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Stability and activity of human immunodeficiency virus protease: Comparison of the natural dimer with a homologous, single-chain tethered dimer

(dimer dissociation/site-specific mutagenesis/fluorogenic substrate/aspartic protease)

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ABSTRACT **A single-chain tethered dimer of human immunodeficiency virus protease (HIV-PR) was produced by expression of a synthetic gene in *Escherichia coli*. The tethered dimer, which consists of two 99-amino acid HIV-PR subunits linked together by a pentapeptide, was isolated from inclusion bodies and refolded as an active protease with enzymatic properties very similar to those of the natural dimer at pH 5.5. In addition to demonstrating that the tethered dimer is active, we have shown that the tethered dimer is more stable than the natural HIV-PR dimer at pH 7.0. This is attributed to dissociation of the natural HIV-PR dimer, for which a surprisingly high dissociation constant, 5×10^{-8} M, was measured. Furthermore, the tethered dimer offers an opportunity to produce asymmetric dimer mutants and thereby determine the effect of changes in one of the two subunits on protease activity. In one such mutant, a single active-site aspartic residue was changed to a glycine residue. This protein was inactive, consistent with a requirement for an aspartic residue from *each* subunit to constitute an active site of HIV-PR.**

The human immunodeficiency virus (HIV) is the etiological agent for the acquired immunodeficiency syndrome (AIDS). HIV, like other retroviruses, encodes an aspartic protease that is synthesized as a polyprotein and is responsible for the processing of gag and the gag–pol polyproteins (1). Genetic studies have shown that the HIV-1 protease (HIV-PR) gene is essential for the replication of HIV (2). HIV-PR has been recognized as a target enzyme for antiviral therapy and has been the subject of a number of recent studies.

HIV-PR and other retrovirus proteases are dimeric molecules consisting of two identical subunits, each contributing a catalytic aspartic residue (3, 4). They differ from cellular aspartic proteases, which consist of a single polyprotein chain that, in turn, is composed of two similar domains, each containing a catalytic aspartic residue. The sequence including the catalytic aspartic residue, Asp-(Thr or Ser)-Gly, is conserved in all aspartic proteases. The importance of these and other amino acid residues for the catalytic activity of HIV-PR has been shown in mutagenesis studies using cloned genes for the HIV-PR subunit (5). However, structure–function studies are limited by this approach because mutational changes result in a change in both identical subunits of HIV-PR.

X-ray crystallographic studies of HIV-PR have shown that the dimer interface of the protease contains the amino- and carboxyl-terminal strands of each monomeric subunit, arranged in a four-stranded, antiparallel β-structure (3, 6). Based on modeling of HIV-PR crystal structure, we have designed a gene consisting of two protease subunits linked together by a DNA segment coding for a 5-residue peptide, Gly-Gly-Ser-Ser-Gly, which can readily form a β-hairpin loop linking the carboxyl terminus of one subunit to the amino terminus of the second subunit. This linking sequence is based on other observed β-hairpin loops found in highly resolved crystallographic structures (7). The gene for the tethered dimer was expressed in *Escherichia coli* as an active enzyme (PR-AA). This tethered dimer gene provides a means of introducing asymmetrical modification for structural studies not possible in systems using HIV-PR monomer. One example is the expression of PR-AA as an inactive protein by changing only one of the two catalytic aspartic residues to a glycine residue.

Furthermore, the greater stability of the tethered dimer has allowed us to distinguish between effects of pH on the catalytic activity and effects of pH on stability of HIV-PR. This has led to measurements of properties of HIV-PR that are consistent with the dissociation of the natural protease into its constituent subunits at neutral pH. Although the substrate specificity and structure of retrovirus proteases have recently been the subject of extensive studies, little attention has been given to the dissociation of HIV-PR to individual subunits and the effect of monomer–dimer transitions on the activity of the enzyme.

MATERIALS AND METHODS

Bacteria, Plasmids, and Clones. *E. coli* K-12 strain JM105, plasmids pET3AM and pTZ18R, and synthetic genes for HIV-PR (gene "*A*") and its amino-terminal flanking 57 codons (gene "*B*") were previously described (8).

Cloning of the Gene "*BAA*." Cloning of gene *BAA* (Fig. 1) used genes *A* and *BA*, which were cloned in the *Eco*RI and *Hin*dIII sites of pTZ18R. The synthetic oligodeoxynucleotide used for the modification of gene *A* to gene *AI* was: 5′-GAATTCGAGCTCAGGCCCGCAGGTTACTCTGTGGCAGCGCT-3′. The oligonucleotide pair used to modify gene *BA* to *BAI* was 5′-CGGCCGTAACCTGCTGACTCAGATCGGTATGACTCTGAACTTTGGCGGGAGCTCAAGCTT-3′. For the expression of the asymmetric mutant tethered dimer, the gene *BAdg* (8), with an $Asp^{25} \rightarrow Gly$ change, was modified as above and joined with the gene *AI* to form the gene *BAdgA*.

Preparation of HIV-PR and the Tethered-Dimer Proteins from Inclusion Bodies. Recombinant JM105 cells were induced for the expression of the genes *BA*, *BAA*, and *BAdgA* by use of the expression vector pET3AM (10). Cells were harvested and lysed with a French pressure cell to prepare the inclusion bodies (8). Inclusion bodies were extracted with

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Abbreviations: HIV, human immunodeficiency virus; HIV-PR, protease encoded by HIV; Z, benzyloxycarbonyl; Phe(NO_2), *p*-nitrophenylalanine.

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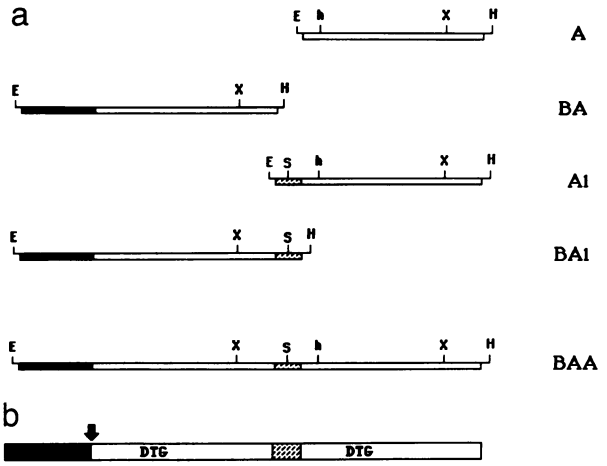

FIG. 1. Strategy for the synthesis and expression of the tethered HIV-PR dimer. (*a*) Synthesis of the gene for the tethered HIV-PR dimer. Gene names are shown at right. Restriction sites are abbreviated: E, *Eco*RI; h, *Hae* II; X, *Xma* III; H, *Hin*dIII; S, *Sst* I. Synthetic genes *A* and *BA* were described previously (8). Gene *A*, shown as open bars, contains, from the 5′ end, an *Eco*RI restriction sequence, an initiation codon, the 99 codons of HIV-PR (nucleotides 1797–2093; ref. 9), two stop codons, and a *Hin*dIII restriction sequence at the 3′ end. Gene *BA* contains the same 99 codons of HIV-PR as gene *A* plus an additional 57 codons (ATGGAATTCATG and nucleotides 1638–1796 of the HIV-1 genome) as shown by the solid bars. Gene *AI* was derived from gene *A* by replacing the *Eco*RI–*Hae* II fragment with a larger synthetic fragment carrying an additional sequence, GAGCTCAGGC, shown in the shaded bar. Similarly, gene *BAI* was derived from gene *BA* by replacing the *Xma* III–*Hin*dIII fragment with a larger fragment with an inserted sequence, GGCGGGAGCTC, shown in the shaded bar. An additional change was made in gene *BAI* by replacing the Cys^{95} codon (TGT) with a methionine codon (ATG). Genes *BAI* and *AI* were joined together at the unique *Sst* I site to generate the gene *BAA*. (*b*) Schematic representation of the predicted protein product of gene *BAA*, PR-BAA, produced by the pET3AM expression vector (8). The amino-terminal flanking peptide of HIV-PR is shown as the solid bar. The two 99-amino acid HIV-PR domains (open bars) are joined by a 5-amino acid linker, Gly-Gly-Ser-Ser-Gly (shaded bar). The sequences for the conserved active site, Asp-Thr-Gly, are indicated as DTG. The expected cleavage site of PR-BAA is shown with an arrow.

67% acetic acid. Extracts were diluted 33-fold with water, dialyzed against water overnight, and dialyzed for 2 hr at 25°C against a pH 5.5 refolding buffer consisting of 20 mM Mes, 1.0 mM dithiothreitol, and 10% (vol/vol) glycerol. For further purification, the refolded protein was concentrated, loaded on a Sephadex G-75 column (2.5 × 95 cm), and eluted with 0.1% acetic acid/1.0 mM dithiothreitol/10% glycerol.

Preparation of Substrates and Inhibitors. The substrates Ala-Thr-His-Gln-Val-Tyr-Phe(NO_2)-Val-Arg-Lys-Ala and anthranilyl-Ala-His-Gln-Val-Tyr-Phe(NO_2)-Val-Arg-Lys-Ala [containing *p*-nitrophenylalanine, Phe(NO_2)] were synthesized on Wang resins by using 9-fluorenylmethyloxycarbonyl-protected amino acids according to the DuPont RaMPS instruction guide. The anthranilyl residue was introduced as Boc-anthranilyl-Ala-OH (Boc is *t*-butoxycarbonyl). The inhibitor Z-Ser-Leu-Asn-PheΨ[CH(OH)CH_2NH]Ile-Val-Ala-OH (Z is benzyloxycarbonyl; the term in brackets replaces the normal Phe-Ile amide bond) was prepared by standard techniques except that the second chiral center of the dipeptide isostere was introduced by the stereospecific reduction of Z-phenylalanine chloromethyl ketone with $Zn(BH_4)_2$ in ether to yield 3(*S*)-benzyloxycarbonylamido-1-chloro-2(*S*)-hydroxy-4-phenylbutane (mp 150–151°C). The composition of all compounds was confirmed by fast-atom bombardment MS and amino acid sequence analyses.

Radioactively labeled substrate, $P55^{gag}$, was prepared as described (11).

Measurement of Kinetic Constants. Rates of hydrolysis of the Tyr-Phe(NO_2) bond of Ala-Thr-His-Gln-Val-Tyr-Phe-(NO_2)-Val-Arg-Lys-Ala at pH 5.5 were determined by measuring the decrease in absorbance at 300 nm and a change in extinction coefficient of 1360 $M^{-1}cm^{-1}$ for complete substrate hydrolysis. Enzyme specificity for this bond was confirmed by identical HPLC profiles of the synthetic peptide Phe(NO_2)-Val-Arg-Lys-Ala and one of the hydrolysis products of the substrate. Kinetic constants for substrate hydrolysis were determined by the method of Lineweaver and Burk, using a substrate range of 0.2–0.02 mM and protease at 5 and 10 μg/ml. Value of k_{cat} were calculated from values of V_m and the active-site concentrations of the proteases determined by the procedure of Morrison (12) using the inhibitor Z-Ser-Leu-Asn-PheΨ[CH(OH)CH_2NH]Ile-Val-Ala-OH. Protease titrations were performed by measuring enzymatic activity in the presence of at least four different concentrations of inhibitor that were at less than stoichiometric levels for enzyme. Linear plots of percent enzyme activity vs. inhibitor concentration were obtained for HIV-PR at 100 and 200 μg/ml and for PR-AA at 50 and 100 μg/ml in assays run in 50 mM Mes buffer, pH 5.5/1.0 mM dithiothreitol/1.0 mM EDTA/0.1 M NaCl/10% glycerol/0.1 mM substrate. The active-site protease concentrations were determined from *x* intercepts of these plots for each protein level. Comparisons of the active-site concentrations and the molar concentration of protein, based on a molecular mass of 22 kDa and the protein concentration (determined from the absorbance at 280 nm and an assumed extinction coefficient of 10 for a 1% solution and 1-cm pathlength), indicated average purities of 53 ± 2% and 98 ± 4% for HIV-PR and PR-AA, respectively. Values of K_i for inhibitor binding were determined from percent activity measurements in the presence of 1.0 μM inhibitor at substrate levels of 0.2 and 0.1 mM and the K_m for substrate hydrolysis.

Hydrolysis of the anthranilyl fluorogenic substrate was monitored by measuring the increase in fluorescence at 420 nm, using an excitation wavelength of 330 nm. Measurements were made on a Perkin–Elmer 650-40 fluorescence spectrometer. Most assays were run using 50 μM substrate and a chart recorder scale of 10 mV for which complete hydrolysis resulted in an increase in fluorescence from 102 to 249 mV for pH 5.5 and 94 to 232 mV for pH 7.0.

RESULTS

Cloning, Expression, and Isolation of the Tethered Dimer. The construction of the gene for the tethered HIV-PR dimer is shown in Fig. 1. Two synthetic genes, *A* and *BA*, were modified for this construction. Gene *A* codes for the monomer of mature HIV-PR. Gene *BA* encodes an HIV-PR precursor consisting of HIV-PR and a 57-codon amino-terminal flanking peptide that is part of the amino-terminal polyprotein of the *pol* gene. Genes *A* and *BA* were modified at the 5′ and 3′ ends, respectively, to generate genes *AI* and *BAI*, which were joined together to form a tethered-dimer gene, *BAA* (Fig. 1*a*). Gene *BAA* also codes for a precursor protein containing the 57-residue amino-terminal flanking peptide that was expected to be cleaved from the tethered dimer (Fig. 1*b*). As discussed previously (8), this flanking peptide portion was expressed to allow one to readily monitor autoprocessing activity.

Another tethered-dimer gene, *BAdgA*, was made. It has a single mutation, $Asp^{25} \rightarrow$ Gly, at the active site of the first domain of the tethered dimer, while the second domain (each domain is equivalent to a subunit of the native dimer) is unaltered (Fig. 1*b*). Proteins expressed from these genes are named PR-BA (from gene *BA*), PR-BAA (from gene *BAA*), and PR-BAdgA (from gene *BAdgA*). Autoproteolytic processing of PR-BA generates the 99-amino acid HIV-PR (8). A

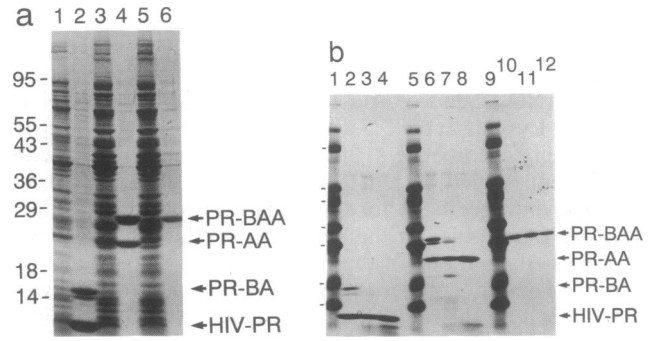

FIG. 2. Isolation and refolding of HIV-PR, the tethered dimer, and an active-site mutant. (*a*) JM105 cells expressing genes *BA* (lanes 1 and 2), *BAA* (lanes 3 and 4), or *BAdgA* (lanes 5 and 6) were lysed, and the protein profiles of the soluble cell extracts (lanes 1, 3, and 5) and inclusion bodies (lanes 2, 4, and 6) were analyzed by SDS/PAGE. (*b*) Acetic acid-soluble proteins were extracted from inclusion bodies of cells expressing genes *BA* (lanes 2–4), *BAA* (lanes 6–8), and *BAdgA* (lanes 10–12) and were refolded by dialysis for 0 hr (lanes 2, 6, and 10), 1 hr (lanes 3, 7, and 11), or 6 hr (lanes 4, 8, and 12). Aliquots were removed from dialysis at the indicated times, clarified by centrifugation for 10 min at 10,000 × *g*, and analyzed by SDS/PAGE. Proteins were stained with Coomassie blue for photography. Protein size standards: 95, 55, 43, 36, 29, 18, and 14 kDa (lanes 1, 5, and 9).

similar autoproteolysis of PR-BAA was expected to generate the 203-residue tethered dimer PR-AA.

Gene *BAA* was expressed at a high level from the expression vector pET3AM (8). This resulted in the accumulation of a 29-kDa and a 22-kDa protein in cellular inclusion bodies (Fig. 2*a*). Sequence analysis identified the expected amino-terminal 30 residues of PR-BAA and PR-AA as MEFMEDLA-F . . . and PQVTLWQRP . . . , respectively. The 22-kDa PR-AA is probably generated by autoproteolysis from its precursor, 29-kDa PR-BAA, prior to the formation of inclusion bodies. This is consistent with the observation that only a single 29-kDa protein, PR-BAdgA, was accumulated in inclusion bodies of recombinant bacteria expressing the mutant tethered-dimer gene *BAdgA* (Fig. 2*a*). The precursor–product relationship of PR-BAA and PR-AA was further confirmed in a "pulse–chase" experiment in *E. coli* (data not shown).

Proteins were extracted from inclusion bodies with 67% acetic acid and refolded by dialysis against a pH 5.5 buffer for various times (Fig. 2*b*). The ratio of the 22-kDa to the 29-kDa protein increased during the dialysis, indicating proteolytic processing of PR-BAA to PR-AA. Similar processing of PR-BA to HIV-PR was observed. Note that the major band in the 29-kDa region of lane 6 has been identified as PR-BAA by sequence analysis, whereas the minor band has not been characterized except to show that it is immunogenically active. The 6-kDa band, observed for both enzymes, is probably the amino-terminal peptide encoded by gene *B*, arising from processing of the precursors. The mutant dimer, PR-BAdgA, which is identical to PR-BAA except for the single change of Asp^{25} to Gly in the first domain, remained intact throughout dialysis, a strong indication that the mutant enzyme is inactive. We have used this method to prepare active HIV-PR and PR-AA directly from bacterial inclusion bodies. The resulting HIV-PR and PR-AA are nearly homogeneous proteins (Fig. 2*b*); the impurities are mostly small proteins that can be removed by chromatography on Sephadex G-75. The amino acid sequence of PR-AA was determined from its tryptic fragments, confirming that PR-AA is composed of two HIV-PR subunits linked in a head-to-tail fashion via a linker peptide, Gly-Gly-Ser-Ser-Gly.

We have also expressed tethered dimers with 3- and 4-amino acid linkers by using the sequences Gly-Ser-Ser, Gly-Ser-Ser-Gly, and Gly-Gly-Ser-Ser. Their proteolytic activities against $P55^{gag}$ were indistinguishable from that of PR-AA with the 5-amino acid linker, Gly-Gly-Ser-Ser-Gly. Independent of our studies, Dilanni *et al.* (13) have recently expressed a tethered dimer, using -Gly-Gly- as a linker, that has properties similar to those we report. Therefore, the above variation in linker length probably does not affect the overall secondary structure of tethered dimers of HIV-PR.

Comparisons of the Properties of PR-AA and HIV-PR. We have compared the proteolytic activities of refolded PR-AA and HIV-PR for both protein and peptide substrates. Radioactively labeled $P55^{gag}$ was cleaved by both enzymes at the p24/p15 and p17/p24 cleavage junctions (11). Both PR-AA and HIV-PR specifically cleaved the Tyr-Pro bond of the synthetic peptide Val-Ser-Gln-Asn-Tyr-Pro-Ile-Val, with respective specific activities of 520 and 470 nmol/min per mg, which are comparable to those reported previously (8). Comparisons of these two enzymes were also made by determining kinetic constants for the hydrolysis of Ala-Thr-His-Gln-Val-Tyr-Phe(NO_2)-Val-Arg-Lys-Ala and by measuring inhibition by Z-Ser-Leu-Asn-PheΨ[$CH(OH)CH_2$-NH]Ile-Val-Ala-OH. As shown in Table 1, kinetic constants for the two enzymes are very similar. Increased ionic

Table 1. Comparison of kinetic constants for HIV-PR and AA-PR

		Substrate* hydrolysis			
Enzyme	NaCl, M	K_m, mM	k_{cat}, sec^{-1}	k_{cat}/K_m, $M^{-1}sec^{-1}$	Inhibitor† K_i, nM
HIV-PR	0.15	0.31 ± 0.02	4.5 ± 0.01	14,700 ± 900	
	1.0	0.11 ± 0.02	4.8 ± 0.96	43,700 ± 3700	55 ± 19
PR-AA	0.15	0.71 ± 0.21	6.1 ± 1.3	8,900 ± 1500	
	1.0	0.14 ± 0.004	4.2 ± 0.14	29,700 ± 1800	44 ± 8

All reported kinetic constants are the average of at least duplicate determinations. Measurements were made at 25°C in 50 mM Mes buffer, pH 5.5/1.0 mM EDTA/1.0 mM dithiothreitol/10% glycerol.

*The substrate, Ala-Thr-His-Gln-Val-Tyr-Phe(NO_2)-Val-Arg-Lys-Ala, has been reported for the protease of myloblastosis-associated virus (14). Nevetherless, it was also cleaved by HIV-PR at the peptide bond between Tyr and Phe(NO_2) with a specific activity comparable to the best substrates containing a Phe(NO_2)-Pro bond for HIV protease (15), but it is hydrolyzed less effectively than other substrates recently reported (16) also containing a Phe(NO_2) residue in the second position.

†Z-Ser-Leu-Asn-PheΨ[$CH(OH)CH_2NH$]Ile-Val-Ala-OH. The amide isostere, [$CH(OH)CH_2NH$], was introduced stereospecifically as the *R* configurational isomer. This isostere was introduced earlier in the development of inhibitors of other aspartic proteases (17), but its use in peptide inhibitors of HIV-PR has not been reported until recently (18, 19). Independently, we have found that, when substituted for the scissile bond of good substrates for HIV-PR, this isostere is among the most effective for inhibitors of the protease. Z-Ser-Leu-Asn-PheΨ[$CH(OH)CH_2NH$]Ile-Val-Ala-OH is of sufficient potency to titrate the active sites of these proteases by using an approach similar to that recently reported (20).

strength results in comparably lower values of K_m while k_{cat} remains essentially unchanged. The similar changes in K_m for both enzymes suggests that high ionic strength increases binding in the Michaelis complex to the same extent. Based on the measurements we have made, HIV-PR and the tethered dimer are functionally similar in autoproteolysis (Fig. 2), substrate specificity, activation by NaCl, and inhibitor sensitivity at pH 5.5

In contrast to the similar properties of PR-AA and HIV-PR at pH 5.5, higher activity was found for PR-AA when assayed at low enzyme concentrations and at higher pH using radiolabeled $P55^{gag}$ as a substrate. To obtain a quantitative comparison at low enzyme levels, the peptide substrate in Table 1 was modified by the introduction of an anthranilyl residue to yield a "fluorescence-quenching" substrate, which increased the sensitivity of the peptide assay at least 10-fold. This modification was made on the basis of principles established previously (21). Fluorescence of the anthranilyl residue is quenched by the Phe(NO_2) residue in the unhydrolyzed substrate. This assay appears to be comparable in sensitivity to the recently reported (22) fluorescence assay that uses different fluorescent and quenching groups.

The activities of PR-AA and HIV-PR were compared at pH 5.5 and 7.0, using the fluorescent substrate at a concentration that reflects values of k_{cat}/K_m (Fig. 3). The slightly higher activity of HIV-PR at pH 5.5 is consistent with the kinetic constants measured in Table 1. At pH 7.0, a clear distinction between the two enzymes was observed. Examination of the progress curves for the two enzymes indicates that initial activities measured in the first 30 sec of the assay are similar, but HIV-PR quickly loses activity in the first 2–3 min of the assay until a much lower steady-state level is obtained. The rate of loss of activity increased in magnitude with increasing pH over a pH range of 6.5–7.5. In contrast, PR-AA at pH 5.0–7.5 and HIV-PR at pH 5.0–6.0 were moderately stable, losing activity with a half-time of 40–60 min. This slow loss of activity is common to both enzymes and occurs only at the low level of enzyme (10 nM) in these assays.

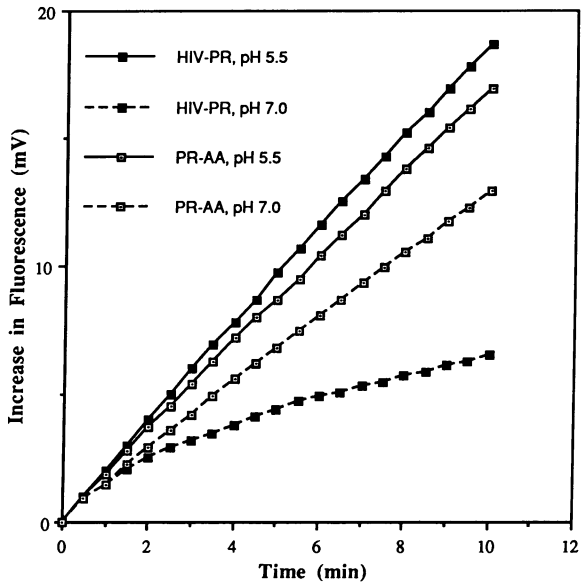

FIG. 3. Comparison of the activity and stability PR-AA and HIV-PR at pH 5.5 and pH 7.0. The hydrolysis of anthranilyl-Ala-His-Gln-Val-Tyr-Phe(NO_2)-Val-Arg-Lys-Ala (25 μM) at 25°C by each enzyme at an active-site concentration of 10 nM was monitored continuously by the increase in fluorescence with time. Assays were run in the Mes buffer described in Table 1 with 1.0 M NaCl. Comparisons of the changes in activities for 10 nM PR-AA and 50 nM HIV-PR with pH follow (pH values and corresponding activities relative to the maximum activity at pH 6.0, in parentheses, are given): 5.0 (0.59), 5.5 (0.87), 6.0 (1.00), 6.5 (0.87), 7.0 (0.70), and 7.5 (0.27) for HIV-PR; 5.0 (0.59), 5.5 (0.80), 6.0 (1.00), 6.5 (0.90), 7.0 (0.85), and 7.5 (0.45) for PR-AA.

Dissociation of HIV-PR to Monomeric Subunits. The behavior of HIV-PR at pH 7.0, monitored by changes in enzymatic activity, is consistent with the dissociation of the natural HIV-PR dimer to monomeric subunits. Following the initial loss of activity, a steady-state or equilibrium level of activity was obtained. No further loss of activity was observed over a period of 30 min except for that arising from slow inactivation of the enzyme (observed for both HIV-PR and PR-AA). In further studies, the effect of initial enzyme concentration on properties of the time-dependent loss of activity was determined (Fig. 4). At the enzyme concentrations of 6.2–25 nM, initial activities were proportional to enzyme levels and steady-state levels of enzymatic activity were measured before substrate depletion significantly effected rate. For each level of protease, dissociation constants (K_d) were calculated based on the initial concentration of protease and percent enzyme activity remaining at the steady-state level of activity. The average value of K_d was 50 ± 20 nM.

Furthermore, the progress curves for the loss of enzymatic activity are consistent with the dissociation of the natural dimer. As expected, consistent values for k, the first-order rate constant for dissociation, were obtained for each concentration of protease over the range 6.2–25 nM. The average value of k is 3.5 ± 0.5 msec^{-1} ($t_{1/2}$ = 3.3 min for dissociation). The association rate constant, based on the dissociation rate

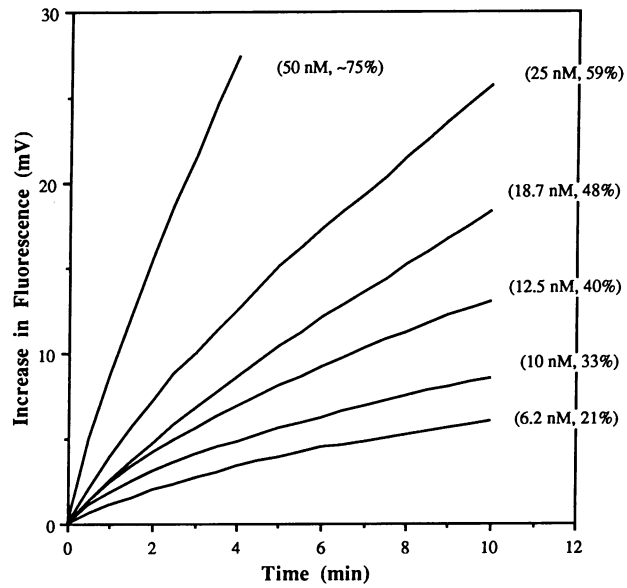

FIG. 4. Effect of HIV-PR concentration on assay progress curves at pH 7.0. Assays were conducted using the conditions in Fig. 3 except that Hepes was substituted for Mes buffer and a substrate concentration of 50 μM was used. The ratio of the initial velocity to the steady-state velocity was determined for each protease concentration and is reported adjacent to individual progress curves as a percent with initial protease concentration. The change in activity with time for each protease concentration was determined at various time intervals by drawing tangents to the curves and fitting the following equation for reversible dimer dissociation (23) to the data graphically: $[A_0 - A_e/A_0 + A_e]\ln[(A - A_e)A_0/(A_0^2 - A_eA)] = -kt$. A_0 and A_e are the initial and steady-state concentration of dimer, respectively; A is the concentration of dimer at time t; k is the first-order rate constant for dimer dissociation. The values of k follow [reported as enzyme concentration, measured value of k (correlation coefficient for linear least-squares fit)]: 6.2 nM, 0.0032 sec^{-1} (0.98); 10 nM, 0.0037 sec^{-1} (0.94); 12.5 nM, 0.0033 sec^{-1} (0.99); 18.7 nM, 0.0045 sec^{-1} (0.97); 25 nM, 0.0027 sec^{-1} (0.98).

constant and the dissociation constant, is 7×10^4 $M^{-1}sec^{-1}$ (0.0035 $sec^{-1}/5 \times 10^{-8}$ M).

Also consistent with the dissociation of the natural HIV-PR dimer, loss of activity due to incubation of the enzyme at pH 7.0, as shown in Fig. 3, can be partially reversed by lowering the pH to 5.5. Reactions identical to those at pH 7.0, except that 10 mM Mes was used, were run for 10 min and then the reaction conditions were adjusted to 50 mM Mes and pH 5.5. After allowing the reaction to proceed for an additional 20 min and correcting for the irreversible loss of activity ($t_{1/2}$ = 40 min), we observed 65–85% of the activity of controls run at pH 5.5.

DISCUSSION

A tethered dimer of HIV-PR was constructed with a 5-amino acid linker and isolated as an active protease with enzymatic properties very similar to those of the natural dimer at pH 5.5. The tethered dimer offers an opportunity to determine the effect of changes in one of the two subunits on properties of HIV-PR. The advantage of the use of the tethered-dimer gene is exemplified in the asymmetric dimer mutant, PR-BAdgA, where a single aspartic residue of a polyprotein containing both domains was changed to a glycine residue. This protein, as expected, was inactive in autoprocessing assays, a result consistent with a requirement of an aspartic residue from each subunit to form the active site of HIV-PR.

We have also found that the tethered dimer is much more stable at pH 7.0 under conditions that would favor dimer dissociation. The differences in properties of the tethered dimer and HIV-PR, measured at pH 7.0 by assays run in 1.0 M NaCl and 10% glycerol, are consistent with the reversible dissociation of HIV-PR for which a K_d of 5×10^{-8} M has been measured. The following observations have been made. (*i*) PR-AA is stable at pH 7.0, whereas HIV-PR loses activity in a time-dependent manner until a steady-state level of activity is reached. (*ii*) Consistent values of K_d were obtained over a range of initial enzyme concentrations. (*iii*) The rate equation (23) for reversible dimer dissociation readily fits data obtained for the loss of enzymatic activity of HIV-PR with time over a range of concentrations yielding consistent values for the first-order rate constant for dissociation. (*iv*) Activity of HIV-PR, after reaching a steady-state, is recovered, at least partially, by adjusting the pH to 5.5, where K_d is much lower. In contrast, subunits of HIV-PR have been linked by crosslinking reagent at pH 8.5, indicating the protease is a dimer under these conditions (4). Our observations suggest that dissociation of the dimer is favored at pH 8.5; however, we have found catalytic activity (4- to 10-fold lower than for PR-AA) can be measured at pH 8.5 for HIV-PR at a concentration of 250 nM. This level of enzyme is comparable to those used in crosslinking experiments and indicates that the dimer forms, albeit with lower affinity.

Measurements of K_d at pH 7.0 were made in solutions containing 1.0 M NaCl and 10% glycerol, conditions that differ considerably from those expected *in vivo*. Preliminary data (not shown) indicate that 10% glycerol has marginal effects on K_d, but a decrease in ionic strength results in an increase in K_d. The latter observation is not unexpected, since hydrophobic interactions are important in the dimer interface (6). Regardless, the magnitude of the value of K_d is surprisingly high, since it is unlikely that intracellular concentration of virus gene products would approach this level. For a multiplicity of viral infection of 1000–5000, the intracellular protease concentration would be in the range 1–5 nM. In the initial generation of the dimeric protease, some mechanism for enhancing dimer formation must be important. Possibly myristoylation and protein–membrane interactions are involved in dimerization, since myristoylation of $P55^{gag}$ is required for assembly of mature virus particles (24). The dimeric molecule would have limited lifetime *in vivo* unless these or other factors were to contribute to its stabilization. We propose that the dissociation of the protease may be a control mechanism for limiting nonspecific intracellular proteolysis during virus maturation.

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