Influence of Flanking Sequences on the Dimer Stability of Human Immunodeficiency Virus Type 1 Protease†

Ewald M. Wondrak‡ and John M. Louis*

Molecular Mechanisms of Development Section, Laboratory of Cellular and Developmental Biology, National Institute of Diabetes and Digestive and Kidney Diseases, The National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: The maturation of the human immunodeficiency virus type 1 protease (PR) from the Gag−Pol polyprotein is dependent on the intrinsic proteolytic activity of the dimeric Gag−Pol. Herein, we report the kinetics and conformational stabilities of two unique fusion proteins of the protease. In one, X_{28}−PR, a random sequence of 28 amino acids (X_{28}) was linked to the N terminus of the mature protease. In the second construct, X_{28}−ΔTF*PR*ΔPol, X_{28} is fused to the protease which is flanked at both its termini by short sequences (Δ) which correspond to the native sequences of the Gag−Pol precursor. Autoprocessing of the latter protein was prevented by inserting an Ala at the native protease cleavage sites. The measured kinetic parameters and the pH-rate profile of both enzymes are nearly identical to those of the mature protease. However, these fusion proteins are more sensitive to acid and urea denaturation than the mature protease. The decrease in the conformational stability of X_{28}−PR and X_{28}−ΔTF*PR*ΔPol is reflected by increases in their apparent dissociation constants (K_d) from <5 nM to approximately 180 and 25 nM, respectively. These results suggest that subunit interactions and hence the dimer stability of the protease domain in the Gag−Pol polyprotein differ from those of the mature protease. The high K_d of X_{28}−PR further suggests that addition of non-native sequences to the N terminus of the protease destablizes the dimer.

The 11 kDa aspartic acid protease of the human immunodeficiency virus type 1 (HIV-1)[1] is synthesized as part of the Gag−Pol precursor (160 kDa) and is responsible for processing the Gag (55 kDa) and Gag−Pol polyproteins into essential structural and functional proteins (Oroszlan & Luftig, 1990; Katz & Skalka, 1994). The complexity of the Gag−Pol polyprotein which contains more than nine protease cleavage sites and the difficulty in purifying the intact precursor have precluded studies of the structural characterization of the protease zymogen and its mechanism of maturation (Co et al., 1994). However, from kinetic investigation of a smaller precursor, MBP−ΔTF−PR−ΔPol, that contains only the two native cleavage sites flanking the protease, we proposed a two-step mechanism for the release of the mature protease from a poorly active homodimeric precursor (Louis et al., 1994). Time dependent hydrolysis of the peptide bond flanking the N terminus of the protease occurs fast, relative to the C-terminal cleavage, and leads to release of the transient intermediate PR−ΔPol and concomitant appearance of mature-like enzymatic activity (Louis et al., 1994; Wondrak et al., 1996).

Extensive subunit interactions of the terminal residues 1−4 and 96−99 of the mature protease may account in part for the low dissociation constant of the dimer (Wlodawer et al., 1989; Weber, 1990; Darke et al., 1994). Previous studies have shown that the activity of the PR−ΔPol intermediate is more sensitive to urea than is the mature protease (Wondrak et al., 1996). This lends experimental support to the hypothesis that interface interactions are weaker in protease precursors than in the mature enzyme due to the ΔPol sequences. Determination of the dissociation constants of the mature protease or PR−ΔPol using simple methods has not been possible due to sensitivity limitation of the assays (Wondrak et al., 1996). Moreover, the low catalytic activity of the model precursor MBP−ΔTF−PR−ΔPol (Louis et al., 1994), further complicated by its large fusion partner MBP and its ability to undergo autoprocessing during assay conditions, has precluded such investigation. Thus, in order to assess the effect of the N-terminal TF and C-terminal RT sequences in modifying subunit interactions within the protease dimer, we synthesized two protease fusion proteins. In one, a random sequence of 28 amino acids (X_{28}) was fused to the N terminus of ΔTF−PR−ΔPol. Alanine insertion mutations were introduced in this construct at the N- and C-terminal cleavage sites of the protease (Phe-Pro changed to Phe-Ala-Pro; X_{28}−ΔTF*PR*ΔPol). We had shown previously that these mutations prevent autoprocessing (Louis et al., 1991). In another construct, the same random sequence (X_{28}) was fused directly to the N terminus of the mature protease domain.

Herein, we describe the construction, expression, and rapid purification of these two protease fusion proteins, define

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* To whom correspondence should be addressed at Bldg. 6, Room B1-16. Telephone: (301) 496-8958. Fax: (301) 496-5239. E-mail: jmlouis@helix.nih.gov.

‡ Present address: P.O. Box 1351, Frederick, MD 21702-0351.

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[1] Abbreviations: DTT, dithiothreitol; HIV-1, human immunodeficiency virus type 1; MBP, maltose binding protein of *Escherichia coli*; PR, HIV-1 protease; ΔPol, 19 amino acids of the reverse transcriptase sequence; ΔTF, 12 amino acids of the transframe protein sequence; X_{28}, random sequence of 28 amino acids encompassing a six-histidine tag; ΔTF*PR*ΔPol, PR fused to ΔTF and ΔPol containing Ala insertion mutations (*) at its cleavage sites; Phe(NO_2), 4-nitrophenylalanine; Nle, norleucine; Tricine, *N*-tris(hydroxymethyl)methylglycine.

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conditions for efficient renaturation, and compare the kinetic parameters and structural stabilities with those of the mature protease. The observed decrease in dimer stability of these fusion proteins now allows studies to measure dissociation constants employing simple assays in a range of protein concentrations that is not feasible with the mature protease.

EXPERIMENTAL PROCEDURES

Cloning and Expression. We had previously constructed an MBP−PR fusion protein that incorporates a factor Xa protease site between the two domains (Louis et al., 1991). Restriction digestion of the DNA construct [similar to clone A in Figure 1B of Louis et al. (1991)] with *Kpn*I and *Hin*dIII and directional ligation into the prokaryotic expression vector PQE-31 containing the His-Tag (Qiagen, Chatsworth, CA) resulted in the construct, X_{28}−PR. Here, the protease is flanked by MRGSHHHHHHTDPHASSVPGRGSIEGRG.

The insert DNA from clone Mut-G (Louis et al., 1991) was similarly isolated using restriction enzymes *Kpn*I and *Hin*dIII and ligated into PQE-31. In this construct (X_{28}−ΔTF*PR*ΔPol), X_{28} was fused to 12 amino acids of the transframe protein (ΔTF) that flank the N terminus of the protease; 19 amino acids of the reverse transcriptase sequence (ΔPol) comprise the C terminus. This construct additionally contains Ala insertion mutations at the N- and C-terminal cleavage sites (Phe-Pro → Phe-Ala-Pro).

Escherichia coli bearing X_{28}−PR and X_{28}−ΔTF*PR*ΔPol constructs were grown and induced for expression as described (Qiagen).

Protein Purification. Cells (5−8 g) were suspended in 40 volumes of buffer A [50 mM Tris-HCl (pH 8.2), 8 M urea or 6 M guanidine hydrochloride, and 1 mM β-mercaptoethanol], homogenized, and lysed by sonication at 4 °C for 15 min with 1 min pulses at 1 min intervals. The lysate was clarified by centrifugation at 15000*g* for 30 min at 20 °C. The supernatant was subjected to column chromatography on Ni-NTA agarose (Qiagen) with a bed volume of 15 mL (2.8 cm in diameter) at a flow rate of 2.5 mL/min and monitored at 280 nm. The protease fusion proteins containing the six-histidine sequence were specifically eluted with 0.2 M imidazole in buffer A. Peak fractions were combined, assayed for protein content (Bio-Rad protein assay, Bio-Rad, Hercules, CA), and subsequently subjected to gel filtration chromatography on Superdex 75 (HiLoad 26/60; Pharmacia Biotech, Uppsala, Sweden) in 50 mM Tris-HCl (pH 8.2), 8 M urea or 4 M guanidine hydrochloride, and 5 mM dithiothreitol at a flow rate of 2.5 mL/min in injections of ≤30 mg protein per run. The fusion proteins in the peak fractions were further purified by RP-HPLC (POROS II R/M resin, PerSeptive Biosystems, Framington, MA) using a linear gradient of 0 to 60% acetonitrile/0.05% trifluoroacetic acid during a period of 12 min and at a flow rate of 4 mL/min. The major peak which corresponds to the protease fusion protein elutes between 6 and 7 min. One-milliliter fractions were collected, and aliquots corresponding to the peak fractions were dried, dissolved in Laemmli sample buffer (Louis et al., 1991), and analyzed by SDS−PAGE (Novex) and Coomassie staining. The desired HPLC fractions were lyophilized, dissolved in buffer B [50 mM Tris-HCl (pH 8.2), 5 M urea, and 0.1 M dithiothreitol] to concentrations between 2 and 10 mg/mL, incubated for 10 min at 25 °C, and stored at −80 °C in aliquots. Before use, aliquots were diluted with 9 volumes of 100 mM acetic acid. Alternatively, HPLC fractions were combined and dialyzed against a 400-fold excess of either 100 mM acetic acid or 50 mM formic acid at 4 °C. The mature protease was isolated as described previously (Wondrak et al., 1996; Louis et al., 1991).

Kinetics. Kinetic parameters were measured using the substrate, Lys-Ala-Arg-Val-Nle-Phe(NO_2)-Glu-Ala-Nle-NH_2 (California Peptide Research, Napa, CA), and the inhibitor, Arg-Val-Leu-(r)-Phe-Glu-Ala-Nle-NH_2 [where (r) denotes a reduced peptide bond; Bachem Bioscience Inc., King of Prussia, PA], in buffer C [100 mM sodium acetate (pH 5.0), 1 mM DTT, 1 mM EDTA, and 0.05% reduced Triton X-100] as described previously (Wondrak et al., 1996). Sodium acetate and formate buffers (100 mM) containing 100 mM NaCl in buffer C were used to obtain k_{cat}/K_m at pH's between 4.2 and 5.6 (Wondrak et al., 1996). Enzyme concentrations were determined by both active-site titration (Wondrak et al., 1996) and Bio-Rad protein assay (Bio-Rad) to ensure a renaturation efficiency of ≥95%.

Intrinsic Protein Fluorescence. An aliquot of the dialyzed protein solution (5 μL) in 50 mM formic acid was quenched in 145 μL of 50 mM formate (pH 4.5) and 2.5 mM DTT or in buffer C (without Triton X-100) in a 100 μL fluorescence cell (Hellma Cells, Inc., Jamaica, NY) at 25 °C. The excitation wavelength was 280 nm with a slit width of 2.5 nm, and the emission wavelength was 348 nm with a slit width of 20 nm. Circular dichroism was measured at 217 and 221 nm in 50 mM sodium formate and 2.5 mM DTT at a protein concentration of 0.76 μM in 1 mm cells (Hellma Cells, Inc.).

RESULTS AND DISCUSSION

Expression and Large-Scale Purification of Intact Protease Fusion Proteins. Induction with isopropyl β-D-thiogalactopyranoside (+ lanes) results in accumulation of full-length proteins X_{28}−ΔTF*PR*ΔPol (Figure 1A) and X_{28}−PR (Figure 1B) in *E. coli* with apparent sizes of 17 and 14 kDa, respectively, as compared to uninduced cell extracts (− lanes). The accumulation of the 17 kDa product (X_{28}−ΔTF*PR*ΔPol) is consistent with previous observations that Ala insertion mutations at the native cleavage sites flanking the protease domain block autoprocessing (Louis et al., 1991). The control construct, X_{28}−ΔTF−PR−ΔPol, without these mutations exhibits complete processing in *E. coli* to release the mature protease with no trace of accumulation of the full-length precursor analyzed by immunoblotting. The steps involved in the purification for these proteins are summarized in Table 1. Denaturing agents were included in the buffers in purification steps 2 and 3 (Table 1) to enhance recovery by eliminating nonspecific binding of the protease to the gel matrix and to prevent autoproteolysis. The affinity purification using Ni-NTA agarose proved to be a rapid and efficient step to enrich the fusion proteins from crude cell lysates (see Figure 1C, 0.2 M imidazole). Typically, both proteins were recovered in high yields, representing 8−13% of total cell lysate. The proteins were >90% homogeneous as determined by SDS−PAGE. Purification step 3 enhances the specific activity of the proteases between 2- and 12-fold. The final purification step by reverse phase HPLC (Table 1; Figure 1D) was introduced to remove minor traces of low-molecular weight proteins which elute in the initial fractions

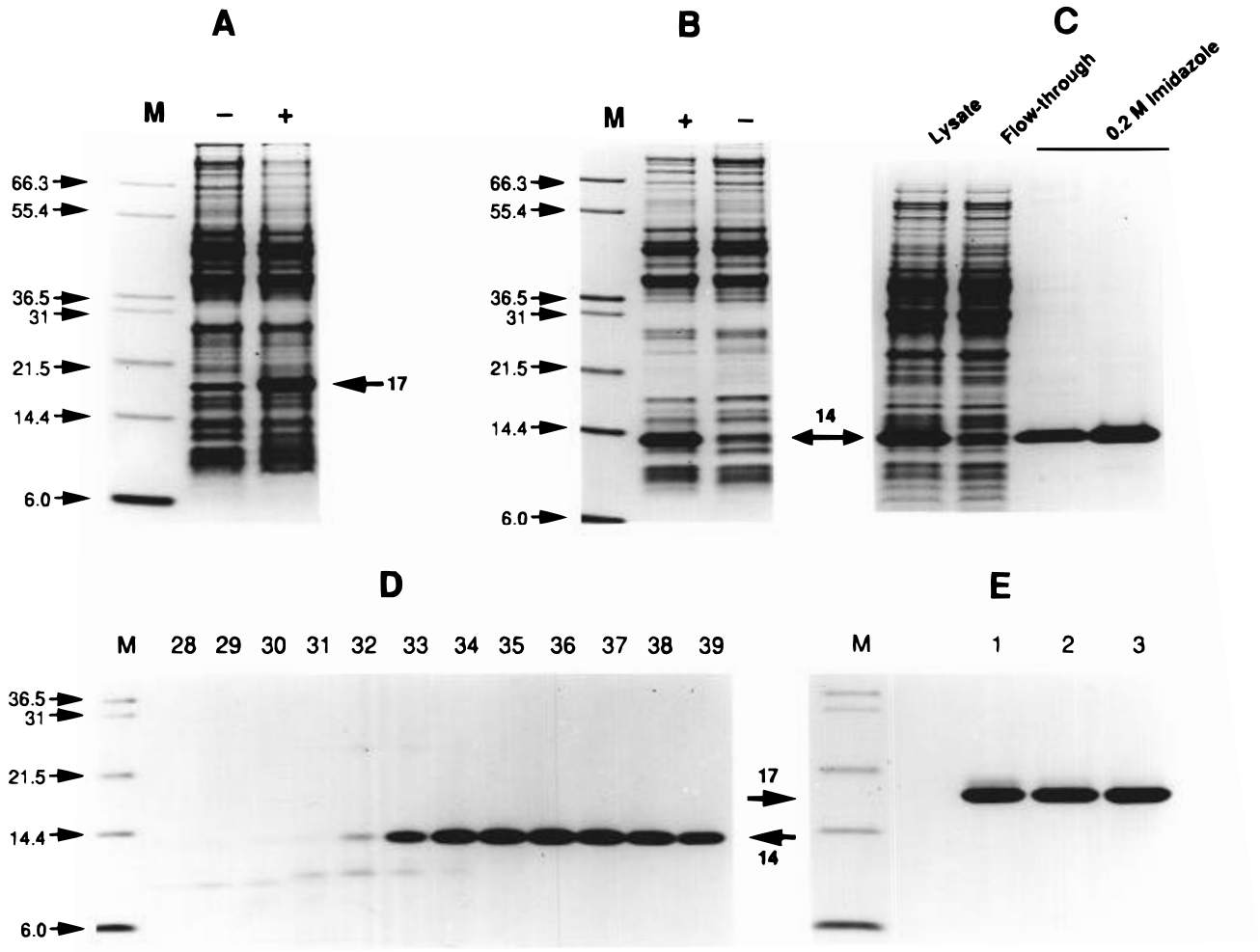

FIGURE 1: Expression and purification of HIV-1 protease fusion proteins. *E. coli* cells expressing X_{28}−ΔTF*PR*ΔPol (A) and X_{28}−PR (B) were lysed in a 10-fold excess of Laemmli sample buffer (Louis et al., 1991). Proteins (10−15 μg) were separated on 10 to 20% Tris-Tricine gradient precast gels (Novex, San Diego, CA) and stained using Coomassie brilliant blue G250. (C) Ni-NTA agarose purification of X_{28}−PR. Cells bearing the expressed X_{28}−PR were lysed and fractionated by Ni-NTA agarose chromatography as described in Experimental Procedure. Lysate, flow-through (23 μg each), and proteins eluted using 0.2 M imidazole (5 and 6 μg) were subjected to SDS−PAGE and stained. Panels D and E represent fractions of X_{28}−PR and X_{28}−ΔTF*PR*ΔPol, respectively, analyzed by SDS−PAGE after RP-HPLC purification. (D) The major protein peak eluting at a gradient concentration of 30 to 45% acetonitrile is shown from fractions 28−39. (E) One to three represent aliquots of pooled fractions from three independent runs. Arrows denote apparent sizes in kilodaltons.

Table 1: Purification of HIV-1 Protease Fusion Proteins

	X_{28}−PR		X_{28}−ΔTF*PR*ΔPol	
purification step	total protein (mg)	specific activity (mmol min^{-1} mg^{-1})	total protein (mg)	specific activity (mmol min^{-1} mg^{-1})
(1) crude lysate	458	nt[a]	744	nt
(2) Ni-NTA agarose chromatography	58	8.7	62	2.8
(3) gel filtration chromatography	38	20.0	31	34.5
(4) reverse phase HPLC	23	21.0	25	30.6

[a] Not tested.

of the major peak of protease fusion proteins. Homogeneous X_{28}−PR fractions (Figure 1D, 34−39) were combined and used for investigations described below. A similar purity was obtained for X_{28}−ΔTF*PR*ΔPol (Figure 1E, 1−3). Thus, both proteins can be produced to a level of about 50 mg/L of culture with a purity reaching >98%.

Conditions for Protein Folding. In a manner similar to that of MBP−ΔTF−PR−ΔPol and PR−ΔPol (Louis et al., 1994; Wondrak et al., 1996), X_{28}−PR and X_{28}−ΔTF*PR*Δ-Pol renature almost instantaneously when diluted 10-fold from 5 to 8 M urea into buffer C at pH 5. After purification steps 2 and 3 (Table 1), both proteins efficiently renature at concentrations of up to 0.1 mg/mL and exhibit full enzymatic activity. However, protein folding at initial concentrations above 0.1 mg/mL results in a decrease of specific enzymatic activity that becomes more pronounced at ≥0.2 mg/mL. Alternatively, proteins stored in 100 mM acetic acid or 50 mM formic acid can be renatured from a concentration of up to 1 mg/mL without any loss of specific enzymatic activity. The protease stored in either of these solutions was stable up to 4 weeks at 4 °C before showing loss of enzymatic activity upon renaturation. Thus, for large-scale preparation of active enzyme, protein fractions after RP-HPLC were directly dialyzed against 50 mM formic acid

Table 2: Kinetic Parameters for Protease Fusion Proteins and Mature Protease-Catalyzed Hydrolysis of the Substrate and Inhibition Constants for the Hydrolytic Reaction with the Inhibitor in Buffer C at 25 °C

protein	k_{cat} (s^{-1})	K_m (μM)	K_i (nM)
X_{28}−PR	0.9 ± 0.05	170 ± 25	60 ± 9
X_{28}−ΔTF*PR*ΔPol	1.5 ± 0.09	140 ± 23	nd[a]
PR	1.9 ± 0.12	130 ± 24	72 ± 16

[a] Not determined.

and then folded with >95% efficiency by dialysis against the desired buffer. The mature protease can be folded under identical conditions.

The dimeric enzymes can be concentrated up to 12 mg/mL in the presence of inhibitor and are monodisperse, making NMR studies feasible (D. I. Freedberg, J. M. Louis, E. M. Wondrak, and D. A. Torchia, unpublished observation). In the absence of inhibitor, these fusion proteins rapidly lose activity due to self-proteolysis similar to that of the mature protease (Rose et al., 1993; Mildner et al., 1994). Consistently, the fragmentation products of X_{28}−ΔTF*PR*Δ-Pol migrate on gels corresponding to sizes that result from proteolysis of the protease domain (Rose et al., 1993; Mildner et al., 1994). For this reason, the fusion proteins were stored in the denatured form either in 5 M urea (buffer B) at −80 °C or in 100 mM acetic acid or 50 mM formic acid at 4 °C.

Protease Fusion Proteins Exhibit Catalytic Activities Similar to That of the Mature Protease. The kinetic parameters k_{cat} and K_m for X_{28}−PR-, X_{28}−ΔTF*PR*ΔPol-, and mature PR-catalyzed hydrolysis of the substrate and the inhibition constant (K_i) for inhibitor are shown in Table 2. The k_{cat}/K_m values for X_{28}−PR and X_{28}−ΔTF*PR*ΔPol are quite similar and exhibit only a 3- and 2-fold difference, respectively, as compared to that of the mature protease- or PR−ΔPol-catalyzed hydrolysis of the same substrate (Wondrak et al., 1996). The measured K_i for the hydrolytic reaction with inhibitor was also similar to that of the mature protease (Table 2). Kinetic measurements below pH 4.0 were not included due to acid instability of the fusion proteins (see below). Thus, from a plot of log k_{cat}/K_m for X_{28}−PR versus pH, a pK_a of 4.9 was derived. This value is in agreement with that obtained for the mature PR (Hyland et al., 1991; Polgar et al., 1994) and PR−ΔPol (Wondrak et al., 1996). These results indicate that the environment of the active sites of X_{28}−PR and X_{28}−ΔTF*PR*ΔPol are not significantly altered by the presence of short native or non-native sequences flanking the protease. These findings are consistent with observations with other fusion proteins of the HIV-1 protease containing short flanking sequences at their termini (Leuthardt & Roesel, 1993; Co et al., 1994; Wondrak et al., 1996). However, the dimeric precursor ΔTF−PR−ΔPol when linked to a large 38 kDa MBP exhibits a catalytic activity at least 600-fold lower even though it can bind inhibitors and substrates with affinities similar to that found for the mature enzyme (Louis et al., 1994). These observations indicate that the length of the sequence flanking the protease influences the catalytic activity of the proteae in the precursor form.

Protease Fusion Proteins Display Decreased Stabilities to Urea and Acid. Equilibria were established by subjecting fully active and denatured mature protease to the desired concentration of urea. Both approaches yielded superimposable transition curves by measuring enzymatic activity or relative protein fluorescence (Figure 2A). The midpoint of this transition occurs at 1.75 M urea. Similar experiments with the fusion proteins yield midpoint transitions at 0.7 and 1.2 M urea for X_{28}−PR and N−ΔTF*PR*ΔPol, respectively (Figure 2A). In all cases, the transition curves were sigmoidal, consistent with a proposed two-state model in which active protease dimers are in equilibrium with unfolded monomers (Grant et al., 1992; Wondrak et al., 1996). We did not observe structural stabilization of the mature protease in the presence of substrate in excess of the K_m, consistent with previous reports (Grant et al., 1992). However, a tight-binding inhibitor (K_i = 12 nM) stabilizes the mature protease from denaturation (Grant et al., 1992). Since the binding affinity of the inhibitor is several orders of magnitude greater than that of the substrate (see Table 2), enzyme stabilization by substrate might not be measurable under these conditions.

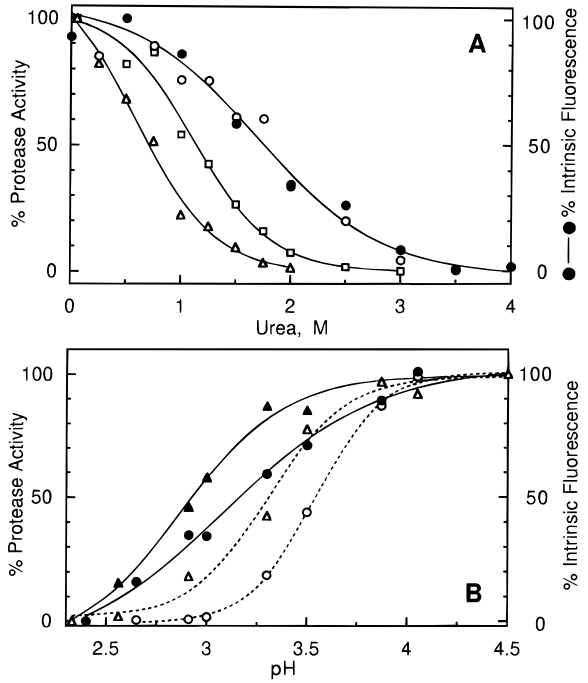

FIGURE 2: Plots of proteolytic activity and intrinsic protein fluorescence versus urea concentration (A) and pH (B). (A) Open symbols denote enzyme activity of X_{28}−PR (triangles), X_{28}−ΔTF*PR*ΔPol (squares), and PR (circles). Enzymes in buffer B were diluted 10-fold in buffer C and assayed for protease activity in the presence of urea at concentrations between 0.05 and 3.0 M and 350 μM substrate. Final concentrations of the enzymes were 0.45 μM PR, 0.69 μM X_{28}−PR, and 0.85 μM X_{28}−ΔTF*PR*ΔPol. (B) X_{28}−PR and PR are indicated as circles and triangles, respectively. Intrinsic protein fluorescences of 0.76 and 0.8 μM of X_{28}−PR and mature protease, respectively, are denoted by closed symbols and continuous lines. Open symbols and dashed lines denote enzymatic activities measured at final concentrations of 0.76 and 0.35 μM X_{28}−PR and mature protease, respectively.

As shown in Figure 2B, the intensity of the intrinsic fluorescence of PR and X_{28}−PR also displays a sigmoidal decline with decreasing pH. The transition occurs over a pH range of 1.0−1.5 units with inflections at pH 2.9 and 3.2 for PR and X_{28}−PR, respectively. The shallow curve of X_{28}−PR could reflect a greater number of transition intermediates between the folded and unfolded states. To confirm that the change in protein fluorescence is due to protein unfolding (Creighton, 1993), we measured the change in ellipticity of X_{28}−PR from pH 2.5 to 4.5 by circular

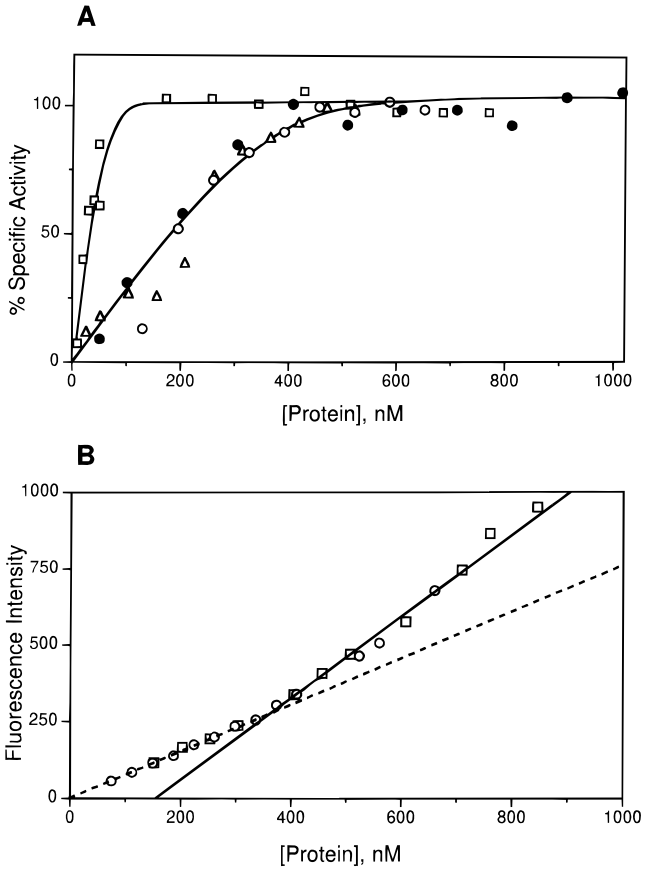

FIGURE 3: Specific activity and intrinsic fluorescence as a function of the dimeric enzyme concentration. (A) X_{28}−PR in 100 mM acetic acid (open and closed circles) and in 50 mM formic acid (triangles) and X_{28}−ΔTF*PR*ΔPol (squares) in 50 mM formic acid were diluted with 10 volumes of 100 mM sodium acetate (pH 5.0) and 50 mM sodium formate (pH 3.5) buffers, respectively, containing 1 mM DTT, 1 mM EDTA, and 350 μM substrate. No decrease in specific activity is observed for the mature protease to 5 nM. (B) X_{28}−PR maintained in 50 mM formic acid was diluted into 50 mM sodium formate (pH 4.0) and 5 mM DTT (circles) or sodium acetate (pH 5.0) and 5 mM DTT (squares). Solid and dashed lines represent two linear fits. The intercept denotes the K_d. The fluorescence intensities obtained at pH 4.0 were multiplied by a factor of 1.2.

dichroism. The titration curves measured by circular dichroism and fluorescence superimpose. The decrease in enzymatic activity as a function of pH assayed between pH 2.3 and 5.0 also shows transitions with inflection at pH 3.3 and 3.6 for the mature protease and X_{28}−PR, respectively. A 50% loss in the enzymatic activities of both X_{28}−PR and the mature PR occurs roughly 0.3−0.4 pH unit higher than for the corresponding change in the fluorescence intensity. Thus, the difference in the pH dependent inactivation of the mature protease and X_{28}−PR could relate to differences in the ionization state of the active-site aspartic acid residues in addition to the pH-induced unfolding of the fusion proteins.

Sequences Flanking the Protease Destabilize Dimer Formation. We had shown that the enzymatic activities of the mature protease and PR−ΔPol are linearly proportional to protein concentrations from 10 to 245 nM (Wondrak et al., 1996). In contrast, the two fusion proteins, X_{28}−PR and X_{28}−ΔTF*PR*ΔPol, which have a higher sensitivity to denaturation do not exhibit this linear relationship. Figure 3A shows a composite plot of the specific enzymatic activities of X_{28}−PR and X_{28}−ΔTF*PR*ΔPol versus protein concentration. Reproducibly, the specific activity of X_{28}−PR decreases below 400 nM, with a 50% loss in specific enzymatic activity at approximately 180 nM, which represents the apparent dissociation constant (K_d) (Jordan et al., 1992). Consistent with this observation, the intrinsic protein fluorescence also undergoes a transition, with a K_d of approximately 360 nM (Figure 3B). The 2-fold higher K_d determined for X_{28}−PR by monitoring the intrinsic protein fluorescence as opposed to enzymatic activity may be due to the different techniques employed. Interestingly, X_{28}−ΔTF*PR*ΔPol, which has a longer sequence at its N terminus than X_{28}−PR, is less sensitive to urea denaturation than X_{28}−PR and forms a more stable dimer (K_d = ∼25 nM; Figure 3A). The mature protease does not show any loss in specific activity measured down to 5 nM, which is the sensitivity limit of the assays used.

CONCLUSION

Dimerization of the HIV-1 protease is a prerequisite for its release and polyprotein maturation, but the extremely low K_d of the mature protease has precluded the development of a simple assay to screen for dissociative inhibitors (Wondrak et al., 1996; Darke, 1994). We have expressed and purified intact fusion proteins containing native and non-native sequences flanking the protease domain to gain insight into the subunit interaction and conformational stability of the protease precursor. Our results suggest that the ΔTF and ΔPol sequences which flank the protease domain in the Gag−Pol precursor have an influence on the overall stability of the dimeric protease precursor. This is reflected in an increase in the apparent K_d for the protease flanked by the ΔTF and ΔPol sequences.

By replacing the transframe (TF) sequence preceding the N terminus of the protease with the non-native X_{28}, we have produced a protease fusion that has catalytic activity nearly identical to that of the mature protease but which dissociates at a much higher concentration than the mature form. This result also indicates that a non-native sequence in place of the native ΔTF further destabilizes dimer formation.

The lowered stability and higher dissociation constant of X_{28}−PR and X_{28}−ΔTF*PR*ΔPol may be due to differences in the subunit interactions at the protease's termini caused by flanking sequences. The purification scheme described has enabled us to prepare sufficient quantities of labeled proteins for ongoing studies to compare the interface contacts of fusion proteins with the mature protease by NMR. Understanding the dimer interface contacts of protease precursors may aid in the design of novel strategies for intervening with dimer formation of Gag−Pol prior to its maturation.

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REFERENCES

Co, E., Koelsch, G., Lin, Y., Ido, E., Hartsuck, J. A., & Tang, J. (1994) *Biochemistry 33,* 1248−1254.

Creighton, T. E. (1993) in *Proteins Structure and Molecular Properties*, pp 287−291, W. H. Freeman and Company, New York.

Darke, P. L. (1994) *Methods Enzymol. 241*, 104−127.

Grant, S. K., Deckman, I. C., Culp, J. S., Minnich, M. D., Brooks, I. S., Hensley, P., Debouck, C., & Meek, T. D. (1992) *Biochemistry 31*, 9491−9501.

Hyland, L. J., Tomaszek, T. A., Jr., & Meek, T. D. (1991) *Biochemistry 30*, 8454−8463.

Jordan, S. P., Zugay, J., Darke, P. L., & Kuo, L. C. (1992) *J. Biol. Chem. 267*, 20028−20032.

Katz, R. A., & Skalka, A. M. (1994) *Annu. Rev. Biochem. 63*, 133−173.

Leuthardt, A., & Roesel, J. L. (1993) *FEBS Lett. 326*, 275−280.

Louis, J. M., McDonald, R. A., Nashed, N. T., Wondrak, E. M., Jerina, D. M., Oroszlan, S., & Mora, P. T. (1991) *Eur. J. Biochem. 199*, 361−369.

Louis, J. M., Nashed, N. T., Parris, K. D., Kimmel, A. R., & Jerina, D. M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 7970−7974.

Mildner, A. M., Rothrock, D. J., Leone, J. W., Bannow, C. A., Lull, J. M., Reardon, I. M., Sarcich, J. L., Howe, W. J., Tomich, C. S., & Smith, C. W. (1994) *Biochemistry 33*, 9405−9413.

Oroszlan, S., & Luftig, R. B. (1990) *Curr. Top. Microbiol. Immunol. 157*, 153−185.

Polgar, L., Szeltner, Z., & Boros, I. (1994) *Biochemistry 33*, 9351−9357.

Rose, J. R., Salto, R., & Craik, C. S. (1993) *J. Biol. Chem. 268*, 11939−11945.

Weber, I. T. (1990) *J. Biol. Chem. 265*, 10492−10496.

Wlodawer, A., Miller, M., Jaskolski, M., Sathyanarayana, B. K., Baldwin, E., Weber, I. T., Selk, L. M., Clawson, L., Schneider, J., & Kent, S. B. (1989) *Science 245*, 616−621.

Wondrak, E. M., Nashed, N. T., Haber, M. T., Jerina, D. M., & Louis, J. M. (1996) *J. Biol. Chem. 271*, 4477−4481.

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