Synthetic “interface” peptides alter dimeric assembly of the HIV 1 and 2 proteases

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Abstract

Retroviral proteases are obligate homodimers and play an essential role in the viral life cycle. Dissociation of dimers or prevention of their assembly may inactivate these enzymes and prevent viral maturation. A salient structural feature of these enzymes is an extended interface composed of interdigitating N- and C-terminal residues of both monomers, which form a four-stranded β -sheet. Peptides mimicking one β -strand (residues 95–99), or two β -strands (residues 1–5 plus 95–99 or 95–99 plus 95–99) from the human immunodeficiency virus 1 (HIV1) interface were shown to inhibit the HIV1 and 2 proteases (PRs) with IC_{50} 's in the low micromolar range. These interface peptides show cognate enzyme preference and do not inhibit pepsin, renin, or the Rous sarcoma virus PR, indicating a degree of specificity for the HIV PRs. A tethered HIV1 PR dimer was not inhibited to the same extent as the wild-type enzymes by any of the interface peptides, suggesting that these peptides can only interact effectively with the interface of the two-subunit HIV PR. Measurements of relative dissociation constants by limit dilution of the enzyme show that the one-strand peptide causes a shift in the observed K_d for the HIV1 PR. Both one- and two-strand peptides alter the monomer/dimer equilibrium of both HIV1 and HIV2 PRs. This was shown by the reduced cross-linking of the HIV2 PR by disuccinimidyl suberate in the presence of the interface peptides. Refolding of the HIV1 and HIV2 PRs with the interface peptides shows that only the two-strand peptides prevent the assembly of active PR dimers. Although both one- and two-strand peptides seem to affect dimer dissociation, only the two-strand peptides appear to block assembly. The latter may prove to be more effective backbones for the design of inhibitors directed toward retroviral PR dimerization in vivo.

Keywords: aspartyl protease; cross-linking; dimer interface; dissociation; enzyme inhibition; HIV1 protease; HIV2 protease; peptide; refolding; tethered dimer

Dimerization is required to create a symmetric active site for aspartyl proteases (PRs) encoded by retroviruses. The crystal structures of the human immunodeficiency virus 1 (HIV1) (Navia et al., 1989; Wlodawer et al., 1989) and Rous sarcoma virus (RSV) (Miller et al., 1989) PRs confirmed the homodimeric nature of these viral enzymes and also defined the regions of interaction between the monomers. The extensive hydrophobic interface present in these PR dimers is dominated by an antiparallel β -sheet formed by the interdigitation of the N-terminal (residues 1–4 in HIV1 and 1–5 in RSV) and the C-terminal (residues 96–99 in HIV1 and 119–124 in RSV) β -strands of each monomer (Fig. 1 and kinemages). These interactions appear to be a major stabilizing force in the enzyme,

since they contribute over 50% of the intersubunit contacts and hydrogen bonds.

The dimerization event of the HIV aspartyl PRs is a prerequisite to enzymatic activity, which leads to self-processing from the gag/pol precursor. Processing at the other cleavage sites within the gag and gag/pol polyproteins produces all the capsid proteins (MA, CA, NC) as well as the other viral enzymes (RT, RNase H, IN) (for a review see Kräusslich & Wimmer, 1988). Because the polyprotein processing steps assigned to the viral PR appear to occur following particle formation and possibly budding, dimerization of the viral PR could be an important regulatory mechanism for viral maturation. Dimerization and activation of the retroviral PRs is presumably concentration dependent and therefore likely to occur once viral capsid assembly has begun increasing the colocalization of viral proteins. Inhibition of this dimerization step would prevent the activation of the viral PR

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Fig. 1. Schematic representation of the four-stranded β -sheet dimeric interface of the HIV1 protease. Main chain and side chain atoms for residues 1–5 and 95–99 are presented in two shades indicating the two different protein monomers.

and, in turn, prevent the polyprotein processing, which is essential for viral maturation and infectivity (Kohl et al., 1988). The ability of oligopeptides to interfere with formation of a protein complex has been previously documented in vitro for two herpes simplex virus systems, the enzyme ribonucleotide reductase (Dutia et al., 1986; McClements et al., 1988), and the regulatory protein VP16 (Haigh et al., 1990).

Our previous use of defective monomers or nonidentical subunits to exchange with wild-type PR homodimers

in vitro produced catalytically defective heterodimers (Babé et al., 1991). This indicated that monomer exchanges were possible and revealed the requirement of a stable interface for enzyme activity, suggesting that a possible mechanism of enzyme inhibition was to disrupt or prevent stable homodimer formation. We have chosen the dimeric interface formed by the N- and C-terminal β -strands of HIV PR as the target region for interfering with the dimerization event. It was proposed that a tetrapeptide composed of the C-terminal residues of HIV1 PR could block dimer formation, since this peptide was involved in extensive atomic interactions (Weber, 1990). Indeed, recent kinetic data were presented where such a peptide showed an effect on dimerization (Zhang et al., 1991). We have synthesized a panel of peptides comprising the region of the interface formed by the N- and C-terminal β -strands. They have been tested for their ability to affect enzymatic activity and dimer dissociation. Their specificity for inactivation of the HIV1 and HIV2 PRs as compared to the RSV PR and pepsin was measured. Their limited ability to inhibit a tethered HIV1 PR polypeptide was confirmed. Cross-linking experiments suggest that these interface peptides alter the monomer/dimer equilibrium of the HIV1 and HIV2 PRs.

Results

Inhibition of HIV1 and HIV2 PR activity

Six interface peptides (Table 1) were synthesized that correspond to regions of the amino and carboxyl termini of the HIV1 PR (1CF, Ac1CF, 1KPL, PF1, AbF1) or the HIV2 PR (ML2). They were made to mimic a single β -strand or a pair of the β -strands that make up the interface of the dimeric PR structures (Fig. 1). Because the distance between the α -carbons of F₉₉ of monomer 1

Table 1. Inhibition of HIV1 and HIV2 proteases (PRs) by synthetic peptides synthesized to represent regions of the four-stranded β -sheet dimeric interface (Fig. 1)^a

Peptide	Sequence	IC ₅₀ (μ M)	
		HIV1 PR	HIV2 PR
1CF	C ₉₅ T ₉₆ L ₉₇ N ₉₈ F ₉₉	150	400
Ac1CF	Ac-C ₉₅ T ₉₆ L ₉₇ N ₉₈ F ₉₉	500	1,000
1KPL	Ac-K-P ₁ Q ₂ I ₃ T ₄ L ₅	>2,000	>2,000
PF1	P ₁ Q ₂ I ₃ T ₄ L ₅ -(G) ₃ -C ₉₅ T ₉₆ L ₉₇ N ₉₈ F ₉₉	40	20
AbF1	Aba ₉₅ T ₉₆ L ₉₇ N ₉₈ F ₉₉ -(G) ₃ -Aba ₉₅ T ₉₆ L ₉₇ N ₉₈ F ₉₉	225	200
ML2	M ₉₅ S ₉₆ L ₉₇ N ₉₈ L ₉₉ -(G) ₃ -M ₉₅ S ₉₆ L ₉₇ N ₉₈ L ₉₉	50	20
1R	PFAAAQQR	No inh.	No inh.

^a The single letter code is used to identify amino acid residues. Residues are numbered according to their positions in the HIV1 PR monomers. A glycine triplet (G)₃ was placed between the two β -strand mimics to provide potential for flexibility. For peptide 1KPL, a lysine was included at the N-terminus to enhance solubility. For peptide AbF1, aminobutyric acid (Aba) was used instead of cysteines to avoid difficulties in synthesis and possible intramolecular disulfide formation. For IC₅₀ determinations, the purified enzymes (10 nM) were assayed with 150 μ M concentrations of substrate 1 and increasing concentrations of peptides (1–500 μ M for PF1, AbF1, ML2, 1R; and 1–200 μ M for 1CF, Ac1CF, 1KPL).

and P₁ of monomer 2 is only 5.5 Å (Wlodawer et al., 1989), a spacer arm of three glycines was used to connect the two strands. Similar short spacer arms have been used in the biosynthesis of active HIV1 PR tethered dimers (this paper and DiIanni et al., 1990).

Table 1 presents the sequence of each peptide and shows the concentration that yields 50% enzyme inhibition (IC₅₀) obtained for the HIV1 and HIV2 PRs using substrate 1. Each of the interface peptides except 1KPL inhibits both enzymes in a concentration-dependent manner. The peptide IR, which is unrelated in sequence to any of the interface peptides but of a hydrophobic nature, showed no inhibition at 5–500 µM concentrations. The inhibitory effects were only observed when the peptides were preincubated with a low concentration of enzyme (1–25 nM) in the absence of NaCl or substrate. These conditions were used to promote PR inhibition by the interface peptides. However, the salt concentration was raised to 1 M NaCl to assay substrate turnover, since high ionic strength increases enzymatic activity several fold (Cheng et al., 1990). Because N-terminal acetylation failed to improve the inhibitory efficacy of the peptide 1CF, all of the two-strand peptides were synthesized with a free N-terminus. A lysine was added to the N-terminal sequence PQITL to increase the solubility of this peptide. Peptide AbF1 was synthesized using α-amino butyric acid in place of both cysteines (position 95 in HIV1 PR) to avoid intramolecular disulfide bond formation during the synthesis and purification of this two-strand peptide. Such a replacement of cysteines has been shown previously to have no effect on the kinetics or structure of the HIV1 PR (Wlodawer et al., 1989).

To assess the degree of specificity of these peptides, their inhibitory effect was measured for the RSV PR, porcine pepsin, and human renin. The results are summarized in Table 2 as the percentage of remaining proteolytic activity in the presence of various concentrations of in-

terface peptides under the assay conditions described above. Using peptide concentrations that showed marked inhibition of either HIV1 or HIV2 PRs (Table 1), a limited amount of inhibition (<15%) is detected for the retroviral RSV PR with peptides PF1, AbF1, or ML2, whereas no effects are observed for the mammalian proteases. The results suggest that these interface peptides affect the interaction of the amino and carboxyl termini of the closely related HIV1 and HIV2 PRs (47.5% identical), whereas they do not affect the more distantly related RSV PR (29% identical to HIV1 PR) or the monomeric, two-domain mammalian proteases: pepsin and renin (7 and 14% sequence identity, respectively, of one domain to the HIV1 PR). The peptide with the lowest IC₅₀ for HIV1 (PF1) was incubated for 1–24 h in the presence of HIV1 PR using a 10-fold greater amount of enzyme than in the inhibition assays, and 1 M NaCl in the reaction to promote proteolysis. Analysis of the reaction mix by reverse-phase high performance liquid chromatography (HPLC) revealed no cleavage of PF1, suggesting that the peptide bound nonproductively or external to the active site.

A more accurate measure of the potency of the peptide inhibitors, as well as an analysis of the mode of inhibition was done for 1CF and PF1. The velocity (*v*) of the enzyme was measured at several fixed concentrations of substrate (*S*) while varying the concentration of the inhibitor (*I*) peptides 1CF and PF1. The two peptides chosen for this analysis were representative of the two classes of peptides synthesized; those that mimic one (1CF) or two (PF1) of the interface β-strands of the PR dimer. A replot of the slope from 1/*v* versus 1/[*S*] plots against [*I*] identifies the apparent *K_i* values as 114 ± 4 µM for 1CF and 52 ± 3.9 µM for PF1 (data not shown). A linear mixed-type inhibition profile was observed for both peptides when 1/*v* was plotted versus [*I*] for varying [*S*] (Dixon plot [Segel, 1975]). The kinetic patterns observed are consistent with linear noncompetitive inhibition, where *E* and *EI* bind *S* with different affinities and the *ESI* complex is nonproductive (mixture of partial competitive inhibition and pure noncompetitive inhibition).

Table 2. Comparison of effect of various peptides on the activity of the Rous sarcoma virus protease (PR), porcine pepsin, and human renin^a

Peptide	Concentration (µM)	Activity (%)		
		RSV PR	Pepsin	Renin
1CF	500	100	100	100
1KPL	500	100	100	ND
PF1	100	90	100	100
AbF1	250	90	100	ND
ML2	50	85	100	ND

^a Equivalent concentrations of enzyme (25 nM) were used to incubate with the interface peptides at the concentrations given, and then the amount of enzymatic activity remaining was determined as compared to untreated samples. All enzyme activities were measured using peptide 1 substrate under conditions mentioned in the text.

Dissociation of HIV1 PR dimers

If a peptide is able to inactivate the PR dimer by interacting with the complementary regions of a PR monomer, its effect on dimerization may be detected as a lowering of the observed dissociation constants (*K_d*) relative to untreated PR dimers. Dissociation constants for the HIV1 PR range between 50 nM, obtained by measuring activity from progress curves of enzyme limit dilutions (Cheng et al., 1990), and 5 nM as determined by kinetic analysis and sedimentation equilibrium measurements (Zhang et al., 1991). We chose to measure the *relative* dissociation concentrations for HIV1 PR by quantitating enzymatic activity in a dilution series in the presence and

absence of interface peptides (Fig. 2). When the molar activity of the enzyme is plotted against enzyme concentration $[E]$, a breakpoint occurs at the dissociation constant for the PR/substrate complex. Presumably, enzyme dimers are no longer the prevalent species below this value, resulting in a marked loss of activity. This breakpoint occurs below 5 nM for the HIV1 PR under our assay conditions in the absence of any interface peptides. In the case of the peptide 1CF, tested at 200 μ M, the breakpoint occurs below 20 nM, showing that this peptide shifts the monomer/dimer equilibrium toward monomers. The effect of peptide PF1, tested at 75 μ M is more difficult to explain, since it reduces enzymatic activity at all the enzyme concentrations tested (1–100 nM), and shifts the inflection point of the curve to about 7 nM. Peptide ML2, tested at 50 μ M behaved similarly to PF1 (data not shown).

The inhibitory effects of the interface peptide 1CF can only be detected when NaCl is not present during the incubation of enzyme and peptide. When identical reactions were carried out in the presence of 1 M NaCl, no inhibition was observed using peptide 1CF, whereas peptides PF1 and ML2 inhibited at enzyme concentrations below 20 nM. It is possible that the tighter binding peptides PF1 and ML2 can overcome the stabilization of the enzyme dimer induced by the high salt concentration. The effects of ionic strength on the interactions within the dimer, as well as between the enzyme, substrate, and interface peptides will require alternative methods of analysis to differentiate. The inhibitory pattern of the one-strand peptide 1CF may reflect its interaction with monomers, or within a weakened dimer interface, since inhibition only occurs at low enzyme concentrations (near the dissociation constant) and in the absence of NaCl, which may help strengthen the hydrophobic interactions of the

dimer. The two-strand peptide PF1 appears to disrupt even tightly associated dimers, since it works at enzyme concentrations much higher than K_d and in the presence of salt.

Similar serial dilution assays of the HIV2 PR resulted in a linear curve between 1 and 100 nM enzyme, suggesting that the apparent dissociation constant for this enzyme under these assay conditions is below 1 nM. The lack of sensitivity of the assay prevented identification of a breakpoint of activity at lower $[E]$. PF1, tested at 50 μ M inhibited the HIV2 PR at all the enzyme concentrations tested (1–100 nM), as observed for the HIV1 PR. Curiously, in the case of the HIV2 PR, peptide 1CF (500 μ M) was inhibitory at all the enzyme concentrations tested (1–100 nM).

Cross-linking of HIV2 PR subunits

Another procedure employed to determine the mode of action of these interface peptides was that of cross-linking reactions of HIV1 or HIV2 PRs in the presence and absence of the peptides. The homobifunctional cross-linker disuccinimidyl suberate (DSS) was chosen because of its reactivity with amines at pH 7.5, because working away from the enzyme pH optimum of 4–5.5 reduces autolysis. Furthermore, the spacer arm length of the linker (11.4 Å) was optimal for the formation of intermolecular covalent bonds between closely associated polypeptides (Coombs & Brown, 1987). Cross-linking reagents with spacer arm lengths of 6.4 Å or shorter showed no ability to cross-link the PR subunits and interface peptides (data not shown).

When a sample of HIV2 PR (50 nM) was cross-linked in the absence of any peptides, the products obtained correspond to the monomer (M, 10.5 kDa), the dimer (D, 21 kDa), and a tetramer (T, >40 kDa) as seen in Figure 3, lane 2 for HIV2 PR. Incubation of a 500–1,000-fold molar excess of interface peptide to enzyme prior to cross-linking resulted in the formation of a new polypeptide band of molecular weight corresponding to one PR monomer and one peptide adduct. The larger peptides PF1, ML2, and β -Nal 1 give larger monomer-peptide adducts (about 11.5 kDa in lanes 3, 4, and 8), whereas the smaller peptide 1CF gives a shorter band of about 11 kDa (lane 5), and 1KPL forms no adduct (lane 7). In the case of peptides 1CF, PF1, and ML2, little or no PR dimer could be detected in these reactions (lanes 3–5), whereas the reaction lacking peptide (lane 2) as well as reactions with 1KPL and β -Nal 1 peptides (lanes 7 and 8) showed similar amounts of PR dimer. Reactions with peptide AbF1 at 500-fold molar excess gave bands corresponding to PR monomer and monomer-peptide adduct (data not shown).

The cross-linking results indicate that in the presence of interface peptides that inhibit the enzyme, the PR monomer/dimer equilibrium is shifted to the monomer form at the enzyme concentration (50 nM) used. The lon-

Fig. 2. Molar activity versus the enzyme concentrations measured in the absence and presence of peptides. The enzymatic activity of the HIV1 protease was measured at various concentrations (1–100 nM) at pH 5.5, in the absence of salts, and the specific activity was determined. The substrate β -Nal 1 was used for these measurements as described in the text. Molar activity was calculated as micromole of substrate digested per minute per micromole of enzyme. Peptides PF1 and 1CF were tested at 75 and 200 μ M concentrations, respectively.

Fig. 3. Immunoblot of the HIV2 protease (PR) following cross-linking reactions with and without peptides. The reactions appear labeled over each lane. All samples were incubated 4 h at ambient temperature except for the lane labeled "no reaction." The labels M, D, and T refer to the PR monomers, dimers, and tetramers, respectively. Proteins were resolved on sodium dodecyl sulfate-16% polyacrylamide gels, transblotted, and probed with antibodies specific for the HIV2 PR.

ger incubation period (4 h) and the higher pH (7.5) for these reactions relative to the conditions used for the dissociation measurements (Fig. 2) presumably result in the greater dissociation of the enzyme and may allow peptide 1CF to react more effectively. In addition, the ratio of monomers to dimers observed after cross-linking suggests that dimers are not the predominant species under the conditions of the reaction. Under the conditions of these cross-linking reactions, autolysis is more severe for the HIV1 PR. Approximately 20% of the PR is specifically degraded to a 94-amino acid polypeptide (Babé et al., 1990), and the cross-linking of this species with the interface peptides gives rise to a pattern of multiple bands of similar molecular weights that are difficult to identify. Nevertheless, a similar polypeptide pattern is observed in reactions with the HIV1 PR (data not shown) where the two-stranded peptides prevented the detection of PR dimers and all peptides except 1KPL formed adducts with the PR monomers.

Limited inhibition of a tethered HIV1 PR dimer by the interface peptides

To further test the mode of action of these interface peptides, a tethered HIV1 PR dimer was created. This molecule has a three-glycine tether constructed between the C-terminal Phe-99 of subunit 1 and the N-terminal Pro-1 of subunit 2 (Fig. 4). In this fashion, the monomers are now covalently bound across one-half of the dimeric interface. This molecule is more restricted at the dimeric interface than the wild-type homodimer and should be much less sensitive to the action of dissociative inhibitors. Similar tethered HIV PRs have been characterized previ-

ously (Cheng et al., 1990; DiIanni et al., 1990) and have shown similar in vitro kinetic parameters to the wild-type enzyme. We have confirmed these similarities on our peptide substrates and have observed an IC_{50} of 1.5 μ M for the competitive inhibitor pepstatin A for both enzymes. When the interface peptides were assayed against this tethered HIV1 PR (Fig. 4) we noted a very significant difference in the inhibitory pattern as compared to the wild-type enzyme. Activity was reduced 5–15% by peptides that otherwise inhibit the dimeric enzyme by 55–85% under similar conditions.

Refolding of HIV1 and HIV2 PR with interface peptides

Because the interface peptides can influence the monomer/dimer equilibrium, they should also affect the ability of monomers to assemble into active homodimers in in vitro refolding experiments. Refolding HIV1 and HIV2 PRs in the presence of the peptides resulted in a reduction of the enzymatic activity recovered. The effects on refolding of HIV1 and HIV2 PRs by a 500-fold molar excess of peptides is presented in Figure 5. A significant reduction (greater than 75%) in the amount of enzymatic activity recovered was observed only for the two-stranded peptides (PF1, AbF1, and ML2), with no effect for the one-stranded peptides (1CF, 1KPL). The substrate 1 peptide as well as bovine serum albumin (BSA) were also tested and showed an enhancement of about twofold of recoverable activity. Less than 20% reduction in activity was observed when the two-stranded peptides were tested

Peptide	Concentration (μ M)	% Activity	
		HIV1 PR dimer	HIV1 PR tethered
1CF	500	15 $\pm$ 1	95 $\pm$ 5
1KPL	500	85 $\pm$ 5	105 $\pm$ 6
PF1	100	35 $\pm$ 2	85 $\pm$ 5
AbF1	250	45 $\pm$ 3	90 $\pm$ 5
ML2	100	35 $\pm$ 2	85 $\pm$ 5
Pepstatin A	5	1.5 $\pm$ 0.1	2.5 $\pm$ 0.1

Fig. 4. Cartoon depicting amino acid sequences of the wild-type HIV1 protease (PR) and the tethered HIV1 PR dimer containing a three-glycine linker. Peptides were assayed against either enzyme using the β -Nal 1 substrate and conditions mentioned in the text. The percent remaining activity was determined as compared to untreated samples and the error margin for the assay (5%) was calculated for each result.

Fig. 5. Enzymatic activity recovered after refolding of the HIV1 and HIV2 proteases (PRs). Purified enzymes were denatured in urea and refolded alone or in the presence of a 500:1 molar ratio of peptides to PR monomers. The products of the reactions were assayed for proteolytic activity using β -Nal 1 substrate, and the activity was expressed as the % recovered for the peptide/BSA-treated samples as compared to those only containing dimethyl sulfoxide.

at 50:1 ratios. Peptides PF1 and AbF1 were more efficient in blocking assembly of the HIV1 than the HIV2 PR, whereas peptide ML2 was more effective toward the HIV2 PR, as expected for a system sensitive to sequence complementarity of peptide to cognate enzyme. Peptide 1CF, which shifted the dimer/monomer equilibrium of the HIV1 PR (Fig. 2), was not able to prevent refolding in vitro, whereas peptide PF1, which affected the enzyme activity at all enzyme concentrations (Fig. 2), was able to affect the folding pathway. Peptide 1CF seems to cause concentration-dependent dissociation of dimers (Fig. 2) and not affect PR assembly (Fig. 5). On the other hand, incubation with the two-stranded peptides causes a concentration-independent enzyme inhibition (e.g., PF1 in Fig. 2) and prevents the assembly of active dimers in refolding reactions (Fig. 5).

Discussion

The strict requirement of homodimer formation for enzyme activity exhibited by the retroviral PRs is a unique feature of enzyme architecture. As a functional homodimer, the enzyme has a single active site where one catalytic aspartic acid is provided by each protein monomer. Dimerization of the PR may be one of the regulatory steps of the viral maturation pathway. The fragile nature of the viral assembly process was shown by the expression and characterization of HIV1 PR tethered dimers (e.g., Cheng et al., 1990). The intermolecularly linked monomers formed a more stable enzyme, which in turn resulted in the premature intracellular release of viral proteins instead of maturation within extracellular particles (Kräusslich, 1991). These results suggest that during the viral life cycle, PR dimerization occurs at a precise time and place. Presumably, disruption or prevention of dimerization of

the viral PR may prevent the entire cascade of polyprotein processing. Our initial demonstration that inactive heterodimers of the HIV PR can be generated in vitro by mixing wild-type and defective monomers (Babé et al., 1991) prompted us to find smaller molecules capable of interacting with PR monomers to prevent formation of active dimers.

A panel of peptides was synthesized that resemble sequences of the four β -strands that interdigitate to form the tight-packed β -sheet composed of the N- and C-termini of both protein monomers. Because this structure seems to possess the most contacts between monomers and is likely to stabilize the dimer, it may also be a critical site for the normal formation of the dimer. Based on analysis of the three-dimensional structure of the PR dimer, it was predicted that a sequence encompassing the C-terminal four residues could serve as an inhibitor of dimerization, presumably by binding to one PR monomer (Weber, 1990). Recently, Zhang and coworkers (1991) showed by kinetic measurements that such a peptide could specifically inhibit the enzyme activity and affect the dissociation constant of the dimer. Their calculated K_d for the enzyme, as well as the apparent fivefold increase for this constant in the presence of the tetrapeptide Ac-TLNF, agrees with our results for the HIV1 PR with or without pentapeptide 1CF (CTLNF). Furthermore, the threefold higher IC_{50} of 1CF on the HIV2 PR shows that this interface peptide has a relative sequence specificity.

The nature of the inhibition by these interface peptides can be expected to be complex, since enzyme dimerization displays second-order kinetics. Indeed, a pattern of mixed-type inhibition was observed for our peptides 1CF and PF1 as expected for a system where both V_{max} and K_m are affected. Consistent with a model for dissociation by the interface peptides, the substrate would have a higher affinity for the enzyme dimer in the absence of inhibitor, and the *ESI* complex would be conformationally disrupted and nonproductive. We observe K_i values of approximately 120 μ M for the one-stranded peptide 1CF, and approximately 50 μ M for the two-stranded peptide PF1. In general, the inhibitory potency of the longer peptides as observed from IC_{50} values suggests that these are better inhibitors than the shorter single-strand 1CF. The absence of any inhibition by peptide 1KPL (tested to 2 mM concentration) suggests that this peptide cannot interact with the PR monomers to interfere with dimerization. The interaction between a C-terminal strand and a monomer yields four H-bonds, whereas the interaction of an N-terminal strand with a monomer only yields two H-bonds (Weber, 1990). Our results therefore agree with the prediction that a peptide encoding the C-terminus would be a more potent inhibitor than an N-terminal peptide. Alternatively, the N-terminal lysine that was added to increase the solubility of the peptide could prevent the specific interaction with the dimer interface.

The peptide 1CF (CTLNF) behaves like the dissociative

inhibitor Ac-TLNF described by Zhang et al. (1991). Using a kinetic analysis, they show this peptide binds to enzyme monomers and prevents dimerization. The effect of 1CF on the enzyme concentration versus activity curve (Fig. 2) is consistent with this behavior. The two-stranded peptide PF1 shows an atypical effect in this experiment. This peptide may bind to PR dimers and cause dissociation. Both one- and two-stranded peptides may be considered dissociative inhibitors, since they affect the final concentration of active dimers by either disruption or prevention of dimerization. The results shown in Figure 2 are consistent with a model where the one-strand peptide 1CF binds to enzyme monomers, and the two-strand peptide PF1 binds primarily to dimers (unpubl.).

The enhanced ability of the longer peptides to affect dimerization is again reflected by their inhibitory effects at the highest concentrations tested (100 nM) in the absence of NaCl, or at reduced enzyme concentrations in the presence of NaCl. In turn, 1CF only inhibited the HIV1 PR in the absence of NaCl and at enzyme concentrations below 25 nM. Because the two-stranded peptides that were synthesized contain one strand from each PR monomer, they could also interact with the complementary strand from two different monomers and therefore have an effect different than the one-strand peptide. It is worth mentioning that the effects of enzyme concentration observed in Figure 2 are particular to those assay conditions. The presence of additives such as Triton X-100, polyethylene glycol, or BSA affect the behavior of the enzyme so that no apparent loss of activity is observed as the enzyme concentration is varied between 0.5 and 100 nM (Jordan et al., 1992; unpubl.). In addition, the effect of these various additives on the relationship between enzyme concentration and activity differs for the HIV1, HIV2, and simian immunodeficiency virus (SIV) PRs tested in our laboratory (data not shown). This emphasizes the exquisite sensitivity of the retroviral PRs to assay conditions. The experiments presented here permit the detection of dimer disruption of the HIV1 and HIV2 PRs. It is yet to be determined whether these *in vitro* observations can be extrapolated to an *in vivo* environment that leads to a reduction of viral maturation.

Previous work on heterodimer formation of HIV1 and HIV2 PRs indicated that interaction among monomers of these two closely related enzymes was possible (Babé et al., 1991), suggesting a degree of complementarity between the regions of interaction formed by these new dimers. The interface of the HIV2 PR consists of N-terminal residues P₁Q₂F₃S₄L₅ and C-terminal residues M₉₅S₉₆L₉₇N₉₈L₉₉, as compared to the HIV1 PR N-terminal residues P₁Q₂I₃T₄L₅ and C-terminal residues C₉₅T₉₆L₉₇N₉₈F₉₉. Peptides of HIV1 and HIV2 sequences can inhibit both HIV1 and HIV2 PRs. The peptides tested displayed specificity for these PRs and did not inhibit the more distantly related retroviral PR from RSV or the mammalian aspartyl PRs pepsin and renin. The

RSV PR also displays a four-stranded β -sheet at its interface, albeit of a different amino acid composition (L₁A₂M₃T₄M₅ interacting with N₁₂₃T₁₂₂L₁₂₁R₁₂₀L₁₁₉) (Miller et al., 1989). Pepsin is a monomeric two-domain aspartyl PR with a six-stranded β -sheet forming at the interface of the two structural domains (Andreeva et al., 1984). The specificity displayed by the peptides tested suggests that sequence complementarity is needed for inhibition, since only the HIV1 and HIV2 PRs were inhibited. The inability of the peptides tested to inhibit RSV PR, pepsin, or renin also supports the idea that these peptides are not binding at the active site. Because all five enzymes have similar substrate preferences, a competitive inhibitor would be expected to show at least some effect on all five.

Our previous mixing experiments with HIV1 PR and either defective HIV1 or wild-type HIV2 PRs resulted in the loss of enzymatic activity using an assay that measured primarily the activity of HIV1 PR. The specific activity of these HIV1/HIV2 heterodimers was not confirmed because of the inability to isolate them, but it was significantly lower than the HIV1 PR activity (Babé et al., 1991). In contrast with these results, Patterson et al. (1992) have shown that tethered dimers of mixed HIV1 and HIV2 PR subunits have significant activity and suggested that covalent linkage of these one-chain dimers may contribute to the alignment and stabilization of the enzymes. Not only could this linkage enhance dimer formation, it could serve to maintain a stable dimeric interface. Based on modeling studies, Gustchina and Weber (1991) proposed that the Phe 99-Leu change would weaken hydrophobic interactions in heterodimers; therefore, covalent linkage of residue 99 to Pro 1 via a short linker could improve stability. Furthermore, the differences observed for the HIV1/HIV2 versus the HIV2/HIV1 constructions may be an indication of the varying stability of the interfaces (Patterson et al., 1992).

The ability to intermolecularly cross-link polypeptides with a reagent of defined length is an indication of the proximity of the macromolecules. The homobifunctional reagent DSS was used to probe for the presence of PR monomers and dimers in reactions containing a variety of peptides. Incubation with the interface peptides that reduce enzymatic activity (PF1, ML2, AbF1, and 1CF) results in a significant reduction of detectable dimers. On the other hand, the peptide 1KPL, which does not inhibit the enzyme, does not affect dimer levels. The substrate β -Nal 1, which is expected to interact with enzyme dimers and has no effect on dissociation, also caused no change in the amount of dimer detected. Polypeptide bands corresponding in molecular weight to the cross-linking of one PR monomer to a peptide molecule could be detected in all cases, except for peptide 1KPL. These results are an indication of the ability of these interface peptides to interact with the PR subunits and cause dissociation. Alternatively, a large distortion of the dimeric conformation

could also lead to inactive monomers that are too distant to be cross-linked by DSS.

A more direct approach to identify the mode of inhibition of these interface peptides was the use of a tethered HIV PR dimer as a target. This polypeptide has shown similar enzymatic properties to the wild-type dimer (data not shown; see Cheng et al., 1990; Dilanni et al., 1990, for examples). The proteins only differ in that the tethered protein is prevented from subunit dissociation because both monomers are covalently attached. Results show a significant reduction in inhibition by all the interface peptides when tested at concentrations that caused significant inhibition of the wild-type enzymes. Because both enzymes have identical active sites, the differential inhibition of the wild-type dimer should be the result of subunit dissociation. The slight inhibition observed (5–15%) could reflect the ability of the interface peptides to interact with the half of the dimer interface that is not covalently bound. We propose the testing of potential PR inhibitors in parallel using both of these HIV PRs. This dual screen could identify dissociative inhibitors and aid in differentiating them from active-site directed inhibitors.

Considering that these interface peptides altered the ratio of detectable PR dimers to monomers, we reasoned that they could also affect the proper folding of PR dimers. Because our goal is to identify molecules that may prevent the association/folding of PR dimers *in vivo*, initial testing of their efficacy in an *in vitro* refolding assay was performed. When purified PR is refolded under the described conditions, approximately 25% of the original proteolytic activity is recovered. When the refolding takes place in the presence of BSA or the substrate 1 peptide, approximately twofold more activity is recovered. When the interface peptides were tested, the two-strand mimics (PF1, AbF1, and ML2) showed a significant reduction in the amount of recoverable active enzyme. At 500:1 molar ratios, these three peptides prevented proper folding of both HIV1 and HIV2 PRs by over 75%. On the other hand, the one-strand peptides 1CF and 1KPL had no inhibitory effect and showed an improvement in recoverable activity. These results suggest that only the two-strand peptides prevent the proper folding of the PR dimers at either the monomer or dimer level. We had expected the one-strand peptide to also prevent refolding, because it was shown to bind protease monomers (Zhang et al., 1991). The inability of peptide 1CF to prevent refolding may be a reflection of its lower affinity for the PRs or an artifact of the denaturing conditions used in this system.

Given the presumed micromolar concentration and inaccessibility of the mature PR in the budding virions, dissociative inhibitors such as 1CF may be ineffective. However, the ability of the two-strand peptides to affect both dimer dissociation and assembly *in vitro*, coupled with their efficacy at all enzyme concentrations may make them a better starting point for the design of dimer

assembly inhibitors than the single-strand peptide mimics. Because dimer assembly is a prerequisite for mature PR formation, the potential to prevent the essential processing of the gag and gag/pol polyproteins by blocking the formation of a dimeric PR may be an alternative to active-site-directed inhibitors. Also, the need to maintain integrity of the dimeric interface may prevent sequence variation from arising within this region in evolving viral strains and therefore preclude mutants resistant to dimer assembly inhibitors.

Materials and methods

Source of PR

The HIV1 and HIV2 PRs were expressed in bacteria as described previously (Babé et al., 1991). The tethered HIV1 dimer was constructed using a synthetic gene for the HIV1 PR monomer described previously (Pichuantes et al., 1989). The polymerase chain reaction was used to generate a gene in which the 3' end was modified to encode three glycine residues, followed by an Apa I site. A second monomer was mutated to contain a 5' Apa I site encompassing the amino-terminal proline. The two monomers were ligated using the Apa I site to produce a dimer gene in which the first monomer is linked by three glycines to the second monomer. This dimer was expressed as a single unit by a vector containing two *tac* promoters derived from the plasmid pMSe5 (Muchmore et al., 1989). The amino-terminal methionine of HIV1 PR expressed from this construction has been shown by N-terminal sequence analysis to be removed in *Escherichia coli* to yield a native amino terminus (J. Rosé, pers. comm.).

Purification of all enzymes now precludes the reverse-phase HPLC separation and refolding steps, using pepstatin-A agarose chromatography instead (R. Salto, unpubl.). RSV PR was a gift from Dr. Moshe Kotler (Hebrew University, Israel), porcine pepsin was purchased from Sigma, and recombinant human renin was a gift from C. Carilli (California Biotechnology, Inc.). Concentrations of active wild-type HIV1, tethered HIV1 dimer, and HIV2 enzymes were determined by active-site titration (Tomasselli et al., 1990) using inhibitor U85548 (a gift of Dr. A. Tomasselli, Upjohn Laboratories).

Peptide synthesis

All amino acid derivatives were purchased from ABI (Applied BioSystems, Inc.) except for *ter*-butyloxycarbonyl- α -amino butyric acid (*t*-Boc- α Aba, Peninsula Laboratories). All peptides were synthesized by solid-phase technique on phenylacetamidomethyl (PAM) resins using *t*-Boc chemistry on a Model 430 ABI peptide synthesizer. Completion of synthesis and coupling efficiencies were measured by a ninhydrin colorimetric test. After comple-

tion of the synthesis, the last N α -Boc group was removed by trifluoroacetic acid (TFA) treatment, and where applicable, the N-terminus was acetylated. Deprotection of the amino acid side chains and cleavage of the peptide from the PAM resin was accomplished by anhydrous liquid hydrogen fluoride (HF) containing 10% anisole at -15°C for 30 min. Following ether extraction, the peptides were solubilized in neat DMSO and purified (>99% purity by absorbance at 215 nm) in one step by preparative HPLC on Vydac C₁₈ or C₈ columns (5.7×30 cm, $15\text{-}\mu\text{m}$ beads) using appropriate linear gradients of water:acetonitrile containing 0.1% TFA. The chemical composition and purity of the peptides were confirmed by mass spectrometry (laser spray ionization mass spectrometry [LSIMS], performed by the UCSF Mass Spectrometry Facility).

Assay conditions

Unless otherwise specified, enzyme inhibition assays involved preincubating the enzyme (10 nM) for 5 min at ambient temperature with the synthetic peptides in a buffer consisting of 50 mM sodium acetate, pH 5.5, 1 mM EDTA, 5 mM dithiothreitol (DTT), 0.1% Triton X-100, and 1% DMSO. Substrate 1 (NH₂-ATLNFPISPW-COOH) was then added to obtain a concentration of $150\text{ }\mu\text{M}$ and NaCl was added to a final concentration of 1 M. Under these conditions the approximate K_m of the substrate is $850\text{ }\mu\text{M}$ and V_{max} is approximately $10\text{ }\mu\text{mol/min/mg}$. Following a 30-min incubation at 37°C , the reactions were quenched by addition of acetonitrile and TFA. The products were analyzed by HPLC as previously described (DesJarlais et al., 1990). Similar assay conditions were used for the determination of inhibitory constants (Dixon plots) except that substrate 1 was used in a range of 0.05–2 mM.

For measurements of dimer dissociation, the more sensitive substrate β -Nal 1 (NH₂-VSQN-beta-naphthyl-alanine-PIV-COOH, a gift from Dr. Paul Darke, Merck-Sharp and Dohme) was used (Heimbach et al., 1989). In this case, the enzyme concentration ranged between 1 and 100 nM and the buffer used was 50 mM sodium acetate, pH 5.5, 1 mM DTT, 1 mM EDTA, 10% glycerol, 1% DMSO. Under these conditions the approximate K_m of the substrate is 1.7 mM. The initial preincubation of enzyme and interface peptide for 5 min at ambient temperature was followed by addition of substrate ($200\text{ }\mu\text{M}$ final concentration) and incubation at 37°C for 5–30 min. However, no NaCl was added in this case because the sensitivity of the substrate precluded NaCl addition. These reactions were also monitored by HPLC fractionation. All the kinetic data were analyzed by the method of least squares using the Enzfitter computer software (DesJarlais et al., 1990).

Recombinant human renin was assayed as described previously (DesJarlais et al., 1990) using angiotensinogen 1–14 as substrate.

Crosslinking reactions

The reaction cocktail consisted of 20 mM sodium phosphate, pH 7.5, 5 mM DTT, 1% DMSO, 0.24 mM DSS, 50 nM HIV1 or HIV2 PR dimers, and peptides ($500\text{ }\mu\text{M}$ 1CF, $500\text{ }\mu\text{M}$ 1KPL, $100\text{ }\mu\text{M}$ PF1, $100\text{ }\mu\text{M}$ ML2, or $200\text{ }\mu\text{M}$ β -Nal 1 substrate). The reactions were allowed to proceed for 4 h at ambient temperature with constant mixing. To analyze the reaction products, the proteins were precipitated by addition of TCA and deoxycholate (10% and 0.5 mg/mL final concentrations, respectively). The pellets were then washed with ice-cold acetone, air dried, and resuspended in Laemmli sample buffer. The proteins were resolved by electrophoresis on sodium dodecyl sulfate–16% polyacrylamide gels, transblotted to nitrocellulose, and probed with antibodies specific for either HIV1 or HIV2 PRs.

Refolding of HIV PRs

A modification of the refolding protocol described by Tomasselli and coworkers (1990) was employed. Purified HIV1 or HIV2 PR (100 ng) was adjusted to 8 M urea, 10 mM DTT, 10% DMSO, and a 500-fold molar excess of peptides (1CF, 1KPL, PF1, AbF1, ML2, substrate 1, or BSA). These samples were heated for 60 s at 55°C to ensure denaturation, then transferred to a 10°C water bath. A 10-fold dilution was performed over 30 min using four aliquots of refolding buffer (200 mM sodium acetate, pH 5.5, 10% glycerol, 5% ethylene glycol, 5 mM DTT, 1 mM EDTA). The final aliquot of refolding buffer also contained 1 M NaCl for a final NaCl concentration of 225 mM in a total volume of $50\text{ }\mu\text{L}$ (100 nM final enzyme concentration). Following a 30-min incubation on ice, the enzymatic activity recovered was determined by assaying the refolded material using the β -Nal 1 substrate.

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