Human immunodeficiency virus type-1 reverse transcriptase and ribonuclease H as substrates of the viral protease

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Abstract

A study has been made of the susceptibility of recombinant constructs of reverse transcriptase (RT) and ribonuclease H (RNase H) from human immunodeficiency virus type 1 (HIV-1) to digestion by the HIV-1 protease. At neutral pH, the protease attacks a single peptide bond, Phe₄₄₀-Tyr₄₄₁, in one of the protomers of the folded, active RT/RNase H (p66/p66) homodimer to give a stable, active heterodimer (p66/p51) that is resistant to further hydrolysis (Chattopadhyay, D., et al., 1992, *J. Biol. Chem.* 267, 14227–14232). The COOH-terminal p15 fragment released in the process, however, is rapidly degraded by the protease by cleavage at Tyr₄₈₃-Leu₄₈₄ and Tyr₅₃₂-Leu₅₃₃. In marked contrast to this p15 segment, both p66/p51 and a folded RNase H construct are stable to breakdown by the protease at neutral pH. It is only at pH values around 4 that these latter proteins appear to unfold and, under these conditions, the heterodimer undergoes extensive proteolysis. RNase H is also hydrolyzed at low pH, but cleavage takes place primarily at Gly₄₃₆-Ala₄₃₇ and at Phe₄₄₀-Tyr₄₄₁, and only much more slowly at residues 483, 494, and 532. This observation can be reconciled by inspection of crystallographic models of RNase H, which show that residues 483, 494, and 532 are relatively inaccessible in comparison to Gly₄₃₆ and Phe₄₄₀. Our results fit a model in which the p66/p66 homodimer exists in a conformation that mirrors that of the heterodimer, but with a p15 segment on one of the protomers that is structurally disordered to the extent that all of its potential HIV protease cleavage sites are accessible for hydrolysis.

Keywords: HIV protease; human immunodeficiency virus; processing; protease specificity; reverse transcriptase; ribonuclease H

The protease encoded within the *pol* gene of retroviruses has been shown to be essential for the processing of viral polyproteins during the late extracellular phase of viral maturation (Kohl et al., 1988; Ashorn et al., 1990). This

processing leads to the liberation of structural proteins and enzymes of the virus, including the protease itself and reverse transcriptase, from their disposition within a polyprotein format. Because of the interest in the protease from human immunodeficiency virus as a potential target for therapeutic intervention in acquired immunodeficiency syndrome (AIDS), considerable effort has been launched in a search for inhibitors of the enzyme (see Hellen et al. [1989], Kay & Dunn [1990], Meek et al. [1990], Oroszlan & Luftig [1990], Tomasselli et al. [1991b], and Wlodawer & Erickson [1993] for recent reviews).

Design of protease inhibitors that might serve as drugs against AIDS is greatly facilitated by a detailed understanding of the structure and function of this enzyme. Part of this understanding has been provided by studies of the substrate specificity of the proteases from both

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Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; RNase H, ribonuclease H (as often used in this text, RNase H refers to our recombinant construct, which begins with a His-rich segment and then extends from Tyr₄₂₇ through Leu₅₆₀ of HIV-1 RT [Fig. 1]); HIV-1 protease, HIV type 1 protease; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; p66/p66, the homodimer of RT having full-length protomer subunits beginning with the His-rich segment and extending through residue 560 at the COOH terminus (Fig. 1); p66/p51, the RT heterodimer in which the p51 component is identical to p66, but which terminates at Phe₄₄₀ (Fig. 1).

types 1 and 2 HIV (Darke et al., 1988; Henderson et al., 1988; Konvalinka et al., 1990; Margolin et al., 1990; Phylip et al., 1990; Tozser et al., 1991; Griffiths et al., 1992). Our laboratory has evaluated a wide variety of nonviral proteins as substrates for the HIV proteases (Hui et al., 1990; Tomasselli et al., 1990a, 1991a,c) and, together with data from other laboratories (e.g., Shoeman et al., 1991; Tomaszek et al., 1992) has compiled an extensive database of octapeptide sequences from known substrates. This database has served as the basis for developing algorithms (Poorman et al., 1991; Chou, 1993; Chou et al., 1993) for predicting bonds likely to be cleaved by the HIV-1 or HIV-2 protease, given appropriate accessibility to the site of attack.

Recently, we reported production of heterodimeric (p66/p51) HIV-1 RT by *in vitro* cleavage of the recombinant homodimeric (p66/p66) HIV-1 RT at neutral pH by the HIV-1 protease (Chattopadhyay et al., 1992). We also demonstrated the existence of two additional HIV-1 protease-sensitive sites in the COOH-terminal ribonuclease H (RNase H) domain of p66; these occur at Tyr₄₈₃-Leu₄₈₄ and Tyr₅₃₂-Leu₅₃₃ within homologous octapeptide sequences (Chattopadhyay et al., 1992). Cleavage at these sites was only observed within the liberated p15 segment resulting from hydrolysis of the Phe₄₄₀-Tyr₄₄₁ bond in one of the two protomers of p66/p66; the RNase H domain of the p66/p51 heterodimer, and p66/p51 itself are refractive to proteolysis at neutral pH (Chattopadhyay et al., 1992). In this paper we describe the cleavage of recombinant constructs of p66/p66 and RNase H with the HIV-1 protease at low pH. Under these conditions of acidic pH, the proteins appear to unfold and become susceptible to hydrolysis by the protease. Several new sequences have been added to the database of substrates for the HIV-1 protease and these serve to reinforce past generalizations with regard to preferences of the enzyme for amino acids in particular P-sites (Poorman et al., 1991; Chou, 1993; Chou et al., 1993). Our results, interpreted with respect to recently published X-ray crystallographic structures for RNase H (Davies et al., 1991; Hostomska et al., 1991; Chattopadhyay et al., 1993) and p66/p51 (Kohlstaedt et al., 1992), are consistent with the view that the RT homodimer adopts a conformation similar to that of the heterodimeric p66/p51, but in which the COOH-terminal RNase H domain of one protomer is disordered.

Results

The amino acid sequences of the RT and RNase H constructs having histidine linker sequences are given in Figure 1. In discussing the course of proteolysis of these substrates by the HIV-1 protease, we adopt a numbering system based upon that of naturally occurring HIV-1 RT. The histidine-rich linkers are at the N-terminus of these

constructs, and these portions of the sequences are not included in the numbering system. Accordingly, RT comprises residues 1–560, and RNase H begins with RT-residue 427 and extends to 560 (see Kinemage 1).

Digestion of RT with HIV-1 protease at pH 5.5 and 4.0

We reported earlier (Chattopadhyay et al., 1992) that hydrolysis of the p66/p66 homodimer by the HIV-1 protease at pH 7.0 results in the cleavage of a single peptide bond in one of the two protomers. This cleavage at Phe₄₄₀-Tyr₄₄₁ results in the release of the COOH-terminal p15 segment from one polypeptide chain, while the corresponding region of the other remains refractive to hydrolysis, presumably due to the fact that it is folded within the three-dimensional structure of the catalytically active homodimer. The released p15 segment is then degraded by cleavage of peptide bonds at Tyr₄₈₃-Leu₄₈₄ and Tyr₅₃₂-Leu₅₃₃ (Chattopadhyay et al., 1992). The same reaction profile was observed when proteolysis was carried out at pH 5.5 (Fig. 2A); that is, hydrolysis with the HIV-1 protease proceeded rapidly to yield p66 and p51 and then stopped. At pH 4.0, however, a different picture emerged as evidenced by SDS-PAGE gels (Fig. 2B). Both of the major RT forms, p66 and p51, were now susceptible to extensive fragmentation and within 3 h had, by and large, disappeared in the gel analysis.

The peptides produced in the pH 4.0 digest of RT were separated by HPLC (Fig. 3). Eleven major fragments were isolated by this procedure, and the N-terminal sequences for each are given in Table 1. The length of peptides enclosed in brackets was inferred from amino acid compositional data. In addition to cleavage sites at the end of p51 (Phe₄₄₀-Tyr₄₄₁) and within the COOH-terminal p15 fragment, six new sites of hydrolysis within the p51 region were documented in this study that have not been heretofore reported: Cys₃₈-Thr₃₉, Phe₈₇-Trp₈₈, Tyr₁₁₅-Phe₁₁₆, Gly₂₃₁-Tyr₂₃₂, Leu₃₀₃-Ala₃₀₄, and Leu₃₂₅-Ile₃₂₆. A previously documented site of hydrolysis within p15, or the RNase H domain, was seen at Tyr₅₃₂, and the high yield of this peptide (#9 in Table 1) supports the idea that it is being generated from both RT chains. There is also indirect evidence for another new site of cleavage at Asn₄₉₄-Ile₄₉₅, since compositional analysis of peptide #2 indicates that the fragment terminates at Asn₄₉₄. Noteworthy by its absence is the cleavage at Tyr₄₈₃-Leu₄₈₄ that was observed earlier in p15 degradation (Chattopadhyay et al., 1992). This sequence occurs in the middle of α -helix A (Davies et al., 1991), and may support the view that the helix is present in the partially unfolded RNase H at pH 4.0 but absent in p15. It should also be noted that the potent HIV-1 protease inhibitor U-75875 (Ashorn et al., 1990) with $K_i < 1$ nM, blocked all of the cleavages documented in this work.

Fig. 1. Amino acid sequences of RT and RNase H constructs having His-rich segments. The His-rich portion of the RT construct is underlined at the top of the figure. Our RT runs, therefore, from Pro₋₁₅ to Leu₅₆₀, and encompasses p66 as defined by the sequence from Pro₁ to Leu₅₆₀. The RNase H construct begins with the sequence: P₋₁₁-I-H-D-H-D-H-P-F-H-G₋₁ as the His-rich leader, and then continues with Tyr₄₂₇ (indicated by |→ in the figure) to the end of the molecule at Leu₅₆₀. An asterisk marks the end of p51 at Phe₄₄₀, and also one of the several sites of cleavage with the HIV-1 protease that are indicated by ↓.

Fig. 2. Time course of proteolysis of RT at pH 5.5 (A) and at pH 4.0 (B) with the HIV-1 protease as monitored by SDS-PAGE. Times in minutes are given at the top of the photographs, and C represents control samples run at the end of the incubation period (240 min). Approximately 10 μ g of protein was applied in each lane; Coomassie stain.

Fig. 3. Separation of peptides generated from RT by cleavage with the HIV-1 protease at pH 4.0 (see Table 1 and Materials and methods). Numbered peptides were analyzed as described in Table 1.

Digestion of the RNase H construct with HIV-1 protease at various pH values

Upon incubation of our RNase H-linker construct (Fig. 1) with HIV-1 protease for 22 h in solutions buffered in the pH range of 4.0–7.0, we observed that the RNase H was refractive to cleavage at any pH value other than pH 4.0. Edman degradation of a 3-nmol sample of the pH 4.0 digestion mixture yielded three easily identifiable sequences: (1) the N-terminus of the construct, Pro₋₁₁-Ile-His-Asp... (2.7 nmol); (2) Ala₄₃₇-Glu-Thr-Phe₄₄₀-OH (2.1 nmol), and (3) Tyr₄₄₁-Val-Asp-Gly... (2.9 nmol) (Fig. 1B). Other PTH-amino acids were seen at each cycle during the course of this analysis, but no other sequences could be discerned or assigned with any confidence.

Table 1. Compositional and sequence analysis of fragments produced by cleavage of RT with the HIV-1 protease at pH 4.0^a

Peptide no. ^b	Peptide identification	Yield (nmol ^c)
2	... A-E-T-F ₄₄₀ -↓-[Y ₄₄₁ -V-D-G-A-... N ₄₉₄]	0.33
4	... V-E-I-C ₃₈ -↓-[T ₃₉ -E-M-E-K-... F ₈₇]	0.23
5	... T-Q-D-F ₈₇ -↓-[W ₈₈ -E-V-Q-L-... Y ₁₁₅]	0.15
7	[H ₂ N-P ₋₁₅ -... G-D-A-Y ₁₁₅]-↓-F-S-V-P-L-...	0.07
8	... L-W-M-G ₂₃₁ -↓-[Y ₂₃₂ -E-L-H-P-... L ₃₀₃]	0.17
9	... E-K-V-Y ₅₃₂ -↓-[L ₅₃₃ -A-W-V-P-... L ₅₆₀ COOH]	0.18
15	... G-D-A-Y ₁₁₅ -↓-[F ₁₁₆ -S-V-P-L-... G ₂₃₁]	0.09
16	... A-E-T-F ₄₄₀ -↓-[Y ₄₄₁ -V-D-G-A-... L ₅₆₀ COOH]	0.03
17	... A-E-T-F ₄₄₀ -↓-[Y ₄₄₁ -V-D-G-A-... L ₅₆₀ COOH]	0.06
21	... E-L-E-L ₃₀₃ -↓-[A ₃₀₄ -E-N-R-E-... L ₅₆₀ COOH]	0.28
22	... S-K-D-L ₃₂₅ -↓-[I ₃₂₆ -A-E-I-Q-... L ₅₆₀ COOH]	0.25

^a Peptides from 1.1 nmol of the RT monomer were injected onto an HPLC column as described in Materials and methods. Sequences enclosed in brackets denote peptides subjected to sequence and compositional analysis (with reference to the sequence given in Fig. 1).

^b Peptide numbers are given in Figure 3.

^c Yields refer to recoveries of peptides as determined by amino acid analysis.

Given the rather extensive hydrolysis of p15 by the protease, we were surprised to see that these same sites in the RNase H construct were resistant to cleavage. This digest afforded us the opportunity to isolate the stable peptide corresponding to p15, which includes most of the sequence of RNase H. To this end, the entire reaction mixture at pH 4.0 was subjected to HPLC (Fig. 4) in order to purify the peptide extending from Tyr₄₄₁ through Leu₅₆₀-OH and test it for RNase H activity. Every peak shown in Figure 4 was collected and subjected to amino acid analysis and automated Edman degradation (Table 2). As expected, the desired peptide, Tyr₄₄₁...Leu₅₆₀-OH, is one of the most abundant (peaks 13, 14, and 15, Fig. 4), along with the N-terminal peptide (peak 7) extending from Pro₋₁₁ through Gly₄₃₆ produced by cleavage of the Gly₄₃₆-Ala₄₃₇ bond. The cleavages that generate these two peptides, Y₄₄₁...L₅₆₀ and P₋₁₁...G₄₃₆, also give rise to the peptide A₄₃₇ETF₄₄₀-OH, which was found by direct sequence analysis of the mixture. Minor cleavages, to the extent of 5–10% of those mentioned above, were also observed (Table 2). One of particular interest is that at Asn₄₉₄-Ile₄₉₅, a site of hydrolysis that was inferred from analysis of peptides derived from RT itself (peptide #2; Table 1). This cleavage was not observed in fragmentation of p15, or of RNase H by the protease at pH 7.0 (Chattopadhyay et al., 1992).

Analysis for catalytic activity of the fragment Tyr₄₄₁ through Leu₅₆₀, one identical to p15 in sequence but not conformation, proved to be inconclusive. The peptide itself had no RNase H activity, but neither did a control containing the RNase H construct with its N-terminal extension (Fig. 1) that had been subjected to pH 4.0. This would imply that the RNase H could not refold from the partial denaturation experienced by the condition of low pH.

Fig. 4. Separation of peptides generated from RNase H by cleavage with the HIV-1 protease at pH 4.0. Numbered peptides were analyzed as described in Table 2.

Table 2. Peptides produced by hydrolysis of RNase H with the HIV-1 protease at pH 4.0^a

Peptide no. ^b	Peptide identification	Yield ^c (nmol)
7	[P ₁₁ -I-H-D-...G ₄₂₃ ...P-I-V-G ₄₃₆]-↓-A ₄₃₇ ...	0.42
8	...A-E-T-F ₄₄₀ -↓-[Y ₄₄₁ -V-D-G-...Y ₄₈₃]	0.07
9	[P ₁₁ -I-H-D-...G ₄₂₃ ...A-E-T-F ₄₄₀]-↓-Y ₄₄₁ ...	0.04
10	...E-K-V-Y ₅₃₂ -↓-[L ₅₃₃ -A-W-V-...L ₅₆₀ COOH]	0.04
11	...E-K-V-Y ₅₃₂ -↓-[L ₅₃₃ -A-W-V-...L ₅₆₀ COOH]	0.04
12	...L-E-V-N ₄₉₄ -↓-[I ₄₉₅ -V-T-D-...Y ₅₃₂]	0.03
13	...A-E-T-F ₄₄₀ -↓-[Y ₄₄₁ -V-D-G-...L ₅₆₀ COOH]	0.04
14	...A-E-T-F ₄₄₀ -↓-[Y ₄₄₁ -V-D-G-...L ₅₆₀ COOH]	0.06
15	...A-E-T-F ₄₄₀ -↓-[Y ₄₄₁ -V-D-G-...L ₅₆₀ COOH]	0.42

^a Peptides were generated from the RNase H construct (0.5 nmol) by cleavage with the HIV-1 protease and were purified by HPLC (Fig. 4). Sequences in brackets were identified by compositional and sequence analysis and are placed in sequence with reference to the structure given in Figure 1.

^b Peptide numbers refer to peaks labeled in Figure 4.

^c Yields are based upon amino acid analysis of the peaks in Figure 4.

Discussion

This paper describes the application of the HIV-1 protease as a probe of protein structure: in this case, of native and partially unfolded forms of the viral RT and RNase H. Our results may be interpreted with respect to recent X-ray crystallographic models of RNase H (Davies et al., 1991; Hostomska et al., 1991; Chattopadhyay et al., 1993) and the RT heterodimer, p66/p51 (Kohlstaedt et al., 1992). Moreover, the new cleavage sites revealed in the study of RT and RNase H as substrates of the enzyme add to our list of known substrate sequences for this important therapeutic target.

For that aspect of the present work that pertains to the specificity of the HIV-1 protease, we have found 10 sites of cleavage in the acid denatured RT (Table 1), 6 of which have not been reported previously. The fact that the new cleavage sites are found at pH 4.0, and not at pH 5.5 or 7.0, implies that the tertiary or quaternary structure of RT loosens up with lowering of the pH, thus exposing sites for hydrolysis by the protease that are normally inaccessible in the native conformation. This is consistent with the fact that polymerases are most active at pH 7.5 and do not show activity at pH 4.0. The activity of the protease is essentially unchanged over this range of pH.

These new sites of proteolysis are listed in Table 3 among a total of 64 HIV-1 protease cleavage sites that have been reported to date in viral and nonviral protein substrates. The striking features that emerge from this list are: (1) the overwhelming presence at P₂' of Glu/Gln (Gln at a lesser extent), which account for over 60% of all the entries at that position, with the remaining percentage shared by β -branched amino acids, and by Leu and Ala; (2) the high incidence of β -branched amino acids at

the P₂ position, provided that P₁' is not a Pro; in the latter case, Asn is preferred in P₂; (3) the absence of aromatic amino acids at either P₂ or P₂', and of Lys in any position from P₂ through P₃'; and (4) the overwhelming presence of Leu, Phe, Tyr, and Met at P₁ and complete absence of β -branched amino acids in that position. Of the P₂-P₁-P₁'-P₂' positions, P₁' appears to be least restrictive. A variety of different amino acids are observed here, but Ala, Pro, Phe, Tyr, and Leu are the most abundant. Arg has been documented at P₁' but has not as yet been seen at P₂, P₁, or P₂'. Pro is similar, but the autocatalytic cleavage of the Pro₆₃-Val₆₄ bond in the HIV-1 protease from mutant SF-2 (Rosé et al., 1993) represents an example of Pro in the P₁ position of a bond susceptible to hydrolysis by this enzyme. Outside the range from P₂ to P₂' it becomes more difficult to discern preferences for, or exclusion of, any particular amino acids. Some inferences concerning the specificity of the HIV protease have come to light from statistical analyses and algorithms that predict sequences that should be susceptible to proteolysis (Poorman et al., 1991; Chou, 1993; Chou et al., 1993). It may be of interest to note that application of the algorithm of Poorman et al. (1991) failed to predict 8 of the 10 new cleavage sites reported herein. However, the recently published algorithm of Chou (1993) was successful in predicting 7 of the 10 cleavages. Clearly, these predictive algorithms will continue to undergo refinement and to increase in accuracy as new parameters and data are incorporated.

The major part of this investigation has concerned the use of the protease as a tool to probe protein accessibility. Although the elements of the three-dimensional structure of proteins that are recognized and cleaved by the HIV-1 protease are still not understood completely, there exists a considerable body of evidence in support of a few generalities. It seems important that (1) the protein loosen up its structure in order to allow easy access to the protease; (2) a stretch of at least seven amino acids encompassing the cleavage site be in a flexible conformation such as to allow its accommodation into the catalytic site in an extended backbone geometry; (3) that stretch of amino acids be free of packing or H-bonded interactions with other parts of the substrate protein; and, perhaps most important from what has been discussed above, (4) the right amino acids be present at the P₄ through P₄' positions.

Our findings relative to processing of RT may provide insights as to the conformation of the p66/p66 homodimer, as well as to the process of viral maturation itself. It is known that the mature form of HIV-1 RT is the heterodimer, p66/p51 (Di Marzo Veronese et al., 1986), and that the enzyme involved in processing of the p66/p66 homodimeric precursor is the HIV-1 protease (Kohl et al., 1988). It follows that the protease can cleave only one of the two p66 subunits in the homodimer, and structural arguments have been put forth to explain this asymmetric

Table 3. P_4 through P'_4 residues in 64 protein substrates of the HIV-1 protease

P_4	P_3	P_2	P_1	↓	P'_1	P'_2	P'_3	P'_4	Protein	P_4	P_3	P_2	P_1	↓	P'_1	P'_2	P'_3	P'_4	Protein
T	Q	I	M	↓	F	E	T	F	Actin	R	K	I	L	↓	F	L	D	G	pol
G	Q	V	N	↓	Y	E	E	F	Calmodulin	G	S	H	L	↓	V	E	A	L	Insulin
P	F	I	F	↓	E	E	E	P	pro-IL1-β	G	G	V	Y	↓	A	T	R	S	Vimentin
S	F	N	F	↓	P	Q	I	T	pol	F	R	S	G	↓	V	E	T	T	gag
D	T	V	L	↓	E	E	M	S	Autolysis	V	E	V	A	↓	E	E	E	E	AAP ^a
A	R	V	L	↓	A	E	A	M	gag	L	P	V	N	↓	G	E	F	S	AAP ^a
A	E	E	L	↓	A	E	I	F	Troponin C	E	T	T	A	↓	L	V	C	D	Actin
S	L	N	L	↓	R	E	T	N	Vimentin	H	L	V	E	↓	A	L	Y	L	Insulin ^b
A	T	I	M	↓	M	Q	R	G	gag	H	Y	G	F	↓	P	T	Y	G	NF-κB ^c
A	E	C	F	↓	R	I	F	D	Troponin C	D	S	A	D	↓	A	E	E	D	AAP ^a
D	Q	I	L	↓	I	E	I	C	Autolysis	G	W	I	L	↓	G	E	H	G	LDH ^d
D	D	L	F	↓	F	E	A	D	pro-IL1-β	G	W	I	L	↓	A	E	H	G	LDH
Y	E	E	F	↓	V	Q	M	M	Calmodulin	Q	A	I	Y	↓	L	A	L	Q	pol ^e
P	I	V	G	↓	A	E	T	F	pol	E	K	V	Y	↓	L	A	W	V	pol ^e
T	L	N	F	↓	P	I	S	P	pol	V	E	I	C	↓	T	E	M	E	pol
R	E	A	F	↓	R	V	F	D	Calmodulin	T	Q	D	F	↓	W	E	V	Q	pol
A	E	T	F	↓	Y	V	D	K	pol	L	W	M	G	↓	Y	E	L	H	pol
A	Q	T	F	↓	Y	V	N	L	pol	G	D	A	Y	↓	F	S	V	P	pol
P	T	L	L	↓	T	E	A	P	Actin	E	L	E	L	↓	A	E	N	R	pol
S	F	I	G	↓	M	E	S	A	Actin	S	K	D	L	↓	I	A	E	I	pol
D	A	I	N	↓	T	E	F	K	Vimentin	L	E	V	N	↓	I	V	T	D	pol
Q	I	T	L	↓	W	Q	R	P	Autolysis	I	I	V	A	↓	C ^f	E	G	N	RNase A
E	L	E	F	↓	P	E	G	G	PE664E	A	A	A	K ^g	↓	F	E	R	Q	RNase A
	A	N	L	↓	A	E	E	A	LysPE40	G	G	N	Y	↓	P	V	Q	H	gag ^h
S	Q	N	Y	↓	P	I	V	Q	gag	A	R	L	M	↓	A	E	A	L	gag
P	G	N	F	↓	L	Q	S	R	gag	P	F	A	A	↓	A	Q	Q	R	gag
K	L	V	F	↓	F	A	E		AAP ^a	P	R	N	F	↓	P	V	A	Q	gag
G	D	A	L	↓	L	E	R	N	LysPE40	G	L	A	A	↓	P	Q	F	S	gag/pol
K	E	L	Y	↓	P	L	T	S	gag	S	L	N	L	↓	P	V	A	K	pol
R	Q	A	N	↓	F	L	G	K	gag	A	E	T	F	↓	Y	T	D	G	pol
S	R	S	L	↓	Y	A	S	S	Vimentin	R	Q	V	L	↓	F	L	E	K	pol
A	E	A	M	↓	S	Q	V	T	gag	Q	M	I	F	↓	E	E	H	G	Fibronectin ⁱ

^a Alzheimer amyloid protein.^b All entries to this point were referenced in Poorman et al. (1991).^c Riviere et al. (1991).^d Tomaszek et al. (1992).^e Chattopadhyay et al. (1992); following *pol* gene entries are from this work.^f Present as carboxamidomethyl Cys; Hui et al. (1990).^g Present as E-succinyl Lys; Hui et al. (1990).^h The following eight entries are from Tozser et al. (1991).ⁱ Oswald and von der Helm (1991).

processing based upon crystallographic models. Davies et al. (1991) and Hostomska et al. (1991) reported the three-dimensional structure of an RNase H variant derived by HIV-1 protease excision from a fusion protein with dihydrofolate reductase. This RNase H construct had the complete structure from Tyr₄₂₇ through Leu₅₆₀ (Fig. 1B) but with an N-terminal extension of four amino acids, Tyr-Ala-Ser-Arg, that are unrelated to the native RT sequence preceding the RNase H domain. The crystal structure of this catalytically inactive molecule was, nevertheless, in complete accord with that of RNase H from *Escherichia coli* (Katayanagi et al., 1990; Yang et al., 1990). Based upon the structural analysis, Davies and co-workers (1991) noted that the Phe₄₄₀ cleavage site that transforms the p66/p66 homodimer to p66/p51 is in a

β-sheet region and is inaccessible to protease binding. This is illustrated in Figure 5 and Kinemage 1, based upon a recently determined structure of our RNase H construct (Chattopadhyay et al., 1993). Davies et al. (1991) reasoned that the p66/p66 homodimer would be asymmetric; one RNase H domain would be properly folded and refractive to protease excision and the other would be at least partially unfolded to allow cleavage at the Phe-Tyr₄₄₁ bond.

The inferred asymmetry of p66/p66 with respect to the RNase H domains has been borne out and extended by Kohlstaedt et al. (1992), who determined the crystal structure of the p66/p51 heterodimer at 3.5 Å resolution. Indeed, not only are the RNase H segments different but the p51 component itself shows a folding pattern that is en-

tirely distinct from that seen in the corresponding segment of the p66 chain. Thus, two identical sequences in residues 1–440 show different tertiary structures depending upon whether they occur in p51 or p66. In any case, it is clear from the analysis of the heterodimer that the RNase H domain in p66 is folded in such a way as to render the Phe₄₄₀-Tyr₄₄₁ bond inaccessible to the protease. We speculate that if the interactions seen in the heterodimer are important in forming the p66/p66 homodimer, then one of the p66 protomers would likely adopt the conformation of p51, with an unraveled RNase H domain.

In an earlier paper we showed that a single bond, Phe₄₄₀-Tyr₄₄₁, is cleaved in one of the two p66 chains in the RT homodimer to liberate one COOH-terminal p15 segment; we also provided evidence that the COOH-terminal residue of the p51 subunit is Phe₄₄₀ (Chattopadhyay et al., 1992). What happens next has bearing on our ideas concerning the nature of folding within the p15 segment. In our hands, the protease rapidly destroys this released p15 segment by two cleavages at Tyr₄₈₃-Leu₄₈₄ and Tyr₅₃₂-Leu₅₃₃ (Chattopadhyay et al., 1992). These bonds lie within homologous sequences, and in the crystal structure of a properly folded RNase H they reside in the middle of a helix and at one edge of a five-stranded β -sheet (Kinemage 1). It has already been suggested (Hostomska et al., 1991) that the p15 segment in the RT homodimer must be at least *partially* loosened to permit cleavage at the Phe₄₄₀ bond. This would correspond to an unwinding of the central β -strand in the sheet. But what about the remainder of the p15 subunit? It could be argued that the remainder of the subunit is still substantially folded prior to excision from p66, and that the further unwinding to permit access to the Tyr₄₈₃ and Tyr₅₃₂ regions only occurs after the Phe₄₄₀ hydrolysis takes place. We believe that this is not the case. Hydrolysis of folded RNase H, even at pH 4, takes place essentially at only two sites, Gly₄₃₆ and Phe₄₄₀, and a high yield is obtained of the full C-terminal peptide from Phe₄₄₀ through Leu₅₆₀ (Table 2, peptide 15). In other words, starting from a properly folded RNase H structure, cleavage at Phe₄₄₀ is not sufficient to cause further unraveling of the remainder of the molecule. The 483 and 532 sites remain stable to hydrolysis. Yet in the p15 fragment released from the p66/p66 form, a molecule with exactly the same sequence but originating from p66/p66, cleavage occurs rapidly at those sites. We can only conclude that in the RT homodimer, the p15 segment that eventually gets liberated is more than just “partially unfolded” to permit access to Phe₄₄₀; p15 must be *substantially* unfolded, with little or none of the tertiary structure seen in the RNase H construct.

Figure 5 and Kinemage 1 summarize the sites of hydrolysis that were observed in the various experiments of the current and previous (Chattopadhyay et al., 1992) studies. In this fully folded structure of our RNase H construct, all five cleavage points are located in regions that appear to be inaccessible to the protease and that do not

Fig. 5. Smoothed backbone representation of the RNase H construct used in the current study. The five locations of HIV-1 protease cleavage in either the RNase H construct or the RNase H domain referred to in the text as p15 are highlighted with color (green = Gly₄₃₆-Ala₄₃₇; blue = Phe₄₄₀-Tyr₄₄₁; yellow = Tyr₄₈₃-Leu₄₈₄; purple = Asn₄₉₄-Ile₄₉₅; red = Tyr₅₃₂-Leu₅₃₃). The apparent inaccessibility of these sites to protease binding is discussed in the text. Coordinates for this diagram are from the X-ray crystal structure of an RNase H dimer (Chattopadhyay et al., 1993; submitted to the Brookhaven Protein Data Bank, PDB entry 1RDH), and are very similar overall to those of Davies et al. (1991; PDB entry 1HRH), with the exception of an ordered region 538–542 in the former structure, which is disordered in the latter. This graphical representation was constructed with the GRASP program (A. Nichols & B. Honig, Columbia University, New York).

exhibit the isolated and extended backbone geometry to which the protease is able to bind. From this, it is quite evident why the RNase H construct is resistant to HIV protease hydrolysis at pH 7. At pH 4, however, cleavages occur at Gly₄₃₆ and Phe₄₄₀, and only very slowly at Tyr₄₈₃, Asn₄₉₄, and Tyr₅₃₂. In fact, it might be that these minor cleavages occur only in a small (<10%) population of molecules that are unfolded, and that the majority of the protein is stable to these points of hydrolysis. Accordingly, one might speculate that cleavage of the Gly₄₃₆-Ala bond at the turn region near the N-terminus may loosen the association of the Phe₄₄₀-Tyr region from its interaction with other β -strands of the sheet. It could also be that the lowered pH condition itself is sufficient to cause exposure of both the Phe₄₄₀ and Gly₄₃₆ regions simultaneously. Regardless of how these residues become exposed to the protease at pH 4, it is clear that essentially no further processing occurs once the two cleavages have com-

pleted. The remainder of the molecule appears to remain folded in a resistant conformation.

One final point needs to be made concerning the straight-line arrangement of three of the cleavage sites in the five-stranded β -sheet, i.e., at Phe₄₄₀, Asn₄₉₄, and Tyr₅₃₂. Although this is visually rather striking, we do not feel that there is anything other than coincidence at work here, given the fact that the three cleavages are from different experiments and that the sheet structure must be disrupted before cleavage can occur.

From the arguments presented above for an extensively unfolded p15 polypeptide in the RT homodimer, it is hard to see how this fragment, alone, could survive in HIV-1 viral lysates and it seems highly improbable that this excised segment would constitute a catalytic entity with RNase activity in the viral particle. Taken as a whole, our results are in general accord with models proposed for the asymmetric processing of the RT homodimer (Davies et al., 1991; Hostomska et al., 1991) and with inferences drawn from the crystallographic analysis of p66/p51 (Kohlstaedt et al., 1992).

Materials and methods

HIV-1 protease, HIV-1 reverse transcriptase, and HIV-1 RNase H were expressed in *E. coli*, purified, characterized, and assayed as described in earlier work (Tomasselli et al. [1990c], Sharma et al. [1991], and Evans et al. [1991], for protease, RT, and RNase H, respectively). RT and RNase H constructs included a histidine-rich segment to facilitate isolation of the proteins from the cell supernatant fractions (Fig. 1). The protease was expressed as the full-length enzyme, residues 1–99; about 5% of the enzyme consisted of chains 100 residues in length with Met at position 1 (Tomasselli et al., 1990c; Hui et al., 1993). Solvents for protein sequencing, amino acid analysis, HPLC, SDS-PAGE, and other reagents used in the present studies, were of the highest grade commercially available.

Analytical methods

Amino acid analysis was performed with the aid of a Beckman model 6300 analyzer. Samples were hydrolyzed for 24 h in vacuo in 6 N HCl at 110 °C, then dried in a Speed Vac concentrator (Savant), and the residues were dissolved in a buffer at pH 2.2 (Na-S; Beckman) for application to the analyzer. Proteins and peptides were sequenced by automated Edman degradation in an Applied Biosystems model 470 gas-phase sequencer fitted with an on-line HPLC analyzer (model 120-A) for identification and quantitation of PTH amino acids. Integration of the peaks from the HPLC was performed with a Nelson Analytical 3000 Series chromatography data system con-

nected in parallel with the recorder to the output of the model 120A HPLC system. SDS-PAGE was performed according to the procedure of Laemmli (1970). Gels (12%) were run under reducing conditions (mercaptoethanol or dithiothreitol) and stained with Coomassie blue for detection of proteins.

Separation of peptides following digestion of RT with HIV-1 protease

Homodimeric p66 RT (200 μ g) in 0.2 M sodium acetate buffer, at pH 5.5 or 4.0, containing 10% glycerol and 5% ethylene glycol was incubated with 5–7 μ g HIV-1 protease at 30 °C. Aliquots were taken at various times and analyzed by SDS-PAGE under reducing conditions and by HPLC. HPLC was carried out with a Vydac C₄ column (0.46 $\times$ 25 cm) eluted with a nonlinear gradient (convex gradient, curve 0.4, in the Perkin-Elmer series 4LC operator manual) of increasing acetonitrile concentration from 0 to 60% in 0.15% TFA. The separation was carried out over a period of 90 min at a flow rate of 1.0 mL/min, and elution of peptides was monitored spectrophotometrically at 230 nm.

Separation of peptides following digestion of RNase H with HIV-1 protease

Cleavage of the RNase H construct by the HIV-1 protease was tested at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 in 0.2 M buffer (sodium acetate, pH 4.0, 4.5, 5.0, and 5.5; MOPS, pH 5.5, 6.0, 6.5, and 7.0) containing 10% glycerol and 5% ethylene glycol. RNase H (11 μ g) and protease (1 μ g) were incubated at 30 °C, and samples withdrawn after 4 h and 22 h were subjected to SDS-PAGE. Only the pH 4.0 sample showed any degradation of the RNase H by the HIV-1 PR. A preparative-scale experiment was carried out at pH 4.0 with 800 μ g (53 nmol) of RNase H and 56 μ g of HIV-1 protease incubated for 22 h at 30 °C in 520 μ L of the sodium acetate buffer solution. Peptides were isolated by HPLC with a Vydac C₄ column (0.46 $\times$ 25 cm) eluted for 15 min in 0.15% TFA and then with a convex gradient (curve 4 in the Waters 625 LC system operator manual) of increasing acetonitrile concentration from 0 to 45% in 0.15% TFA. Separation was carried out over a period of 70 min at a flow rate of 1 mL/min, and the effluent stream was monitored continuously for peptide content at 230 nm.

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their RNase H X-ray structure prior to publication (Chattopadhyay et al., 1993).

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