Removal of the His side chain from the enzyme allowed the P4 or P4′ His side-chains in the substrate to be accommodated. While this effect was relatively small compared to the effects seen in subsites nearer to the catalytic Asp residues, it was reproducible.

Activity of the RSV PR (*S10*). To further explore the effect of the R10K substitution on substrate selection, this mutation was cloned into the RSV S9 PR. This enzyme contains nine amino acid substitutions (S38*T*, I42*D*, I44*V*, M73*V*, A100*L*, V104*T*, R105*P*, G106*V* and S107*N*) that alter the substrate selectivity and drug sensitivity of the RSV PR to those of HIV-1 PR (*7*). It has a specific activity of 1347 pmoles/min/μg using the RSV NC-PR peptide substrate. The RSV S9 PR when combined with the R10K mutation, referred to as the RSV S10 PR, was active on the RSV NC-PR peptide, but with a reduced specific activity of 198 pmoles/min/μg. This reduction was similar to that observed when the single

Table 4: Summary of Crystal Structures of RSV Protease Mutants[a]

RSV PR mutant[b]	resolution (Å)	*R*−factor	C_α rmsd (Å) with WT	C_α rmsd (Å) with RSV S9 PR
H7A	2.6	0.165	0.279	0.787
R10K	2.4	0.183	0.309	0.825
I44*V*	2.4	0.19	0.319	0.803
I71V	2.2	0.189	0.377	0.749
I108V	2.4	0.168	0.273	0.762

[a] The root-mean-square (rms) differences on C_α atoms were calculated after superimposing the pairs of dimers. The dimer structures were compared for residues 1−59 and 71−124 in subunit 1 and residues 1−58 and 71−124 in subunit 2, due to the disordered flap residues in the crystal structure of wild-type RSV protease. [b] Single amino acid substituted RSV PR mutants.

R10K substitution was cloned into RSV PR (Table 6). When Phe was substituted for Ser in the P1 position of the NC-PR peptide substrate, the RSV S10 PR showed a 3-fold increase in activity that was not observed with the RSV S9 PR (Table 7 in the appendix). This behavior was again similar to that observed with RSV R10K PR. Thus, while the R10K substitution alters substrate preference for the P1 position, it has an overall deleterious effect on activity. In this property, it differs from the other substitutions in the RSV S9 PR, which individually alter substrate selection while increasing or at least preserving the catalytic rate of the enzyme (*7*).

RSV S9 and S10 Mutants Cleave the NC-PR Peptide at Two Sites. The cleavage products of both RSV S9 and S10 on the unsubstituted and P1 Phe- and P1 Trp-substituted NC-PR peptides were analyzed by direct amino terminal sequencing, and the results are summarized in Table 8 in the Appendix. RSV S9 primarily favors the alternative Met-Thr site preferred by HIV-1 PR; 85% of the cleavage products corresponded to this site. RSV S10 PR cleaved this substrate solely at this alternate site, manifesting full HIV-1 PR behavior. In contrast, RSV S10 PR cleaved the P1 Phe and P1 Trp substituted NC-PR peptides exclusively at the Ser-Phe or Ser-Trp bonds, respectively, typical of RSV PR. The RSV S9 PR cleaved the latter two peptides almost equally at both junctions. From this pattern, it again can be inferred that RSV PR residue 10 (HIV-1 PR residue *8*) plays a role in the determination of substrate selection.

The RSV I44V Mutant Cleaves the NC-PR Peptide at Two Sites. RSV PR residue 44 (*Val32* in HIV-1 PR) was previously shown to influence the S2 and S2′ subsites (*5*). The RSV PR I44*V* mutant tolerated P2 or P2′ Leu substitutions in the NC-PR peptide with at least 60% and 30% activity, respectively, relative to the unsubstituted peptide (*5*, *7*, *25*). We reexamined this enzyme with NC-PR substrates that changed either Val in P2 or Ala in P2′ to Leu, or a combination of both (Table 6). As observed before (*5*), wild-type AMV PR did not cleave these substrates, which place a larger hydrophobic amino acid side chain into either or both of these positions. Only the I44*V* mutant cleaved the substrates, but with a lower efficiency than was reported previously (*5*).

The nature of the products resulting from cleavage of these peptides by the RSV PR I44*V* mutant was established by direct amino acid sequencing (summarized in Table 8). Cleavage occurred on the wild-type NC-PR peptide predominantly (83%) between the Ser-Leu bond, as expected. About 17% of the cleavage, however, occurred at a previously undetected cleavage site between the Met-Thr bond. The NC-PR P2′ Leu substrate was cleaved at the usual Ser-Leu bond, but when Leu was substituted into P2, cleavage occurred primarily at the alternative Met-Thr site. The I44V mutant also cleaved the double P2/P2′ Leu substituted peptide primarily between the Met-Thr bond.

The Met-Thr cleavage site was originally missed due to technical reasons. Large amounts of proteolytic products were prepared for sequencing by cleaving the starting peptide with significantly higher concentrations of PR than those used in routine assays. Before sequencing, reaction products were purified by HPLC using a reverse phase C4 column to remove salt. Cleavage at two sites should have resulted in four product elution peaks, but only two were observed. Cleavage at the major Met-Thr site releases a short hydrophilic Thr-Met-Arg-Arg peptide, which most probably eluted in the HPLC column pass-through obscured by the salt and solvent peak. The other fragment, PPAVSLAM, was not distinguished from the PPAVS or LAMTMRR fragments resulting from cleavage at the Ser-Leu site. This led to the incorrect conclusion that RSV PR I44*V* mutant cleaved these NC-PR peptides exclusively between the Ser-Leu site as is observed with wild-type and each of the other PR mutants. With the exception of HIV-1 PR, RSV S9 and S10 PRs, and now the RSV I44*V* mutant, cleavage of the NC-PR peptide substrates by the current and previously analyzed RSV mutants was between the Ser-Leu site, as expected.

Structure Determination for RSV PR Mutants. Selected single substitution mutants of RSV protease were crystallized and their structures solved by molecular replacement. A summary of the crystallographic results is found in Table 4. The mutants analyzed include those selected by analysis of the crystal structures of RSV and HIV proteases, such as H7A, which alters a residue in a surface loop that is not present in HIV-1 PR, and I44*V*, which introduces the residue present in HIV-1 protease. The other mutants analyzed have sites equivalent to those found in drug resistant mutants of HIV-1 PR, such as R10K, I71V, and I108V. The effects of the mutations were analyzed by comparing the crystal structures of mutants with the wild-type enzyme (*26*) and with the RSV S9 PR complexed with an inhibitor (*6*). The mutants had root-mean-square (rms) differences of 0.27−0.38 Å on alpha carbon atoms compared with the wild type enzyme. These differences are less than the mean difference between alpha carbon atoms of different crystal forms of the same protein (*27*), suggesting that the mutant proteases had structures that are essentially identical to that of the unliganded wild-type RSV protease. Larger differences of 0.75−0.83 Å were observed on comparison of the unliganded mutant proteases with the RSV S9 protease structure. However, these differences are probably due to the inhibitor bound to RSV S9 protease. Thus, overall, the crystal structures of these mutants obtained without bound inhibitor were essentially identical, suggesting that the substitutions do not induce any significant conformational changes in the unliganded protein. Therefore, the observed changes in activity noted above are likely to arise from the change in type of amino acid and its effect on substrate binding and hydrolysis.

DISCUSSION

Effect of Protease Mutations on Substrate Selection. Wild-type HIV-1 PR and six mutant enzymes (*R*8K, *V*32I, *V*82T, *I*84V, *V*82T/*I*84V and *G*48V/*L*90M) were analyzed using a new HIV-1 CA-NCa peptide library. The need for an HIV-1 PR-specific peptide library was predicated upon the finding that although HIV-1 PR can cleave the RSV NC-PR peptide as previously described (*2*−*5*), it does so at a previously unrecognized site between the Met and Thr. Despite this fact, substitutions of aromatic side chains in the P1 position of the NC-PR substrate can switch the HIV-1 PR Met-Thr cleavage site to the RSV recognition site (Ser-Leu). Thus, the introduction of an amino acid into the substrate that optimally fits into a given subsite will cause the site of cleavage of the substrate to shift. Analyses of product peptides from reactions with the RSV S9 and S10 PRs on the natural and P1 substituted NC-PR peptides (Table 8) also demonstrates that these enzymes behave like HIV-1 PR in choice of site of cleavage.

Of the six HIV-1 PR mutants analyzed, only the *R8*K and *V32*I mutants showed significantly less activity than wild type with the unmodified HIV-1 CA-NCa peptide substrate. In further analyzing the HIV-1 PRs with the substituted CA-NCa peptide library, it was found that all of the enzymes displayed increased relative activities with peptides substituted with large hydrophobic residues in the P1′ position, particularly the *V32I* mutant. This mutant cleaved the P1′ Ala to Phe substituted peptide 56 times more efficiently than the wild type CA-NCa peptide, as compared to the 6-fold improvement in cleavage efficiency observed for the wild type HIV-1 enzyme with the same peptide (Tables 2 and 5). These increases were primarily due to increases in k_{cat}, although decreases in K_m values were also observed for the *V32*I and *R8*K mutants.

Interestingly, the preference for large hydrophobic residues at the P1′ position of natural cleavage sites by HIV-1 PR drug resistant mutants has been observed to be of biological significance. Doyon et al. (*28*) reported the appearance of mutations in the HIV-1 Gag cleavage sites of viral isolates in culture, concomitant with the development of mutations in the PR that together lead to drug resistance. One of the mutations observed was a P1′ Leu to Phe change in the p1/p6 cleavage site. These cleavage site mutations were shown to improve Gag processing, compensating for the impaired protease activity in protease-mutated virions. Zhang et al. (*29*) recently reported the observation of similar Gag cleavage site mutations (p7/p1 and p1/p6), that accompany protease mutations, in viral isolates from patients resistant to Indinavir. They found that while the earliest mutations in the viral protease did not confer significant resistance to the drug, additional mutations in the Gag cleavage sites gave these mutated viruses a growth advantage over the wild-type virus in the presence of the drug.

The wild-type and seven RSV mutants (H7A, R10K, L12V, I44*V*, A60*M*, I71V, and I108V) were analyzed with the RSV NC-PR peptide library. With the exception of the RSV PR I44*V* mutant, wild type and all other mutant enzymes cleaved the NC-PR peptide substrates almost exclusively at the expected Ser-Leu site. In general, all of the mutant enzymes were active on their respective peptide substrates (Tables 5 and 6). This was consistent with the fact that crystal structures for several selected RSV mutant enzymes indicated that the mutations did not significantly perturb the three-dimensional structure of the enzyme (Table 4). However, the A60*M*, R10K, I44*V*, and I108V enzymes had specific activities on the homologous wild-type substrates that were less than those seen with wild-type. Only the RSV R10K enzyme displayed a dramatically increased preference for aromatic side chains in the P1 and to a lesser extent P3 positions of the RSV NC-PR peptide substrate.

No corresponding effect on substrate selection for the HIV-1 enzymes was present for substituted HIV-1 CA-NCa P1 peptides. This is most likely due to the presence of Leu in P1 in the unsubstituted CA-NCa peptide. This residue is the optimal size for the S1 subsite, thus, substitutions of larger hydrophobic residues at P1 would not be expected to dramatically increase activity as seen with the P1′ substitutions. This is consistent with the finding that the introduction of a smaller residue at P1, such as Ala, resulted in a significant reduction in activity. For the RSV R10K mutant increases in activity for substituted NC-PR P1′ peptides were not detected since, again, Leu is found in the P1′ position of the wild-type NC-PR peptide, resulting in decreases in cleavage of peptides with smaller residues substituted at this position. NC-PR Peptides with the larger Phe and Met substituted in P1′ were not tested. By selectively mutating the S2 and S2′ subsites of RSV PR, we have shown previously that asymmetry is introduced into the symmetric retroviral PR by substrate (or inhibitor) binding, so that the corresponding subsites in the enzyme are not equivalent (*7*).

HIV-1 R8K/RSV R10K PR Mutants. HIV-1 PR *R8*K corresponds to the same mutation introduced into the RSV PR R10K mutant. Based upon our data, it can be argued that the *R8*K mutation in HIV-1 PR had the same effect on recognition of substrates as the R10K mutation in RSV PR. The introduction of the R10K mutation into RSV S9 PR, which contains nine other amino acid substitutions that alter substrate selection and drug sensitivity (*7*), resulted in an enzyme (RSV S10 PR) with radically reduced activity (Table 7). However, it retained the enhanced preference for P1 Phe and Trp substituted peptides, observed with the RSV R10K mutant (Tables 6 and 7), which was absent in RSV S9 PR. Taken together, these results suggest that residue Arg10/*8*, conserved between the RSV and HIV-1 PRs, has a marked influence on substrate selection.

The reduced catalytic rate seen upon the substitution of lysine at position *8* in HIV-1 PR, or position 10 in RSV PR, may be due to the disruption of the ionic interactions observed between wild-type HIV-1 PR Arg *8*/Arg 10 in RSV PR and the Asp residues at positions *29* and 41, respectively, in the HIV-1 and RSV PRs (*30*). Lys10′ in the RSV R10K structure is too short to form the ionic interaction that is observed between Arg10′ and Asp41 in the RSV PR structure (Arg8′ and Asp 29 in HIV-1 PR) (*32*). In addition, analysis of the RSV S9 PR crystal structure with inhibitor showed that the Arg10 side chain has moved away from the inhibitor compared to its position in the wild-type enzyme without inhibitor (*6*). Furthermore, comparison of RSV S9 and wild-type PR structures suggests that the wild-type Arg105 side chain must move to accommodate the inhibitor. This suggests that Arg10 and Arg105 will affect the binding of large side chains in S1 and S1′ subsites, consistent with the altered selection. Incidentally, the RSV L12V mutant

residue, which has no direct contact with inhibitor, shows indirect effects via loss of van der Waals interactions with the adjacent residues Leu35 and Arg10 that contact inhibitor, consistent with the lower catalytic efficiency of this mutant compared to wild-type enzyme (Table 3).

HIV-1 V32I/RSV I44V PR Mutants. The RSV mutant, I44*V*, was predicted to have decreased activity toward the NC-PR peptide due to decreased substrate binding efficiency caused by alterations in the van der Waals contacts in the S2 and S2′ subsites (*5*). While the activity was indeed reduced, the effect was in k_{cat} rather than K_m (Table 3). In fact, the K_m for the NC-PR peptide is 50% lower than that observed for wild-type RSV PR. An interesting comparison can be made between the HIV-1 *V32*I mutant and the structurally equivalent but inverse RSV I44*V* mutant. The Val to Ile mutation introduced a methylene group into the S2 and S2′ subsites of HIV-1 *V32*I PR decreasing their already small size. A reduction in size of the S2/S2′ subsites is postulated to result in the enlargement of the adjacent S1/S1′ subsites, enabling them to accommodate larger residues comfortably. This mutant displayed a negative S2/S2′ effect, tolerating only the P2 Ile substitution of the CA-NCa peptide and none of the P2′ substitutions (Table 5). An enhanced P1′ effect was also observed for the *V32*I mutant. The opposite was true for the RSV I44*V* mutant; a methylene group was removed from its S2/S2′ subsites, resulting in larger P2 and P2′ subsites. This mutant was the only one to significantly tolerate substitutions in the P2 position of the NC-PR peptide (Table 6), as was shown previously (*5*). The crystal structure of this mutant enzyme showed that the side chains of the Val44 in both subunits are in similar conformations to those in the wild-type RSV PR Ile44, therefore, the major effect of the I44*V* mutation is to enlarge the small S2 and S2′ subsites, as predicted. In contrast to the HIV-1 PR *V32*I mutant, a slightly negative P1 effect was observed for large hydrophobic residues, compared to the wild-type enzyme. This suggested that the enlarged S2 subsite in the RSV I44*V* enzyme might have restricted the size of the adjacent S1 subsite so that binding larger residues was unfavorable. These results demonstrate that changes that affect one subsite may also affect adjacent subsites, suggesting that subsite interactions with the substrates are not always independent of each other, in agreement with previous studies (*25*, *32*).

HIV-1 PR V82T Mutant. Like the other HIV-1 PR enzymes, the *V82*T mutant displayed increased activity for P1′ substituted peptides, however, the magnitude of the response was considerably smaller than anticipated for this substitution. Residue 82 has been predicted to form part of the S1/S1′ subsites (see Table 1). Substitution of Thr for *Val* at position *82* introduced a polar −OH group into the relatively large S1 and S1′ subsites of HIV-1 PR. The presence of this hydrophilic side chain might be predicted to destabilize van der Waals interactions between the PR residues and the CA-NCa peptides substituted with hydrophobic residues in the P1 and P1′ positions, which would explain the above result.

Other Protease Mutants. None of the other HIV-1 PR or RSV PR substitutions resulted in any significant detectable change in selection compared to wild-type PR toward their respective peptide sets. For example, the RSV PR side chains of I71V and I108V (which correspond to HIV-1 PR positions *54* and *84*, respectively) were found to have similar conformations to those in the wild-type enzyme in both subunits. These results suggest that either these amino acid substitutions found in drug-resistant HIV-1 PRs have very subtle effects on substrate selection or have significant

Table 5

substrate position[b]	substituted amino acid	rel sp act. of HIV-1 PR mutants[a] (% wild-type) WT	*R8*K	*V32*I	*V82*T	*I84*V	*G48*V/*L90*M	*V82*T/*I84*V
	none	100	100	100	100	100	100	100
P4 (Ala)	Pro	59	50	46	73	81	57	71
	Arg	67	46	83	84	79	59	80
	Glu	45	45	81	62	57	58	70
	Met	50	37	33	62	68	57	38
P3 (Arg)	Phe	335	717	543	232	410	207	379
	Thr	72	80	76	73	67	66	52
	Gly	6	6	15	15	7	9	13
	Gln	71	43	83	102	95	75	85
	Lys	21	11	31	48	22	74	58
	Ala	42	7	19	68	43	70	47
P2 (Val)	Ile	106	28	188	132	111	131	102
	Asn	0	0	0	0	0	2	0
	Ala	12	0	4	14	14	14	10
	Gly	2	0	1	0	3	0	3
	Ser	0	0	0	0	0	0	2
	Arg	2	0	0	0	0	0	0
P1 (Leu)	Ala	5	0	3	5	1	8	0
	Met	113	185	133	133	101	116	169
	Phe	142	118	193	118	113	80	124
	Asn	3	0	7	7	0	0	0
	Val	0	0	0	5	4	0	2
	Ile	4	0	0	3	0	0	0
	Tyr	73	38	37	157	nd	40	68
P1′ (Ala)	Met	432	1319	1851	476	422	781	865
	Leu	305	607	953	276	245	242	583
	Phe	661	1319	2750	188	167	786	690
	Val	902	953	2531	692	615	881	1069
	Ile	158	125	385	252	224	209	434
	Asn	39	16	37	51	31	40	18
	Gly	4	2	0	13	0	0	0
P2′ (Glu)	Gln	0	0	5	7	11	9	0
	Leu	12	0	19	20	22	63	9
	Gly	0	0	3	9	0	0	0
	Ala	0	0	5	13	0	10	8
	Val	5	6	10	8	8	21	17
	Asp	0	10	0	6	0	0	6
	Asn	0	0	3	1	0	0	0
P3′ (Ala)	Arg	98	55	125	96	102	101	91
	Gly	81	48	79	59	88	59	74
	Ser	40	30	67	23	12	49	21
	Asp	0	0	9	9	0	3	0
	Glu	29	16	23	4	0	37	12
	Phe	41	17	66	19	62	32	33
P4′ (Met)	Glu	56	0	34	52	50	39	42
	Arg	24	12	24	0	0	21	18
	Lys	18	3	9	0	0	19	0
	Gly	57	5	3	0	54	48	79
	Pro	27	0	4	0	0	27	19
	Ala	32	0	8	0	53	37	60

[a] The relative specific activity values (pmol min^{-1} μg^{-1}) were determined using 20 μM substrate concentration so that substrate was probably not saturating in all instances. 100% is defined as 188 pmol min^{-1} μg^{-1} for wild-type HIV-1 PR. For HIV-1 PR mutants *R8*K, *V32*I, *V82*T, *I84*V, *G48*V/*L90*M, and *V82*T/*I84*V, 100% is defined as 96, 29, 220, 378 159, and 105 pmol min^{-1} μg^{-1}, respectively. [b] The wild-type amino acid in the CA-NCa peptide is indicated in parentheses in the first column.

Table 6

substrate position[c]	substituted amino acid	rel sp act. of RSV PR mutant[a] (% wild-type)							
		WT[b]	H7A	RIOK	A60*M*	I108V	I71V	L12V	I44V
	none	100	100	100	100	100	100	100	100
P4 (Pro)	Gly	20	22	14	15	10	18	17	27
	Glu	30	27	28	39	12	29	27	41
	Arg	36	48	23	49	10	33	51	61
	His	32	46	33	37	21	37	26	41
	Val	18	15	10	0	26	20	4	52
P4 (Thr)	Glu	37	36	28	40	34	42	30	57
	Arg	58	82	33	38	52	59	52	72
	His	98	173	88	111	99	104	104	111
	Val	280	269	286	341	289	163	227	320
P4 (Pro), P4′ (Thr)	Gly	3	1	0	0	0	0	2	10
	Glu	14	11	5	14	7	18	8	19
	Arg	20	32	9	0	20	21	18	34
	His	26	56	34	35	28	36	34	60
P3 (Ala)	Phe	291	216	609	286	429	236	202	171
	Arg	110	99	90	176	177	128	97	139
	Gly	16	20	18	28	17	28	15	46
	Ser	158	143	246	145	177	154	125	166
	Thr	213	182	319	218	299	203	175	211
P2 (Val)	Ser	1	4	0	5	0	3	1	0
	Gly	3	6	0	0	9	4	2	0
	Leu	2	9	3	0	0	3	8	28
	Ala	11	19	12	4	0	17	19	14
	His	7	6	5	2	0	0	9	25
P2 (Val), P2′ (Ala)	Leu	0	nd	nd	nd	nd	nd	nd	28
P1 (Ser)	Phe	761	682	6700	1310	1110	719	543	572
	Trp	664	408	2015	559	750	403	256	418
	Gly	2	3	4	5	2	5	4	9
	Ala	129	126	144	182	121	134	110	131
	Val	56	38	110	64	63	35	28	89
	Ile	60	38	93	84	70	32	28	93
P1′ (Leu)	Val	143	153	137	145	117	130	152	197
	Ala	6	13	16	7	5	10	42	22
	Ile	95	113	98	87	61	70	82	145

[a] The relative specific activity values (pmol min^{-1} μg^{-1}) were determined using 40 μM substrate concentration so that substrate was probably not saturating in all instances. For P2-substituted peptides, 100 μM substrate was used, and the incubation time was 20 min for all enzymes except the A60M mutant, which was 40 min. 100% is defined as 294 pmol min^{-1} μg^{-1} for wild-type AMV PR. For RSV PR mutants H7A, R10K, A60*M*, I108V, I71V, L12V, and I44*V*, 100% is defined as 341, 83, 22, 78, 390, 244, and 176 pmol min^{-1} μg^{-1}, respectively. nd, not determined. [b] WT is AMV PR, which is enzymatically indistinguishable from RSV PR. [c] The wild-type amino acid in the NC-PR peptide is indicated in parentheses in the first column.

influence only in combination with other amino acid substitutions in the enzyme.

Insight into the mechanism of decreased catalytic activity for the RSV PR A60*M* mutant is offered by computational studies with HIV-1 PR. Molecular dynamics simulations of HIV-1 PR suggest that an amino acid substitution at the structurally equivalent position (Ile for *Met46*) can alter flap flexibility and shift its conformation between the closed and open states that have been observed for the PR dimer (*31*). While the RSV A60*M* mutation does not represent the same Ile substitution in HIV PR, it does represent substitution of a larger hydrophobic side chain at the structurally related position and alterations of flap flexibility may contribute to the observed decreased catalytic efficiency. In addition, RSV PR has extended flaps compared to those of HIV-1 PR, which may be more flexible and sensitive to alterations (*6*). Steady-state kinetic measurements indicate that the decreased activity of the A60*M* mutant is due to a decrease in k_{cat} value.

The RSV PR H7A substitution may alter substrate recognition of the S3, S4 and S3′, S4′ subsites (Table 4). The H7A substitution removed the His side chain and eliminated the hydrogen bond interactions of His 7 and 7′ with the carbonyl oxygens of Asp 41′ and 41, respectively, that are observed in the structures of both wild-type enzyme and the RSV S9 protease with inhibitor (*26*, *6*). These interactions of His7 are expected to be important for stabilizing the protease-substrate complex, since Asp 41 and 41′ in RSV S9 PR also form hydrogen bond interactions with the inhibitor (*6*).

CONCLUSION

Previously, the influence of resistance mutations on the catalytic efficiency of HIV-1 PR was found to be correlated with the selective advantage of a particular mutant/drug combination. This led Gulnik et al. (*19*) to define a new property, Vitality, for resistant enzymes that combines the relative K_{cat}/K_m and K_i values for a given mutant. The present results underscore the compensatory influence that single residue mutations in a native cleavage site can have on drug resistant mutant enzymes; particularly those with overall reduced catalytic efficiency toward native substrates. More-

Table 7

	substituted amino acid	rel sp act. of RSV PR mutant[a] (% wild-type)		
		WT	S10	S9
	none	100	100	100
P1 (Ser)	Phe	761	2095	392
	Trp	664	1018	160
	Gly	2	0	20
	Ala	129	79	82
	Val	56	122	104
	Ile	60	256	104

[a] The relative specific activity values (pmol min^{-1} μg^{-1}) were determined using 40 μM substrate concentration. 100% is defined as 294 for wild-type AMV PR, 1347 for RSV S9 PR, and 198 for RSV S10 PR, respectively.

over, our data suggest that alterations in the protease activity on substrates may also contribute to drug resistance. Therefore, mechanisms of drug resistance (*33*) may include PR mutations with reduced affinity for specific inhibitors, PR mutations with increased rates of hydrolysis of substrates, and/or cleavage site mutations to form better substrates.

APPENDIX

Activity of Mutant HIV-1 Proteases on Substituted CA-NCa Peptides. The HIV CA-NCa (PARVL-AEAMR) or CA-NCa substrates with amino acid substitutions in the P4 to P4′ positions as indicated were incubated with purified HIV-1 PR or HIV-1 PR mutants. The extent of cleavage was determined using the fluorescamine assay described under Experimental Procedures. (See data in Table 5.)

Activity of Mutant RSV Proteases on Substituted RSV NC-PR Peptides. The RSV NC-PR (PPAVS-LAMTMRR) or NC-PR substrates with amino acid substitutions in the P4 to P4′ positions as indicated were incubated with purified AMV PR or RSV PR mutants with single amino acid substitutions. The extent of cleavage was determined using the fluorescamine assay described under Experimental Procedures. (See data in Table 6.)

Activity of Mutant RSV Proteases on P1-Substituted RSV NC-PR Peptides. The RSV NC-PR (PPAVS-LAMTMRR) or NC-PR substrates with amino acid substitutions in the P1 position were incubated with purified AMV PR or RSV S9 or S10 PR mutants as indicated. The extent of cleavage was determined using the fluorescamine assay described under Experimental Procedures. (See data in Table 7.)

Recognition of Two Cleavage Sites in the NC-PR Peptide Substrate by the RSV 144V, S9, and S10 PR Mutants. The indicated peptide substrates were incubated with RSV PR mutants under standard assay conditions described under "Experimental Procedures" . Products were subjected to two cycles of amino terminal peptide sequencing to quantitate the relative amount of product produced upon cleavage at the two recognition sites. Cleavage sites are indicated by the hyphen. The natural NC-PR substrate sequence is indicated at the top in nonbold letters. Substitutions in the P1, P2, and P2′ positions are highlighted in large boldface letters. (See data in Table 8.)

Table 8

substrate sequence	ratio of N-terminal Leu to Thr among cleavage products (%) for RSV PR		
	I44*V*	S9	S10
PPAVS-LAM-TMRR	83:17	15:85	0:100
PPA**L**S-LAM-TMRR	18:82	−	−
PPAVS-L**L**M-TMRR	93:7	−	−
PPA**L**S−L**L**M-TMRR	30:70	−	−
PPAV**F**-LAM-TMRR	−	64:36	100:0
PPAV**W**-LAM-TMRR	−	59:41	100:0

ACKNOWLEDGMENT

We thank Dr. E. Houts from Molecular Genetic Resources for AMV protease. We also thank Jill Adomat for her assistance in purification of mutants for crystallography and analysis of the alternative site of cleavage of the NC-PR peptide by the HIV-1 and RSV S9 PRs. In addition, we thank Charles Reed for preparation of the RSV S9 and HIV-1 PR structural models shown in Figure 1.

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BI980612K