Identification of a Loop Outside the Active Site Cavity of the Human Immunodeficiency Virus Proteases Which Confers Inhibitor Specificity†

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Received October 31, 1996; Revised Manuscript Received December 31, 1996⊗

ABSTRACT: We have investigated the inhibitor specificity for the proteases of the human immunodeficiency viruses, types 1 and 2. Using a series of related inhibitors, the P1′ side chain was confirmed to play a significant role in determining both the absolute and relative affinity for the enzymes. To further define the residues in the enzymes responsible for the difference in affinity, chimeric proteins were constructed in which domains of the respective proteases were exchanged at the genetic level. The results of these studies demonstrated that inhibitor affinity is conferred by a combination of the active site residues (32, 47, and 82) along with a loop comprised of residues 31 and 33−37, which lies outside of the active site cavity. These results are discussed in terms of existing structural data.

The human immunodeficiency viruses, types 1 and 2 (HIV-1 and HIV-2),[1] are the etiological agents of acquired immune deficiency syndrome (AIDS) in humans. Both HIV-1 and HIV-2, as well as the related simian immunodeficiency virus (SIV), encode their own protease. The protease encoded by these viruses is responsible for processing the nonfunctional viral polyprotein precursors Pr^{GAG} and $Pr^{GAG-POL}$ into functional structural proteins and enzymes (Oroszlan & Luftig, 1990). This proteolytic activity has been shown to be essential to the viral life cycle (Kohl et al., 1988). Although the retroviral proteases belong to the ubiquitous aspartyl protease family, the enzymes possess unique structural and functional properties that distinguish them from their cellular and microbial counterparts (Fitzgerald & Springer, 1991). For example, a competitive inhibitor specific for the HIV-1 protease was shown not to affect human monocyte function *in vitro*, for which cellular aspartyl proteases are thought to play an essential role (Bugelski et al., 1992). Thus, the HIV protease has been an attractive therapeutic target for the identification of antiviral agents to combat AIDS.

Studies of enzyme activity and inhibitor sensitivity of the proteases encoded by HIV-1, HIV-2, and SIV have revealed significant functional differences between these proteases (Fan et al., 1995; Bugelski et al., 1992; Grant et al., 1991; Mulichak et al., 1993a; Tomasselli et al., 1992; Tong et al., 1993; Zhao et al., 1993; Stebbins & Debouck, 1994). However, the structure−activity relationships underlying these functional differences are not completely understood (Sardana et al., 1994; Hoog et al., 1996; Stebbins et al., 1996). For example, the primary structure of the HIV-2 protease is more closely related to the SIV enzyme than to the HIV-1 enzyme (the amino acid sequences of the HIV-2 ROD and SIV Mn251 proteases are 89% identical whereas HIV-1 and HIV-2 proteases are only 45% identical) (Myers et al., 1990). Consistent with this homology, the activity and inhibitor sensitivity of the HIV-2 and SIV proteases are approximately identical (Fan et al., 1995; Grant et al., 1991; Tomasselli et al., 1992). In contrast to their primary structure, X-ray crystallographic analysis has revealed that the secondary and tertiary structures are mostly conserved in retroviral proteases (Fitzgerald & Springer, 1991; Wlodawer & Erickson, 1993). Comparative structure analysis of HIV-1 and HIV-2 or SIV complexed with the identical inhibitor reveals only minimal differences in the protease structure (Priestle et al., 1995; Tong et al., 1993; Hoog et al., 1996). In fact, even structures in which the inhibitor shows marked differences in binding modes between HIV-1 and SIV, the protease polypeptide chain is remarkably similar (Hoog et al., 1996). In the active site cavity, the HIV-1 and HIV-2 proteases differ at residues 32, 47, and 82 (Figure 1). However, studies have suggested that both the structural and kinetic specificities of the HIV proteases are conferred, at least in part, by residues outside the active site cavity (Sardana et al., 1994; Hoog et al., 1996; Stebbins et al., 1996). To discover drugs active against HIV-1 and HIV-2 proteases and possibly other HIV protease genetic variants, it is essential to understand the fundamental determinants of inhibitor specificity (Gustchina & Weber, 1991).

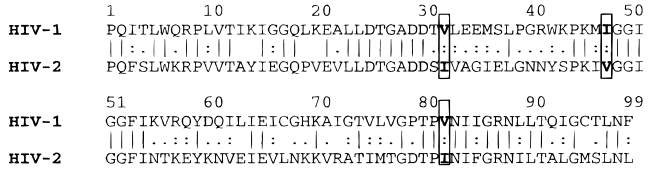

FIGURE 1: Comparison of the 99 amino acid sequence for the protease from the HIV-1 (III_b strain) and the HIV-2 (*rod* strain). Lines between the sequences indicate amino acid identity. Dots between the sequences indicate amino acid homology. Bold, enclosed letters denote the amino acids in the active site which differ between the two enzymes.

† This work was supported by NIH RO1 Grant GM50579 to C.D.

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⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1997.

[1] Abbreviations: BOC, *tert*-butoxycarbonyl; *E. coli*, *Escherichia coli*; HIV, human immunodeficiency virus; MLV, murine leukemia virus.

S0006-2960(96)02729-8 CCC: $14.00

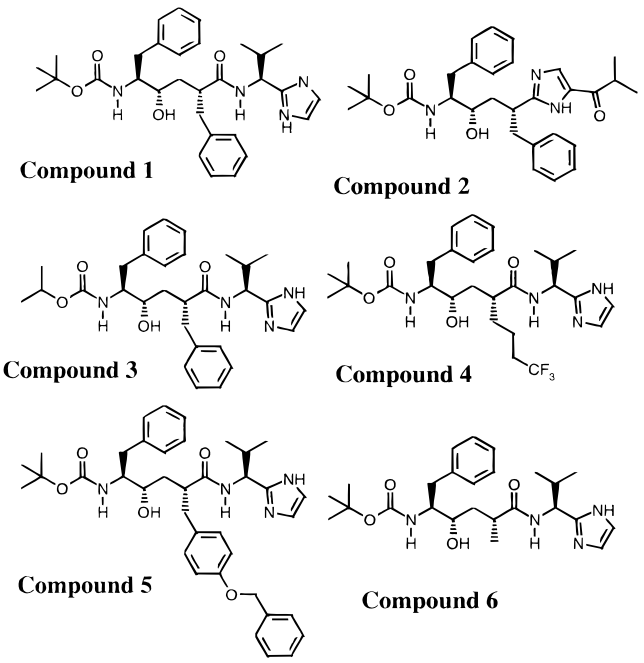

FIGURE 2: Chemical structure of compounds used in this study. Compound **1** is also known as SB203386 (Abdel-Meguid et al., 1994). Compound **2** is also known as SB205891 (Thompson et al., 1994).

(Sardana et al., 1994; Hoog et al., 1996; Dorsey et al., 1994; Kempf et al., 1995; Mulichak et al., 1993b; Chen et al., 1994; Gustchina & Weber, 1991; Tomasselli et al., 1990). We investigated this further by testing a series of related inhibitors (Figure 2). Using these inhibitors, we confirmed that the P1′ side chain [as previously defined (Berger & Schechter, 1970)] plays a significant role in determining both the absolute and relative affinity of an inhibitor for the HIV proteases. To further define the residues in the HIV enzymes responsible for the differences in affinity, chimeric proteins were constructed in which one domain or more of the respective proteases were exchanged. The results of these studies show that inhibitor specificity is conferred by a combination of the active site residues (32, 47, and 82) along with a loop comprised of residues 31 and 33−37, which lies outside of the active site cavity. These results are discussed on the basis of existing structural data.

MATERIALS AND METHODS

Materials. Enzyme grade buffers and reagents were purchased from Baxter Scientific (McGaw Park, IL). Phenylmethanesulfonyl fluoride was purchased from Sigma Chemical Co. (St. Louis, MO). Microsampler tubes and caps were purchased from Alltech Associates, Inc. (Deerfield, IL). Dithiothreitol was purchased from Research Organics (Cleveland, OH).

Enzyme Preparation and Kinetic Characterization. The HIV-1(IIIb) and HIV-2(ROD) protease expression plasmids have been described previously (Stebbins & Debouck, 1994). Chimeric proteases were constructed by insertions of silent *Age*I, *Sac*I, and *Bcl*I DNA restriction sites into each plasmid at codons 25, 37, and 59 of the mature protease, respectively, using site-directed mutagenesis (Towler et al., 1996) followed by subcloning the relevant regions according to standard molecular biological procedures (Sambrook et al., 1989). Additional site-directed mutagenesis was as described (Towler et al., 1996). Proteases were purified to apparent homogeneity, and peptidolysis reactions were performed as previously described (Grant et al., 1991; Hoog et al., 1996). Kinetic values (shown in Tables 1 and 3) represent the average of three or more independent assays. Michaelis constant values (K_m's) above 15 mM were obtained by extrapolation of data due to the limits of substrate solubility. All inhibitors were determined to be competitive by Dixon analysis (Dixon, 1953). Genetic construction was aided by the Program Manual for the Wisconsin Package,Version 8.0, September 1994 (Genetics Computer Group, 575 Science Drive, Madison, WI 53711). Kinetic analysis was aided by the program Kaleidograph (Synergy Software). Theoretical free energy of binding was calculated as $\Delta G = -RT \ln K$, where ΔG is the free energy, R is the gas constant, T is the absolute temperature, and K is the kinetic K_i value (Fersht, 1985).

Table 1: Kinetic Parameters of Wild-Type and Substituted Proteases

	k_{cat}[a] (mol s^{-1} mg^{-1})	K_m[b] (mM)	k_{cat}/K_m
HIV-1	1487	10.2	145.8
HIV-2	187	3.3	56.8
HIV-1 (2: 1−25)[c]	113	8.2	13.7
HIV-1 (2: 1−37)	223	32.0	7.0
HIV-1 (2: 1−59)	382	13.6	28.0
HIV-1 (2: 31−37)	1122	64.1	17.5
HIV-1 (2: 32)	346	11.1	31.1
HIV-1 (2: 31, 33−37)	1450	22.7	63.9
HIV-1 (2: 32, 47, 82)	952	9.0	106.1
HIV-1 (2: 31−37, 47, 82)	330	20.7	15.9
HIV-2 (1: 32, 47, 82)	1268	19.1	66.3

[a] Kinetic constants determined as described in Materials and Methods. [b] Values above 15 mM are extrapolated. [c] The numbers in parentheses indicate which residues are substituted. For example, HIV-1 (2: 1−25) is HIV-1 protease with HIV-2 residues at positions 1−25.

Inhibitor Synthesis. Compound **1** was prepared as described previously (Abdel-Meguid et al., 1994), and compounds **3**-**6** were prepared by methods analogous to those described for compound **1**. Compound **2** was prepared as described previously (Thompson et al., 1994).

RESULTS AND DISCUSSION

Inhibitor Sensitivity of the HIV Protease. Despite the high degree of structural similarity between the HIV proteases, it has been shown that the HIV-2 protease has a lower affinity than the HIV-1 protease for a number of inhibitors (Sardana et al., 1994; Hoog et al., 1996; Dorsey et al., 1994; Kempf et al., 1995; Mulichak et al., 1993b; Chen et al., 1994; Gustchina & Weber, 1991; Tomasselli et al., 1990). We investigated this further by testing a series of inhibitors, all related to the previously characterized compound **1** (Abdel-Meguid et al., 1994; Hoog et al., 1996). All six inhibitors are transition-state analogues in which the scissile bond is replaced by a nonhydrolyzable hydroxyethylene isostere (Figure 2). These inhibitors allowed us to test the role of the P1′ side chain, the placement of the imidazole ring, and the amino-terminal BOC moiety in determining inhibitor selectivity.

Purified HIV-1 and HIV-2 proteases were kinetically characterized (Table 1) and analyzed for their affinity for the six inhibitors tested (Table 3). As previously determined,

compound **1** has a K_i of 20 nM for the HIV-1 enzyme and 1.2 μM for the HIV-2 enzyme, giving a K_i(HIV-2)/K_i(HIV-1) ratio of approximately 70 (Abdel-Meguid et al., 1994; Hoog et al., 1996). Altering the BOC moiety of compound **1** to an isopropyl carbamate (compound **3**) had no significant effect on the individual K_i's or the K_i(HIV-2)/K_i(HIV-1) ratio, indicating that this position has little effect on the absolute or relative affinity of compound **1**. Even changing this position to an imidazolyl moiety had little effect on these values (T. Tomaszek, unpublished). Moving the imidazole ring of compound **1** from the P3′ to the P2′ position (compound **2**) raised the K_i's of the two enzymes but had little effect on the K_i(HIV-2)/K_i(HIV-1) ratio, suggesting this change disrupted the same productive binding in both enzymes. This result is in agreement with the finding that both proteases have similar substrate specificity at these subsites (Poorman et al., 1991). Increasing the size of the P1′ phenylalanine (compound **5**) resulted in no change in the K_i with the HIV-1 enzyme or in the K_i(HIV-2)/K_i(HIV-1) ratio, demonstrating the ability of HIV-1 protease to accommodate large hydrophobic side chains in the P1′ subsite. On the other hand, substitution of the P1′ phenylalanine with trifluorobutylglycine (compound **4**) caused a significant increase in the K_i of HIV-1 but no change in the K_i(HIV-2)/K_i(HIV-1) ratio, demonstrating the preference for the hydrophobic group at the P1′ subsite for both enzymes. Finally, decreasing the size of the P1′ side chain by substituting an alanine for phenylalanine at the P1′ position (compound **6**) drastically decreased the K_i(HIV-2)/K_i(HIV-1) ratio, as shown previously for a related compound (Zhao et al., 1993).

Taken together, the inhibitor studies with the wild-type enzymes confirmed an important role for the P1′ side chain (Table 3). The K_i's of compounds **1**, **4**, and **5** with the wild-type enzymes indicated that the nature of the P1′ side chain plays a significant role in determining the absolute affinity, as shown previously (Roberts et al., 1990). The role of the P1′ side chain in determining the relative affinity is evident in that the calculated increase in binding energy (Fersht, 1985) for HIV-2 as compared to HIV-1 protease is approximately 10 kJ/mol for all compounds except compound **6**, which gives only a 5 kJ/mol difference. Since compound **6** differs from the other five inhibitors in that the P1′ side chain is smaller, this demonstrated that the size of the P1′ side chain plays a major role in determining the relative affinity of the inhibitors for the enzymes. This conclusion is consistent with previous models suggesting that the larger size of the HIV-1 active site cavity is the underlying cause for the differences observed in inhibitor and substrate specificity of the HIV-1 and HIV-2 enzymes (Hoog et al., 1996; Gustchina & Weber, 1991; Stebbins et al., 1996; Mulichak et al., 1993b; Tomasselli et al., 1990).

Inhibitor Sensitivity of the Chimeric HIV Proteases. The HIV-1 and HIV-2 proteases differ only at residues 32, 47, and 82 in the active site. Given the high degree of structural homology, one would predict that these residues are responsible for the observed differences in inhibitor affinities. However, previous work has shown that exchanging the active site residues alone between the HIV proteases is not sufficient to confer inhibitor specificity (Sardana et al., 1994; Hoog et al., 1996). Therefore, to identify other domains responsible for inhibitor affinity, we created a series of chimeric proteases. Interestingly, half of the protease constructs made in this study produced inactive protease (Table 2). A similar result has been obtained in saturation mutagenesis of the HIV-1 protease (Loeb et al., 1989) and in comparing the substrate binding pockets of the MLV and HIV-1 proteases (Menendez-Arias et al., 1996). These results alone demonstrate the complex structure−activity relationships that exist in the viral proteases.

Table 2: Inactive Substituted Proteases[a]

HIV (1: 1−61. 2: 62−99)
HIV (1: 1−25. 2: 26−99)
HIV (2: 1−25. 1: 26−61. 2: 62−99)
HIV (1: 1−25. 2: 26−59. 1: 60−99)
HIV (2: 1−15. 1: 16−25. 2: 26−59. 1: 60−99)
HIV (1: 1−5. 2: 6−59. 1: 60−99)
HIV-1 (2: 1−59, 82)
HIV-2 (1: 31−37, 47, 82)
HIV-1 (2: 32, 34, 47, 82)

[a] Proteases were expressed in *E. coli* and determined to be inactive by their inability to auto process from a larger precursor to the mature size, as previously described (Stebbins & Debouck, 1994).

Three active chimeric proteases were initially constructed. The substitutions are shown graphically in Figure 3. The three chimeric proteases were purified and kinetically characterized against all six inhibitors. As shown in Table 3, substituting the first 25 amino acids of HIV-1 protease with those of HIV-2 protease to produce HIV-1 (2: 1−25) had little effect on the inhibitor affinity. In contrast, substituting the next 12 residues for those of HIV-2 protease to produce HIV-1 (2: 1−37) created a chimeric protease which approximates HIV-2 protease in terms of inhibitor affinity for all six compounds. Lastly, little or no effect was seen by substituting the subsequent 22 residues for those of HIV-2 protease to produce HIV-1 (2: 1−59). Since residues 25−30 are identical between the two enzymes (Figure 1), these results suggested that amino acids 31−37 play a role in inhibitor affinity. This conclusion was substantiated by the fact that substituting residues 31−37 alone, to produce HIV-1 (2: 31−37), resulted in resistance to a representative subset of the inhibitors (since the changes represented by compounds **3** and **5** had no effect on either absolute or relative affinity, these inhibitors were eliminated from further study).

A possible explanation for these results was that the effect of residues 31−37 was due solely to the valine-to-isoleucine substitution at position 32, which lies in the active site. Indeed, the substitution at residue 32 alone has been shown to be important in conferring inhibitor specificity. Reports have shown that the active site mutant, HIV-1 (2: 32), arises in inhibitor-resistant variants of HIV-1 in response to HIV-1 protease inhibitors (Borman et al., 1996; Kaplan et al., 1994) and that the purified mutant enzyme approximates the K_i values of HIV-2 against a diverse set of inhibitors (Sardana et al., 1994). Therefore, to determine the role of the isoleucine at position 32 in the HIV-1 (2: 31−37) protease, further substituted proteases were constructed and tested against a representative subset of the inhibitors. As seen in Table 3, both HIV-1 (2: 32) and HIV-1 (2: 31, 33−37) gave K_i values higher than HIV-1 protease but not as high as HIV-1 (2: 31−37). These results demonstrate that the effects of substitutions at residue 32 and at residues 31 and 33−37 (the "30's loop") were separable. In addition, since the K_i of the HIV-1 (2: 31, 33−37) protease is not as high as that of HIV-2 protease, it also can be concluded that the

Table 3: Inhibition of Wild-Type and Substituted Proteases by Test Inhibitors

	compound **1**		compound **2**		compound **3**		compound **4**		compound **5**		compound **6**[c]	
	K_i[a] ± sd	R[b]	K_i ± sd	R	K_i ± sd	R	K_i ± sd	R	K_i ± sd	R	K_i ± sd	R
HIV-1	0.02 ± 0.00	1	0.14 ± 0.07	1	0.01 ± 0.00	1	0.15 ± 0.02	1	0.01 ± 0.00	1	0.01 ± 0.00	1
HIV-2	1.25 ± 0.05	70	5.65 ± 0.93	40	0.55 ± 0.03	64	9.51 ± 4.18	62	0.48 ± 0.05	45	0.06 ± 0.02	6
HIV-1 (2: 1−25)[d]	0.02 ± 0.00	1	0.12 ± 0.01	1	0.02 ± 0.00	2	0.55 ± 0.04	4	0.01 ± 0.01	1	0.02 ± 0.01	2
HIV-1 (2: 1−37)	0.51 ± 0.10	28	3.49 ± 0.96	25	0.42 ± 0.06	49	9.66 ± 0.27	63	0.30 ± 0.04	29	0.11 ± 0.01	11
HIV-1 (2: 1−59)	0.69 ± 0.03	38	1.90 ± 0.12	13	0.44 ± 0.09	51	6.97 ± 0.24	46	0.33 ± 0.07	31	0.11 ± 0.02	11
HIV-1 (2: 31−37)	1.41 ± 0.18	78	1.83 ± 0.03	13	ND[e]		5.65 ± 0.48	37	ND		0.24 ± 0.05	24
HIV-1 (2: 32)	0.28 ± 0.03	16	0.69 ± 0.10	5	ND		1.65 ± 0.08	11	ND		0.10 ± 0.01	10
HIV-1 (2: 31, 33−37)	0.21 ± 0.02	12	0.31 ± 0.05	2	ND		1.06 ± 0.16	7	ND		0.08 ± 0.01	8
HIV-1 (2: 32, 47, 82)	0.11 ± 0.05	6	0.44 ± 0.05	3	ND		1.05 ± 0.16	7	ND		0.07 ± 0.00	7
HIV-1 (2: 31−37, 47, 82)	0.46 ± 0.02	26	1.23 ± 0.16	9	ND		2.77 ± 0.16	18	ND		0.15 ± 0.09	15
HIV-2 (1: 32, 47, 82)	0.94 ± 0.04	53	2.94 ± 0.33	21	ND		9.03 ± 0.49	59	ND		0.13 ± 0.04	13

[a] K_i values (μM) determined as described in Materials and Methods. [b] Ratio of K_i(enzyme studied)/K_i(HIV-1) for the identical inhibitor. [c] The actual K_i values for compound **6** could not be determined for technical reasons. Absolute values for compound **6** are based on that of HIV-1 wild-type which was arbitrarily given the value of 0.01 μM. [d] The notation indicates HIV-1 protease with HIV-2 residues at positions 1−25. [e] ND = not determined.

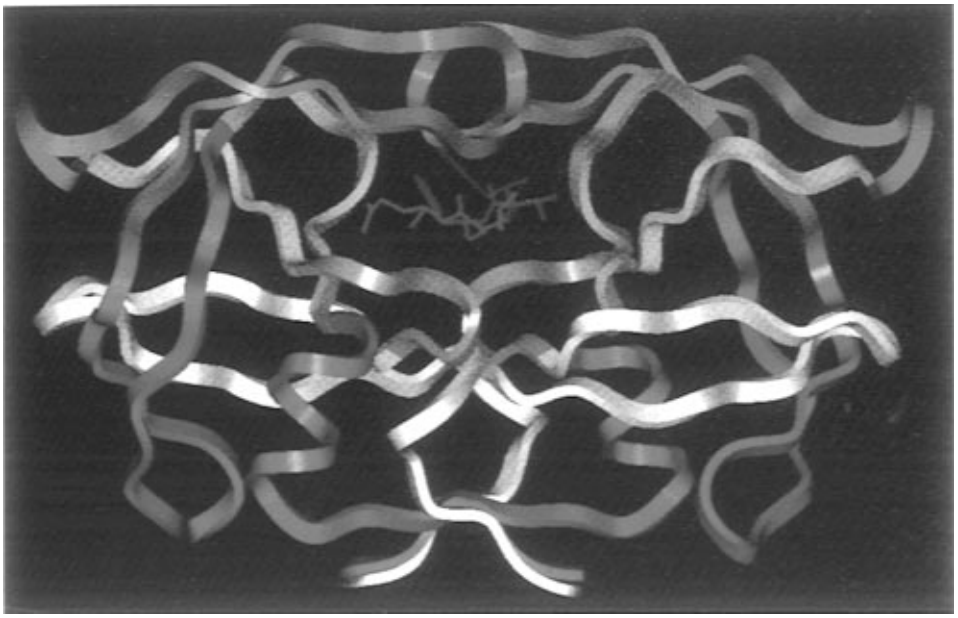

FIGURE 3: Ribbon diagram of the HIV-2 protease dimer in complex with a reduced amide inhibitor, shown as a red stick diagram (Tong et al., 1993). Residues which were substituted in the chimeric proteases are color coded as follows (the identical residues of each monomer are colored alike): The amino-terminal residues 1−24 are colored white. Residues 25−30, which are identical between the HIV-1 and HIV-2 proteases, are colored pink. Residues 31−37, which contain the 30's loop, are colored yellow. Residues 38−59 are colored green. Residues 78−85, which constitute the 80's loop, are colored blue. The remaining residues are colored red.

30's loop alone is not responsible for conferring the lower inhibitor affinity of HIV-2 protease.

To examine the possibility that the active site and the 30's loop together confer inhibitor specificity, these substitutions were assayed separately and together against a representative subset of the inhibitors. As shown in Table 3, the HIV-1 active site substitution, HIV-1 (2: 32, 47, 82), or the 30's loop substitution, HIV-1 (2: 31, 33−37), had roughly the same effect on inhibitor affinity. The combined effect of these substitutions, HIV-1 (2: 31−37, 47, 82), approximated the values obtained for the HIV-2 enzyme for all inhibitors tested. The converse substitutions in HIV-2 protease were constructed. On the basis of the results with the active site substituted HIV-1 enzyme, HIV-1 (2: 32, 47, 82), the K_i for HIV-2 (1: 32, 47, 82) was expected to be lowered approximately 5-fold. Interestingly though, the HIV-2 (1: 32, 47, 82) enzyme gave values that were indistinguishable from the HIV-2 enzyme. Nevertheless, the data are consistent with the finding that the active site alone is not able to confer inhibitor specificity. Unfortunately, the mutant HIV-2 (1: 31−37, 47, 82) was inactive, and therefore the effect of the 30's loop could not be assayed. These results demonstrate that the combination of active site residues and the 30's loop of HIV-2 protease together is able to confer HIV-2 inhibitor specificity on the HIV-1 protease.

Structural Role of the 30's Loop. The role of the 30's loop residues (colored yellow in Figure 3) in determining inhibitor affinity in the HIV proteases can be explained on the basis of existing structural data. In crystal structures of the proteases of HIV-1 and HIV-2 or SIV with the identical inhibitor, an asymmetric peptide chain conformation is seen at residues 78−85 (the "80's loop", colored blue in Figure 3) (Priestle et al., 1995; Hoog et al., 1996). In both HIV-2/SIV protease structures, this region extends more than 1 Å further into the active site than in the corresponding HIV-1 protease structure. This has the effect of decreasing the overall size of the active site cavity, particularly at the S1/S1′ subsite. It has been hypothesized that this shift is due to a main chain interaction present only in the HIV-1 enzyme (Hoog et al., 1996; Stebbins et al., 1996). These authors

suggest that the side chain of glutamate-34 interacts with a backbone carbonyl oxygen of the 80's loop to pull this region away from the active site. In HIV-2/SIV protease, residue 34 is replaced by an alanine/threonine, respectively, which would not permit such an interaction. Our results are consistent with this hypothesis. Unfortunately, this model could not be tested directly since the substituted protease, HIV-1 (2: 32, 34, 47, 82), was inactive. The inactivity of HIV-1 (2: 32, 34, 47, 82) itself suggests that complex interactions within the 30's loop may be important for structural stability.

Conclusions. Naturally occurring substitutions in the 30's loop in the presence and absence of inhibitor selection have been reported. However, these substitutions have been shown to be nonessential for inhibitor resistance (Jacobsen et al., 1995; Borman et al., 1996; Lech et al., 1996; Ridky & Leis, 1996). Mutations in this region appear later during selection, apparently acting only to stabilize the primary mutations. Given its relative role in determining inhibitor affinity, this seems intuitively surprising. One possible explanation for this may be that the entire 30's loop plays an important structural role, limiting the number of possible substitutions. This is supported by the fact that a number of the substituted proteases involving this region were inactive (Table 2) and by the fact that nonconservative substitutions at residues 31−33 and 36 have been shown to inactivate the HIV-1 protease (Loeb et al., 1989). If this hypothesis is correct, the only plausible way of generating a substituted protease such as those in this study would be through a recombination event between HIV-1 and HIV-2. Recombination between two HIV-1 molecules has been reported (Moutouh et al., 1996) . In fact, a recent report suggests the presence of an HIV-1/HIV-2 chimeric protease from a clinical isolates (Winslow et al., 1995). Consistent with the results presented here, the hybrid protease from this report contains HIV-2 sequence homology in the carboxy-terminal region but shows HIV-1 protease inhibition patterns.

In conclusion, we have used a molecular genetic approach to further our understanding of the fundamental determinants of inhibitor specificity of the HIV proteases. The use of chimeric HIV enzymes has been exploited previously, but only in mapping the interaction sites of *noncompetitive* inhibitors of reverse transcriptase (Condra et al., 1992). The lack of any previous reports using chimeric proteases to map the specificity domains for *competitive* inhibitors presumably reflects the assumption that specificity for the inhibitors would lie completely within the active site (Sardana et al., 1994; Hoog et al., 1996). We have shown that inhibitor specificity is dictated as much by residues outside the active site as those within the cavity itself. Further evaluation of these substituted proteases by X-ray crystallography will determine if those residues which dictate inhibitor specificity also determine structural specificity. These studies should greatly aid in the rational design of protease inhibitors.

ACKNOWLEDGMENT

We acknowledge Thomas Carr, Balan Chenera, and Melvin Rutledge for their valued technical assistance in the synthesis of the inhibitors used in this study. We thank Dr. John Erickson of NCI Frederick, Dr. Manal Swairjo and Dr. Jun Li of SmithKline Beecham, Dr. Jeff Stebbins of NIH, and Dr. Patricia A. Towler for their valuable discussion and for critical reading of the manucript.

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BI962729J