Purification and Biochemical Characterization of Recombinant Simian Immunodeficiency Virus Protease and Comparison to Human Immunodeficiency Virus Type 1 Protease†

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ABSTRACT: Simian immunodeficiency virus protease (SIV-PR) was produced in *Escherichia coli* with a recombinant expression system in which the mature enzyme autoprocessed from a precursor form. Recombinant SIV and HIV-1 (human immunodeficiency virus, type 1) proteases were purified from bacterial cell lysates by use of sequential steps of ammonium sulfate precipitation and size-exclusion and ion-exchange chromatography. The amino acid composition, amino-terminal sequence, and molecular weight (monomer) of the recombinant SIV-PR were in accord with that of the 99 amino acid polypeptide predicted from the SIV_{Mac}-PR nucleotide sequence. The active form of SIV-PR was shown to be dimeric by gel filtration chromatography. Inhibition by pepstatin A, time-dependent inactivation by 1,2-epoxy-3-(4-nitrophenoxy)propane, and pH rate profiles using oligopeptide substrates demonstrated that SIV-PR behaves as an aspartic protease. Recombinant HIV-1 $Pr55^{gag}$ precursor was processed in vitro by SIV-PR and HIV-1 PR with indistinguishable proteolytic patterns upon $NaDodSO_4$–polyacrylamide gel electrophoresis. Oligopeptide substrates for HIV-1 PR were found to be suitable substrates for recombinant SIV-PR with the exception of a peptide containing the site identified for p66/p51 cleavage (Phe*Tyr) within HIV-1 reverse transcriptase (RT). Several synthetic peptide analogue inhibitors of HIV-1 PR were also potent inhibitors of SIV-PR, indicating that SIV infection in macaques and rhesus monkeys should be useful models for the preclinical evaluation of acquired immunodeficiency syndrome (AIDS) therapeutics targeted toward the virally encoded HIV-1 protease.

Simian immunodeficiency virus (SIV)[1] is a member of the lentivirus subgroup of retroviruses and has closely related morphology and genomic sequence and organization to the human immunodeficiency viruses, type 1 (HIV-1) and type 2 (HIV-2) [for reviews see Desrosiers (1988) and Desrosiers and Ringler (1989)]. SIV infection of rhesus monkeys has been shown to cause disease states that parallel acquired immunodeficiency snydrome (AIDS) and related disorders in humans (Kestler et al., 1990). Since symptoms of the HIV-1 disease have failed to be demonstrated to date in nonhuman primates infected with this virus, SIV infections of macaques and rhesus monkeys represent the closest animal models currently available for AIDS. This disease model should greatly facilitate the characterization of lentiviral infection and pathogenesis and also allow the preclinical evaluation of compounds developed to block specific steps in the HIV-1 life cycle.

One such important step that has been the focus of intense investigation is the proteolytic processing of retroviral polyprotein precursors by the virally encoded protease. The primary structures of the retroviral proteases are composed of less than 130 amino acids. As was predicted (Pearl & Taylor, 1987) and has been shown for the proteases of the Rous sarcoma virus (Miller et al., 1989) and HIV-1 (Wlodawer et al., 1989; Meek et al., 1989), they have homodimeric quaternary structures. These enzymes are catalytically and structurally related to the class of aspartic proteases that includes pepsin and renin. SIV protease is of interest in the context of animal models, but as yet, characterization of this enzyme has not been reported. Therefore, we undertook the purification and characterization of recombinant SIV-PR. Since the primary sequence of SIV_{Mac} protease (Myers et al., 1990) differs significantly from that of HIV-1 protease (50% identity; 73% similarity), we were also interested in comparing recombinant SIV-PR to HIV-1 PR with respect to substrate specificity and kinetic parameters.

Proteolytic processing by HIV-1 PR is necessary for viral maturation and therefore represents a potential target for anti-AIDS drugs (Kohl et al., 1988; Meek et al., 1990a). Recent reports have demonstrated that rationally designed inhibitors of HIV-1 PR deter viral maturation and replication in HIV-infected cells (Meek et al., 1990b; McQuade et al., 1990; Erickson et al., 1990; Roberts et al., 1990). In an effort to further evaluate the usefulness of the SIV infection model

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[1] Abbreviations: CH_3CN, acetonitrile; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPNP, 1,2-epoxy-3-(4-nitrophenoxy)propane; HIV, human immunodeficiency virus; kDa, kilodalton; MES, 2-(*N*-morpholino)ethanesulfonic acid; NaCl, sodium chloride; $NaDodSO_4$ or SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PR, protease; RP-HPLC, reversed-phase high-performance liquid chromatography; RSV, Rous sarcoma virus; RT, reverse transcriptase; SIV, simian immunodeficiency virus; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane. Amino acids are designated by the one-letter code.

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for AIDS chemotherapy, we also tested the ability of synthetic peptide analogue inhibitors developed against HIV-1 PR to inhibit recombinant SIV-PR.

Materials and Methods

Biochemicals. The oligopeptide substrates Ac-RA-SQNYPVV-NH_2, Ac-SQNYPVV-NH_2, Ac-RKILFLDG-NH_2, and Ac-AETFYVD-NH_2 were prepared by solid-phase synthesis on benzhydrylamine resin as previously described (Moore et al., 1989). The peptides were cleaved from the resin with anhydrous liquid HF at 0 °C and were purified by gel filtration, countercurrent distribution, and/or preparative reversed-phase high-performance liquid chromatography (RP-HPLC) as appropriate. Ac-AETYYTD-NH_2 was prepared by American Peptide Company. Preparations of all peptides were found to be of ≥95% purity upon analysis by RP-HPLC and thin-layer chromatography. Their structures were confirmed by amino acid analysis and fast atom bombardment mass spectroscopy. The syntheses of peptide analogue inhibitors are described elsewhere: Ala-Ala-PheΨ-[CH(OH)CH_2]Gly-Val-Val-OCH_3 (Dreyer et al., 1989), Ala-Ala-PheΨ[CH(OH)CH_2]Phe-Val-Val-OCH_3, and Ala-Ala-PheΨ[CH(OH)CH_2]Ala-Val-Val-OCH_3, in which PheΨ[CH(OH)CH_2]Gly designates the incorporated hydroxyethylene dipeptide isostere of (4*S*,5*S*)-6-phenyl-5-amino-4-hydroxyhexanoic acid (G.B.D. et al., manuscript in preparation). Q-Sepharose Fast Flow and S-Sepharose Fast Flow analytical and preparative chromatographic columns and packing material were purchased from Pharmacia LKB. RP-HPLC C_4 and C_{18} columns were obtained from Vydac. Dimethyl sulfoxide (Aldrich, Gold label), glycerol (LKB, Ultrograde), 1,2-epoxy-3-(4-nitrophenoxy)propane (Sigma, used without further purification), and other biochemicals were of the highest purity available.

The following buffers were used: AED buffer, 50 mM sodium acetate, pH 5.0, 5 mM EDTA, 10 mM DTT; AEND buffer, same as AED buffer with 0.35 M NaCl; FAMTNE buffer, 50 mM sodium formate, 50 mM sodium acetate, 50 mM MES, 50 mM Tris-HCl, 0.2 M NaCl, 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; FAMTNEDT buffer, same as FAMTNE buffer with 1 mM DTT and 0.1% Triton X-100; MENDT buffer, 50 mM MES, pH 6.0, 1 mM EDTA, 0.2 M NaCl, 1 mM DTT, 0.1% Triton X-100; TED buffer, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM DTT; TEDG buffer, same as TED buffer with 10% glycerol; TEDP buffer, same as TED buffer with 1 mM phenylmethanesulfonyl fluoride; TEND buffer, same as TED buffer with 0.2 M NaCl; and TENDG buffer, same as TEND buffer with 10% glycerol.

Recombinant Proteins. Recombinant proteins were expressed in *Escherichia coli* strain AR58 containing the following plasmids: SIVSKF, expressing SIV-PR (I.C.D., S. Richardson, F. Watson, R. C. Craig, and C.D., manuscript in preparation) from SIV_{Mac} BK28 strain [Kornfield et al., 1987; referred to as SIVMM251 in compilation of retroviral sequences by Myers et al. (1990)], and PRO4, expressing HIV-1 PR from HIV-1 (HTLVIIIB, BH10 clone) (Ratner et al., 1985; Debouck et al., 1987). In these plasmids, the protease expression is directed by the λ P_L promoter and induced by temperature increase (Shatzman & Rosenberg, 1987).

Recombinant $Pr55^{gag}$ (p55) was cloned and expressed as described before (Debouck et al., 1987). Recombinant p55 in bacterial lysates was solubilized in TEDG buffer containing 8 M urea and purified by successive steps of cation-exchange chromatography with S-Sepharose FF (eluant, 50 mM MES (pH 6.0), 1 mM DTT, 8 M urea; linear gradient, 0–0.5 M NaCl) and gel filtration chromatography on Superose-12 (Pharmacia) (eluant, 50 mM Tris-HCl (pH 7.2), 1 mM DTT, 2 M guanidine hydrochloride; flow rate = 0.5 mL/min).

Protein Analysis. Amino acid composition of purified, recombinant SIV-PR (500 pmol) was performed as described previously (Strickler et al., 1989). The molecular mass of purified SIV-PR was determined by mass spectroscopic analysis. Electrospray mass spectra were recorded on a Sciex API-III triple-quadrupole mass spectrometer fitted with a standard pneumatically assisted nebulization probe and an atmospheric pressure ionization source (Sciex, Ontario). Protein samples were desalted prior to mass spectrometric analysis by RP-HPLC on a Vydac C_4 column (4.6 × 250 mm, 5 μm packing) with use of the linear gradient: t = 0–10 min, 10% B; t = 20 min, 100% B, where A = 0.1% TFA and B = 90% CH_3CN, 0.1% TFA (v/v). Molecular weight determination under denaturing conditions on SDS–PAGE was performed with a PhastImage Gel Scanner with a calibration curve constructed from Pharmacia protein molecular weight standards. Molecular weight determination under nondenaturing conditions was performed by size-exclusion chromatography as described previously (Meek et al., 1989). Briefly, heat-induced bacterial cells (0.5 g) containing the SIVSKF expression plasmid were disrupted by freeze-thawing, followed by sonication and centrifugation for 10 min at 10000*g*, 4 °C, in 1 mL of TEND buffer. The supernatant fraction (1 mL, 30 mg of protein) was applied to a column (2.5 × 23 cm) of Sephacryl S-200 superfine (Pharmacia) and equilibrated with TEND buffer, and the sample was eluted at a flow rate of 8.0 mL/h. The protein standards used were thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (45 kDa), myoglobin (17.5 kDa), and vitamin B_{12} (1.25 kDa). SIV protease activity was determined in the fractions by using the peptidolytic assay as described below.

Protease concentrations were determined by 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) using a Vydac C_{18} (4.6 × 250 mm, 300-Å pore size) column with a linear 25–75% CH_3CN gradient in 0.1% TFA, over 20 min at a flow rate of 1 mL/min. In this latter method, protease concentrations in purified preparations are determined from a calibration curve composed of peak integrations prepared from various aliquots of a solution of SIV-PR in which its concentration had been previously determined by amino acid analysis.

Enzyme Assays. Assays of the peptidolytic activities of SIV-PR and HIV-1 PR were routinely performed as described previously for HIV-1 PR (Meek et al., 1989; Moore et al., 1989). During enzyme purification, enzyme activity was measured in MENDT buffer (pH 6) by incubation of enzyme-containing fractions with either 1–2 mM Ac-RA-SQNYPVV-NH_2 or Ac-SQNYPVV-NH_2 at 37 °C in a total volume of 50 μL, which was then quenched after 10 min by the addition of 50 μL of 5% TFA. Some oligopeptides were solubilized in DMSO and added to give a final concentration of 10% DMSO in assay solutions. For initial velocity studies, substrate concentrations were varied over a range of 0.1–10 mM. Peptidolytic cleavage products were separated with use of a Beckman C_{18} column (4.5 × 250 mm, 5 μm) on a Hewlett-Packard HP1090 high-performance liquid chromatograph equipped with a diode array detector, a ternary solvent delivery system, a HP3392 digital integrator, and a HP89500 ChemStation. Initial rates were measured at single time points

($t \leq 10$ min) at which <15% of substrate had been converted to product. The time course of the peptidolytic reaction at a 10-fold range of substrate concentrations was determined to be linear between 0 and 20 min under these assay conditions. The pH dependence of initial velocity for the peptidolytic reaction was determined in FAMTNEDT held at constant ionic strength and adjusted to pH values between pH 3.5 and 7.5. The enzyme activity was found to be stable following preincubation at these pH values. Proteolysis of recombinant HIV-1 p55 was performed by incubating 1.5 μg of p55 with 30 ng of either HIV-1 PR or SIV-PR in 50 μL of MENDT buffer, for 90 min. The reaction was quenched by boiling for 3 min.

Protease inhibitors/inactivators were preincubated for 30 min (37 °C) in a 50-μL reaction mixture containing MENDT (pH 6) buffer and 40–50 ng of either SIV-PR or HIV-1 PR, from which 10-μL aliquots were withdrawn and assayed in 50-μL reaction mixtures as described above. For experiments with time-dependent loss of activity, the enzyme was incubated with several fixed levels of inactivator and assayed after various preincubation times (Meek et al., 1989). Reversibility of protease inhibition/inactivation was determined by assaying preincubation samples after microdialysis with two 100-mL changes of MENDT buffer using a Pierce microdialyzer system 100 and Spectra/Por dialysis tubing (2000-kDa cutoff).

Active Site Titration. Reaction mixtures (50 μL) at 37 °C, containing MENDT buffer (pH 6), 10% DMSO, 0–60 nM Ala-Ala-PheΨ[CH(OH)CH_2]Gly-Val-Val-OCH_3, and 0.8 mM Ac-RASQNYPVV-NH_2, were initiated by the addition of SIV-PR or HIV-1 PR at final concentrations ranging from 5 to 50 nM and quenched after 60 min by the addition of an equal volume of 3% TFA. The reaction products were analyzed by RP-HPLC as described above.

Purification of Recombinant SIV-PR and HIV-1 PR. The procedures for the purification of both recombinant HIV-1 and SIV proteases were identical. Cell pellets (300 g) of *E. coli* induced for HIV-1 PR or SIV-PR expression were resuspended in a total volume of 1 L of TEDP buffer. The suspension was lysed by passing twice through a Manton-Gaulin cell homogenizer at 6000–8000 psi. After centrifugation of the lysate at 30000*g* for 30 min at 4 °C, the supernatant (900 mL) was decanted. Solid ammonium sulfate was slowly added to the supernatant to 35% saturation, which was then stirred for 1 h at 4 °C. Following centrifugation (30000*g*, 30 min, 4 °C), the supernatant was discarded and the pellet was dissolved in 100 mL of TEDP buffer. This solution was dialyzed overnight at 4 °C against 4 L of TENDG buffer. The retentate was filtered and loaded (25 mL/min) onto a Superose-12 column (11 × 46 cm) that had been preequilibrated with TENDG buffer at 4 °C. The fractions containing the majority of the purified protease (as determined by elution on RP-HPLC) were pooled and diluted (1:2) in TEDG buffer. The pooled fractions (950 mL) were applied to a Q-Sepharose fast flow (QSFF) column (5 × 15 cm) that had been preequilibrated in TEDG buffer (15 mL/min). Under these conditions protease was not retained on the QSFF column. The flow-through fraction was collected, adjusted to pH 5 with 10% acetic acid, and diluted (1:2) with AED buffer. The pooled fraction was loaded onto a S-Sepharose fast flow (SSFF) column (2.2 × 12 cm), and protease activity eluted as a single peak at a flow rate of 10 mL/min with AEND buffer. While the QSFF and SSFF purification steps effect no further purification of the proteases, being highly purified following the Superose-12 chromatography step, these steps, respectively, were useful for removing contaminating nucleic acids and residual ammonium sulfate and for concentrating the resulting enzyme preparation without significant loss in specific activity. Concentration by SSFF chromatography provided more effective recovery of activity than by membrane filtration.

Electrophoresis and Isoelectric Focusing. SDS–PAGE was performed on a PhastSystem electrophoresis apparatus (Pharmacia) using 20% polyacrylamide gels. Proteins were visualized by silver staining (Merril et al., 1984) with staining reagents from Bio-Rad and protein standards of Pharmacia: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Isoelectric points for SIV-PR and HIV-1 PR were determined under nondenaturing conditions by analytical isoelectric focusing (IEF) using a LKB 2117 Multiphor II electrophoresis unit and preformed polyacrylamide electrofocusing gels (Ampholine PAGPLATE, pH 3.5–9.5, from Pharmacia LKB). The IEF protein standards of Bio-Rad were used. Samples of concentrated protein (5 μg) were applied to the IEF gels, which were focused at 200 V at 10 °C until the prestained standard proteins became immobile (2 h). Gels were fixed in a solution consisting of 3.5% sulfosalicyclic acid and 11.5% TFA, stained in Coomassie Brillant Blue G-250 (Bio-Rad) for 15 min at 60 °C, and destained in a solution of 8% acetic acid and 25% ethanol. Theoretical isoelectric points were calculated from the amino acid composition of SIV_{Mac}-PR [SIVMM251 in compilation of retroviral sequences by Myers et al. (1990)], HIV-1 PR from HIV-1 (HTLVIIIB, BH10 clone) (Ratner et al., 1985; Debouck et al., 1987), and HIV-2 PR (ROD isolate) (Guyader et al., 1987) with use of the Genetics Computing Group Sequence Analysis Software Package (Devereux, 1989).

Data Analysis. Kinetic data was analyzed by use of the Fortran programs of Cleland (1979). Initial velocity data were fitted to

$$v = VA/(K + A) \quad (1)$$

where A is the substrate concentration, K is the Michaelis constant, v is initial velocity, and V is the maximum velocity. Initial velocity data for pH profiles were first fitted to eq 1, and the resulting values of V/K and V were evaluated with respect to pH by fitting to eq 2 for "bell-shaped" patterns or eq 3 for "wave-shaped" patterns:

$$\log y = \log [c/(1 + H/K_1 + K_2/H)] \quad (2)$$

$$\log y = \log \{[Y_L + Y_H(K_3/H)]/(1 + K_3/H)\} \quad (3)$$

where y is V/K or V, c is the pH independent value of y, K_1, K_2, and K_3 are acid dissociation constants, H is the hydrogen ion concentration, and Y_L and Y_H are the values of y at low and high pH, respectively. Inhibition data was analyzed by the method of Dixon (1953), assuming competitive inhibition. Kinetic parameters from the time-dependent inactivation of protease activity were determined by fitting enzymatic activity remaining after preincubation with inactivator to

$$\ln v_i/v_0 = -k_{obs}t \quad (4)$$

where v_i and v_0 are remaining activity in the presence and absence of inhibitor, respectively, k_{obs} is the observed pseudo-first-order inactivation rate, and t is preincubation time in minutes. A half-maximal inactivation constant, K_{inact}, and the maximal rate of inactivation, k_{inact}, were obtained by a replot of $1/k_{obs}$ versus $1/[EPNP]$ by fitting to a form of eq 1 (Kitz & Wilson, 1962). For active site titration or tight-binding inhibition of the retroviral proteases, initial rates of the pep-

Table I: Purification of Recombinant SIV-PR[a]

purification step	total volume (mL)	total protein[b] (mg)	total units[c] (μmol/min)	sp act. (units/mg)	% yield	x-fold purification
(1) crude lysate	500	13000	660	0.05	100	1.0
(2) 35% $(NH_4)_2SO_4$ fractionation	125	5125	340	0.07	52	1.4
(3) Superose-12 chromatography	400	10	120	12.0	18	240
(4) QSFF chromatography	725	11	98	9.0	15	180
(5) SSFF chromatography	50	9	76	8.0	12	160

[a] These results are typical for the purification of recombinant SIV-PR from 300 g of *E. coli* cell paste. [b] Protein concentration was measured by the BCA method or by RP-HPLC. [c] Peptidolytic assay was conducted with 2 mM Ac-RASQNYPVV-NH_2 as described under Materials and Methods, where 1 unit is 1 μmol of Ac-RASQNY-OH produced per minute.

tidolytic reactions were obtained at variable concentrations of enzyme, at changing fixed levels of inhibitor, and at a single fixed concentration of substrate. Titrations of the active sites were obtained by fitting of initial velocities to

$$v = k_{cat}A\{[\alpha E_t - I_t - K_i' + [(K_i' + I_t + \alpha E_t)^2 - 4I_t\alpha E_t)]^{1/2}\}/2(K + A) \quad (5)$$

where v is the initial rate, k_{cat} is the enzymatic turnover number in units of min^{-1}, α is the fraction of active enzyme, and $K_i' = K_i\,(1 + A/K)$ where K_i is the inhibition constant, A is the fixed substrate concentration, E_t and I_t are total concentrations of enzyme (based on protein concentration) and inhibitor, respectively (Ackermann & Potter, 1949; Morrison, 1969; Cha, 1975). Apparent inhibition constants for tight-binding inhibitors were obtained by fitting to the equation (a rearranged form of eq 5)

$$v_i/v_0 = \{\alpha E_t - I_t - K_i' + [(\alpha E_t - I_t + K_i')^2 + 4K_i'I_t]^{1/2}\}/2\alpha E_t \quad (6)$$

where v_i and v_0 are initial velocities in the presence and absence of inhibitor, respectively. Equations 5 and 6 were fitted by use of the SUPERFIT package, a nonlinear regression analysis utilizing the method of Marquardt (1983). Double-reciprocal plots of inhibition data were fitted to eqs 7 and 8, respectively,

$$v = VA/\{K[1 + (I/K_{is})] + A\} \quad (7)$$

$$v = VA/\{K[1 + (I/K_{is})] + A[1 + (I/K_{ii})]\} \quad (8)$$

for competitive and noncompetitive inhibition patterns, in which K_{is} and K_{ii} are the slope and intercept inhibition constants, with use of the Fortran programs of Cleland (1979).

Results and Discussion

Purification of Recombinant SIV-PR and HIV-1 PR

Recombinant SIV-PR and HIV-1 PR were purified from *E. coli* cells, expressing the proteases as precursor forms from which the mature enzymes were autocatalytically processed (Debouck et al., 1987; I.C.D., S. Richardson, F. Watson, R. C. Craig, and C.D., manuscript in preparation). The recovery of protease activity during the purification of SIV-PR is summarized in Table I. The specific activity of purified SIV-PR was about 160 times that of SIV-PR as a crude lysate. As previously reported, peptidolysis of the substrate used to monitor purification (Ac-RASQNYPVV-NH_2) was not observed in bacterial lysates containing the products of the SKF vector with no protease insert (Meek et al., 1989). Recombinant SIV-PR and HIV-1 PR were purified to homogeneity with SIV-PR migrating as a single band of 11.6 ± 0.3 kDa on SDS–PAGE as visualized by silver staining (Figure 1). This was similar to recombinant HIV-1 PR, which migrated on SDS–PAGE at 11 kDa (Strickler et al., 1989). The purification of these proteases were greatly aided by the use of size-exclusion chromatography on Superose-12, where the proteases eluted much later than the majority of contaminating proteins (Strickler et al., 1989). The proteases were apparently homogeneous following purification through the Superose-12 chromatography step (Figure 1). Contaminating nucleic acids were removed by chromatography on a QSFF column as evidenced by an increase in the 280:260 nm absorbance ratio in the eluate. Proteases were concentrated by 12-fold in a final step by ion-exchange chromatography on a SSFF column. SSFF-purified SIV-PR and HIV-1 PR eluted as single peaks upon analytical RP-HPLC, which further indicated that both enzymes were purified to homogeneity. SIV-PR demonstrated sensitivity to ionic strength similar to that described for HIV-1 PR (Richards et al., 1990), where peptidolytic activity increased with increasing salt concentration up to 1 M NaCl.

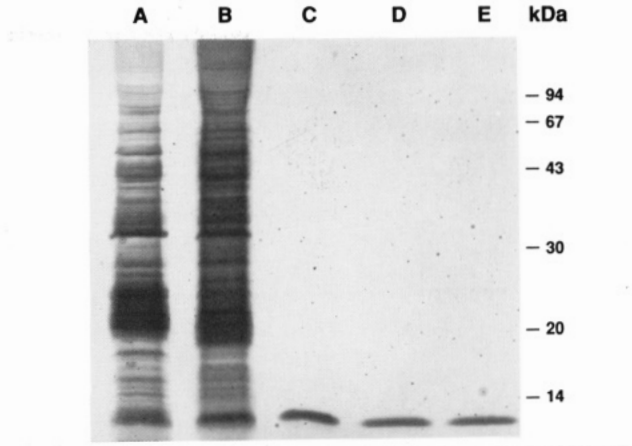

FIGURE 1: Analysis of recombinant SIV-PR from *E. coli* by SDS–PAGE (20% polyacrylamide) PhastGel (Pharmacia). Proteins were visualized by silver staining. Lanes A–E represent 1 μg of protein from cell lysate (A), dissolved 35% ammonium sulfate pellet (B), Superose-12 chromatography eluate (C), QSSF chromatography elutate (D), and SSFF chromatography eluate (E).

While providing no additional purification of the HIV-1 and SIV proteases, the QSFF and SSFF chromatographic steps were used to eliminate contaminating nucleic acids and to concentrate the enzymes, respectively, which was found to be essential for maintaining stable enzyme activity. Purified SIV-PR and HIV-1 PR were also unstable due to autoproteolysis. Degradation products were observed in the RP-HPLC profiles in 24 h at 4 °C with corresponding loss of peptidolytic activity. However, peptidolytic activity could be maintained for several months when the proteases were stored at −70 °C in AEND buffer, pH 5, 40% (v/v) glycerol (Strickler et al., 1989).

Primary Structure of Recombinant SIV-PR

Amino Acid Analysis. As shown in Table II, the amino acid composition of purified, recombinant SIV-PR was consistent, except for slight discrepancies of amino acids Val and Ile, with the 99 amino acid polypeptide (Figure 2) that would result from autoprocessing of the recombinant precursor beginning at Pro-1 (corresponding to nucleotide 2631 of BK28 SIV_{Mac}) and ending at Leu-99 in the *pol* coding region of SIV_{Mac} [SIVMM251 in Myers et al. (1990)]. While some of the discrepancies in the composition are likely to be attributable to incomplete hydrolysis, as was observed for Ile and Val in

Table II: Amino Acid Composition of Recombinant SIV-PR[a]

amino acid	residue/mol	
	experimental	predicted[b]
Asx	9.5	9
Thr	8.7	9
Ser	3.6	3
Glx	8.4	7
Pro	6.0	6
Gly	13.0	13
Ala	4.2	3
Val	6.8	8
Met	1.5	2
Ile	9.3	11
Leu	10.1	10
Tyr	1.4	2
Phe	3.4	3
His	2.7	2
Lys	6.4	6
Arg	3.1	4
Trp	ND[c]	1
Cys	ND	0
total	98.1	99

[a] Duplicate analyses were run as described under Materials and Methods. [b] SIV-PR sequence beginning at nucleotide 2631 from the SIV_{Mac} BK28 strain (SIVMM251; Myers et al., 1990). [c] ND, not determined.

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                DOMAIN 1                                  DOMAIN 2
HIV-1 PR    PQITLWQRPL  VTIKIGGQLK  EALLDTGADD 30
            ||  || ||   ||  |  ||    | ||||||||
SIV-PR      PQFSLWRRPV  VTAHIEGQPV  EVLLDTGADD

                                    DOMAIN 3
HIV-1 PR    TVLEEMSLPG  RWKPKMIGGI  GGFIKVRQYD 60
                 |         ||  |||   ||||    |
SIV-PR      SIVTGIELGP  HYTPKIVGGI  GGFINTKEYK

                                    DOMAIN 4
HIV-1 PR    QILIEICGHK  AIGTVLVGPT  PVNIIGRNLL 90
              ||  |       ||   | |   | ||  |||||
SIV-PR      NVKIEVLGKR  IKGTIMTGDT  PINIFGRNLL

            DOMAIN 5
HIV-1 PR    TQIGCTLNF 99
            |  |  ||
SIV-PR      TALGMSLNL
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FIGURE 2: Primary sequence alignment and homology comparison between HIV-1 PR (BH10 clone; Ratner et al., 1985) and SIV_{Mac}-PR (BK28 clone, SIVMM251; Myers et al., 1990). Vertical lines denote exact matches or identity between aligned amino acids. The five domains of homology are indicated above the corresponding amino acid residues.

the analysis of recombinant HIV-1 PR (Strickler et al., 1989), the slight overestimation of Ala cannot be explained.

The primary structure was further characterized by determination of the sequence of the amino terminus and by accurate measurement of the molecular weight of the purified, recombinant SIV-PR. The amino-terminal sequence for the first 11 amino acid residues of the purified protein was found to be H_2N-PQFSLWRRPVV, corresponding exactly to the predicted SIV_{Mac}-PR amino-terminal sequence. Electrospray mass spectroscopy provided a highly accurate molecular weight of the purified SIV protein, which was used to distinguish the correct C-terminal sequence from among several possible ones when the corresponding molecular weights were calculated from the predicted C-terminal sequences; e.g., -SLNL (M_r = 10715), -SLNLP (M_r = 10812), -SLNLPI (M_r = 10925). The molecular weight of monomeric, recombinant SIV-PR as measured by this method was 10712.9 ± 1.0, which is in excellent agreement with the calculated molecular weight of 10715 for the predicted 99 amino acid sequence (Pro-1–Leu-99). This value is in good agreement with the molecular weight value of 11600 obtained by SDS–PAGE (see Figure 1) and demonstrates that the amino acid composition of recombinant SIV-PR is identical with the predicted composition shown in Table II, despite the small experimental discrepancies determined by amino acid analysis. From this information and by analogy with HIV-1 PR, it can be concluded that mature SIV-PR was produced in bacteria by autoprocessing from a fusion protein precursor where processing at the amino and carboxyl termini occurred at Ala*Pro and Leu*Pro bonds, respectively. This is in contrast with the observed Phe*Pro cleavage sites found at both termini of HIV-1 protease.

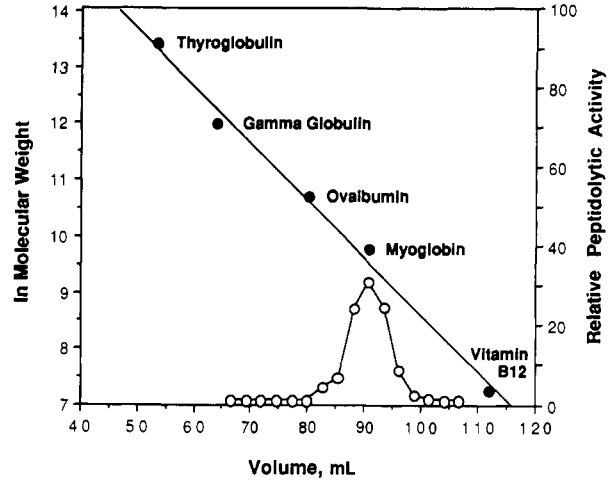

FIGURE 3: Analytical gel filtration of SIV-PR on Sephacryl S-200. The S-200 column was calibrated with protein standards, and SIV-PR elution was monitored by relative peptidolytic activity (O) given as percentage of conversion of substrate to product with 2 mM Ac-RASQNYPVV-NH_2 in MENDT buffer, pH 6, as described under Materials and Methods.

Demonstration of Dimeric Structure. As was suggested by molecular modeling of retroviral proteases (Pearl & Taylor, 1987), demonstrated by size-exclusion chromatography, ultracentrifugation, and cross-linking studies (Meek et al., 1989), and by crystallographic characterization (Wlodawer et al., 1989; Lapatto et al., 1989), HIV-1 PR exists as a dimer of two identical 11-kDa subunits. In order to determine the native molecular weight of active SIV-PR, the recombinant protein was also analyzed by size-exclusion chromatography. A crude extract of induced *E. coli* cells containing the SIVSKF plasmid was subjected to analytical gel filtration on Sephacryl S-200. The peak of peptidolytic activity eluted just prior to the protein standard myoglobin, giving an apparent molecular weight of 18000–19000 (Figure 3). The elution volume for HIV-1 PR (Meek et al., 1989) from a similar column of Sephacryl S-200 was identical with that of SIV-PR. This result demonstrates that the active form of SIV-PR, like HIV-1 PR, is a homodimer.[2]

Isoelectric Focusing. Isoelectric focusing of the purified proteases, under nondenaturing conditions, was used to identify possible isozymic forms of the enzymes as well as to determine isoelectric points. After electrofocusing for 2 h and staining by Coomassie blue, a single protein band was observed for samples of both SIV-PR and HIV-1 PR, indicating the presence of only one charged species for each enzyme. These bands corresponded to isoelectric points of 8.2 for SIV-PR and 8.6 for HIV-1 PR (Figure 4). The p*I* value determined for HIV-1 PR agreed well with the predicted isoelectric point of 8.6, while the p*I* observed for SIV-PR was about 1 p*I* unit less than the predicted value of 9.2. Although there is good

[2] Preliminary studies of purified SIV-PR using analytical ultracentrifugation have confirmed its homodimeric, native structure.

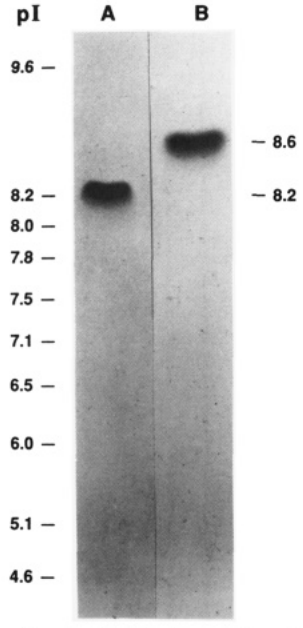

FIGURE 4: Analytical isoelectric focusing of 5 μg of purified SIV-PR (A) and HIV-1 PR (B). Samples were focused for 2 h on preformed Ampholine PAGPLATE, pH 3.5–9.5, and visualized by Coomassie staining. The p*I* values were obtained by using the protein p*I* standards of Bio-Rad.

agreement between the isoelectric points determined under nondenaturing conditions and predicted values, this may be fortuitous since predicted values would more accurately reflect a fully denatured protein in which all ionizable residues are exposed. While it was not surprising to see disparity between the isoelectric points of SIV_{Mac}-PR and HIV-1 PR since there is only 50% identity between their primary sequences, it is interesting to note that there is a large difference between the isoelectric points of SIV_{Mac}-PR (p*I* = 8.2) and HIV-2 PR (p*I* = 5.1; Devereux, 1989; Pichuantes et al., 1990), which share 87% identity. Of the 13 nonidentical amino acid residues, a basic residue of SIV-PR is substituted by an acidic group in HIV-2 PR (SIV_{Mac}/HIV-2; Lys-63/Glu-63), which may account for this difference in p*I*.

Biochemical Characterization

Classification. The primary sequences of the HIV-1 protease (Ratner et al., 1985) and SIV_{Mac} protease (Myers et al., 1990) (Figure 2) exhibit strong homology in regions that comprise important structural domains of the enzymes as indicated by the crystal structure of HIV-1 PR (Wlodawer et al., 1989; Lapatto et al., 1989): the active site (domain 2), the dimer interface regions (domains 1 and 5), the substrate-binding region or "flaps" (domain 3), and the α-helix (domain 4). It is therefore reasonable to conclude that SIV-PR should be structurally and mechanistically similar to HIV-1 PR. In order to examine the protease class of SIV_{Mac}-PR, a series of inhibitors that selectively inactivate metalloproteases and aspartic, thiol, and serine proteases were investigated for inhibitory activity against recombinant SIV-PR. As in the case of HIV-1 PR, preincubation with two serine protease inhibitors, *N*-tosylphenylalanine chloromethyl ketone (0.1–1 mM) and phenylmethanesulfonyl fluoride (0.1–1 mM), led to no loss of SIV-PR peptidolytic activity. Likewise, the metal chelating reagents EDTA and 1,10-phenanthroline (0.1–10 mM) had no effect on activity. One significant difference between SIV-PR and HIV-1 PR was the effect of sulfhydryl-specific reagents on peptidolytic activity. *N*-Ethylmaleimide and iodoacetamide (0.1–10 mM) did not cause any measurable loss of SIV-PR activity, which is in contrast to the report by Meek et al. (1989), in which these reagents were potent inactivators of HIV-1 PR. This is probably due to the reaction with Cys-95 in HIV-1 PR (Debouck et al., 1989), while there are no cysteinyl residues present in SIV-PR (see Figure 2).

Several natural product protease inhibitors were also tested, including leupeptin (Aoyagi et al., 1969), amastatin (Aoyagi et al., 1978), chymostatin (Umezawa et al., 1970a), and pepstatin A (Umezawa et al., 1970b). Leupeptin, a serine and cysteine protease inhibitor, was found not to inhibit either SIV-PR or HIV-1 PR peptidolytic activity at concentrations ≤1 mM. Amastatin and chymostatin, inhibitors of aminopeptidase and chymotrypsin, respectively, demonstrated weak inhibitory activity toward SIV-PR (K_i = 0.3 mM). The fact that these two compounds inhibit SIV-PR, albeit weakly, may reflect its preference for aromatic and hydrophobic residues in the P_1 position of substrates, similar to chymotrypsin or HIV-1 PR, or that possibly these aldehydes or their hydrates may bind as transition-state analogues. In contrast, pepstatin A, a naturally occurring inhibitor for aspartic proteases, bound more tightly to these retroviral proteases with apparent K_i values of 3 μM for both SIV-PR and HIV-1 PR under identical assay conditions (pH 6, 0.2 M NaCl). Enzymatic activity could be reversibly restored by microdialysis. These results are in agreement with inhibition data previously reported for HIV-1 PR (Dreyer et al., 1989; Darke et al., 1989) that were interpreted as evidence for aspartic protease activity.

As an additional test of aspartic protease activity, the affinity label 1,2-epoxy-3-(4-nitrophenoxy)propane (EPNP) was examined against SIV-PR. For porcine pepsin and penicillopepsin, EPNP was shown to be a specific and irreversible inactivator by the esterification of one or both of the active site aspartic acid groups (Hartsuck & Tang, 1972; James et al., 1977). For HIV-1 PR, the pH dependence of time-dependent inactivation by EPNP demonstrates that an enzymatic residue of pK = 3.8 (Meek et al., 1989) must be unprotonated for inactivation. This group is most likely one of the catalytic aspartyl residues and suggests that inactivation of HIV-1 PR proceeds by alkylation of this residue in a mechanism that is analogous to that of porcine pepsin. Time-dependent inactivation of SIV-PR peptidolytic activity upon preincubation treatment with EPNP (data not shown) exhibited a half-maximal inactivation concentration of K_{inact} = 8.6 ± 2.8 mM and a maximum inactivation rate of k_{inact} = 0.026 ± 0.008 min^{-1}. These inactivation parameters are very similar to those reported for HIV-1 PR (K_{inact} = 11 ± 2 mM, k_{inact} = 0.040 ± 0.005 min^{-1}; Meek et al., 1989) and suggest that this enzyme is inactivated by EPNP through a mechanism common to HIV-1 PR and other aspartic proteases. Inactivation by EPNP was also found to be irreversible since microdialysis of the enzyme–inhibitor solution did not restore peptidolytic activity. This inactivation and inhibition data for SIV-PR is consistent with the conclusion that SIV-PR is an aspartic protease.

pH Studies. In order to probe its chemical mechanism, the pH dependence of SIV-1 PR activity was investigated. The pH optima for SIV-PR with two synthetic HIV-1 PR substrates of disparate sequence, Ac-SQNYPVV-NH_2 (Moore et al., 1989) and Ac-RKILFLDG-NH_2, were both observed at pH 5.5 (data not shown). This value is much higher than the pH optimum observed for porcine pepsin (ca. pH 2; Fruton, 1976) but similar to that reported for human renin (ca. pH 6; Cumin et al., 1987) and HIV-1 PR (pH 5.5; Meek et al., 1989; Darke et al., 1989; Richards et al., 1990). The plot of log (V/K) versus pH for SIV-PR with Ac-SQNYPVV-NH_2 (Figure 5) was a "bell-shaped" curve, in which the value of

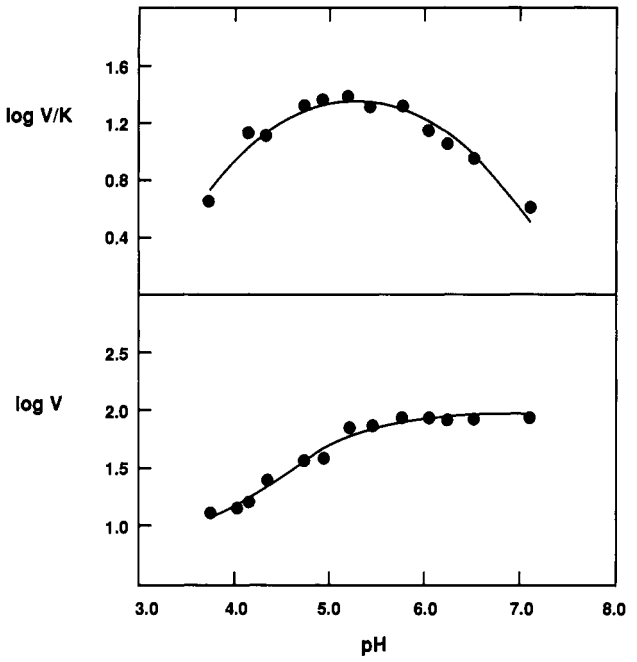

FIGURE 5: Dependence of kinetic parameters of SIV-PR-catalyzed cleavage of Ac-SQNYPVV-NH_2 with pH. Initial velocities were determined in FAMTNE buffer with 0.5–10 mM substrate as described under Materials and Methods. Kinetic parameters were calculated according to eq 1 and plotted as log (V/K) and log V versus pH. The curves drawn through the experimental data points were generated by fitting these data to eqs 2 and 3, respectively.

log (V/K) decreased at acidic and basic pH with asymptotic slopes of 1 and −1, respectively. The pK values, obtained by the fitting of these data to eq 2, were pK_1 = 4.3 ± 0.1 and pK_2 = 6.2 ± 0.1. Since Ac-SQNYPVV-NH_2 has no titratable groups in the experimental pH range, these pK values must correspond to enzymatic groups. These results indicate that the substrate binds to an enzyme form in which two residues of pK = 4.3 and 6.2 must be unprotonated and protonated, respectively. These two enzymatic residues are most likely the active site aspartyl residues, Asp-25 and Asp-25′, in opposite states of protonation. These acid dissociation constants reflect an upward shift by nearly 1 log unit compared to those observed for HIV-1 PR with Ac-SQNYPVV-NH_2 as the substrate: pK_1 = 3.3 and pK_2 = 5.5 (Hyland et al., 1990).

Similar to the pH rate profiles for the aspartic proteases chymosin (Suzuki et al., 1989) and HIV-1 PR (Richards et al., 1990), protonation or deprotonation of the enzymatic groups observed in the log (V/K) profile of SIV-PR does not lead to suppression of V in the log V versus pH profile (Figure 5). Instead, the protonation of an enzymatic residue of apparent pK_3 = 5.00 ± 0.08 (upon fitting to eq 3) shifted V to a constant, lower value, resulting in a "wave-shaped" profile of log V versus pH. A similar profile of log V versus pH was observed for Ac-SQNYPVV-NH_2 with HIV-1 PR (pK_3 = 4.7; Hyland et al., 1990). The results of the pH rate profiles of log (V/K) and log V suggest that the oligopeptide substrate may only bind to the enzyme form in which the residue of pK = 4.2 is protonated and the other of pK = 6.2 is unprotonated and that protonation of an enzyme group (pK = 5.0) in the enzyme–substrate (or an enzyme–product complex) results in a parallel, but slower, reaction pathway. In addition, it is evident from the crystal structures of HIV-1 protease–inhibitor complexes (Miller et al., 1989; Jaskólski et al., 1991) that in the enzyme–substrate complex, as exemplified by the log V profile, the active site aspartyl residues, putatively apparent in the log (V/K) profile, are deeply buried beneath the bound substrate and are likely to be inaccessible to solvent.

Table III: Prediction of Proteolytic Cleavage Sites within SIV *gag–pol* Polyproteins from HIV-1 Sequences

	cleavage site[a]	HIV-1 sequence[b]	SIV sequence[c]
(1)	MA/CA	SQNY*PIVQ	GGNY*PVQQ[d]
(2)	CA/X	ARVL*AEAM	ARLM*AEAL[d]
(3)	X/NC	ATIM*MQRG	PFAA*AQKR[d]
(4)	NC/p6	PGNF*LQSR	PRNF*PMAQ[d]
(5)	TF/PR	SFNF*PQIT	GFAA*PQFS
(6)	PR/RT	TLNF*PISP	SLNL*PIAK
(7)	RT/RN	AETF*YVDG	EETY*YTDG
(8)	RT/IN	RKIL*FLDG	RQVL*FLEK

[a] Products from processing of HIV-1 *gag* and *gag–pol* polyproteins by HIV-1 PR; CA is capsid protein, IN is integrase, MA is matrix protein, NC is nucleocapsid protein, PR is viral protease, RN is RNase-H, RT is reverse transcriptase, TF is transframe protein, X is undefined protein, and the asterisk indicates sites of cleavage (Leis et al., 1988). [b] Cleavage sites found for HIV-1 *gag* and *gag–pol* polyproteins (Henderson et al., 1988) are shown here for the BH10 clone of HIV-1 (Ratner et al., 1985). [c] Cleavage sites predicted from alignment of HIV-1 polyprotein cleavage sites with homologous sequences in SIV_{Mac} (SIVMM251 in Myers et al., 1990). [d] Cleavage sites determined by protein sequencing for SIV_{Mne} (Henderson et al., 1988).

The value of pK_3 indicates that the protonation of a carboxylic residue is responsible for this shift in V. This carboxylic group could be that of one of the active site aspartyl residues (Asp-25 or Asp-25′) or the carboxylic product Ac-SQNY-OH. If the two catalytic aspartyl groups assume opposing roles in general acid–general base catalysis, then a reasonable chemical mechanism would have one of these aspartyl groups unprotonated both before and after peptidolysis. Protonation at pH < 5.0 of the aspartyl group in the enzyme–product complex, which presumably would have previously imparted a proton to the neutral amine product, could result in a change in the rate-limiting step. This change in the rate-limiting step could be from a chemical one to the release of the amine product, if the proximal, protonated aspartyl group subsequently protonates this amine and forms an ionic pair with this product. Alternatively, protonation of another enzymatic residue could elicit a conformational change in an enzyme–substrate or enzyme–product complex that results in a slower and different rate-limiting step. A possible candidate for this residue is Asp-29, which is conserved among the retroviral proteases and is located adjacent to the substrate binding site, as defined by the X-ray structure of HIV-1 PR (Miller et al., 1989; Wlodawer et al., 1989). Precedence for the simultaneous observation of a "bell-shaped" V/K versus pH and a "wave-shaped" V versus pH profiles has been reported for the glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteriodes* (Viola, 1984).

Characterization of Protein and Peptide Substrates. Since the results from the primary structure and kinetic characterization of SIV-PR with Ac-SQNYPVV-NH_2 were similar to those of HIV-1 PR, the substrate binding sites and therefore the substrate cleavage specificity of these two proteases should also be alike. We aligned the eight HIV-1 PR cleavage sites that have been identified within HIV-1 $Pr^{gag-pol}$ (Henderson et al., 1988) to the sequence of SIV_{Mac} $Pr^{gag-pol}$ (SIVMM251; Myers et al., 1990; Henderson et al., 1988; Graves et al., 1990; Mizrahi et al., 1989) in order to predict potential cleavage sites for SIV-PR. While the amino acid sequences for the MA/CA, NC/p6, PR/RT, RT/RN, and RT/IN cleavage sites predicted for SIV $Pr^{gag-pol}$ were highly homologous to those in HIV-1 (Table III), the predicted sequences of the CA/X, X/NC, and TF/PR sites of SIV were less similar to those of HIV-1. In order to characterize the substrate specificity of SIV-PR, we compared HIV-1 PR and SIV-PR proteolysis of

Table IV: Kinetic Parameters for Synthetic Peptide Substrates with SIV-PR and HIV-1 PR[a]

peptide	protease	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
(1) Ac-SQNY*PVV-NH_2	SIV-PR	4.3 ± 1.6	6.4 ± 1.1	1.5 ± 0.2
	HIV-1 PR	5.4 ± 2.8	13.2 ± 3.4	2.4 ± 0.1
(2) Ac-RASQNY*PVV-NH_2	SIV-PR	3.1 ± 1.6	6.2 ± 1.4	2.0 ± 0.2
	HIV-1 PR	3.3 ± 1.2	12.3 ± 3.7	3.7 ± 0.1
(3) Ac-RKIL*FLDG-NH_2	SIV-PR	0.3 ± 0.1	1.3 ± 0.2	4.3 ± 1.3
	HIV-1 PR	1.0 ± 0.3	23 ± 6.0	23 ± 6.0
(4) Ac-ATLNF*PISPIE-NH_2	SIV-PR	4.6 ± 0.6	0.6 ± 0.1	0.1 ± 0.01
	HIV-1 PR	6.7 ± 1.1	9.1 ± 1.5	1.4 ± 0.2
(5) Ac-AETF*YVD-NH_2	SIV-PR	ND[b]	ND	0.172 ± 0.006[c]
	HIV-1 PR	4.3 ± 0.2	4.9 ± 0.1	1.1 ± 0.03
(6) Ac-AETY*YTD-NH_2	SIV-PR	ND	ND	0.036 ± 0.002[c]
	HIV-1 PR	ND	ND	0.040 ± 0.003[c]

[a] Determined in MENDT buffer (pH 6), 10% DMSO, as described under Materials and Methods, where values for k_{cat} and k_{cat}/K_m are corrected for the fraction of active enzyme as determined by active site titration. [b] ND, not determined. [c] Approximate values of k_{cat}/K_m obtained from plots of initial rates versus substrate concentration under conditions where [A] $\ll K_m$, in units of mM^{-1} s^{-1}.

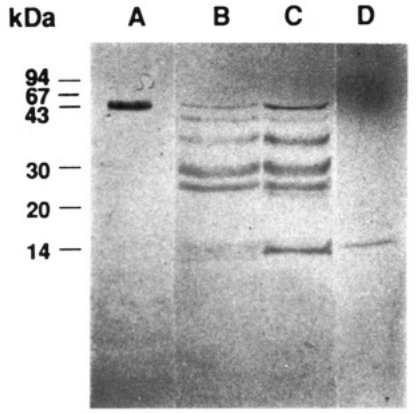

FIGURE 6: SDS–PAGE (20% polyacrylamide) PhastGel (Pharmacia) analysis of cleavage products for proteolysis of recombinant HIV-1 p55 by HIV-1 PR and SIV-PR. Proteins were visualized by silver staining. Lanes A–D represent approximately 1 μg of untreated p55 (A), reaction products of p55 incubated with 30 ng of HIV-1 PR (B) or with 30 ng of SIV-PR (C), and 30 ng of SIV-PR alone (D).

recombinant HIV-1 p55 and determined kinetic parameters for each protease with synthetic peptides on the basis of the similar sequences shown in Table III.

The recombinant p55 protein of HIV-1 $Pr55^{gag}$ contains most of the MA protein (p17) fused to 56 codons of bacterial galactokinase, all of the CA protein (p24), the X protein, and most of the NC protein (p7) (Debouck et al., 1987). SIV-PR and HIV-1 PR were incubated separately with recombinant HIV-1 p55, and the cleavage products were analyzed by separation on SDS–PAGE. Identical proteolytic patterns were obtained for both HIV-1 PR (lane B) and SIV-PR (lane C) on SDS–PAGE with protein bands at 54, 47, 39, 29, 24, and 11 kDa (Figure 6). The sizes of these proteins were consistent with those of uncleaved p55 (54 kDa), protease (11 kDa), the CA–X–NC product (site 1, 39 kDa), the MA–CA product (site 2, 47 kDa), the CA product (site 3, 29 kDa), and the MA-fusion product (24 kDa). Although the identity of the sites of cleavage was not confirmed by sequencing, it appears that SIV-PR recognizes HIV-1 $Pr55^{gag}$ cleavage sites in p55 and suggests that (1) SIV-PR might process SIV_{Mac} $Pr^{gag-pol}$ at the homologous cleavage sites indicated in Table III and (2) synthetic peptides based on the HIV-1 protease cleavage sites might be suitable substrates for SIV-PR. In a similar manner, protein and peptide substrates of HIV-1 PR were shown to be suitable substrates for HIV-2 PR, whose primary structure is nearly identical with that of SIV-PR (Myers et al., 1990; Pichuantes et al., 1990; Le Grice et al., 1989).

Several synthetic peptide substrates of HIV-1 PR were tested as possible substrates for recombinant SIV-PR. A comparison of the kinetic parameters determined for the peptidolysis of several HIV-1 $Pr^{gag-pol}$ cleavage site mimics by SIV-PR and HIV-1 PR is given in Table IV. These synthetic peptides were cleaved by either SIV-PR or HIV-1 PR, in MENDT buffer (pH 6), 10% DMSO, to give products with indistinguishable HPLC profiles (data not shown). Peptides with the cleavage sequences Tyr*Pro (peptides 1 and 2), Leu*Phe (peptide 3), and Phe*Pro (peptide 4) were suitable substrates for SIV-PR. Of these substrates, peptide 3 had the lowest Michaelis constant and the highest k_{cat}/K_m value with SIV-PR, similar to the trend with HIV-1 PR. It should be noted that although cleavage of these synthetic peptides by SIV-PR supports the predicted cleavage sites by alignment with HIV-1 PR cleavage sites (see Table III), this data should not be viewed as confirmation of these sequences as the cleavage sites within SIV_{Mac} $Pr^{gag-pol}$.

Values of k_{cat} and k_{cat}/K_m for the oligopeptide substrates were between 2- and 18-fold higher with HIV-1 PR than with SIV-PR. While substrates containing the scissile Tyr*Pro bond (peptides 1 and 2) exhibited comparable Michaelis constants for the two proteases, both k_{cat} and k_{cat}/K_m values for HIV-1 PR were approximately twice the values measured for SIV-PR. This result is strikingly similar to the 2-fold variation observed in these kinetic parameters when a peptide substrate of nearly identical sequence was tested with HIV-2 PR and compared to HIV-1 PR (Tomasselli et al., 1990). Since the corresponding K_m values were relatively invariant for peptides 1 and 2, it follows that the proportionally lower values of k_{cat} and k_{cat}/K_m with SIV-PR reflect a mechanism in which a chemical step is more rate-limiting for these substrates.

Peptide 5, whose scissile bond corresponds to the p66/p51 cleavage site (site 7) within the HIV-1 reverse transcriptase (RT), was not a good substrate for SIV-PR, exhibiting apparent, nonsaturating, first-order kinetics up to 15 mM compared to K_m = 4.3 mM for HIV-1 PR. A decrease in binding affinity of SIV-PR for substrates containing the Phe*Tyr cleavage site was also observed in bacteria for the processing of recombinant HIV-1 RT and another recombinant polypeptide containing this cleavage site (I. C. D., S. Richardson, F. Watson, R. C. Craig, and C.D., manuscript in preparation). This may represent variance in substrate specificity between SIV-PR and HIV-1 PR where smaller amino acid residues are preferred in P_1 and P_1' for SIV-PR, similar to the difference reported between HIV-1 PR and recombinant (Le Grice et al., 1989; Tomasselli et al., 1990) and synthetic (Wu et al., 1990) HIV-2 PR.

Since SIV_{Agm}-RT exists as a heterodimer (Lüke et al., 1990; Kraus et al., 1990) similar to HIV-1 RT (Mizrahi et al., 1989,

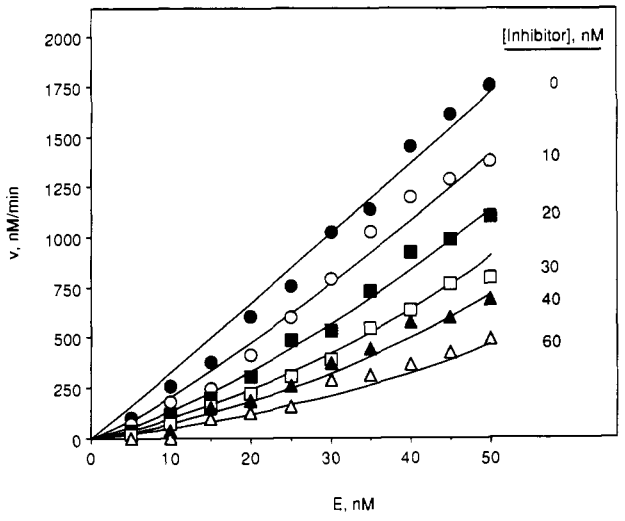

FIGURE 7: Ackerman–Potter plot for competitive, tight-binding inhibition of SIV-PR with Ala-Ala-PheΨ[CH(OH)CH_2]Gly-Val-Val-OCH_3. Peptidolytic activity at 0.8 mM Ac-RASQNYPVV-NH_2 (where v = nM Ac-RASQNY-OH formed per minute) was obtained at variable concentrations of SIV-PR (5–50 nM) and at changing fixed levels of inhibitor (0–60 nM) in MENDT buffer (pH 6), 37 °C. The curves drawn through the experimental data points were generated by fitting the data to eq 5.

and references therein), it was of interest to see if recombinant SIV_{Mac}-PR would cleave a synthetic peptide that mimics the homologous cleavage sequence in SIV_{Mac}-RT (site 7 in Table III). Peptide 6 (Tyr*Tyr) was a poor, but comparable, substrate for both SIV-PR and HIV-1 PR with a rate for peptidolysis by SIV-PR of one-fourth that of peptide 5. These results suggest that (1) the site of cleavage in SIV-RT (to form the heterodimer) may not be Tyr*Tyr and/or (2) the cleavage may occur from proteolysis by SIV-PR and/or an endogenous protease.

The k_{cat} and k_{cat}/K_m values for these peptide substrates were uniformly lower for SIV-PR than for HIV-PR, ranging from 2- to 10-fold less. Interestingly, substrates that contain a Phe residue either in the P_1 or P_1' position (peptides 3–5) were hydrolyzed much more poorly by SIV-PR than by HIV-1 PR, as indicated by the 5- to 18-fold decreases in both k_{cat} and k_{cat}/K_m. These differences were particularly apparent in the case of peptide 4, which contains a scissile Phe*Pro bond. While it is similar in sequence to peptide 1 and its K_m values are comparable for both proteases, the values of k_{cat} and k_{cat}/K_m for peptide 4 were nearly equal to those of peptide 1 for HIV-1 PR but were 10-fold lower for SIV-PR. This finding is consistent with the comparison of peptide 5 and 6, which differ in sequence only in two positions (P_1 and P_2') and for which the effects of substituting Phe for Tyr at P_1 may be reasonably compared. As above, the k_{cat}/K_m values for peptide 6 (Tyr at P_1) were similar for both proteases, while k_{cat}/K_m for peptide 5 (Phe at P_1) was 7-fold lower for SIV-PR. The apparent preference of SIV-PR for a Tyr residue as opposed to a Phe at P_1 indicates that, in addition to smaller amino acid residues, the S_1 binding site of SIV-PR may also better accommodate a less hydrophobic residue than does HIV-1 PR and that the *p*-hydroxyl group of Tyr may effect more favorable interactions within the S_1 binding site of SIV-PR than Phe. Since the residues that comprise the S_1 binding site of HIV-1 PR (Leu-23, Asp-25, Gly-27, Ala-28, Gly-49, Ile-50, Val-82, and Ile-84; Wlodawer et al., 1989) are identically conserved in the primary sequence of SIV-PR (except for Ile-82, see Figure 2), it is not clear what subtle differences in SIV-PR may account for this preference.

Synthetic Peptide Analogue Inhibitors. We also tested the sensitivity of SIV-PR toward several potent, peptide analogue inhibitors of HIV-1 PR containing a hydroxyethylene isostere replacement for the scissile amide bond (Dreyer et al., 1989). These compounds are presumed transition-state analogues, which incorporate elements of substrate sequence and the proposed tetrahedral adduct formed during hydrolysis. Ala-Ala-PheΨ[CH(OH)CH_2]Gly-Val-Val-OCH_3 has been shown to effect potent, competitive inhibition of HIV-1 PR (Dreyer et al., 1989). This compound also exhibited apparent competitive inhibition in a double-reciprocal pattern of inhibitor concentration versus Ac-RASQNYPVV-NH_2 concentration. The plot of v versus E_t at changing fixed levels of Ala-Ala-PheΨ[CH(OH)CH_2]Gly-Val-Val-OCH_3 is shown in Figure 7. Fitting of these data to eq 5 resulted in values of k_{cat} = 190 ± 20 min^{-1}, α = 0.87 ± 0.09 for SIV-PR, and K_i = 8.4 ± 1.0 nM for SIV-PR. This inhibition constant was similar to that obtained for HIV-1 PR, where K_i = 11.7 ± 0.9 nM. The sensitivity of SIV-PR toward other tight-binding inhibitors of HIV-1 PR was also tested, including Ala-Ala-PheΨ[CH(OH)CH_2]Phe-Val-Val-OCH_3 (K_i = 1.6 ± 0.4 nM) and Ala-Ala-PheΨ[CH(OH)CH_2]Ala-Val-Val-OCH_3 (K_i = 1.9 ± 0.3 nM). These results demonstrate that in vitro these peptide analogue inhibitors designed for HIV-1 PR are also potent inhibitors of SIV-PR. In accord with these results is the recent report of the blocking of HIV-1, HIV-2, and SIV replication in cells with a similar peptide analogue inhibitor of HIV-1 PR (Ashorn et al., 1990).

The stoichiometric titration of active SIV-PR and HIV-1 PR was determined by the method of Ackermann and Potter (1949), Morrison (1969), and Cha (1975) using Ala-Ala-PheΨ[CH(OH)CH_2]Gly-Val-Val-OCH_3 (as shown in Figure 7). If one assumes that the inhibitor does not bind to inactive enzyme, then 87% of the active sites within the apparently homogeneous preparation of SIV-PR are functional. Accordingly, the k_{cat} and k_{cat}/K_m values reported in Table IV have been corrected for the fraction of active enzyme. Analysis of HIV-1 PR with 14 different tight-binding inhibitors revealed that typical preparations of this protease exhibited values for the fraction of active enzyme of α = 1.0 ± 0.2 (G.B.D. et al., manuscript in preparation).

CONCLUSIONS

From biochemical characterization of recombinant SIV-PR, a tertiary structure similar to HIV-1 PR can be proposed for SIV-PR in which an Asp-Thr-Gly sequence from each subunit of the active, dimeric form of the enzyme contributes to a symmetrical active site thought to be much like that observed in the crystal structures of the RSV and HIV-1 proteases (Wlodawer et al., 1989; Lapatto et al., 1989; Miller, et al., 1989). Hydrolysis of oligopeptide substrates is most likely catalyzed by aspartic acid (Asp-25) assisted attack of water upon the scissile bond with subsequent protonation by the second aspartic acid (Asp-25′) group (Meek et al., 1989). These results demonstrate that SIV-PR can be effectively used to evaluate inhibitors directed toward HIV-1 PR activity and give further support for the usefulness of SIV infection as a model for the preclinical evaluation of HIV-1 PR inhibitors as anti-AIDS drugs. Further goals for the development of a simian model include structure–activity studies of substrates and inhibitors and the demonstration of the blockade of viral maturation of SIV in infected cells.

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The Histone Core Exerts a Dominant Constraint on the Structure of DNA in a Nucleosome†

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ABSTRACT: We have examined the structures of unique sequence, A/T-rich DNAs that are predicted to be relatively rigid [oligo(dA)·oligo(dT)], flexible [oligo[d(A-T)]], and curved, using the hydroxyl radical as a cleavage reagent. A 50-base-pair segment containing each of these distinct DNA sequences was placed adjacent to the T7 RNA polymerase promoter, a sequence that will strongly position nucleosomes. The final length of the DNA fragments was 142 bp, enough DNA to assemble a single nucleosome. Cleavage of DNA in solution, while bound to a calcium phosphate crystal, and after incorporation into a nucleosome is examined. We find that the distinct A/T-rich DNAs have very different structural features in solution and helical periodicities when bound to calcium phosphate. In contrast, the organization of the different DNA sequences when associated with a histone octamer is very similar. We conclude that the histone core exerts a dominant constraint on the structure of DNA in a nucleosome and that inclusion of these various unique sequences has only a very small effect on overall nucleosome stability and structure.

Nucleosomes are positioned in vivo with respect to DNA sequence (Simpson, 1991). Considerable effort has been made to understand what elements of DNA structure and histone composition within the nucleosome are responsible for the phenomenon of positioning and how nucleosome position might be influenced by trans-acting factors. Our most substantial insight into this problem concerns the influence of DNA structure on the rotational and translational position of the DNA helix with respect to the histone core (Travers, 1989).

Within a nucleosome core particle, 146 base pairs (bp) of DNA are wrapped in 1.8 turns around a histone core (Richmond et al., 1984). This constraint of DNA has two consequences. First, the histone core exhibits a preference for DNA that has an intrinsic curvature or anisotropic flexibility with a periodicity of 10.0 bp/turn (Schrader & Crothers, 1990). Second, rigid or straight DNA, such as tracts of oligo(dA)·oligo(dT) (>12 bp), is not favored for incorporation into a

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