Use of Protein Unfolding Studies To Determine the Conformational and Dimeric Stabilities of HIV-1 and SIV Proteases†

Stephan K. Grant,‡,§ Ingrid C. Deckman,∥,⊥ Jeffrey S. Culp,# Michael D. Minnich,# Ian S. Brooks,○ Preston Hensley,○ Christine Debouck,∥ and Thomas D. Meek*,‡

Department of Medicinal Chemistry, Department of Molecular Genetics, Department of Protein Biochemistry, and Department of Macromolecular Sciences, SmithKline Beecham Pharmaceuticals, 709 Swedeland Road, King of Prussia, Pennsylvania 19406-0939

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ABSTRACT: The free energies of dimer dissociation of the retroviral proteases (PRs) of human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) were determined by measuring the effects of denaturants on the protein fluorescence upon the unfolding of the enzymes. HIV-1 PR was more stable to denaturation by chaotropes and extremes of pH and temperature than SIV PR, indicating that the former enzyme has greater conformational stability. The urea unfolding curves of both proteases were sigmoidal and single phase. The midpoints of the transition curves increased with increasing protein concentrations. These data were best described by and fitted to a two-state model in which folded dimers were in equilibrium with unfolded monomers. This denaturation model conforms to cases in which protein unfolding and dimer dissociation are concomitant processes in which folded monomers do not exist [Bowie, J. U., & Sauer, R. T. (1989) *Biochemistry 28*, 7140–7143]. Accordingly, the free energies of unfolding reflect the stabilities of the protease dimers, which for HIV-1 PR and SIV PR were, respectively, $\Delta G_u^{H_2O}$ = 14 ± 1 kcal/mol (K_u = 39 pM) and 13 ± 1 kcal/mol (K_u = 180 pM). The binding of a tight-binding, competitive inhibitor greatly stabilized HIV-1 PR toward urea-induced unfolding ($\Delta G_u^{H_2O}$ = 19.3 ± 0.7 kcal/mol, K_u = 7.0 fM). There were also profound effects caused by adverse pH on the protein conformation for both HIV-1 PR and SIV PR, resulting in unfolding at pH values above and below the respective optimal ranges of 4.0–8.0 and 4.0–7.0.

The human and simian immunodeficiency viruses are members of the lentiviral subfamily of retroviruses and are the etiological agents for immunodeficiency diseases of humans and primates. A vital step in the life cycle of human immunodeficiency virus type 1 (HIV-1),[1] SIV, and all retroviruses is the posttranslational processing of viral polyprotein precursors to the active viral enzymes and structural proteins as catalyzed by the retroviral protease (Dickson et al., 1984; Kohl et al., 1988; Meek et al., 1990b). The retroviral proteases belong to the family of aspartic proteases (Meek et al., 1989; Wlodawer et al., 1989; Grant et al., 1991), which also contains the pepsins, cathepsin D, and renin. The retroviral proteases and the monomeric aspartic proteases apparently have common secondary and tertiary structural features, but differ in quaternary structure. The retroviral proteases are composed of two polypeptides folded into noncovalently associated dimers (Wlodawer et al., 1989; Miller et al., 1989a). Accordingly, the active sites of the retroviral proteases contain two catalytic aspartyl residues, which are absolutely conserved in the monomeric aspartic proteases, and each monomer contributes one of these residues.

Previously, we reported that the quaternary structure and biochemical properties of recombinant SIV PR (Grant et al., 1991) were very similar to those of HIV-1 PR (Meek et al., 1989). The overall sequence homology of recombinant SIV PR (Myers et al., 1990; Desrosiers, 1988, 1990; Grant et al., 1991) and HIV-1 PR (Strickler et al., 1989) is also high (50% identity, 73% similarity), and the two proteases display nearly total primary sequence identity in five regions. These regions constitute important structural elements of these proteins, as identified in HIV-1 PR: (1) proximal to and including the active-site triad, Asp_{25}-Thr_{26}-Gly_{27}; (2) at the substrate-binding "flaps" (Gly_{48}-Gly_{49}-Ile_{50}-Gly_{51}-Gly_{52}); (3) at an α-helical region, Gly_{86}-Arg_{87}-Asn_{88}-Leu_{89}-Leu_{90}; (4) at the N-terminal [Pro_1-Gln_2-Ile(Phe)$_3$-Thr(Ser)$_4$] and (5) at the C-terminal [Thr(Ser)$_{96}$-Leu_{97}-Asn_{98}-Phe(Leu)$_{99}$] segments which constitute the major part of the dimeric interface. Having established the commonality of the primary and quaternary structures of HIV-1 PR and SIV PR, we compare herein the secondary and tertiary structures of these two proteases by assessing their conformational stabilities and to use these physical properties to further ascertain the stabilities of the protein homodimers.

Recently, values have been obtained for the dissociation constants of the homodimers of HIV-1 PR (Zhang et al., 1991) and HIV-2 PR (Holzman et al., 1991) by using equilibrium sedimentation and for HIV-1 PR by examining enzyme inactivation by a peptide fragment comprising one of the interdimeric strands (Zhang et al., 1991). In a different approach, one might expect that disruption of the quaternary structure of the proteases would accompany their unfolding by chaotropes, as seen for the Arc (Bowie & Sauer, 1989) and

† Supported in part by NIH Grants AI 24845 and GM 39526.
* Author to whom correspondence should be addressed.
‡ Department of Medicinal Chemistry.
§ Present address: Merck Sharp & Dohme Research Laboratories, PO Box 2000, Rahway, NJ.
∥ Department of Molecular Genetics.
⊥ Present address: Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ.
Department of Protein Biochemistry.
○ Department of Macromolecular Sciences.
[1] Abbreviations: DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HIV-1, human immunodeficiency virus, type 1; kDa, kilodalton(s); Mes, 2-(*N*-morpholino)-ethanesulfonic acid; PR, protease; RP-HPLC, reversed-phase high-performance liquid chromatography; SIV, simian immunodeficiency virus; Tris, tris(hydroxymethyl)aminomethane.

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trp (Gittleman & Matthews, 1990) repressor proteins. If so, methods that could be used to quantify the unfolding of these homodimeric proteins could also provide measurements of the dissociation constants of the homodimers. There are two tryptophan residues in the primary sequence of HIV-1 PR (Trp-6 and Trp-42) and a single tryptophan residue in SIV PR (Trp-6). As revealed by crystallographic data for HIV-1 PR, and presumably for SIV PR, Trp-6 is proximal to the dimer interface of the native enzyme (Wlodawer et al., 1989). Denaturation, which would result in subunit dissociation, should be accompanied by changes in the protein emission fluorescence spectrum for each protein as the tryptophan residues become more exposed to solvent. We report here the use of protein fluorescence and activity measurements to assess the conformational stabilities of HIV-1 PR and SIV PR and the use of unfolding studies to provide a measurement of the stability constants of the homodimers.

MATERIALS AND METHODS

Reagents and Proteins. The oligopeptide substrate Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 was synthesized and characterized as described previously (Moore et al., 1989; Hyland et al., 1991a). The synthesis and characterization of the peptide analogue inhibitor Ala-Ala-PheΨ[CH(OH)CH_2]-Gly-Val-Val-OCH_3 are described elsewhere (Dreyer et al., 1992). Urea (ICN Biomedicals, ultra pure) was used for denaturation experiments. Solutions of urea were passed through a Poly-Prep chromatography column (Bio-Rad) containing 3 g of an ion retardation resin (AG 11A8, 50–100 mesh, analytical grade, Bio-Rad) before use in order to remove possible ionic contaminants. Glycerol (LKB, Ultrograde) and other biochemicals were of the highest purity available. The following buffers were used: MEND buffer, 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.0, 0.2 M NaCl, 1 mM EDTA, and 1 mM DTT; MENDT buffer, same as MEND buffer with the addition of 0.1% Triton X-100; FAMTEN buffer, 50 mM sodium formate, 50 mM sodium acetate, 50 mM Mes, 50 mM Tris-HCl, 0.2 M NaCl, and 1 mM EDTA, pH adjusted with 4 M HCl or 4 M NaOH and then brought to constant conductance by the addition of small aliquots of 4 M NaCl; and FAMTENDT buffer, same as FAMTEN buffer with 1 mM DTT and 0.1% Triton X-100.

Recombinant HIV-1 PR and SIV PR were expressed in *Escherichia coli* and purified as described previously (Debouck et al., 1987; Grant et al., 1991). The proteases, purified to apparent homogenity, were stored at concentrations of 20–400 μg/mL at −80 °C in 50 mM sodium acetate (pH 5.0), 1 mM dithiothreitol, 1 mM EDTA, 0.35 M NaCl, and 20% glycerol.

Enzyme Assays. Protein concentrations were determined by using either the BCA protein assay reagent (Pierce) with bovine serum albumin as a standard or by analytical RP-HPLC as previously described (Strickler et al., 1989; Grant et al., 1991). The apparent inhibition or inactivation of HIV-1 PR and SIV PR by fixed concentrations of chaotropes, or by variation of temperature or pH, was quantified by using the peptidolytic assay as described (Meek et al., 1989; Moore et al., 1989), with Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 as the variable substrate. The reactions [50 μL (final volume) in either MENDT or FAMTENDT buffer] were initiated by the addition of either HIV-1 PR or SIV PR; after incubation at 37 °C for 30 min, reactions were quenched with 50 μL of 5% (v/v) trifluoroacetic acid. Peptide substrates and products were separated and analyzed by RP-HPLC (Hyland et al., 1991a; Moore et al. 1989). Initial velocities, obtained at a single time point, were determined by digital integration of the substrate and product peaks in the chromatograms.

Time-dependent inactivation of the enzymes at extremes of pH was similarly evaluated by separately preincubating 50-μL mixtures containing HIV-1 PR (0.4 μg) or SIV-PR (0.6 μg) in FAMTENDT buffer (pH 3–9) at 22–23 °C for 5–60 min. Peptidolytic activity was assayed by adding 5-μL aliquots of these preincubation mixtures to 30-μL mixtures containing 5 mM peptide substrate in MENDT buffer (pH 6.0), followed by reaction at 37 °C for 10 min. The temperature dependence of k_{cat} and k_{cat}/K_m was determined by fitting the initial rate data to eq 1, obtained at variable concentrations of the peptide

$$v = k_{cat}E_tA/(K_m + A) \quad (1)$$

substrate. Reaction mixtures (40 μL) containing FAMTENDT buffer (pH 4.7), 0.5–5 mM Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2, and 40 ng of either HIV-1 or SIV protease were incubated for 10 min at 10–80 °C. The reactions were quenched by the addition of 40 μL of 3% (v/v) trifluoroacetic acid and analyzed as described above. In eq 1, v is the initial velocity, E_t is the total enzyme concentration, and A is the substrate concentration. Equation 1 was fitted by using the FORTRAN program of Cleland (1979).

Possible precipitation of the enzymes from urea-containing solutions was examined by incubating 50-μL mixtures containing either HIV-1 PR (0.4 μg) or SIV-PR (0.3 μg) at room temperature for 30 min in 0–8 M urea (0.25 M increments) and MEND buffer (pH 6.0), followed by centrifugation at 17 000 rpm (4 °C) for 10 min. Supernatants were decanted, and 50 μL of fresh buffer was added, followed by mixing to resuspend any precipitate. Supernatant and resuspended solutions were transferred onto nitrocellulose using a Bio-Dot microfiltration apparatus (Bio-Rad). HIV-1 PR or SIV PR was probed using a rabbit polyclonal antibody which reacts with both proteases. Protease–antibody complexes were detected as previously described (Meek et al., 1990a).

Analytical Ultracentrifugation. Equilibrium sedimentation experiments of HIV-1 PR were performed with a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner and a temperature control system. Double-sector cells with charcoal-filled epon centerpieces and sapphire windows were used. Data were collected using an IBM AT with an analog-to-digital interface attached to the scanner. Data were analyzed using nonlinear least-squares methods (Johnson & Frasier, 1985) under the control of a modified version of IGOR (Wavemetrics, Lake Oswego, OR) running on a Macintosh IIfx computer computer (Brooks et al., unpublished results). Data sets were collected at a rotor speed of 20 000 rpm for 18 h.

Sedimentation experiments were performed at 4 °C in FAMTEN buffer (pH 5.0). The initial concentration of HIV-1 PR was 0.4 mg/mL, resulting in an initial absorbance at 280 nm of 0.46, assuming an extinction coefficient of 12 500 M^{-1} cm^{-1} for monomer concentrations of the proteases (Nutt et al., 1988). The molecular weight of the protease monomer was calculated to be 10 775 from the amino acid sequence of HIV-1 PR (Strickler et al., 1989). The partial specific volume, v, was calculated using the partial specific volumes of the component amino acids (Zamyatnin, 1972), yielding a value of 0.749 mL/g. The solvent density, ρ, was estimated to be 1.007 g/mL.

At sedimentation equilibrium, the concentration of a single, homogeneous species is given by eq 2, in which c_r and c_0 are, respectively, the concentrations of the protein at a radial

$$c_r = c_0 \exp\{M[(1 - v\rho)\omega^2(r^2 - r_m^2)]/2RT\} \quad (2)$$

position r and at a reference position, usually the meniscus. In addition, M is the protein molecular weight, v is the partial specific volume of the protein, ρ is the solvent density, ω is the angular velocity, r is the distance in centimeters from the center of rotation, r_m is the radial position of the reference position in centimeters, R is the universal gas constant, and T is the absolute temperature. The data in terms of a monomer–dimer equilibrium were analyzed as described by Hensley et al. (1975).

Protein Unfolding Studies. The denaturation of HIV-1 PR and SIV PR under the condition of either increasing concentrations of urea or changing pH was monitored at equilibrium by recording changes in the intrinsic fluorescence of these proteins. Protein fluorescence measurements were performed with a Perkin-Elmer MPF-66 fluorescence spectrophotometer equipped with a Perkin-Elmer 7500 professional computer. Constant temperature at 25 °C was maintained in 1-mL quartz cells by a Forma water bath that circulated water through jacketed cell holders. The following spectral conditions were used: 280-nm excitation wavelength, 10-nm excitation slit width, 10-nm emission slit width, and 2.0-s response. Emission spectra were recorded from 300 to 400 nm at 120 nm/min. Typically, 5–20 μL of protease (0.3 mg/mL for SIV PR or 0.4 mg/mL for HIV-1 PR) was added to give mixtures of 1-mL final volume, which contained either FAMTEN buffer (pH 3–9) or MEND (pH 6) buffer and 0–8 M urea. Fluorescence spectra were recorded at 2-min intervals until equilibrium was established (30–60 min) as indicated by the attainment of invariant emission intensity (Pace, 1986). Spectra were corrected by subtraction of the buffer solution in the absence of protein. Transition curves of protein unfolding at increasing urea concentrations were constructed from the measured protein fluorescence intensities, y_{obs}, determined for each concentration of urea. The transition curves were obtained at several fixed concentrations for each of the two proteases (Pace, 1986; Gittleman & Matthews, 1990). For each value of y_{obs}, the apparent fraction of unfolded protein, f_u, was calculated from $f_u = (y_{obs} - y_f)/(y_u - y_f)$, where y_f and y_u are the fluorescence intensities, respectively, of the fully folded proteins (acquired in the absence of denaturant) and the fully unfolded proteins (acquired at high urea concentrations). The values of y_f and y_u were obtained at the base lines of the transition curves, at which y_{obs} became invariant at changing urea concentrations.

In addition to fluorescence measurements, the urea-mediated unfolding of HIV-1 PR was assessed by use of the peptidolytic assay to determine the remaining fraction of folded protease following preincubation with variable urea concentrations. In 50-μL reaction mixtures (25 °C) containing MEND buffer (pH 6.0) and 0–7 M urea, HIV-1 protease was added and incubated for 30 min at 25 °C. After incubation, the remaining active (folded) protease was determined from residual peptidolytic activity by the addition of 50 μL of 30 mM Ac-Arg-Ala-Ser-Gln-Tyr-Pro-Val-Val-NH_2 to a final concentration of 15 mM, and reaction was allowed to proceed at 25 °C for an additional 10 min. Residual enzyme activities were determined from the peptidolytic products as described above. From these data, the fraction of unfolded protease f_u was calculated using the above equation in which y_{obs}, y_f, and y_u were, respectively, the remaining protease activities at each urea concentration, in the absence of urea, and zero.

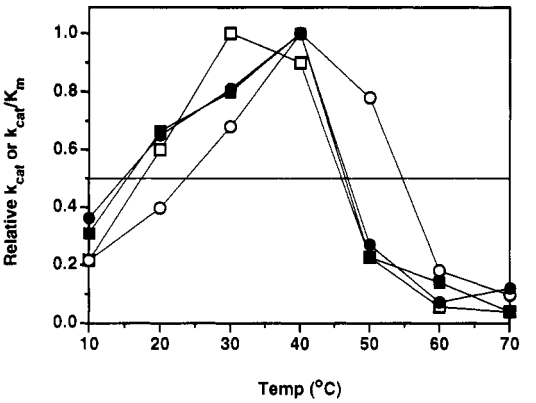

FIGURE 1: Effect of temperature on the apparent k_{cat} and k_{cat}/K_m of HIV-1 PR (open symbols) and SIV PR (filled symbols). Initial rate data were obtained at the indicated temperatures from reaction mixtures (10-min incubations) containing FAMNTENDT buffer (pH 4.7), 0.5–5.0 mM Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2, and 0.18 μM either protease. Apparent values of k_{cat} (circles) and k_{cat}/K_m (squares) were obtained by fitting of the data to eq 1 at each temperature.

RESULTS

Effects of Temperature on the Peptidolytic Activities of HIV-1 PR and SIV PR. Heat denaturation has frequently been used as a probe of protein stability (or unfolding). Here we assessed the temperature dependence of the peptidolytic activities of the lentiviral proteases to measure their respective solution stabilities. The effects of temperature on k_{cat} and k_{cat}/K_m were ascertained for SIV PR and HIV-1 PR (Figure 1) using 0.5–5.0 mM concentrations of Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 (10-min incubations, pH 4.7). Enzymatic reactions of mixtures maintained at the indicated temperatures were initiated by the addition of minimal aliquots of enzyme. Both k_{cat} and k_{cat}/K_m for either SIV PR or HIV-1 PR increased to optimal values at about 40 °C, but above this temperature, apparent inactivation of the enzymes was evident in the marked decreases in these values between 50 and 60 °C. All the apparent midpoints for protein "melting" based on k_{cat}/K_m values for both enzymes and on k_{cat} for SIV PR occurred at approximately 45–47 °C, while for HIV-1 PR, the midpoint of k_{cat} was 55 °C. This indicates that the substrate-bound form of HIV-1 PR is somewhat more stable than the free enzyme, while for SIV PR, these two forms of the enzyme are of equal stability, which is less than that of the substrate-bound HIV-1 PR.

Sedimentation Equilibrium of HIV-1 PR. Figure 2A,B shows the results of equilibrium analytical ultracentrifugation of HIV-1 PR in the absence and presence (0.54 mM) of the inhibitor Ala-Ala-PheΨ[CH(OH)CH_2]Gly-Val-Val-OCH_3 [K_i = 12 nM (pH 6.0), molecular mass = 578 g/mol]. The best fit of these data to a single sedimenting species by eq 2 yields apparent molecular weights of 21 190 ± 260 and 22 180 ± 270 in the absence and in the presence of the inhibitor, respectively. Attempts to fit the data with a model allowing for dimer dissociation failed to give better fits. No dissociation of the protease homodimer is detectable, so an upper limit of the monomer–dimer dissociation constant can be estimated at 10^{-7}–10^{-8} M, on the basis of the extinction coefficient of HIV-1 PR and the lowest concentration of protein detected. The true dissociation constant may be significantly lower than this estimated value.

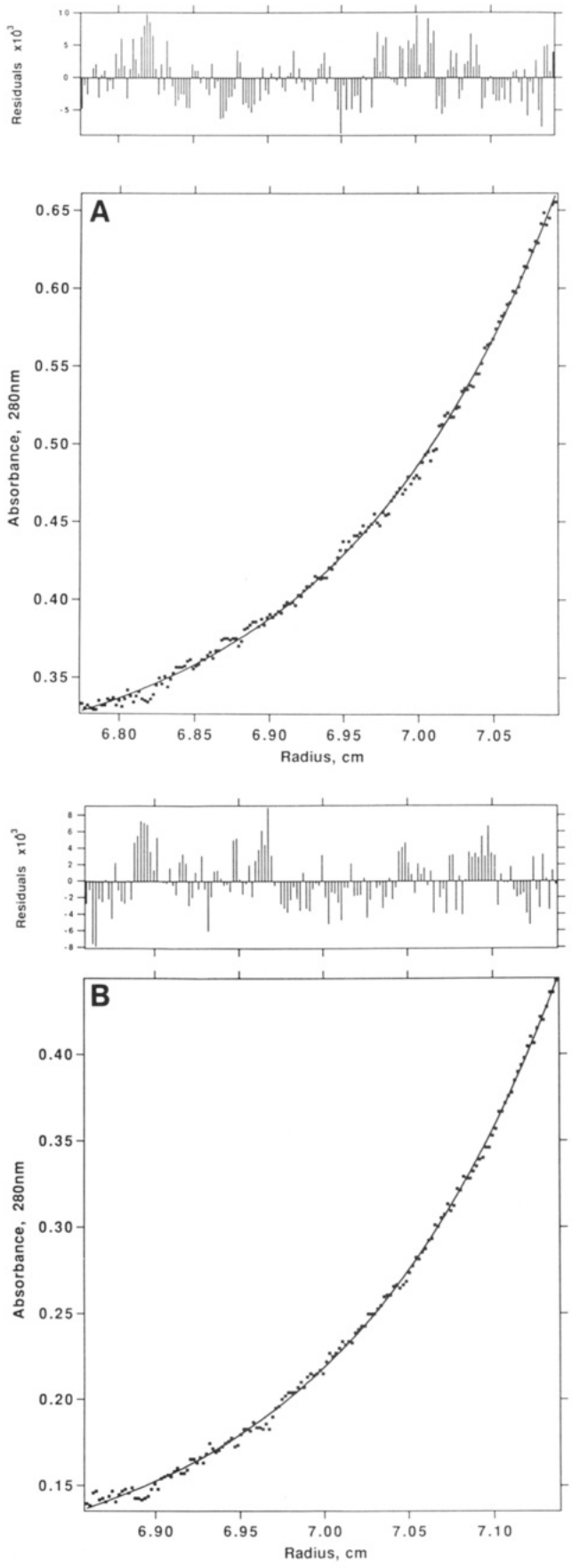

FIGURE 2: Sedimentation equilibrium analysis of (A) unbound and (B) inhibitor-complexed HIV-1 protease at pH 5.0, 4.0 °C, as described under Materials and Methods. Curves drawn through the experimental data points of absorbance vs radial position r were obtained by fitting the data to eq 2. The residual differences of observed and fitted data for each point are shown in the top sections of each panel.

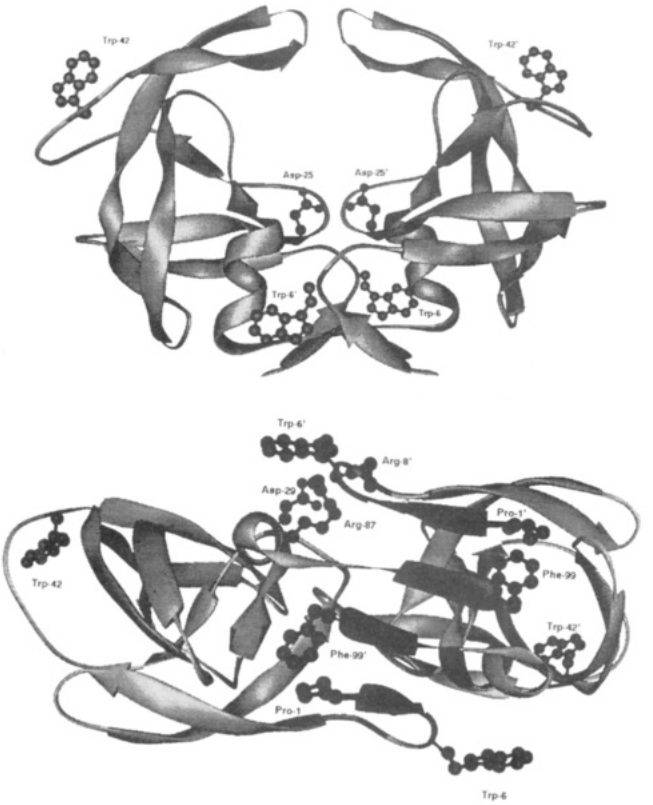

FIGURE 3: Ribbon drawings of two orientations (front and bottom views) of the uncomplexed form of the HIV-1 PR homodimer (Wlodawer et al., 1989), in which the active-site aspartyl residues (Asp-25 and Asp-25′), the interdigitating β-sheets of the amino and carboxyl termini that comprise the dimeric interface, Trp residues in each monomer, and Asp-29, Arg-8′, and Arg-87 are labeled.

Urea Unfolding As Quantified by Changes in Protein Fluorescence. The HIV-1 PR and SIV PR homodimers contain four and two tryptophanyl residues, respectively. In the HIV-1 PR structure (Wlodawer et al., 1989), the Trp residues are found near or in β-sheet structures (Figure 3). The Trp-6 and Trp-6′ residues, conserved within both HIV-1 and SIV PR, are located proximal to the two N-terminal β-strands that constitute half of the dimer interface in HIV-1 PR (Wlodawer et al., 1989). In addition, HIV-1 PR contains Trp-42 and Trp-42′, which are found in external loops located near the substrate-binding "flaps". Denaturation of either protease would be expected to increase the solvent exposure of these Trp residues, potentially altering their intrinsic fluorescence. In particular, fluorescence changes of Trp-6 and Trp-6′ for either protease should prove to be sensitive probes of dimer dissociation, since separation of the monomers would likely change the solvent environment of these residues. Therefore, we determined the effects of urea denaturation on the protein fluorescence of both HIV-1 and SIV PR in order to measure the strength of the monomer-monomer interactions.

Treatment of either HIV-1 PR or SIV PR with 7 or 8 M urea (pH 6) resulted in similar changes of the fluorescence emission spectra (Figure 4). Native HIV-1 PR displayed more than twice the intrinsic fluorescence intensity of SIV PR; this is attributable to twice the number of tryptophan residues per dimer of HIV-1 PR. Addition of 8 M urea caused a 50% quenching of the fluorescence emission for both HIV-1 PR and SIV PR and a red-shifted emission maximum (from 347 to 351 nm). The quenching of the fluorescence intensity and the red-shifting of the λ_{max} characteristically accompany protein unfolding (Pace et al., 1989). In order to determine if the decrease in fluorescence emission intensity was due to

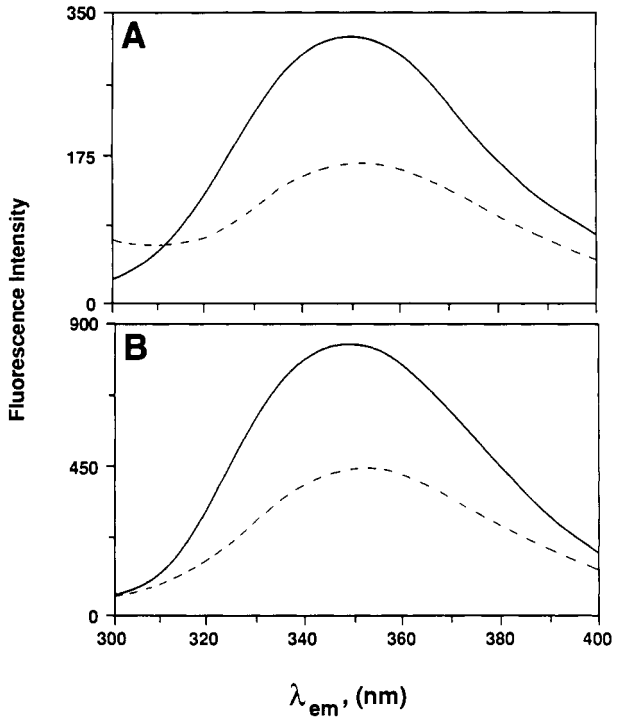

FIGURE 4: Fluorescence emission spectra for native (folded, solid lines) and denatured (unfolded, dashed lines) states for (A) 8 μg/mL (0.53 μM monomer) SIV PR (obtained, respectively, at 0 and 7 M urea) and (B) 8 μg/mL (0.73 μM monomer) HIV-1 PR (obtained, respectively, at 0 and 8 M urea) in MEND buffer (pH 6) at 25 °C.

possible precipitation of the enzymes, each protease was incubated in urea (0–8 M) and centrifuged, and the resulting supernatant fractions and the resuspended pellets were separately immobilized onto nitrocellulose. Upon immunoblotting, HIV-1 and SIV proteases were detected in the supernatants at all urea concentrations (data not shown). Neither protease was detected in the resuspended pellets, indicating either that the decrease in fluorescence emission intensity was not caused by protein precipitation or that precipitated protein could not be resuspended.

The reversibility of unfolding of the retroviral proteases as measured by changes in protein fluorescence was also investigated. HIV-1 protease (0.5 μM monomeric concentration) was allowed to equilibrate in 5.0 M urea for 30 min, at which point the protein fluorescence intensity had decreased to a stable level which, at the λ_{max} range of 347–351 nm, was 70% of that of an identical protein sample equilibrated in 0.5 M urea (Figure 5A). Ten-fold dilution into urea-free buffer of a sample of HIV-1 protease which had been similarly preincubated for 30 min in 5.0 M urea (final monomeric concentration of 0.5 μM) resulted in an instantaneous recovery of protein fluorescence to 70% of the intensity of the protein sample in 0.5 M urea (Figure 5A). This recovery of protein fluorescence intensity and the blue-shifting of the λ_{max} to 347 nm upon the dilution of the chaotrope indicate that HIV-1 protease had significantly refolded. The enzymatic activity within the samples which produced the three curves in Figure 5A was also assessed by the addition of a minimal volume of 20 mM Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 to each sample (2.5 μL of substrate to 47.5 μL of protein sample solution to produce a final substrate concentration of 1 mM), followed by incubation at 37 °C for 15 min. The relative enzymatic activities of the HIV-1 protease within the samples which resulted in curves 1–3 in Figure 5A were 100%, 0%, and 70%, respectively.

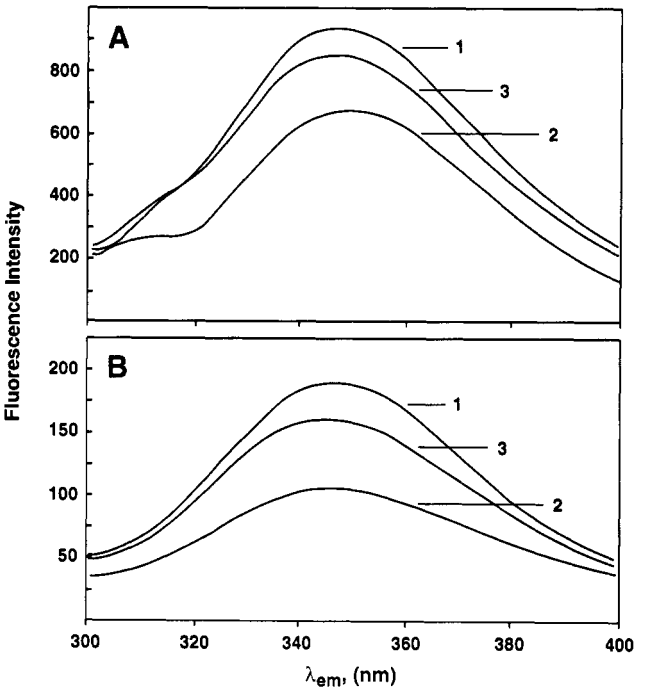

FIGURE 5: Reversibility of urea-induced or temperature-dependent unfolding of the retroviral proteases. (A) Fluorescence emission spectra obtained in MEND buffer (pH 6.0, 25 °C) for 0.5 μM monomers of HIV-1 protease equilibrated in 0.5 M urea for 30 min (curve 1), for HIV-1 protease equilibrated in MEND buffer (pH 6.0)–5.0 M urea (30 min, 25 °C) and then diluted 10-fold to a final concentration of 0.5 μM in MEND buffer containing 5.0 M urea (curve 2), and for HIV-1 protease equilibrated in MEND buffer (pH 6.0)–5.0 M urea (30 min, 25 °C) and then diluted 10-fold to a final protease concentration of 0.5 μM in MEND buffer containing no urea. (B) Fluorescence emission spectra for 0.45 μM monomers of SIV protease in MEN (pH 6.0) at 25 °C (curve 1), after heating to 70 °C (curve 2), and then upon cooling of the heated sample to 25 °C (curve 3).

That this recovery of HIV-1 protease protein fluorescence upon its dilution from 5.0 M urea is equivalent to the recovery in enzymatic activity confirms that the increase in protein fluorescence reflects refolding of the protein to its active form. Also, addition of substrate to the sample containing 5.0 M urea led to no recovery of enzymatic activity. Attempts to refold HIV-1 protease following its exposure to 5.0 M urea for 1 h or more led to lower, but equivalent, recoveries of both protein fluorescence and enzymatic activity (30%). Urea-mediated unfolding of HIV-1 protease is therefore largely reversible upon its dilution in a single step from a concentrated solution of urea, but this reversibility diminishes as the incubation period in urea is prolonged. This may result from slow precipitation of the unfolded form of this highly hydrophobic protein. However, as described above, no precipitation could be detected after urea treatment for 30 min, after which time refolding was nearly complete. In all cases, 30 min was a sufficient period for protein fluorescence measurements to become stable. Consequently, protein fluorescence data for urea unfolding studies described below were acquired at 0–30-min periods.

Similarly, refolding of SIV protease could be demonstrated by nearly total restoration of protein fluorescence upon cooling to 25 °C a sample of the enzyme which had been unfolded at 70 °C (Figure 5B). Incremental heating of SIV protease from 25 to 70 °C resulted in a progressive decrease in protein fluorescence to 60% of its original level (curves 1 and 2 in Figure 5B). Subsequent cooling of the unfolded SIV protease to 25 °C was accompanied by an increase in protein

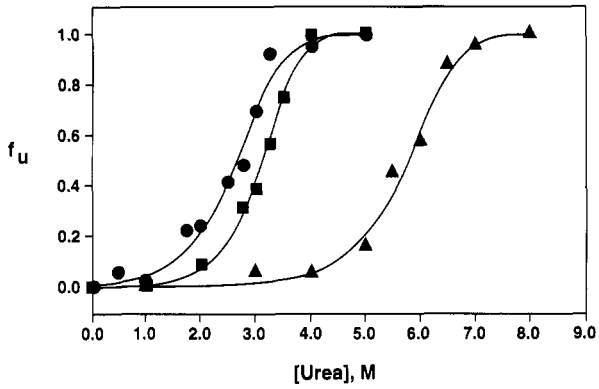

FIGURE 6: Urea denaturation curves for free and inhibitor-bound forms of HIV-1 protease as determined by changes in protein fluorescence (MEND buffer, pH 6.0, 25 °C). The fluorescence intensity at 347 nm was obtained after equilibrium was achieved (fluorescent intensity became stable). The apparent fractions of unfolded protein (f_u) were obtained as described under Materials and Methods. Samples contained 1.2 μg [0.11 μM monomer (circles)] and 12 μg of HIV-1 protease [1.1 μM monomer (squares)] and 1.2 μg of HIV-1 PR mixed with 10 μM Ala-Ala-PheΨ[CH(OH)-CH_2]Gly-Val-Val-OCH_3 (triangles). Curves drawn through the experimental points were obtained by fitting to eq 8 as described under Results.

fluorescence (curve 3) which attained an intensity equivalent to 66% of the original fluorescence in the unfolded protein at 25 °C. This again demonstrates that the unfolding of a retroviral protease was nearly completely reversible upon removal of the denaturing condition.

The changes in protein fluorescence intensity with increasing urea concentrations were used to generate equilibrium unfolding curves for each protease at 25 °C (Pace, 1986; Pace et al., 1989). For both HIV-1 PR (Figure 6) and SIV PR (data not shown), there was a sharp sigmoidal decrease in fluorescence intensity between 2.0 and 3.0 M urea, indicating an increase in the fraction of unfolded protein forms, f_u. These curves demonstrated that both proteins achieved unfolded states through a single-phase transition, suggesting no discrete or stable unfolding intermediates. Urea-mediated unfolding also depended on the total protein concentration of either protease. The unfolding transition regions occurred at higher concentrations of urea as the fixed concentration of either protease was increased, as shown for HIV-1 PR in Figure 6. The midpoint of the transition from folded to unfolded protein species, $D_{1/2}$, increased as the fixed concentration of proteases increased. Over a 10-fold and 2-fold range of HIV-1 PR and SIV PR concentrations, $D_{1/2}$ increased by 19% and 47%, respectively (Table I). Therefore, the stability of these proteins with respect to urea denaturation increased at increasing concentrations of total protein, as would be expected if the urea mediated unfolding of both HIV-1 PR and SIV PR involved a bimolecular process, that is, a folded dimer–unfolded monomer equilibrium, as opposed to a unimolecular process (folded dimer–unfolded dimer or folded monomer–unfolded monomer equilibrium).

The urea-induced unfolding of HIV-1 PR was also investigated in the presence of a competitive inhibitor, Ala-Ala-PheΨ[CH(OH)CH_2]Gly-Val-Val-OCH_3 (10 μM, K_i = 12 nM, pH 6.0). The fluorescence emission spectrum of the HIV-1 protease–inhibitor complex was identical to that of the free enzyme (data not shown). There was, however, a significant difference in the urea denaturation curve obtained for the HIV-1 PR–inhibitor complex (Figure 6). Whereas uncomplexed enzyme unfolded with a sharp transition between 2 and 3 M urea, the enzyme–inhibitor complex yielded a single-phase transition with a midpoint urea concentration of 5.8 M. The concentration dependence of the unfolding of the protease–inhibitor complex was not investigated.

Table I: Free Energy of Unfolding of HIV-1 and SIV Proteases[a]

	$[P_t]$ (μM)	$\Delta G_u^{H_2O}$ (kcal/mol)	$D_{1/2}$ (M)	K_u (M)
(1) HIV-1 PR	0.11	14.4 ± 0.6	2.6	3.9×10^{-11}
	0.18	15.6 ± 0.4	2.6	
	0.34	12.6 ± 0.4	2.6	
	1.1	15.4 ± 0.5	3.1	
		(av 14.2 ± 1.4)		
(2) HIV-1 PR/10 μM inhibitor	0.11	19.3 ± 0.7	5.8	7.0×10^{-15}
(3) SIV PR	0.20	12.4 ± 0.7	1.9	1.8×10^{-10}
	0.54	14.2 ± 0.4	2.8	
		(av 13.3 ± 1.3)		

[a] Data were obtained in MEND buffer (pH 6.0) containing 2% glycerol at 25 °C as described under Materials and Methods. Protease concentrations are expressed as monomer concentrations. Values of $\Delta G_u^{H_2O}$ were obtained by fitting of data from urea denaturation curves to eq 8, and values of K_u were calculated from averaged values of $\Delta G_u^{H_2O}$ by use of eq 7. Values of $D_{1/2}$, the urea concentrations at which $f_u = 0.5$, were calculated from eq 9.

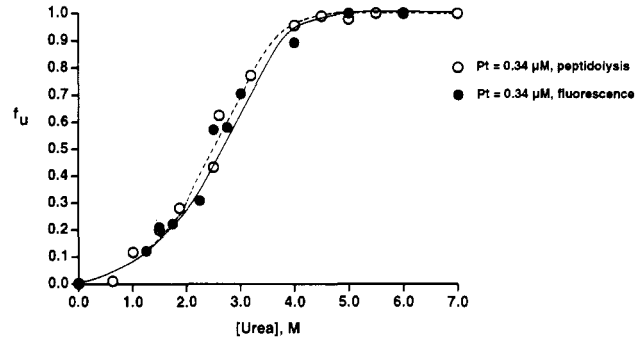

FIGURE 7: Urea denaturation curves of HIV-1 PR in which the fraction of unfolded protein was determined by protein fluorescence (filled circles, solid line) and enzymatic assay (open circles, dashed line). Samples contained MEND buffer (pH 6.0) and 0.34 μM HIV-1 protease monomer. The peptidolytic activities and protein fluorescence measurements were determined after incubation at 25 °C for 30 min as described under Materials and Methods. Curves drawn through the experimental points were obtained by fitting to eq 8.

The urea-mediated unfolding of HIV-1 PR was also investigated by use of the peptidolytic assay to determine the fractions of unfolded protein at each urea concentration. Values of f_u were calculated from the measured fraction of remaining enzyme activity as $f_u = 1 - v_u/v_0$, where v_u and v_0 are enzyme activities in the presence and absence of urea, respectively. The protease activity measurements were performed by 2-fold dilutions of the urea-containing enzyme solutions by the addition of substrate, followed by incubation at 25° for an additional 10 min. As shown above, the 2-fold dilution of urea (especially at lower urea concentrations) may result in some refolding of HIV-1 protease during the course of the peptidolysis, which would result in inaccuracy in the determination of the fraction of unfolded protein. It is also possible that urea at high concentrations is a competitive inhibitor of HIV-1 protease, which would also obscure the determination of the fraction of unfolded (inactive) enzyme.

Urea denaturation curves using the peptidolytic assay and fluorescence measurements were obtained under identical conditions for a common concentration of HIV-1 protease (0.34 μM monomer), as shown in Figure 7. The curves are nearly superimposable ($D_{1/2}$ values of 2.6 M urea were obtained for both the fluorescence and enzymatic measurements, respectively). However, given the caveats of partial refolding

and competitive inhibition in the peptidolytic assay as described above, from the apparent coincidence of the urea unfolding curves in Figure 7 we may only conclude that concentrations of urea which result in the single-phase transition of quenching of HIV-1 protease protein fluorescence are commensurate with urea concentrations which diminish enzymatic activity.

Calculation of Free Energies of Protease Unfolding. The characteristics of urea denaturation of the two lentiviral proteases shown here in Figures 6 and 7 and in Table I are strikingly similar to the denaturation data for two other small dimeric proteins, the Arc repressor (Bowie & Sauer, 1989) and the *trp* aporepressor (Gittleman & Matthews, 1990). Both of these repressor proteins displayed single-phase transitions in urea-induced unfolding, which in both cases depended on the concentration of protein. The authors concluded that the unfolding of these proteins occurs through a two-state model in which two populations of protein exist at equilibrium, namely, folded dimers (D) and unfolded monomers (M). The two-state model is described by eq 3 and 4. We propose that

$$D \overset{K_u}{\rightleftharpoons} 2M \tag{3}$$

$$K_u = [M]^2/[D] \tag{4}$$

this two-state model is also consistent with the urea denaturation data of HIV-1 PR and SIV PR. Two conditions of this model are that the unfolding of the dimer is freely reversible and that the unfolding curves demonstrate a dependence on protein concentration. For both proteases, we have established above the partial reversibility of unfolding; for HIV-1 protease, reversibility upon dilution of urea has been demonstrated under the experimental conditions used for generation of the urea unfolding curves. The dependence of the urea unfolding curves on protein concentration is consistent with an equilibrium established between folded protease dimers and unfolded monomers (described by eq 3 and 4) as opposed to that of folded dimers–unfolded dimers or folded monomers–unfolded monomers. Given these prerequisites, the unfolding constant of the protease homodimers, K_u, can be evaluated from the urea denaturation curves, and since unfolding and dimer dissociation are inexorably linked, values of K_u are tantamount to measurements of the dimer dissociation constants of these proteins.

The total concentration of protease monomers, P_t, at any concentration of urea can be described in terms of the fraction of unfolded protein as obtained from fluorescence measurements by eq 5 (Bowie & Sauer, 1989; Gittleman & Matthews, 1990):

$$P_t = 2D + M = (1 - f_u)P_t + f_u P_t \tag{5}$$

From eq 4 and 5, K_u can be expressed in terms of the measurable quantities P_t and f_u (eq 6):

$$K_u = 2P_t(f_u)^2/(1 - f_u) \tag{6}$$

As shown by Pace (1986), the free energy of unfolding (ΔG_u) for the two-state denaturation model is a linear function of urea concentration (eq 7), in which $\Delta G_u^{H_2O}$ is the

$$\Delta G_u = \Delta G_u^{H_2O} + m[U] = -RT \ln K_u \tag{7}$$

extrapolated free energy of unfolding in the absence of urea, m is the slope of a plot of ΔG_u vs urea concentration, [U], and R and T are the gas constant and the absolute temperature, respectively.

By combining eq 6 and 7 and rearranging, values of $\Delta G_u^{H_2O}$ in terms of the fraction of unfolded protein and urea concentration can be obtained directly from the urea denaturation curves by fitting of these curves to eq 8:

$$f_u = (\{\exp[-(\Delta G_u^{H_2O} + m[U])/RT]^2 + 8P_t \exp[-(\Delta G_u^{H_2O} + m[U])/RT]\}^{1/2} - \exp[(\Delta G_u^{H_2O} + m[U])/RT])/4P_t \tag{8}$$

Equation 8 can be rearranged and solved to determined $D_{1/2}$ (the urea concentration at which 50% of the protein is in the unfolded form; $f_u = 0.5$), as shown in eq 9. Denaturation

$$D_{1/2} = -\{(\ln P_t)RT + \Delta G_u^{H_2O}\}/m \tag{9}$$

curves were fitted to eq 8 using the SUPERFIT package, a nonlinear regression analysis package developed in-house, which uses the approach of Marquardt (1983). Results of fitting the urea denaturation data to eq 8 and 9 are summarized in Table I. Values of $\Delta G_u^{H_2O}$ of 14.2 ± 1.4 kcal/mol (HIV-1 PR) and 13.3 ± 1.3 kcal/mol (SIV PR) were determined by averaging $\Delta G_u^{H_2O}$ values calculated at each protein concentration. The values of $\Delta G_u^{H_2O}$ among the protein concentrations were within experimental error (10%) of each other for both proteases. This indicates that $\Delta G_u^{H_2O}$ values for both proteases were effectively invariant over a range of protein concentrations, as would be expected for the two-state denaturation model. The $\Delta G_u^{H_2O}$ values for HIV-1 PR and SIV PR indicate that HIV-1 PR possesses a slightly more stable conformation than SIV PR, as has been indicated in the studies of temperature and pH stabilities (see below). For the HIV-1 PR–inhibitor complex, $\Delta G_u^{H_2O}$ was much greater, 19 kcal/mol, indicating that the protease dimer is made much more stable upon the binding of an inhibitor.

From the averaged values of $\Delta G_u^{H_2O}$ (Table I) and by using eq 7, apparent K_u values for the lentiviral protease dimers were calculated. At 25 °C (pH 6.0, 0.2 M NaCl), the unfolding dissociation constants of the homodimers of HIV-1 PR and SIV PR were found to be 39 pM and 180 pM, respectively, while that of the HIV-1 PR–inhibitor complex was 7.0 fM.

Fitting the urea denaturation curve obtained by use of the peptidolytic assay (Figure 7) to eq 8 and 9 yielded $\Delta G_u^{H_2O}$ and $D_{1/2}$ values of 12.8 ± 0.3 kcal/mol and 2.6 M, respectively, which are nearly identical to values obtained from fluorescence measurements at the same protease concentration ($\Delta G_u^{H_2O}$ = 12.6 ± 0.4 kcal/mol, $D_{1/2}$ = 2.6 M; Table I). Typically, we found that the peptidolytic assay indicates unfolding of the protease at lower concentrations of urea than that required to cause fluorescence quenching; this may reflect inhibition of the remaining folded protease by the high urea concentrations. Attempts were made to correct for the potential inhibition of HIV-1 PR due to urea by determining the time dependence of urea inactivation of HIV-1 PR. Diminution of enzymatic activity was observed in samples of urea and protease that were not preincubated prior to the addition of substrate; the true cause of this loss of activity remains unclear since unfolding/refolding of the enzyme should be quite rapid and indistinguishable from reversible inhibition. The binding of inhibitors to HIV-1 PR requires the formation of numerous hydrogen bonds between carbonyl and amide groups of both the protein polypeptide backbone and the inhibitor; therefore, the possibility of inhibition of this enzyme by urea is not unexpected.

Stability of HIV-1 PR and SIV PR as a Function of pH. The effect of pH on protease stability was examined by first

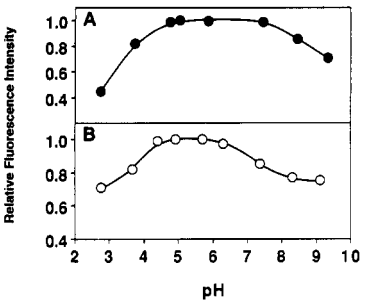

FIGURE 8: Plots of the pH dependence of the relative fluorescence emission intensity (347 nm) for HIV-1 PR (180 nM monomer, A) and SIV PR (440 nM monomer, B) at equilibrium. Aliquots of the proteases were added to 1-mL samples (FAMTEN buffer, pH 2.0–9.0) at 25 °C, and fluorescence emission spectra were recorded as described under Materials and Methods at 30–60 min after the addition of the enzymes.

measuring enzymatic activities following preincubation. The pH-dependent inactivation of HIV-1 PR and SIV PR was assessed by measurement at pH 6.0 (37 °C) of the remaining peptidolytic activity after preincubation for 5–60 min in buffers adjusted to pH values of 3.0–8.0 (data not shown). Neither HIV-1 PR nor SIV PR activities were significantly changed (≥90% activity remaining) following preincubation of either enzyme at pH 3.0–6.0 (Grant et al. 1991). However, the activities of both enzymes were slightly diminished (≥70% activity remaining) after preincubation at pH values between 7.0 and 8.0, indicating that both protease activities were slightly less stable at alkaline pH. These results suggested that either denaturation of the two proteases at adverse pH occurred to only a small extent or it was readily reversible upon returning the proteins to a pH of 6.0 and the presence of substrate for activity measurements.

Protein fluorescence experiments with the two proteases were also undertaken over this pH range to ascertain the role of protein unfolding in the observed inactivation at these extremes of pH. Fluorescence emission spectra of SIV PR and HIV-1 PR were obtained over a pH range of 2.8–9.1 after 30–60 min at 25 °C, at which the fluorescence emission spectra stabilized. As in the urea unfolding studies, both enzymes exhibited about a 50–65% decrease in emission intensity at 347 nm at pH values above and below 4.0–6.5. Changes in the fluorescence spectra also included shifts in the maximum emission wavelength with pH. A red-shift to about 350 nm, similar to that observed in the urea denaturation studies, was observed at pH 8.0–9.0, while a blue-shift to 340 nm was seen below pH 3.5 (data not shown). These shifts were more pronounced for HIV-1 PR than for SIV PR.

By plotting the relative fluorescence intensities of each protease after incubation for 30 min at pH 2.0–9.0, plots of fluorescence intensity vs pH were obtained for both HIV-1 PR and SIV PR (Figure 8). Fluorescence intensities for both proteases decreased markedly below the inflection points of pH 4.5. At alkaline pH, the inflection points occurred above pH 7.5 and 6.5 for HIV-1 PR and SIV PR, respectively. Consistent with the urea denaturation results, these data suggested that both proteins were unfolding at high and low pH. Once again, HIV-1 PR was slightly more stable to adverse solution conditions than was SIV PR. At high and low pH, the fluorescence intensity of SIV PR decreased to plateau levels that were 65% of the level for the fully folded protein at neutral pH. These plateau regions of SIV PR fluorescence at high and low pH values resembled the sigmoidal urea unfolding profiles. This suggests that unfolded and folded species of SIV PR existed in equilibrium at these extremes of pH. In contrast, at low pH, the fluorescence intensity of HIV-1 PR, which was characterized by a blue-shift in λ_{max}, decreased linearly below this 65% threshold. This fluorescence change at acidic pH suggested that a process other than or in addition to unfolding accounts for this shift in λ_{max}, and this process has a first-order dependence on proton concentration.

The pH-mediated unfolding of HIV-1 protease was found, by recovery of both protein fluorescence and enzymatic activity, to be partially reversible. Incubation of 0.5 μM HIV-1 protease at pH 3.0, 6.0, and 9.0 for 30 min resulted in relative fluorescence intensities of 13%, 100%, and 8%, respectively, and enzymatic activities of 10%, 100% and 0%, respectively (data not shown). However, upon readjustment of the pH 3.0 and 9.0 mixtures to pH 6.0, respective fluorescent intensities of 41% and 43% and enzymatic activities of 52% and 55% (relative to the pH 6.0 sample) were attained. Therefore, HIV-1 protease could be partially refolded and reactivated from these conditions of adverse pH, but less so than the refolding observed following urea and heat treatment.

DISCUSSION

To date, transition-state analogues based on peptide substrates have provided a class of potent and specific inhibitors of HIV-1 PR [for reviews, see Huff (1991) and Meek (1991)]. Since HIV-1 PR is a homodimer in which the active site is formed from side chains of amino acids from both monomers, the inactivation of the enzyme by disruption of the dimers is in principle a highly selective alternative approach to its inhibition. However, to determine the viability of this approach the stability of the dimer interface must first be ascertained.

From the three-dimensional structure of HIV-1 PR (Wlodawer et al., 1989), 34 hydrogen bonds and 4 salt bridges constitute the necessary interactions between the 2 99 amino acid polypeptides to afford the homodimer (Weber, 1990). Roughly half of these intermonomer interactions occur in the four interdigitating N-terminal and C-terminal β-strands (residues 1–4, 96′–99′, 96–99, and 1′–4′) (Figure 3). These N- and C-terminal sequences are highly conserved between HIV-1 PR and SIV PR. Four of the eight terminal residues that are nonidentical are functionally similar (HIV-1 PR/SIV PR: Ile-3/Phe-3; Thr-4/Ser-4; Thr-96/Ser-96; Phe-99/Leu-99) (Grant et al., 1991). Given that the active form of SIV PR has also been shown to be a 22-kDa homodimer (Grant et al., 1991) it is highly likely that the dimeric interface of this protease is composed of the same monomer–monomer interactions in HIV-1 PR. From modeling of the structure of HIV-2 PR, which has 87% sequence identity with that of SIV PR, the dimeric interface of HIV-2 PR is, as expected, structurally similar to that of HIV-1 PR (Gustchina & Weber, 1991). For HIV-1 PR, ionic interactions are likely between the protonated N-terminal Pro residues and the carboxylates of Phe-99 and Phe-99′. The carboxylate of Phe-99 may also form an ionic interaction with His-69 (Weber, 1990) at pH values at which that residue is protonated. Folding of the two subunits of HIV-1 PR into dimers results in an approximately 25% decrease in the solvent-accessible surface area of the subunits. This suggests that separation of the dimers might promote a significant unfolding of the protease, causing greater solvent exposure to hydrophobic residues, possibly including the conserved Trp-6 and Trp-6′ (Weber, 1990). The fact that these two Trp residues occur on separate polypeptide chains and are proximal to the dimeric interface (Figure 3) suggests that protein fluorescence may be a sensitive method for probing the disruption of the quaternary structure of HIV-1 and SIV PRs. For the latter enzyme, the observed quenching of protein

fluorescence upon denaturation must reflect the environmental changes of Trp-6 and Trp-6′, since these are the only Trp residues in the protein.

Determination of the Conformational Stabilities of HIV-1 PR and SIV PR by the Use of Denaturants, and Effects of pH and Temperature. The comparative conformational stabilities of the retroviral proteases of HIV-1 and SIV have been investigated in terms of the effects of denaturants, temperature, and pH on protein fluorescence and enzymatic activities. These studies suggested that under certain conditions of temperature and pH, the protease dimers may dissociate to unfolded monomers. However, such results were inconclusive, and no definitive measure of dimer stabilities could be determined except to note that in all cases HIV-1 PR was found to be qualitatively more stable than SIV PR. In contrast, urea denaturation studies using the method of Pace (1986) gave direct quantitation of the stabilities of the folded dimers, yielding values of 39 and 180 pM for the unfolding dissociation constants of HIV-1 PR and SIV PR, respectively. Comparison of the corresponding $\Delta G_u^{H_2O}$ values determined for the two proteases revealed that HIV-1 PR is more stable to unfolding than SIV PR by 0.85 kcal/mol; this indicates that the subunits of HIV-1 PR are held together with an affinity that is greater by 5-fold than that of SIV PR. It is presently impossible to pinpoint the tertiary interactions that render SIV PR less stable. However, Gustchina and Weber (1991) have proposed that the Leu residue found at the C-termini of HIV-2 PR (also present in SIV PR) may provide an intermonomer hydrophobic interaction with its counterpart that is weaker than that of the Phe-99 found in HIV-1 PR. Other explanations of the observed differences in conformational stabilities of the two proteases are discussed below.

Partially-reversible inactivation of peptidolytic activity was observed for both SIV PR and HIV-1 PR after preincubation at nonoptimal pH values lower or greater than pH 4–6. The source of this inactivation was suggested by an analysis of changes in protein fluorescence. These results suggested that losses of activity of HIV-1 PR and SIV-PR were caused by pH-dependent protein unfolding. For both proteases, protonation of an enzymatic group or groups at pH <4.5 resulted in protein unfolding as measured by fluorescence quenching. At high pH, HIV-1 PR and SIV PR apparently unfolded upon deprotonation of one or more enzymatic groups at pH values of 7.5 and 6.5, respectively.

Are specific residues on these proteases involved in the pH-dependent denaturation? One would expect that disruption of the ionic interactions between Pro-1/Phe-99′, Asp-29/Arg-87, and Asp-29/Arg-8′ would destabilize the protease homodimers. Protonation of these conserved aspartyl residues and the C-terminal carboxylate of Phe-99′ below pH 4.0 may cause this to occur, although hydrogen-bonding interactions would still be possible. The alkaline instability of either protease occurs at a sufficiently low pH (less than 8.0) such that its cause more likely lies in charge repulsion between deprotonated carboxylates than in the loss of ionic interactions due to deprotonations of amines or guanidino groups. For either enzyme, the denaturation at basic pH may result in part from charge repulsion between the adjacent active-site Asp-25 and Asp-25′ residues when both are deprotonated [pK values of the less acidic Asp-25 residue were measured as 5.3 and 6.2 for HIV-1 PR (Hyland et al., 1991b) and SIV SR (Grant et al., 1991), respectively].

Thermal inactivation of globular proteins is usually the result of denaturation caused by increasing vibrational and rotational motions (Privalov, 1979, 1982). Accordingly, proteins of comparable tertiary structure would be expected to exhibit similar thermal stability. SIV PR exhibits only 50% amino acid sequence identity with HIV-1 PR (Myers et al., 1990). Hence, there was a possibility that these two proteases could exhibit different thermal stabilities despite an overall similar tertiary structure. Proteins with small variations in primary structure, such as single-point mutations, can have profound differences in thermal stability. HIV-1 PR proved to be more stable than SIV PR to thermal inactivation. Single-point mutations of two residues within HIV-1 PR, Pro-79 and Lys-45, have been shown to render the enzyme temperature-sensitive (Loeb et al., 1989; Rockenbach et al., 1990; Baum et al., 1990). The lack of conservation of the Pro-79 residue in SIV PR (an Asp is found in that position) may in part account for the differences in thermal stabilities.

Use of Protein Unfolding Studies To Measure Dimer Dissociation Constants of HIV-1 PR and SIV PR. The denaturation of compact, globular proteins by chaotropes most likely occurs by the disruption of the protein conformational structure by increasing the solubility of nonpolar amino acid residues (Kauzmann, 1959; Jaenicke & Rudolph, 1989). The process of chaotrope-induced unfolding of proteins can be monitored by a number of biophysical methods, such as changes in circular dichroism, protein fluorescence, and ultraviolet difference spectrophotometry. For enzymes, activity assays could also be used for the sensitive detection of conformational changes caused by denaturants, provided that the chaotrope does not affect the catalytic reaction. Partial, reversible disruption of the conformational structure of a protein by denaturants may be observable as a loss of enzymatic activity, whereas such subtle structural changes may be undetectable by biophysical measurements of protein conformation. In this report, we have demonstrated that the monitoring of the urea-dependent unfolding of HIV-1 protease both by fluorescence changes and by enzyme activity measurements produced similar results.

We have shown that both HIV-1 PR and SIV PR are inactivated and unfolded at urea concentrations of 2–4 M, with transition midpoints ranging from 1.9 to 3.1 M urea. Dilanni et al. (1990a) have reported unfolding of wild-type and mutant forms of HIV-1 PR at similar urea concentrations by using nondenaturing electrophoresis. In contrast, the monomeric aspartic protease, porcine pepsin, has been reported to be active at 6 M urea (Steinhardt, 1938; Fruton, 1976). Recently, recombinant forms of HIV-1 PR have been prepared in which the two subunits are covalently linked by a short polypeptide chain (Cheng et al., 1990; Dilanni et al., 1990b). Cheng et al. found that the covalently-linked HIV-1 PR dimer was considerably more stable than the unconnected wild-type form. These authors calculated a value of K_d = 50 nM for the wild-type enzyme (pH 7.0, 25 °C) by comparing the enzyme activities of the wild-type and linked forms of HIV-1 PR upon dilution. In kind, Dilanni and co-workers have found that a covalently-linked form of HIV-1 PR is considerably more stable to urea inactivation than is the wild-type enzyme (C. Dilanni, personal communication). Given these findings, the two-state unfolding model we have observed for HIV-1 and SIV PRs is consistent with a process in which unfolding is tantamount to dissociation of the protein homodimers. Indeed, urea denaturation of several other dimeric proteins, including phosphoglucose isomerase (Blackburn & Noltmann, 1981) and the DNA binding proteins *arc* repressor (Bowie & Sauer, 1989) and *trp* aporepressor (Gittleman & Matthews, 1990), has demonstrated that unfolding of these proteins by chaotrope is concomitant with subunit dissociation.

In addition, the significant stabilization of HIV-1 PR ($\Delta\Delta G_u^{H_2O}$ = 5.1 kcal/mol) upon binding of the competitive inhibitor Ala-Ala-PheΨ[CH(OH)CH_2]Gly-Val-Val-OCH_3 also attests to the validity of the two-state unfolding model. These findings are very similar to the observed stabilization of porcine pepsin to thermal denaturation by 4.3 kcal/mol upon the binding of pepstatin (Privalov, 1982). The K_u of the HIV-1 PR–inhibitor complex is, therefore, nearly 6000-fold lower than that of the uncomplexed enzyme. On the basis of structural studies of inhibitor-complexed HIV-1 PR (Miller et al., 1989b), one would expect the inhibitor to bind only to the active, dimeric form of the enzyme. Moreover, since inhibitor binding involves hydrogen-bonding interactions between the protease and inhibitor, with equitable contributions from each of the monomers, the bound inhibitor in effect serves as an intermediary for additional interactions between the monomers. Undoubtedly, these protease–inhibitor–protease interactions impart considerable stability to the HIV-1 PR homodimer. In this regard, it is interesting to note that in a sedimentation equilibrium analysis of HIV-2 protease, Holzman et al. (1991) have observed only a 14-fold increase in the affinity of the monomer–monomer interaction upon the addition of a high-affinity inhibitor.

The unfolding dissociation constants for the homodimers of HIV-1 PR (K_u = 39 pM) and SIV PR (K_u = 180 pM) that we have obtained from $\Delta G_u^{H_2O}$ values are considerably lower than reported dimer dissociation constants obtained by other methods. As mentioned above, Cheng et al. (1990) have obtained a K_d value of 50 nM at pH 7.0, 25 °C, by measurement of protease activity upon dilution of the enzyme. In addition, Zhang et al. (1991) recently reported a K_d value of 3.6 nM for HIV-1 PR at pH 5.0, 37 °C, obtained by kinetic analysis of the inactivation of 2–130 nM concentrations of protease by a tetrapeptide that comprises residues 96–99 of the monomer and is proposed to disrupt the dimeric interface. From sedimentation equilibrium analysis, Holzman et al. (1991) have calculated K_d values for HIV-2 protease of 28–87 μM at pH 4.5 and 7.5, 8.8 °C. From our sedimentation equilibrium studies, detailed above, we may only conclude that the K_d value for the free and inhibitor-complexed forms of HIV-1 PR dimer is $\leq$0.1 μM at pH 5.0 (4 °C), since the low extinction coefficient of the protein impairs the sensitivity of this method. Which of these experimental approaches is the most appropriate for the measurement of the dissociation constant for the monomer–dimer equilibrium of HIV-1 PR? All of these measured values have been obtained with different protein preparations and very different experimental conditions, and these variations likely contribute significantly to the lack of agreement. For example, the value obtained by Zhang et al. (1991) was obtained at 1 M NaCl, 37 °C, and pH 5.0, at which the HIV-1 PR dimer is likely to be less stable to urea than at 25 °C and pH 6.0. Furthermore, the results of Cheng et al. were obtained at pH 7.0. We have shown here that the protease is slightly less stable at pH 7.0 than at pH 6.0.

However, measurements of HIV-1 PR activities at solution concentrations of $\leq$1 nM (Heimbach et al., 1989) would be greatly complicated by a significant amount of dimer dissociation, which would be the case if the operant value of $K_d \geq$ 10 nM. At protease concentrations that are equivalent to or below the K_d value, plots of protease activity vs total enzyme concentration would be concave-up and display negative y intercepts. In the kinetic analysis of tight-binding inhibitors, we have observed a linear dependence of both HIV-1 PR and SIV PR activities (pH 6.0, 37 °C) at 5–60 nM concentrations of the enzyme (Dreyer et al., 1992), which would not been seen if the K_d value was in this concentration range. In addition, the determination of the dimeric dissociation constant of HIV-1 PR by measurement of enzyme activity upon dilution of subnanomolar concentrations might lead to spuriously high values due to potential loss of the highly dilute protein to surface adsorption or denaturation. For the measurement of the dimeric dissociation constant of HIV-1 PR, the sedimentation equilibrium method suffers from two important disadvantages: (1) the ability of the method to measure protein subunit interactions of high affinity requires a large extinction coefficient for the protein or the attainment of very concentrated protein solutions; (2) the lengthy time of analysis and the necessity for high protein concentrations, both of which invite the potential for autoproteolysis (Strickler et al., 1989), could lead to misleading experimental results.

The determination of the dimeric stability for HIV-1 PR by a direct physical measurement in which autoproteolysis is not an issue, such as the urea unfolding method presented here, should provide more reliable values. A clear advantage of protein unfolding studies to assess dimer stability is that subunit association of extremely high affinity may be quantified without the requirement of high dilution of the protein. This method could be used to examine destabilization of the dimeric interface by oligopeptides and other small molecules. However, the picomolar and subpicomolar values of the dimer dissociation constants measured here for free and inhibitor-complexed forms of HIV-1 PR indicate that inactivation of this enzyme by disruption of its quaternary structure will be difficult to achieve. This would be especially true within the environment of the maturing HIV virion particle, where the local concentrations of viral polyprotein substrates, which would serve to stabilize the homodimer, as well as the unprocessed monomers of HIV-1 protease may exceed 1 μM (Meek et al., 1990b). This range of concentrations is a full 9 orders of magnitude higher than the apparent stability constant for HIV-1 protease when complexed to a peptide analogue inhibitor.

Finally, adherence of the retroviral proteases to a model of folded dimer–unfolded monomer equilibrium suggests that the unprocessed, monomeric form of HIV-1 protease within the $Pr160^{gag\text{-}pol}$ polyprotein of HIV-1 is also unfolded. HIV-1 protease apparently catalyzes its own autoprocessing from this precursor (Debouck et al., 1987); from structural considerations, this autoprocessing is probably intermolecular (Meek et al., 1990b). It is likely then that during virion maturation, pairs of unfolded protease domains assemble into active dimeric domains within intact chains of $Pr160^{gag\text{-}pol}$ which may then proteolyze a similar domain on a third polyprotein chain. Kaplan and Swanstrom (1991) have recently demonstrated activation of the HIV-1 protease from viral polyproteins in the cytoplasm of infected T-lymphocytes, an environment in which the high dilution of the viral polyproteins would render the assembly of unprocessed protease dimers more difficult to achieve than in sealed and densely-packed immature virions. However, this cytosolic activation of HIV-1 protease would be facilitated in the event that the high stability of the processed dimers of the retroviral proteases, as shown above, is also retained within the polyprotein protease domains.

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