# Human Immunodeficiency Virus-1 Protease. 1. Initial Velocity Studies and Kinetic Characterization of Reaction Intermediates by $^{18}O$ Isotope Exchange†

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*Received March 25, 1991; Revised Manuscript Received May 29, 1991*

ABSTRACT: The peptidolytic reaction of HIV-1 protease has been investigated by using four oligopeptide substrates, Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-$NH_2$, Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-$NH_2$, Ac-Ser-Gln-Ser-Tyr-Pro-Val-Val-$NH_2$, and Ac-Arg-Lys-Ile-Leu-Phe-Leu-Asp-Gly-$NH_2$, that resemble two cleavage sites found within the naturally occurring polyprotein substrates $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$. The values for the kinetic parameters $V/KE_t$ and $V/E_t$ were 0.16–7.5 $mM^{-1}$ $s^{-1}$ and 0.24–29 $s^{-1}$, respectively, at pH 6.0, 0.2 M NaCl, and 37 °C. By use of a variety of inorganic salts, it was concluded that the peptidolytic reaction is nonspecifically activated by increasing ionic strength. $V/K$ increased in an apparently parabolic fashion with increasing ionic strength, while $V$ was either increased or decreased slightly. From product inhibition studies, the kinetic mechanism of the protease is either random or ordered uni–bi, depending on the substrate studied. The reverse reaction or a partial reverse reaction (as measured by isotope exchange of the carboxylic product into substrate) was negligible for most of the oligopeptide substrates, but the enzyme catalyzed the formation of Ac-Ser-Gln-Asn-Tyr-Phe-Leu-Asp-Gly-$NH_2$ from the products Ac-Ser-Gln-Asn-Tyr and Phe-Leu-Asp-Gly-$NH_2$. The protease-catalyzed exchange of an atom of $^{18}O$ from $H_2{}^{18}O$ into the re-formed substrates occurred at a rate which was 0.01–0.12 times that of the forward peptidolytic reaction. The results of these studies are in accord with the formation of a kinetically competent enzyme-bound amide hydrate intermediate, the collapse of which is the rate-limiting chemical step in the reaction pathway.

The initial translation products of the *gag* and *pol* genes of the human immunodeficiency virus type 1 (HIV-1)[1] are two precursor polyproteins, $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$. These polyproteins respectively contain, in nascent form, the structural *gag* proteins of the virion core (p17, p24, p7, and p9) and the enzymes of retroviral replication (protease, reverse transcriptase, endonuclease, and ribonuclease H). The retroviral protease, encoded at the 5′-end of the *pol* reading frame, apparently processes its own maturation from $Pr160^{gag\text{-}pol}$ (Debouck et al., 1987) and subsequently releases the *gag* proteins and the retroviral enzymes by effecting specific cleavages at eight peptide sequences within the polyprotein substrates (Darke et al., 1988; Henderson et al., 1988; Mizrahi et al., 1989; Meek et al., 1990a). A primary class of cleavage sequence contains an aromatic amino acid and a proline at the P1 and P1′ sites,[2] while a more general secondary class contains a leucine or methionine at the P1 position (Henderson et al., 1988). Three of the eight cleavage sites of the HIV-1 polyproteins are of the primary class.

The virions formed from HIV-1 proviruses in which the protease has been inactivated by a single point mutation are of immature morphology, due to their inability to proteolyze these polyproteins, and contain decreased or negligible reverse transcriptase activity (Kohl et al., 1988; Peng et al., 1989; Gottlinger et al., 1989). These studies demonstrated that inactivation of HIV-1 protease resulted in noninfectious virions and therefore suggested that the blockade of HIV-1 protease activity by specific inhibitors should provide a therapeutic approach for the treatment of the acquired immunodeficiency syndrome. Indeed, the antiviral effect of specific inhibitors of HIV-1 protease has been demonstrated in human T-lymphocytes that are either chronically or acutely infected with HIV-1 (Meek et al., 1990b; McQuade et al., 1990; Roberts et al., 1990; Erickson et al., 1990). These findings clearly confirm the importance of HIV-1 protease as a target for rationally designed therapeutic agents.

HIV-1 protease is an aspartic protease that relies on the assembly of two 99 amino acid polypeptides into a homodimer, which results in molecular architecture analogous to those of the monomeric aspartic proteases pepsin, renin, and penicillopepsin (Pearl & Taylor, 1987; Meek et al., 1989; Navia et al., 1989; Wlodawer et al., 1989). Its active site is formed at the interface of the homodimer and consists of two aspartyl residues, Asp-25 and Asp-25′, one contributed by each subunit. The catalytic mechanism of the nonviral aspartic proteases has been extensively studied by kinetic methods, affinity labeling studies, and X-ray crystallographic methods [for reviews, see Fruton (1976), Kostka (1985), Fruton (1987), and Polgar (1987)]. Although this vast compendium of data is most consistent with a "general acid–general base" mechanism

† Supported in part by a grant from the National Institutes of Health (GM-39526) to S.A.C.

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[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FAB-MS, fast atom bombardment mass spectrometry; HIV-1, human immunodeficiency virus type 1; HPLC, high-performance liquid chromatography; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; amino acids designated by the one-letter code.

[2] The nomenclature of Berger and Schechter (1970) is used throughout.

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of the catalytic aspartyl residues, questions regarding the participation of the aspartyl residues in the formation of covalent reaction intermediates have not been completely resolved. Recently solved X-ray crystal structures of HIV-1 protease complexed with various oligopeptide inhibitors have demonstrated that the inhibitors bind in the active-site cleft of the protease in a similar fashion to those of the monomeric aspartic proteases and that the aspartyl residues, Asp-25 and Asp-25′, are similarly positioned for catalysis (Miller et al., 1989; Swain et al., 1990; Erickson et al., 1990; Fitzgerald et al., 1990; Jaskolski et al., 1991). In this report, we have used four oligopeptide substrates (three of the primary class of cleavage site and one of the secondary class) to kinetically characterize the reaction intermediates of HIV-1 protease by isotope exchange reactions and to investigate the chemical rate-limiting steps of the enzymatic reaction.

## Experimental Procedures

*Enzymes and Chemicals.* Recombinant HIV-1 protease was obtained from the PRO4 expression vector in *Escherichia coli* strain AR58 (Debouck et al., 1987; Strickler et al., 1989). The protease was purified to >95% homogeneity, as judged by both amino acid analysis and $NaDodSO_4$–polyacrylamide gel electrophoresis by the method of Strickler et al. (1989) from the supernatants of bacterial crude extracts using successive steps of ammonium sulfate precipitation and size exclusion chromatography on a TSK G2000 SW column (Bio-Rad). Some of the protease was purified by using a modified procedure in which a Sepharose-12 column (Pharmacia) replaced the TSK G2000 column in the gel filtration step (Grant et al., 1991). Briefly, the protease-containing fractions (≤100 mL) from the ammonium sulfate fractionation step in 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM dithiothreitol, 0.2 M NaCl, and 10% glycerol (TEDNG buffer) were loaded at 25 mL/min onto a Sepharose-12 column (11 × 46 cm), and the eluted enzymatically active fractions were pooled and diluted 1:2 with the same buffer containing no NaCl. The fractions were next applied to a column of Q-Sepharose Fast Flow (Pharmacia; 5 × 15 cm) equilibrated with TEDG buffer to remove contaminating nucleic acids. The protease is not retained on this column and is immediately eluted with TEDG buffer at a flow rate of 15 mL/min. The active fractions (≤950 mL) were pooled and adjusted to pH 5.0 with 10% acetic acid, and protease activity was concentrated by chromatography on S-Sepharose Fast Flow (Pharmacia; 2.2 × 12 cm). The bound enzyme was eluted in a single peak with a buffer of 50 mM sodium acetate (pH 5.0), 5 mM EDTA, 10 mM dithiothreitol, and 0.35 M NaCl at a flow rate of 10 mL/min. The purified protease was routinely stored at −20 °C in 50 mM Tris-HCl (pH 7.5), 1 or 10 mM dithiothreitol, 1 or 5 mM EDTA, 200 mM NaCl, and 40% glycerol at protein concentrations of 10–30 μg/mL. Typically, specific activities measured at pH 6.0, 37 °C for these enzyme preparations were 75–90 nmol of product·$min^{-1}$·(μg of protease)$^{-1}$ with 5 mM of the substrate Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-$NH_2$.

Protease concentrations were measured either by using the BCA protein reagent of Pierce Chemical Co. or by analytical reverse-phase HPLC [Vydac $C_{18}$ column, 4.6 × 250 mm, 300 Å pore size; linear gradient of acetonitrile (25–75%) in 0.1% trifluoroacetic acid over 20 min at 1 mL/min] according to the method of Strickler et al. (1989). In this chromatographic method, protease concentrations in purified preparations were determined from a standard curve of peak integrations constructed from variable aliquots of a solution of purified HIV-1 protease in which the concentration had been accurately determined by amino acid analysis. The concentrations of active protease in the purified enzyme preparations were routinely determined by active-site titration (Cha, 1975; Ackermann & Potter, 1949) as described (Grant et al., 1991) with potent competitive peptide analogue inhibitors, such as Ala-Ala-PheΨ(CHOHCH$_2$)Gly-Val-Val-OMe [$K_i$ = 12 nM; PheΨ(CHOHCH$_2$)Gly designates the incorporated dipeptide isostere, (4*S*,5*S*)-5-amino-4-hydroxy-6-phenylhexanoic acid] (Dreyer et al., 1989). Typically, 80–100% of the active sites of HIV-1 protease were found to be active in the enzyme preparations used in these studies, as well as those of the accompanying paper (Hyland et al., 1991), and the values of $V/E_t$ and $V/KE_t$ were corrected for the concentration of active enzyme.

$H_2{}^{18}O$ (97 atom % $^{18}O$) was obtained from MSD Isotopes. EDTA (Gold Label grade) was obtained from Aldrich Chemical Co. Trifluoroacetic acid (Sequenal grade) was purchased from Pierce Chemical Co. Solvents used for high-performance liquid chromatography were purchased from Burdick-Jackson Laboratories. Glycerol (Ultrograde) was a product of LKB Instruments, Inc. Buffers, Triton X-100, and dithiothreitol were obtained from Sigma Chemical Co. All other chemicals were of the highest available quality.

*Oligopeptide Substrate and Inhibitors.* The following oligopeptides were prepared by solid-phase synthesis on benzhydrylamine resin as previously described (Moore et al., 1989): Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-$NH_2$ (Ac-SQNYPVV-$NH_2$), Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-$NH_2$ (AcRASQNYPVV-$NH_2$), Ac-Ser-Gln-Ser-Tyr-Pro-Val-Val-$NH_2$ (AcSQSYPVV-$NH_2$), Pro-Val-Val-$NH_2$, (PVV-$NH_2$), Ac-Arg-Lys-Ile-Leu-Phe-Leu-Asp-Gly-$NH_2$ (AcRKILFLDG-$NH_2$), and Phe-Leu-Asp-Gly-$NH_2$, (FLDG-$NH_2$). Solid-phase synthesis on Merrifield resin was similarly used to prepare Ac-Ser-Gln-Asn-Tyr-OH (Ac-SQNY) and Ac-Arg-Lys-Ile-Leu-OH (AcRKIL). The peptides were cleaved from the resin with anhydrous liquid HF at 0 °C and were purified by either gel filtration, countercurrent distribution, and/or preparative reverse-phase HPLC, as appropriate. All peptides were homogeneous by reverse-phase HPLC and thin-layer chromatography, and their structures were confirmed by amino acid analysis and FAB-MS. The competitive inhibitor, Ala-Ala-PheΨ[CHOHCH$_2$]Gly-Val-Val-OMe was synthesized and characterized as described (Dreyer et al., 1989).

*Tritiated Oligopeptides.* [*tyrosyl*-3′,5′-$^3H_2$]Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-$NH_2$ was prepared by catalytic tritiation of [*tyrosyl*-3′,5′-diiodo]Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-$NH_2$ as described (Hyland et al., 1990). The resulting product contained 52.5 mCi of [*tyrosyl*-3′,5′-$^3H_2$]Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-$NH_2$ ([$^3$H]AcSQNYPVV-$NH_2$, 52 Ci/mmol), which was stored as a solution in 90% aqueous ethanol at −80 °C. The tritiated product [$^3$H]Ac-Ser-Gln-Asn-Tyr-OH was prepared by complete enzyme-catalyzed peptidolysis of the substrate by HIV-1 protease with reaction conditions as described (Hyland et al., 1990). The product was purified by preparative HPLC, lyophilized, and redissolved in water (final specific activity = 0.05–0.14 mCi/mmol).

*Enzyme Assays.* The peptidolytic activity of HIV-1 protease with the oligopeptide substrates AcSQNYPVV-$NH_2$, AcRASQNYPVV-$NH_2$, AcSQSYPVV-$NH_2$, and AcRKILFLDG-$NH_2$ was measured by using an HPLC-based assay under conditions similar to those that were previously described (Meek et al., 1989; Moore et al., 1989). A Hewlett-Packard 1090 high-performance liquid chromatograph equipped with a ternary solvent delivery system, an autosampler, a diode

array spectrophotometric detector, and a digital integrator was used to analyze all samples. Products and substrates were separated on an octyldecylsilane column (Beckman Ultrasphere ODS, 4.5 × 250 mm, 5 μm) with a mobile phase (flow rate = 1.5 mL/min) composed of 0.05% trifluoroacetic acid and the following gradients of acetonitrile: 5–20% (7 min), 20% (5 min) (gradient 1); 5–20% (15 min), 20% (5 min) (gradient 2); 5–40% (20 min), 40% (5 min) (gradient 3); and 10–20% (5 min), 20–75% (20 min) (gradient 4). The fraction of reaction was calculated as the ratio of product formed/(product formed + substrate remaining) from digital integration of the following pairs of substrate and product peaks detected at 220 nm: AcSQNY (4.9 min)/AcSQNYPVV-$NH_2$ (9.8 min) (gradient 1); AcRASQNY (5.5 min)/AcRASQNYPVV-$NH_2$ (9.9 min) (gradient 1); AcSQSY (8.4 min)/AcSQSYPVV-$NH_2$ (16.9 min) (gradient 2); FLDG-$NH_2$ (10.5 min)/AcRKILFLDG-$NH_2$ (17.8 min) (gradient 3); and AcRKIL (12.1 min)/AcRKILFLDG-$NH_2$ (gradient 3). The product PVV-$NH_2$ was difficult to detect at 220 nm, indicating that the tyrosyl-containing products used to quantify the extent of reaction contained most of the absorbance found in the corresponding substrates. Relative molar absorbtivities at 220 nm of AcRKILFLDG-$NH_2$, AcRKIL, and FLDG-$NH_2$ were 0.53, 0.19, and 0.24, respectively. No corrections were made in values of peak integrations due to differences in molar absorbtivities of the substrate–product pair used to calculate kinetic rates.

Aqueous solutions (10–50 mM) of each oligopeptide substrate were freshly prepared for each experiment from lyophilized material. The solution concentrations of the substrates were determined from their UV spectra assuming extinction coefficients of $\epsilon^{275nm}$ = 1420 $M^{-1}\cdot cm^{-1}$ and $\epsilon^{257nm}$ = 197 $M^{-1}\cdot cm^{-1}$ for tyrosyl- and phenylalanyl-containing peptides, respectively.

The following buffers were used for enzymatic assays: 80 mM sodium acetate (pH 5.0), 1 mM EDTA, and 1 mM DTT (AED buffer); 80 mM sodium acetate (pH 5.0), 1 mM EDTA, 1 mM DTT, and 0.2 M NaCl (AEDN buffer); 50 mM Mes (pH 6.0), 1 mM EDTA, 0.2 M NaCl, 1 mM DTT, and 0.1% (v/v) Triton X-100 (MENDT buffer); 50 mM each of glycine, sodium acetate, Mes, Tris-HCl (variable pH), 1 mM DTT, 1 mM EDTA, 0.2 M NaCl, and 0.1% (v/v) Triton X-100 (GAMT-NEDT buffer).

In general, peptidolytic assays were conducted in 50-μL reaction mixtures at 37 °C. Mixtures containing buffer, substrates (0.1–10 mM), activators or inhibitors, were preincubated for 10 min at 37 °C prior to initiation of reaction by the addition of 2–6-μL aliquots of HIV-1 protease (5–160 ng), which was kept at room temperature prior to its addition to the reaction mixtures. Substrates were varied over a concentration range equivalent to 0.2–2.0 times the values of their Michaelis constants. Reactions were quenched at various times ($t \leq 40$ min) by the addition of 50 μL of 3% (v/v) trifluoroacetic acid, and the samples were then analyzed by reverse-phase HPLC as outlined above. At the low substrate concentrations (<1 mM), larger sample volumes (50 μL as opposed to 10 μL) were chromatographed in order to obtain signals for the substrate and products that were comparable to those observed at >1 mM substrate.

The time course of the protease-catalyzed peptidolytic reaction for each oligopeptide substrate was ascertained under all of the experimental conditions described below. Time courses were uniformly linear at reaction times of less than 40 min and at which substrate turnover had not proceeded beyond 20%. Initial rate data were in most cases obtained from single time points ($t \leq 20$ min) at which substrate turnover was ≤18%. Typically, substrate turnover at the higher substrate concentrations (3–10 mM) proceeded to <8%, while at lower substrate concentrations (<3 mM) substrate turnover had proceeded to 8–18%.

*Initial Velocity and Inhibition Studies.* Initial velocity studies of peptidolytic reactions of HIV-1 protease were conducted in MENDT buffer (pH 6.0) for the substrates AcSQNYPVV-$NH_2$, AcRASQNYPVV-$NH_2$, AcSQSYPVV-$NH_2$, and in AEDN buffer (pH 5.0) or MENDT buffer (pH 6.0) for AcRKILFLDG-$NH_2$ using protease concentrations of 5–40 nM. Initial rate data were obtained as double-reciprocal plots at 4–5 concentrations of the substrates. The effects of various inorganic salts on HIV-1 protease activity were conducted in AED buffer (pH 5.0) with variable concentrations of the substrates, AcRKILFLDG-$NH_2$ and AcRASQNYPVV-$NH_2$, and changing fixed levels of NaCl, KCl, $(NH_4)_2SO_4$, $NH_4Cl$, $Na_2SO_4$, LiCl, NaBr, NaF, or LiBr. The changing fixed molar concentrations used for each of these salts corresponded to comparable values of ionic strength. That constant ionic strength was maintained was confirmed by measurement of solution conductivities.

Product inhibition studies of AcSQNY and PVV-$NH_2$ were conducted with AcRASQNYPVV-$NH_2$ as the variable substrate in MENDT buffer (pH 6.0) or GAMT-NEDT (pH 3.5). The monitored product, AcRASQNY, was well separated chromatographically from AcSQNY by using gradient 2. AcRKILFLDG-$NH_2$ was used as the variable substrate for product inhibition studies of AcRKIL and FLDG-$NH_2$ in MENDT buffer (pH 6.0), in which enzymatic initial rates were monitored by the formation of FLDG-$NH_2$ and AcRKIL, respectively.

*Reverse-Reaction and Isotope-Partitioning Studies.* The ability of HIV-1 protease to catalyze peptide synthesis was investigated in GAMT-NEDT buffer (pH 3.6 and 6.0) with both the unlabeled peptidolysis products and [*tyrosyl*-$^3H_2$]-AcSQNY. In a 50-μL reaction mixture containing buffer and 5% dimethyl sulfoxide, the following pairs of reaction products were incubated with 4 μg (3.3 μM) of HIV-1 protease at 37 °C for 120 and 300 min: 5 mM AcRKIL and 10 mM FLDG-$NH_2$; 4 mM AcSQNY and 10 mM PVV-$NH_2$; and 4 mM AcRASQNY and 10 mM PVV-$NH_2$. The quenched reaction mixtures were analyzed by reversed-phase HPLC as described above, and the peaks corresponding to unreacted products and synthesized substrates were quantified by integration. The retention time of the substrates were verified by the subsequent addition of authentic material to the reactions mixtures.

The protease-catalyzed synthesis of AcSQNYPVV-$NH_2$ was also investigated under identical conditions with [*tyrosyl*-$^3H_2$]AcSQNY (0.14 mCi/mmol; 30000 dpm; 2 mM) and 5 mM PVV-$NH_2$. The products were incubated with 360 ng (0.33 μM) HIV-1 protease over a period of 120 min and then subjected to reverse-phase HPLC (gradient 1). Fractions (0.75 mL) were collected at the elution positions of both AcSQNYPVV-$NH_2$ and AcSQNY, and their tritium content was determined by liquid scintillation counting. Likewise, reaction mixtures containing 2 mM FLDG-$NH_2$, 4 mM [$^3H$]AcSQNY, 5% dimethyl sulfoxide, and 0.3–0.6 μM HIV-1 protease at pH 3.6 and 6.0 were incubated for 300 min and subjected to HPLC using gradient 4, and tritium content within the collected fractions were determined.

In addition, the isotope exchange of [$^3H$]AcSQNY was investigated by addition of 10 mM [$^3H$]AcSQNY (0.14 mCi/mmol; 150000 dpm) to a 50-μL reaction mixture con-

taining 2 mM AcSQNYPVV-$NH_2$, with or without 5 mM PVV-$NH_2$ (5% dimethyl sulfoxide in final mixture) and 0.33 $\mu$M protease in GAMT-NEDT buffer (pH 6.0 and 3.6). The reaction was quenched after 60–300 min, and, following preparative HPLC as described, the tritium content of both AcSQNYPVV-$NH_2$ and AcSQNY was determined by liquid scintillation counting.

Analysis of the enzyme-catalyzed incorporation of $^{18}O$-labeled $H_2O$ into both AcSQNYPVV-$NH_2$ and AcRK-ILFLDG-$NH_2$ was conducted as follows: Reaction mixtures (100 $\mu$L) contained GAMT-NEDT (pH 6.0), 5 mM Ac-SQNYPVV-$NH_2$, or 5 mM AcRKILFLDG-$NH_2$ in 78% (v/v) $H_2{}^{18}O$ (37 °C). Reactions were initiated by the addition of HIV-1 protease ($\leq$0.16 $\mu$M, final concentration) and were quenched with an equal volume of 3% trifluoroacetic acid at 0–300 min. Control reaction mixtures contained all components and included 10 $\mu$M of the competitive inhibitor Ala-Ala-Phe$\Psi$[CHOHCH$_2$]Gly-Val-Val-OMe ($K_i$ = 12 nM) and were incubated for matching time intervals. In no case were peptidolysis products observed in the presence of this inhibitor. Additional control samples contained all reaction mixture components except enzyme and, following incubation at 37 °C for 300 min, were quenched, and enzyme was then added. The extent of exchange of $^{18}O$ into the carboxylic peptidolytic product AcSQNY was determined from a sample containing enzyme, $H_2{}^{18}O$, and 5 mM AcSQNY, which was incubated at 37 °C for 300 min. The peptides AcSQNYPVV-$NH_2$, AcSQNY, and AcRKILFLDG-$NH_2$ were purified by semipreparative reverse-phase HPLC using conditions described above. In a similar fashion, $^{18}O$ incorporation into the products of a reaction mixture containing 3 mM FLDG-$NH_2$, 3 mM AcSQNYPVV-$NH_2$, 5% dimethyl sulfoxide, and 3.3 $\mu$M protease in 78% $H_2{}^{18}O$ was investigated. The fractions containing the peptides were subsequently lyophilized prior to analysis by FAB-MS.

*Mass Spectrometry.* Fast atom bombardment (FAB) mass spectra were obtained on either (a) a VG ZAB-HF magnetic deflection mass spectrometer (accelerating voltage 8 kV, mass range 3000) equipped with a standard FAB ion source and a fast atom gun (Ion Tech) or (b) the first double focusing portion (MS-1) of a VG ZAB SE-4F tandem magnetic deflection mass spectrometer (accelerating voltage 10 kV, mass range 12 500) equipped with a standard FAB ion source and a high voltage Cs ion gun. The Ion Tech FAB gun was operated at 8 kV, and a discharge current of 1 mA was obtained by using xenon while the voltage Cs gun was operated at 35 kV with an emission of 2–4 $\mu$A. A 2-$\mu$L aliquot of a solution (approximately 2 $\mu$g/mL) of each sample in 10% acetonitrile containing 0.1% trifluoroacetic acid was dispersed on the stainless steel target in a matrix of monothioglycerol or a combination of monothioglycerol/DTT/dithioerythritol (1:2:2, v/v/v). Data were acquired over either a mass range of $m/z$ 1000–500 at 70 s/dec with a resolution of 1500 (10% valley definition) or $m/z$ = 800–840 at 15 s/dec with a resolution of 10000 (10% valley definition). To obtain the best signal-to-noise ratios, seven sequential scans were summed in the peak profile multichannel acquisition mode. The mass range was calibrated by using the $[Cs(CsI)_n]^+$ peaks generated by FAB-MS of CsI. A VG 11-250J data system was used to acquire and process all data. Calculations were performed by using peak values from either the $MH^+$ or the $MNa^+$ pseudomolecular ion species.

*Data Analysis.* Kinetic data were fitted to the appropriate rate equations by using the FORTRAN programs of Cleland (1979). Values of $V/K$ and $V$ at a single fixed concentration of inorganic salt were obtained by fitting of initial velocity data at variable concentrations of the oligopeptide substrates to eq 1. In the equations that follow, $v$ is the initial velocity, $V$ is the maximum velocity, $K$ is the Michaelis constant, $A$ is the concentration of variable substrate, and $K_{is}$ and $K_{ii}$ are slope and intercept inhibition constants, respectively. The nomenclature used in the following rate equations is that of Cleland (1963).

$$v = \frac{VA}{K + A} \tag{1}$$

Patterns conforming to linear competitive inhibition, linear noncompetitive inhibition, or linear uncompetitive inhibition were fitted to eqs 2–4, respectively.

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \tag{2}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \tag{3}$$

$$v = \frac{VA}{K + A(1 + I/K_{ii})} \tag{4}$$

When the type of kinetic pattern was in doubt for an experiment, data were fitted to all of the appropriate equations, and a comparison of the resulting $\sigma$ values (square root of the average residual least-square) was used to determine to best fit.

*Molecular Modeling.* Two molecular models of the proposed enzyme-bound amide hydrate intermediate (EXH and EXH′ in Figure 3) were constructed from the crystal structure (Swain et al., 1990) of a complex of the synthetic HIV-1 protease (Schneider & Kent, 1988) and a hydroxyethylamine peptide analogue inhibitor, JG-365 (Rich et al., 1990), and subjected to a series of molecular dynamics calculations in order to assess whether the models were energetically reasonable. The structure of the inhibitor JG-365 (P1 and P1′ residues are substituted by Phe-CHOH-$CH_2$-Pro) was converted into the amide hydrate intermediate structure of a Tyr-Pro containing substrate (such as AcSQNYPVV-$NH_2$) by removal of the methylene group between Phe and Pro, replacement of the CHOH at the scissile position by CH-$(OH)_2$, addition of a $p$-hydroxy group on Phe and conversion of the C-terminal methyl ester to an amide. The bond lengths and angles in the initial models were not optimal but were adjusted to reasonable values in the first few cycles of minimization.

Molecular dynamics calculations were performed with AMBER 3.0 revision A (Seibel et al., 1989) and the parameter set parm89a. The amide hydrate intermediate required the addition of two angle parameters: OH–CT–OH, force constant = 80.0 kcal/mol·deg$^2$, $\theta$-eq = 109.5°; and OH–CT–N3, force constant = 63.0 kcal/mol·deg$^2$, $\theta$-eq = 109.5°), which were chosen by analogy with existing parameters. Partial atomic changes for the amide hydrate intermediate were calculated with MOPAC 5.0 modified to fit partial atomic changes to the electrostatic potential (Bessler et al., 1990) and adjusted so that the Y–$\Psi$(amide hydrate)–P fragment would have neutral charge. The system was solvated and counter ions were placed on charged amino acids to create an electrically neutral system. A series of molecular mechanics and dynamics were then performed on these models as outlined in Table I. These calculations were designed to permit relaxation of the model-built portion of the system [the substrate (intermediate) and the solvent] without allowing large deviations of the protein–inhibitor crystal structure and maintaining the scissile bond of the substrate proximal to the active-site aspartyl residues.

Table I: Summary of Molecular Mechanics and Dynamics Calculations[a]

| function | mobile atoms | constraints |
|---|---|---|
| 1. MIN | water molecules | none |
| 2. MD | same as 1 | none |
| 3. MIN | protein site,[b] amide hydrate, water molecules, counterions | protein heavy atoms, 50 kcal/mol, hydrogen bonds[c] |
| 4. MD | same as 3 | same as 3 |
| 5. MD | same as 3 | protein heavy atoms, 30 kcal/mol, hydrogen bonds,[c] |
| 6. MD | same as 3 | protein heavy atoms, 10 kcal/mol |
| 7. MD | same as 3 | protein main chain, 5 kcal/mol |
| 8. MD | same as 3 | none |
| 9. MIN | same as 3 | none |

[a] MIN, energy minimization; minimizations were run for 300 steps with distance-dependent dielectric constants and a 8.0-Å nonbonded cutoff. MD, molecular dynamics; MD calculations were run for 1 ps with a 1-fs time step, a distance-dependent dielectric constant, and a 8.0-Å nonbonded cutoff. Temperature was held constant at 298 K by using the temperature coupling algorithm of Berendsen et al. (1984) with a coupling constant of 0.1 ps. [b] The protein site consisted of all residues with at least one atom within 8.0 Å of the amide hydrate. [c] For the EXH model, the hydroxyls of the amide hydrate were constrained to hydrogen bond to the oxygens of Asp-25 (left), and Asp-25′ (right) was constrained to hydrogen bond to the proline nitrogen of the amide hydrate. For the EXH′ model, one hydroxyl of the amide hydrate was constrained to hydrogen bond to Asp-25, the other to Asp-25′, and Asp-25 was constrained to hydrogen bond to Asp-25′. All constraints held the hydrogen and the heavy atom 1.9 Å apart with a force constant of 50 kcal/mol·Å$^2$.

## Results and Discussion

*Kinetic Mechanism.* Three of the four oligopeptides used in these studies, AcSQNYPVV-$NH_2$, AcRASQNYPVV-$NH_2$, AcSQSYPVV-$NH_2$, are analogues of the cleavage site (Ser-Gln-Asn-Tyr*Pro-Ile-Val, scissile bond denoted throughout by *) found between the p17–p24$^{gag}$ proteins of the naturally occurring polyprotein substrate of HIV-1 protease, Pr55$^{gag}$ (Casey et al., 1985). As expected, HIV-1 protease cleaved these three substrates between the tyrosyl and prolyl residues. The fourth substrate, AcRKILFLDG-$NH_2$, contains the cleavage site between the reverse transcriptase–endonuclease activities [Arg-Lys-Ile-Leu*Phe-Leu-Asp-Gly (Lightfoote et al., 1986)] in the other polyprotein substrate, Pr160$^{gag\text{-}pol}$, and is cleaved between the leucyl and phenylalanyl residues. The use of a single-point stopped-time assay for measurement of initial rates of HIV-1 protease was justified following two observations: (1) the time courses for the oligopeptide substrates were apparently linear from 0 to 40 min at all concentrations used (0.1–10 mM) at which product formation had not exceeded 18%, such that a single time point obtained within these constraints should accurately reflect an initial rate; (2) a comparison of the kinetic parameters $V/KE_t$ and $V/E_t$ for the substrate Ac-Arg-Lys-Ile-Leu*(*p*-nitro)Phe-Leu-Asp-Gly-$NH_2$ obtained by a continuous spectrophotometric assay of peptidolysis agreed within experimental error to values determined for this same substrate by use of the HPLC-based stopped-time assay under identical conditions (Tomaszek et al., 1990).

Kinetic parameters of the four peptide substrates obtained in MENDT buffer (pH 6.0) were AcSQNYPVV-$NH_2$, $K$ = 5.0 ± 0.5 mM, $V/E_t$ = 29 ± 2 s$^{-1}$, $V/KE_t$ = 5.7 ± 0.5 mM$^{-1}$ s$^{-1}$; AcRASQNYPVV-$NH_2$, $K$ = 3.9 ± 0.9 mM, $V/E_t$ = 29 ± 4 s$^{-1}$, $V/KE_t$ = 7.5 ± 0.9 mM$^{-1}$ s$^{-1}$; AcSQSYPVV-$NH_2$, $K$ = 1.5 ± 0.2 mM, $V/E_t$ = 0.24 ± 0.01 s$^{-1}$, $V/KE_t$ = 0.16 ± 0.01 mM$^{-1}$ s$^{-1}$; and AcRKILFLDG-$NH_2$, $K$ = 2.1 ± 0.2 mM, $V/E_t$ = 7.68 ± 0.04 s$^{-1}$, $V/KE_t$ = 3.6 ± 0.3 mM$^{-1}$ s$^{-1}$. The $V/KE_t$ and $V/E_t$ values reported here for AcSQNYPVV-$NH_2$ and AcRASQNYPVV-$NH_2$ are lower than originally reported for these substrates (Moore et al., 1989; Meek et al., 1989) and may reflect either a loss of enzymatic activity upon storage or that the concentration of active enzyme has been more accurately determined in the present analysis.

Given the similarity of $V/K$ and $V$ for AcRASQNYPVV-$NH_2$ and AcSQNYPVV-$NH_2$, the additional two residues in the nonapeptide substrate appear to have little effect on substrate binding. While the Michaelis constants for all four substrates fall within 1–5 mM, the $V/K$ and $V$ values for AcSQSYPVV-$NH_2$ are considerably lower than the comparable values obtained for the other three substrates, which demonstrates that the asparginyl residue at the P2 position greatly contributes to the competence of this type of substrate.

Both AcRKIL and FLDG-$NH_2$ were competitive inhibitors of HIV-1 protease vs AcRKILFLDG-$NH_2$ (pH 6.0, MENDT buffer, 10% dimethyl sulfoxide): $K_{is}$ = 22 ± 1 mM and 3.3 ± 0.3 mM, respectively. Patterns of AcSQNY vs AcRASQNYPVV-$NH_2$ and AcRASQNY vs AcSQNYPVV-$NH_2$ were linear noncompetitive at pH 6.0 [MENDT buffer; $K_{is}$ = 3.6 ± 1.6 mM, $K_{ii}$ = 1.2 ± 0.39 mM (AcSQNY); $K_{is}$ = 12 ± 1.7 mM and $K_{ii}$ = 4.4 ± 1.3 mM (AcRASQNY)]. The noncompetitive inhibition of the protease by the product inhibitors AcSQNY and AcRASQNY indicates that these products bind to an enzyme–product complex in an ordered manner. However, AcSQNY more potently inhibits HIV-1 protease at pH 3.5 and is in fact a linear uncompetitive inhibitor of the enzyme at this pH ($K_{ii}$ = 0.23 ± 0.04 mM). Plots of $1/v$ vs both AcRASQNY and AcSQNY concentration at a fixed level of AcSQNYPVV-$NH_2$ and AcRASQNYPVV-$NH_2$, respectively, at pH 3–6 demonstrated that inhibition by both products increased linearly with decreasing pH to a plateau value at pH 3.3. These results indicate that the carboxylic acid form of Ac(RA)SQNY (p$K$ = 3.5)[3] binds more tightly to HIV-1 protease than the carboxylate or that the protonation of an enzymatic residue of similar p$K$ enhances the binding of Ac(RA)SQNY. No inhibition was observed by PVV-$NH_2$ (≤20 mM). Also, there was no enhancement of inhibition by variable levels of AcSQNY (0–2.5 mM) at changing fixed levels of PVV-$NH_2$ (0–16 mM).

The observation of competitive inhibition by AcRKIL and FLDG-$NH_2$ and noncompetitive inhibition by both AcRASQNY and AcSQNY are consistent with a uni–bi kinetic mechanism in which AcRKIL and FLDG-$NH_2$ are released randomly from central complexes, while the peptidolytic products of the substrates AcRASQNYPVV-$NH_2$ and AcSQNYPVV-$NH_2$ desorb in an ordered manner in which the release of the carboxylic products AcRASQNY and AcSQNY precedes that of PVV-$NH_2$ (Cleland, 1970). Similar results have been observed in product inhibition studies of porcine pepsin. Noncompetitive inhibition was effected by the Ac-Phe product of the substrate Ac-Phe-Phe-Gly (Kitson & Knowles, 1971), while competitive inhibition resulted from the amino peptide product of a similar substrate Boc-His-(*p*-nitro)Phe-Phe-OMe (Inouye & Fruton, 1968). Alternatively, the products of AcRKILFLDG-$NH_2$ may be released by a rapid-equilibrium ordered mechanism. That AcSQNY and PVV-$NH_2$ do not bind randomly to free enzyme or enzyme–product complexes is further supported by the inability to elicit inhibition of the protease by PVV-$NH_2$ in the presence of

[3] For AcSQNY, a p$K$ value of 3.45 was determined by pH-stat titration.

Table II: Effects of Inorganic Salts on the Peptidolytic Activity of HIV-1 Protease[a]

| salt (mM) | variable substrate | $K$ (mM) | $V_{rel}$ | $V/K_{rel}$ |
|---|---|---|---|---|
| NaCl (200) | AcRASQNYPVV-$NH_2$ | 1.5 ± 0.2 | 1.00 | 1.00 |
| NaCl (800) | | 0.4 ± 0.1 | 1.37 | 2.64 |
| NaBr (200) | AcRASQNYPVV-$NH_2$ | 2.0 ± 0.2 | 0.98 | 0.73 |
| NaBr (800) | | 0.7 ± 0.2 | 0.75 | 1.68 |
| $(NH_4)_2SO_4$ (67) | AcRASQNYPVV-$NH_2$ | 1.2 ± 0.2 | 0.75 | 1.13 |
| $(NH_4)_2SO_4$ (267) | | 0.7 ± 0.3 | 0.74 | 1.98 |
| LiCl (200) | AcRASQNYPVV-$NH_2$ | 2.2 ± 0.2 | 0.90 | 0.63 |
| LiCl (800) | | 0.7 ± 0.1 | 0.66 | 1.55 |
| $NH_4Cl$ (200) | AcRASQNYPVV-$NH_2$ | 1.3 ± 0.1 | 1.20 | 0.63 |
| $NH_4Cl$ (800) | | 0.49 ± 0.08 | 0.92 | 1.88 |
| NaCl (200) | AcRKILFLDG-$NH_2$ | 0.21 ± 0.04 | 1.00 | 1.00 |
| NaCl (800) | | 0.07 ± 0.02 | 1.34 | 4.03 |
| $(NH_4)_2SO_4$ (67) | AcRKILFLDG-$NH_2$ | 0.8 ± 0.2 | 1.02 | 0.97 |
| $(NH_4)_2SO_4$ (267) | | 0.3 ± 0.1 | 0.95 | 2.36 |
| KCl (800) | AcRKILFLDG-$NH_2$ | 0.06 ± 0.01 | 0.96 | 3.21 |

[a] In AED buffer (pH 5.0), 37 °C, as described under Experimental Procedures.

Scheme I

$$EH \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EAH \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EAH' \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EXH \xrightarrow{k_7} EPQH' \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EPQH \xrightarrow{k_{11}} EQH \xrightarrow{k_{13}} EH$$

changing fixed levels of AcSQNY. Since the E–PVV-$NH_2$ complex does not form at pH 6.0 and the ordered release of products does not allow for the formation of the ternary complex E–PVV-$NH_2$–AcSQNY, inhibition of the protease by PVV-$NH_2$ cannot be effected by the presence of the other product inhibitor.

The conversion of a noncompetitive pattern of a product inhibitor to an uncompetitive one at a more favorable pH is unusual, but interpretable. If upon the binding of AcSQNY (P) to E–PVV-$NH_2$ (EQ) the EPQ complex cannot reverse to form EA (that is, peptidolysis is essentially irreversible on the enzyme surface as is demonstrated below), then P should act as a product analogue inhibitor and effect uncompetitive inhibition vs A.[4] As such, the formation of E–AcSQNY–PVV-$NH_2$ complex will not affect the apparent value of $V/K_{AcRASQNYPVV\text{-}NH_2}$, thereby producing an apparently uncompetitive inhibition pattern at all values of pH. The noncompetitive pattern observed at pH 6.0 must then result from mixed dead end-product inhibition, in which the anionic form of AcSQNY combines weakly with both free enzyme ($K_{is}$ = 4 mM) and the E–PVV-$NH_2$ complex, EQ, ($K_{ii}$ = 1 mM), but upon protonation of AcSQNY (or a residue on the enzyme, or both) AcSQNY acts only as a product inhibitor, binding more tightly to E–PVV-$NH_2$ ($K_{ii}$ = 0.2 mM) at the lower pH.

A kinetic mechanism consistent with these data is the uni–bi mechanism shown in Scheme I. The release of the products [P and Q are the products Ac(RA)SQNY and PVV-$NH_2$, respectively] is apparently ordered for the substrates AcSQNYPVV-$NH_2$ and AcRASQNYPVV-$NH_2$ and random for the substrate AcRKILFLDG-$NH_2$ (P and Q are either AcRKIL or FLDG-$NH_2$). EH is free enzyme in its catalytically competent "monoprotonated" form [see Hyland et al. (1991)], A is substrate, EAH′ and EPQH′ are conformational isomers of EAH and EPQH, and X is an intermediate.

Under initial velocity conditions, product release steps ($k_{11}$, $k_{13}$) are considered irreversible, as is the breakdown of the intermediate complex EXH to the ternary EPQH′ complex (the $k_7$ step), on the basis of the discussion below.

*Effects of Ionic Strength on Initial Velocity.* The effects of changing fixed levels of various inorganic salts on the peptidolytic activity of HIV-1 protease were investigated by comparison of the values of $V/K$ and $V$ obtained for AcRASQNYPVV-$NH_2$ and AcRKILFLDG-$NH_2$ (Table II). In these studies, the ionic strength, rather than the concentration of a specific ion, was held constant at 0.2 or 0.8 M. For both substrates, $V/K$ increased 2–4-fold as the ionic strength of each salt was quadrupled, while $V$ was either increased or decreased ≤37%. Relative to the values obtained for AcRASQNYPVV-$NH_2$ at 0.2 M NaCl, no significant changes in the kinetic parameters $V$ and $V/K$ were observed upon substitution of $Na^+$ by $K^+$, $NH_4^+$, or $Li^+$, or substitution of $Cl^-$ by $Br^-$ or $SO_4^{2-}$. This lack of specific activation of HIV-1 protease by an inorganic salt suggests that the activity of the enzyme is dependent on the ionic strength of the reaction mixture, rather than the binding of a specific ion to an enzymatic site.

The activation of HIV-1 protease at increasing ionic strength of NaCl was further investigated. From fitting of initial velocity data (eq 1) of AcRKILFLDG-$NH_2$ (0.1–2.0 mM) in AED buffer (pH 5.0) and AcRASQNYPVV-$NH_2$ (0.5–2.0 mM) in MENDT buffer (pH 6.0) at changing fixed levels of NaCl (0.2–2.0 M), $V/KE_t$ was increased in a nonlinear (concave up) fashion at increasing concentration of NaCl, while $V$ was only slightly increased (Figure 1). Above 0.8 M NaCl, the plots of $V/KE_t$ vs [NaCl] for both peptide substrates sharply departed from linearity, to yield a concave-upward apparently parabolic plot. These results indicate that NaCl is not a saturable activator of HIV-1 protease activity and therefore probably does not bind specifically to the enzyme.

The activation of HIV-1 protease by inorganic salts has been previously reported (Richards et al., 1990). Similar to our findings, Richards et al. have also observed that increasing ionic strength had no effect on $k_{cat}$ while the corresponding $K_m$ values decreased for a chromogenic oligopeptide substrate. Thus, an increase in the solution ionic strength enhanced the specificity constants, $V/K$, of oligopeptide substrates of HIV-1 protease. In view of the fact that HIV-1 protease and many of its oligopeptide substrates and inhibitors contain a preponderance of hydrophobic residues, the increased binding of these oligopeptides to the protease possibly results from an enhancement of favorable hydrophobic interactions between

[4] From Scheme I, the expression for $V/K$ in the presence of a fixed level of AcSQNY (P) is $k_1k_3A/[(k_2 + k_3)[1 + k_2k_4k_6k_8k_{10}(k_{12}P + k_{13})/(k_2 + k_3)k_5k_7k_9k_{11}k_{13} + k_2k_4k_6k_8/(k_2 + k_3)k_5k_7k_9 + k_2k_4k_6/(k_2 + k_3)k_5k_7]]$, when the $k_7$ step is freely reversible and $k_8$ is the rate constant for the reverse direction, and $k_1k_3A/[(k_2 + k_3)[1 + k_2k_4(k_6 + k_7)/(k_2 + k_3)k_5k_7]]$ when the $k_7$ step is irreversible.

Table III: Reverse Reaction and $^3H$ Isotope Exchange of Peptidolysis of AcSQNYPVV-$NH_2$[a]

| reaction conditions | pH | reaction time (min) | recovered tritium (dpm) AcSQNYPVV-$NH_2$ | recovered tritium (dpm) AcSQNY | $F_{cat}$ | $v_{cat}$ $s^{-1}$ |
|---|---|---|---|---|---|---|
| (A) Reverse Peptidolytic Reaction | | | | | | |
| 2 mM [$^3$H]AcSQNY<br>5 mM PVV-$NH_2$<br>0.33 μM HIV-1 protease | 6.0<br>3.6 | 120<br>120 | 14<br>4 | 45 006<br>44 284 | 0.0003<br>0.0001 | 0.00025<br>0.00008 |

| | pH | reaction time (min) | recovered tritium (dpm) Ac-SQNYP-VV-$NH_2$ | recovered tritium (dpm) AcSQNY | $F_x$ | $F_{cat}$ | $v_{ex}/v_{cat}$ |
|---|---|---|---|---|---|---|---|
| (B) Isotope Exchange of [$^3$H]AcSQNY | | | | | | | |
| 10 mM [$^3$H]AcSQNY | 6.0 | 0 | 77 | 37 401 | 0.0 | 0.0 | 0.0 |
| 2 mM AcSQNYPVV-$NH_2$ | | 150 | 112 | 46 956 | 0.0007 | 0.58 | 0.0062 |
| 0.33 μM HIV-1 protease | | 300 | 93 | 31 019 | 0.0005 | 0.96 | 0.0025 |
| | | | | | | | av 0.004 ± 0.002 |
| | 3.6 | 150 | 91 | 42 159 | 0.0003 | 0.17 | 0.009 |
| | | 300 | 97 | 48 318 | 0.0004 | 0.39 | 0.0051 |
| | | | | | | | av 0.007 ± 0.003 |
| 10 mM [$^3$H]AcSQNY<br>2 mM AcSQNYPVV-$NH_2$<br>5 mM PVV-$NH_2$<br>0.33 μM HIV-1 protease | 6.0 | 300 | 95 | 22 109 | 0.0008 | 0.90 | 0.0043 |

[a] $F_x$ = dpm [$^3$H]AcSQNYPVV-$NH_2$/(dpm[$^3$H]AcSQNYPVV-$NH_2$ + dpm [$^3$H]AcSQNY); $F_{cat}$ = mole fraction of product formed from substrate for either forward or reverse peptidolytic reactions, $v_{cat}$ = [[$^3$H]AcSQNYPVV-$NH_2$ (μM)]($F_{cat}$)/[$t$ (s)][HIV-1 protease (μM)], $v_{ex}/v_{cat}$ is nmol of [$^3$H]AcSQNYPVV-$NH_2$ formed/nmol of AcSQNYPVV-$NH_2$ hydrolyzed at the indicated reaction time.

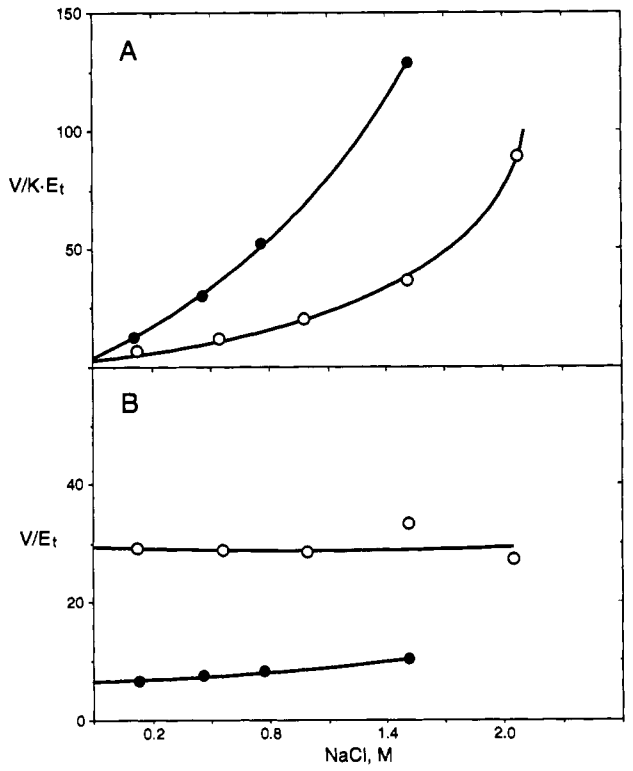


FIGURE 1: Dependence of $V/KE_t$ (A) and $V/E_t$ (B) on changing fixed levels of NaCl concentration for the substrates, AcRKILFLDG-$NH_2$ (pH 5.0; closed circles) and AcRASQNYPVV-$NH_2$ (pH 6.0; open circles).

enzyme and substrates. At high ionic strengths, the oligopeptides may actually be "salting in" to the enzyme's active site, as has been described for chymotrypsin (Paberit, 1990).

*Reverse Peptidolytic Reaction and Isotope Exchange.* The ability of HIV-1 protease to catalyze a synthetic reaction from its peptidolytic products was investigated (Table III). [$^3$H]AcSQNY at 2 mM ($K_{is}$ = 3.6 mM) and 5 mM PVV-$NH_2$ were incubated with HIV-1 protease (360 ng) for 2 h at pH 3.6 and 6.0. Under all conditions, the recovery of tritium in the chromatographic fractions collected in the Ac-SQNYPVV-$NH_2$ peak was negligible, while recovery of the label in the AcSQNY fractions was equal to that applied to the column. Incubation of millimolar concentrations of the product pairs AcRKIL/FLDG-$NH_2$, AcSQNY/PVV-$NH_2$, and AcRASQNY/PVV-$NH_2$ for 120 and 300 min, with a concentration of HIV-1 protease (3.6 μg; 3.3 μM) 100-fold higher than that used in the forward reaction, failed to yield detectable levels of resynthesized AcRKILFLDG-$NH_2$, Ac-SQNYPVV-$NH_2$, or AcRASQNYPVV-$NH_2$ upon analysis by reverse-phase HPLC. Since this method would allow the detection of substrate peaks that are ≥0.5% of those of the products, the rate of synthesis of substrates from the three sets of peptidolysis products by HIV-1 protease was less than 0.0004 $s^{-1}$. This result is unsurprising for Ac(RA)-SQNYPVV-$NH_2$, given the lack of inhibitory activity of PVV-$NH_2$. In contrast, both products of AcRKILFLDG-$NH_2$ are competitive inhibitors of the forward reaction and therefore bind to the enzyme at the concentration ranges used in this experiment. The HIV-1 protease-catalyzed cleavage of these substrates is apparently irreversible at pH 6.0.

However, HIV-1 protease does catalyze the synthesis of AcSQNYFLDG-$NH_2$ from the product inhibitors AcSQNY and FLDG-$NH_2$, as evidenced by the time-dependent formation of a tritium-containing product in the HPLC profile (retention time = 10.5 min; gradient 4) of a reaction mixture containing [$^3$H]AcSQNY, FLDG-$NH_2$, and protease. Upon isolation of this product and analysis by FAB-MS, the molecular weight was found to be $m/z$ = 1006.6, consistent with the $MNa^+$ ion of AcSQNYFLDG-$NH_2$. HIV-1 protease also catalyzed the synthesis of AcSQNYFLDG-$NH_2$ in samples containing only AcSQNYPVV-$NH_2$ and FLDG-$NH_2$, which may be construed as a transpeptidation reaction. The rate of synthesis of AcSQNYFLDG-$NH_2$ from the product inhibitors alone was determined from the time-dependent formation (0–10 min) of this new chromatographic peak in reaction mixtures consisting of 3 mM FLDG-$NH_2$, 4 mM AcSQNY, 5% dimethyl sulfoxide, 3.3 μM HIV-1 protease, and

Table IV: Isotope Partitioning of $^{18}O$ from $H_2{}^{18}O$ in the Peptidolytic Reaction of HIV-1 Protease[a]

| reisolated substrate/product | reaction time (min) | $E_t$ ($\mu$M) | $F_{cat}$[b] | $[M^{18}O]/([M^{16}O] + [M^{18}O])$ | $F_x$[c] | $v_{ex}$ ($s^{-1}$)[c] | $v_{ex}/v_{cat}$ |
|---|---|---|---|---|---|---|---|
| AcSQNY | 300 | 0.16 | 0.98 | 0.76 | | | |
| AcSQNYPVV-$NH_2$ | 60 | 0.16 | 0.45 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 300 | 0.10 | 0.80 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 300 | 0.16 | 0.97 | 0.06 ± 0.007 | 0.08 ± 0.009 | 0.04 ± 0.005 | 0.08 ± 0.01 |
| AcRKILFLDG-$NH_2$ | 300 | 0.06 | 0.40 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 300 | 0.08 | 0.73 | 0.01 | 0.01 | 0.03 | 0.01 |
| | 300 | 0.16 | 0.93 | 0.085 ± 0.007 | 0.11 ± 0.009 | 0.071 ± 0.004 | 0.12 ± 0.008 |
| AcSQNY/AcSQNYFLDG-$NH_2$[d] | 300 | 3.3 | 0.99 | 0.74/0.50 ± 0.13 | | | |

[a] Reactions conditions and isolation of substrates and products for FAB-MS analysis are as described under Experimental Procedures. Samples contained 5 mM substrate, 78% $H_2{}^{18}O$, and GAMT-NEDT buffer (pH 6.0), 37 °C. [b] $F_{cat}$ is the mole fraction of product formed from substrate; values of $[M^{18}O]/([M^{16}O] + [M^{18}O])$ ($F_1$) were obtained in duplicate or in triplicate; M represents either the $MNa^+$ or the $MH^+$ species: $m/z$ = 869.9 for AcSQNYPVV-$NH_2$ ($MNa^+$) and $m/z$ = 1002.6 for AcRKILFLDG-$NH_2$ ($MH^+$). Subtracted from these values were identical control samples that contained 10 $\mu$M Ala-Ala-Phe$\Psi$[CHOHCH$_2$]Gly-Val-Val-OMe. In addition control samples were performed in 100% $H_2{}^{16}O$ in which the reaction had proceeded to 90%, and data from the samples in $H_2{}^{18}O$ were corrected for the M + 2 peak intensities found in these control samples. [c] $F_x = [F_1(t) - F_1(t = 0)]/[F_1(t = eq) - F_1(t = 0)]$, in which $F_1(t = 0) = 0.0$ and $F_1(t = eq) = 0.76$ in reaction mixtures containing 78% $H_2{}^{18}O$, respectively. $v_{ex}/v_{cat}$ is nmol of [$^{18}O$]AcSQNYPVV-$NH_2$/nmol of AcSQNYPVV-$NH_2$ hydrolyzed at the indicated reaction time and pH value, and $v_{ex} = [F_{cat}/\ln(1 - F_{cat})][\text{AcSQNYPVV-NH}_2(\mu M)][\ln(1 - F_x)]/[t\text{ (s)}][\text{HIV-1 protease }(\mu M)]$ (Litwin & Wimmer, 1979). [d] $^{18}O$ content determined in samples of AcSQNY ($m/z$ for $MNa^+$ species of $M^{16}O$ = 575) and AcSQNYFLDG-$NH_2$ ($m/z$ for $MNa^+$ species of $M^{16}O$ = 1006.6) recovered from a reaction mixture containing 3 mM AcSQNYPVV-$NH_2$, 3 mM FLDG-$NH_2$, and 5% dimethyl sulfoxide (pH 6.0). Values for $[M^{18}O]/([M^{18}O + M^{16}O])$ were obtained from duplicate determinations and were corrected for the corresponding peak intensities found in a replicate sample prepared in $H_2{}^{16}O$.

GAMT-NEDT buffer (pH 6.0) and was found to be 0.014 $s^{-1}$. With the addition of 4 mM AcSQNYPVV-$NH_2$, this rate of peptide synthesis was considerably higher ($v$ = 0.036 $s^{-1}$), possibly due to the increased amounts of AcSQNY available upon cleavage of the substrate. In this second experiment, the ratio of nanomoles of AcSQNYPVV-$NH_2$ hydrolyzed/nanomole of AcSQNYFLDG-$NH_2$ formed was calculated from the individual chromatographic peaks to be 0.0031. It is clear from these results that AcSQNYPVV-$NH_2$ is not necessary for the synthesis of AcSQNYFLDG-$NH_2$, thereby demonstrating that the synthesis of AcSQNYFLDG-$NH_2$ cannot uniquely be the result of a transpeptidation process.

The ability of HIV-1 protease to catalyze isotope exchange of [$^3$H]AcSQNY into AcSQNYPVV-$NH_2$ was also investigated (Table III). Isotope exchange was determined at both pH 3.6 and 6.0 in reaction mixtures containing 10 mM AcSQNYPVV-$NH_2$, 2 mM [$^3$H]AcSQNY and 360 ng of HIV-1 protease, with or without 5 mM PVV-$NH_2$, at various fractions of complete peptidolysis ($F_{cat}$ = 0–0.96). The exchange of tritium into the chromatographically purified AcSQNYPVV-$NH_2$ was negligible in all cases ($v_{ex}/v_{cat} \leq 0.009$). Since incorporation of [$^3$H]AcSQNY into substrate was either invariant or actually decreased at increasing incubation time (150 and 300 min), it is unlikely that the tritium found in the isolated substrate results from enzyme-catalyzed exchange but instead could be due to artifactual coelution of [$^3$H]AcSQNY in the AcSQNYPVV-$NH_2$ chromatographic peak. For this reason, the values of $v_{ex}/v_{cat}$ reported in Table III may be considered upper limits for the exchange reaction rate. Thus, the binding of AcSQNY to the putative E–PVV-$NH_2$ complex at either pH 6.0, or more favorably at pH 3.6, failed to produce detectable AcSQNYPVV-$NH_2$. Although AcSQNY apparently binds to form an E–AcSQNY–PVV-$NH_2$ central complex, HIV-1 protease does not catalyze peptide synthesis or isotope exchange in the AcSQNYPVV-$NH_2$–AcSQNY substrate–product pair, and therefore the HIV-1 protease-catalyzed cleavage of the tyrosyl–prolyl bond of AcSQNYPVV-$NH_2$ is apparently irreversible, while the formation of a tyrosyl–phenylalanyl bond was observable.

*Isotope Partitioning of $H_2{}^{18}O$.* The extent of incorporation of $^{18}O$, derived from $H_2{}^{18}O$, into an oligopeptide substrate as catalyzed by HIV-1 protease would provide a sensitive measure of the partitioning of the presumed enzyme-bound tetrahedral adduct of substrate and $H_2O$ between substrate and product formation. Porcine pepsin was found to catalyze $^{18}O$ exchange from $H_2O$ into its transpeptidation products (Antonov et al., 1978) and into "nonhydrolyzable" peptide substrates (Antonov et al., 1981). However, no data exist for $^{18}O$ exchange from $H_2{}^{18}O$ into the scissile carbonyl oxygen of a competent substrate of an aspartic protease. Hence isotope partitioning was investigated by using mass spectrometric analysis of the reisolated substrates AcSQNYPVV-$NH_2$ and AcRKILFLDG-$NH_2$ following treatment of these substrates by HIV-1 protease in reaction mixtures highly enriched in $H_2{}^{18}O$ (Figure 2, Table IV). The increase in relative intensities of the $M^{18}O$ species of AcSQNYPVV-$NH_2$ and AcRKILFLDG-$NH_2$ (molecular ion peak + 2) was used to monitor enrichment of $^{18}O$ into either substrate during peptidolysis.

Incubation of the product AcSQNY in 78% $H_2{}^{18}O$ at pH 6.0, 37 °C for 300 min in the absence of enzyme resulted in relative peak intensities of 100, 12, and 0 for the M, M + 2, and M + 4 species, respectively. These intensities reflect the naturally occurring distribution of these isotopic species and nonenzymatic exchange. Exchange of $^{18}O$ into 5 mM AcSQNY (pH 6.0, 37 °C) occurred to the extent of 3% [[M + 2]/([M + 2] + [M]) = 0.03] following incubation with 0.16 $\mu$M HIV-1 protease over 300 min. Therefore, the enzyme catalyzed some exchange of $^{18}O$ from $H_2{}^{18}O$ into AcSQNY, as has been observed for the carboxylic products of pepsin peptidolysis (Antonov et al., 1978).

Under identical conditions, the protease-catalyzed hydrolysis of AcSQNYPVV-$NH_2$, ($\leq$0.16 $\mu$M protease) in 78% $H_2{}^{18}O$ resulted in the incorporation of 1 mol of $^{18}O$ per mole of AcSQNY formed, as indicated by the mole fractions of $^{18}O$ in the resulting AcSQNY: [M + 2]/([M] + [M + 2]) = 0.76 (Table IV). Analysis of the reisolated [$^{18}O$]AcSQNY by FAB tandem MS (Biemann, 1990) demonstrated that as expected, all of the $^{18}O$ resided in the tyrosyl residue of this peptide, as indicated by the appearance of a consistent M + 2 species accompanying each successive fragment which contained the tyrosyl residue. The average fractional enrichment of the M + 4 species in AcSQNY (which reflects the incorporation of two atoms of $^{18}O$ in the product) is 0.04; this value actually decreased at an increasing extent of peptidolytic reaction. This result suggests that the M + 4 species arises from either nonenzymatic exchange or the enzyme-catalyzed incorporation

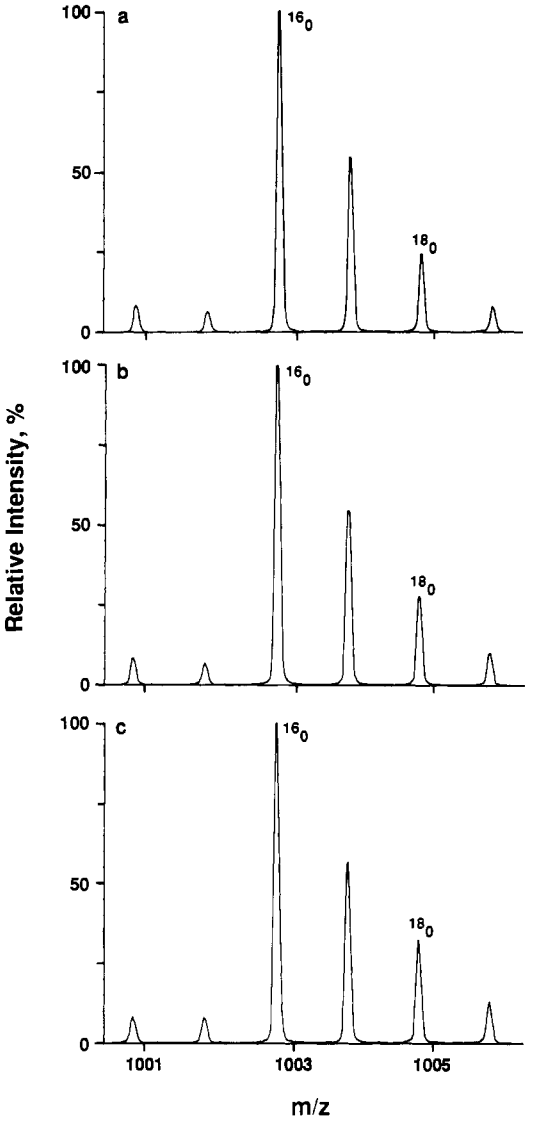


FIGURE 2: Protease-catalyzed incorporation of $^{18}O$ from $H_2{}^{18}O$ into AcRKILFLDG-$NH_2$. FAB-MS of the reisolated AcRKILFLDG-$NH_2$ were obtained at an instrumental resolution of 10000 as described under Experimental Procedures. The relative intensities of the $MH^+$ pseudomolecular ion species are shown ($m/z$ = 1002.6 and 1004.6 for the $^{16}O$- and $^{18}O$-containing substrates, respectively). (a) Control sample (5 mM substrate, 0.16 $\mu$M protease, 10 $\mu$M Ala-Ala-Phe$\Psi$-[CHOHCH$_2$]Gly-Val-Val-$NH_2$, pH 6.0, $t$ = 300 min, $F_x = F_{cat}$ = 0.0); (b) 5 mM substrate, 0.08 $\mu$M protease, pH 6.0, $t$ = 300 min, $F_x$ = 0.01, $F_{cat}$ = 0.73; (c) 5 mM substrate, 0.16 $\mu$M protease, pH 6.0, $t$ = 300 min; $F_x$ = 0.11, $F_{cat}$ = 0.93.

of a second atom of $^{18}O$ into the [$^{18}O$]AcSQNY product that is formed during the course of the reaction, in accord with the 3% exchange observed when the product was incubated with enzyme alone.

In the reisolated substrates, protease-catalyzed incorporation of $^{18}O$ into both AcSQNYPVV-$NH_2$ and AcRKILFLDG-$NH_2$ was observed after incubation for 300 min at increasing concentrations of HIV-1 protease and only at high values of substrate turnover ($F_{cat} \geq 0.7$) (Figure 2; Table IV). Protease-catalyzed incorporation of $^{18}O$ into these two substrates was indicated by the enrichment of the M + 2 (M$^{18}$O) species relative to those of matching control samples that contained a potent competitive inhibitor of the protease (in these control samples, the relative intensities of the M and M + 2 species were 100 and 13–22, respectively). For either substrate, the enzyme-catalyzed isotopic exchange occurred to a small extent (1–11%, Table IV), and its detection apparently required the depletion of most of the $^{16}O$-labeled substrate. The fraction of $^{18}O$ exchange into AcSQNYPVV-$NH_2$ ($F_x$) was determined from the fraction of uncatalyzed incorporation [$F_1$ ($t$ = 0); the fraction of $^{18}O$ incorporated in the presence of the inhibitor) and the calculated $F_1$ at equilibrium ($F_{eq}$ = fraction of $^{18}O$ in solvent $H_2O$). The exchange velocities $v_{ex}$ (average values: 0.04–0.07 $s^{-1}$) for the two substrates were calculated by using the equation of Litwin and Wimmer (1979), which corrects for depletion of the substrates during the exchange reaction (Table IV). The partitioning of the enzyme-bound adduct of substrate and $H_2{}^{18}O$ between re-formation of the substrate and breakdown to products was obtained from the ratio $v_{ex}/v_{cat}$ and equaled 0.08 ± 0.01 for AcSQNYPVV-$NH_2$ and 0.01–0.12 for AcRKILFLDG-$NH_2$. In samples containing 3 mM each of FLDG-$NH_2$ and AcSQNYPVV-$NH_2$ in 78% $H_2{}^{18}O$, the protease-catalyzed incorporation of one atom of $^{18}O$ into AcSQNY and AcSQNYFLDG-$NH_2$ was found to be 74% and 50 ± 13 %, respectively, on the basis of the ratio [M$^{18}$O]/([M$^{18}$O] + [M$^{16}$O]) obtained in $H_2{}^{18}O$ (and corrected by use of identical samples conducted in $H_2{}^{16}O$), in which M is the $MNa^+$ molecular ion for the respective reisolated products (Table IV). These results demonstrated that incorporation of $^{18}O$ into the product of peptide synthesis, [$^{18}O$]AcSQNYFLDG-$NH_2$, was 65% of that found in the peptidolysis product [$^{18}O$]AcSQNY.

*Kinetic Characterization of the Enzyme-Bound Reaction Intermediates.* From Tables III and IV, a comparison of the average values of $v_{ex}/v_{cat}$ for isotope exchange of [$^3$H]AcSQNY (≤0.004 at pH 6.0; $F_{cat}$ = 0.96) and $^{18}O$ isotope partitioning from $H_2O$ into AcSQNYPVV-$NH_2$ (0.08 ± 0.01 at pH 6.0; $F_{cat}$ = 0.97) revealed that the rate of incorporation of $^{18}O$ into substrate from $H_2{}^{18}O$ exceeded the rate of isotope exchange of [$^3$H]AcSQNY into substrate by at least 20-fold. For AcRKILFLDG-$NH_2$, a value as high as $v_{ex}/v_{cat}$ = 0.12 was obtained, while the resynthesis of this substrate from its products could not be demonstrated. This indicates that the enzyme-bound intermediate (X in Scheme I) that gives rise to the $^{18}O$ exchange is kinetically competent (Rose, 1979), since its rate of formation is faster than either the reverse peptidolytic reaction or the isotope exchange reaction in which enzyme-bound [$^3$H]AcSQNY is incorporated into AcSQNYPVV-$NH_2$. Furthermore, the $^{18}O$ exchanged into AcSQNYPVV-$NH_2$ could not have come from [$^{18}O$]AcSQNY as indicated by the absence of isotope exchange or peptide synthesis. Product inhibition studies have demonstrated that AcSQNY binds to an enzyme–product complex to form a ternary E–AcSQNY–PVV-$NH_2$ complex (EPQH in Scheme I).

Under experimental conditions of tritium isotope exchange, AcSQNYPVV-$NH_2$ is turned over, such that steady-state levels of all enzyme transitory complexes are established. The binding of added [$^3$H]AcSQNY (P) to the steady-state level of EQH results in an enzyme–products complex (EPQH ↔ EPQH′) from which the formation of EAH proceeds at a rate which is at most 1/20 of that observed for the EXH complex. Thus, the formation of the EXH complex is depicted as reversible in Scheme I, while the EPQH complex cannot return to EAH. It is reasonable to assume that the isomerization of the E–AcSQNY–PVV-$NH_2$ (EPQH) complex to EPQH′ is reversible. Given that, the lack of isotope exchange of [$^3$H]AcSQNY into AcSQNYPVV-$NH_2$ would result from the inability of the enzyme to catalyze the re-formation of the tyrosyl–prolyl bond (reverse of the $k_7$ step).

The observation of enzyme-catalyzed transpeptidation reactions by porcine pepsin led to the proposal that two types

of covalent intermediates could occur in the reaction pathway of this aspartic protease: an "acyl–enzyme", resulting from the acylation of an active-site aspartyl residue by the scissile carbonyl group of the peptide substrate, and an "amino–enzyme", resulting from the subsequent rearrangement of this acyl–enzyme to form an amide of the enzymic $\beta$-carboxylate and the amino peptide fragment (Takahashi et al., 1974; Newmark & Knowles, 1975; Wang & Hofmann, 1976). For HIV-1 protease, the observation of transpeptidation of the carboxylic peptide product would require that the amino peptide product desorb from the acyl–enzyme complex and be replaced by a second amine, in lieu of hydrolysis of the acyl–enzyme to form the carboxylic product. Such a mechanism could account for the formation of AcSQNYFLDG-$NH_2$ from FLDG-$NH_2$ and AcSQNYPVV-$NH_2$. However, Antonov and co-workers subsequently demonstrated facile $^{18}O$ exchange from $H_2{}^{18}O$ into transpeptidation products of pepsin, thereby providing direct evidence against the participation of covalent intermediates in the transpeptidation activity of pepsin since the acylation of an amine product by the acyl–enzyme would necessarily preclude the involvement of $H_2{}^{18}O$ (Antonov et al., 1978, 1981).

Similarly, the present results allow us to address the possibility of covalent catalysis in the chemical mechanism of HIV-1 protease. The observation of $^{18}O$ exchange into substrate under conditions in which the re-formation of the tyrosyl–prolyl bond cannot be measured argues strongly against the involvement of a covalent intermediate based on the following grounds: nucleophilic attack of Asp-25, instead of $H_2O$, on the scissile carbonyl of the peptide substrate would result in the formation of an acyl–enzyme intermediate and the amino peptide product in the EXH complex. Minimally, the subsequent hydrolysis of this mixed anhydride to form the carboxylate peptide product dictates that the attack of $H_2{}^{18}O$ on this enzyme-bound intermediate only occurs after the scission of the tyrosyl–prolyl bond. In such a sequence of events, the rate of incorporation of $^{18}O$ into re-formed substrate would be no faster than that of the resynthesis of the tyrosyl–prolyl bond unless this hydrolysis of the acyl–enzyme was irreversible. In that case, $^{18}O$ incorporation into substrate would result from the reversal of the tetrahedral adduct of the acyl–enzyme and $H_2{}^{18}O$, which should be an oxyanion, and presumably the free aspartyl residue would serve to deprotonate $H_2{}^{18}O$ to facilitate formation of this intermediate. By microscopic reversibility of this complex, $^{16}O$, the anionic oxygen, should be retained in the substrate since its loss as $H_2O$ would require two protons where only one would be available from the aspartyl residue that was protonated by the lytic $H_2{}^{18}O$.

The protease-catalyzed "transpeptidation" of AcSQNYFLDG-$NH_2$ from AcSQNYPVV-$NH_2$ and FLDG-$NH_2$ could either result from a pathway involving an acyl–enzyme intermediate or from a noncovalent reverse reaction of the available AcSQNY and FLDG-$NH_2$. From the discussion above, the observation that the relative rate of enzyme-catalyzed exchange of $^{18}O$ into AcSQNYPVV-$NH_2$ compared to substrate hydrolysis ($v_{ex}/v_{cat} = 0.08$) exceeds by 30-fold the relative rate of synthesis of AcSQNYFLDG-$NH_2$ (0.003 nmol formed/nmol of AcSQNYPVV-$NH_2$ hydrolyzed) argues against an acyl–enzyme intermediate in the catalytic pathway. Assuming that the kinetics of the formation of AcSQNYFLDG-$NH_2$ reflect the re-formation of a carbon–nitrogen bond in an enzyme complex, then the greater rate of $^{18}O$ exchange into substrate supports a mechanism in which the involvement of the lytic $H_2O$ precedes the cleavage of a carbon–nitrogen bond. As additional evidence against an acyl–enzyme intermediate, the incorporation of $^{18}O$ into AcSQNYFLDG-$NH_2$ demonstrates that AcSQNYFLDG-$NH_2$ is formed from the products [$^{18}O$]AcSQNY and FLDG-$NH_2$ and not from a covalent enzyme–AcSQNY complex and FLDG-$NH_2$. This finding is similar to that of porcine pepsin (Antonov et al., 1981). Moreover, since the incorporation of $^{18}O$ into AcSQNYFLDG-$NH_2$ was within experimental error of 50% (65 ± 17%) of that $^{18}O$ found in the peptidolytic product AcSQNY, a random incorporation of isotope from the singly labeled carboxylic group of [$^{18}O$]AcSQNY is occurring, consistent with the reaction of the free products FLDG-$NH_2$ and AcSQNY to form AcSQNYFLDG-$NH_2$.

A reasonable interpretation of the results in this report is that the chemical mechanism of HIV-1 protease is identical with the noncovalent mechanism proposed for pepsin and penicillopepsin by numerous investigators (Suguna et al., 1987; Bott et al., 1982; James & Sielecki, 1985; Polgar, 1987), in which the two active-site aspartyl residues play opposing general acid–general base roles to activate the scissile carbonyl as an electrophile and $H_2O$ as a nucleophile. The enzyme-bound intermediate in the EXH complex of Scheme I would therefore be the amide hydrate product of peptide and $H_2O$. In addition, the incorporation of an atom of $^{18}O$ from $H_2{}^{18}O$ into substrate requires that the rate of formation and breakdown of the EXH complex exceeds that of its decomposition to products. As derived from Scheme I by the method of net rate constants (Cleland, 1975), the ratio of the rate of substrate turnover to the rate of $^{18}O$ incorporation into AcSQNYPVV-$NH_2$, $v_{cat}/v_{ex} = 12.5$, is a partitioning constant that provides an estimate of the forward catalytic commitment ($c_f$; Northrop, 1977; Cook & Cleland, 1981) according to

$$v_{cat}/v_{ex} = k_7/k_{6'} = k_7/k_6[1 + k_5/k_4(1 + k_3/k_2)] = 12.5 \quad (5)$$

Shown in Figure 3 is a proposed chemical mechanism for HIV-1 protease that accounts for the incorporation of $^{18}O$ into substrate. The experimental results from which this mechanism is derived are discussed in detail in the accompanying paper (Hyland et al., 1991). In the EAH′ complex, the aspartyl residues Asp-25 and Asp-25′ catalyze the formation of the amide hydrate intermediate in EXH by two concerted proton transfers. The transfer of a proton from Asp-25 to the carbonyl oxygen to form an amide hydrate intermediate, as opposed to a tetrahedral oxyanionic one, is crucial to the observation of $^{18}O$ incorporation. In EXH, the general acid–general base roles of the aspartyl groups are now reversed from those in the free enzyme, such that $^{16}O$ would be retained in the re-formed substrate upon dehydration via a pathway of microscopic reversibility (reaction steps defined by $k_{6'}$). Collapse of the tetrahedral intermediate (pathway a, the $k_7$ step) results in the incorporation of one atom of $^{18}O$ into product.

We propose that in order for the $^{18}O$ atom to be incorporated into substrate, the protonation states of the aspartyl residues in the EXH complex must be reversed, such that the resulting carboxylate can deprotonate the $^{18}O$-bearing hydroxyl group of the intermediate while protonation of the $^{16}O$-bearing hydroxyl group by the catalytic carboxylic acid results in its loss as $H_2O$. The symmetrical nature of both the active-site aspartyl groups of the enzyme, which share a proton in the free enzyme, and the putative amide hydrate intermediate suggests that this may be accomplished in the EXH complex by (1) proton transfer from Asp-25′ (pathway b) to Asp-25 in EXH′, followed by (2) positioning of the proton on Asp-25 for transfer to the $^{16}O$-containing hydroxyl group. Since the

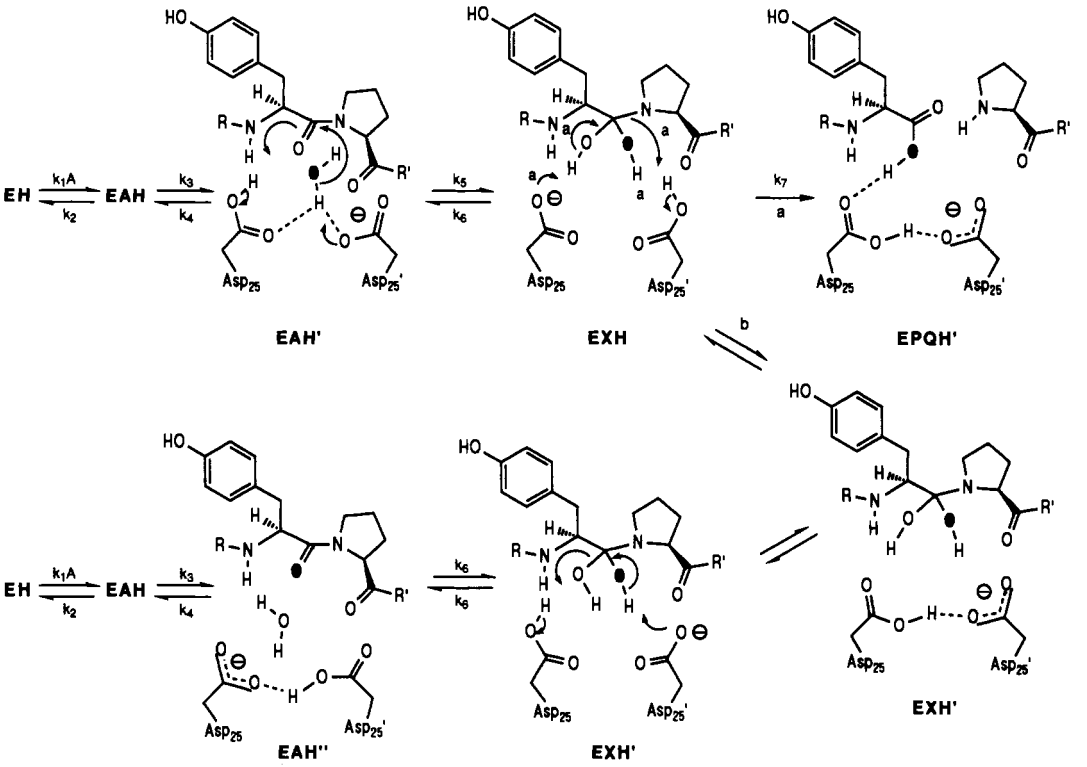


FIGURE 3: Proposed mechanism for protease-catalyzed incorporation of $^{18}O$ (filled oxygen atom) from $H_2{}^{18}O$ into peptide substrate. The chemical mechanism is based on results in this and the accompanying paper. Enzyme complexes are as described in Scheme I. Pathways a and b depict those mechanistic steps that result in product formation and isotope incorporation, respectively.

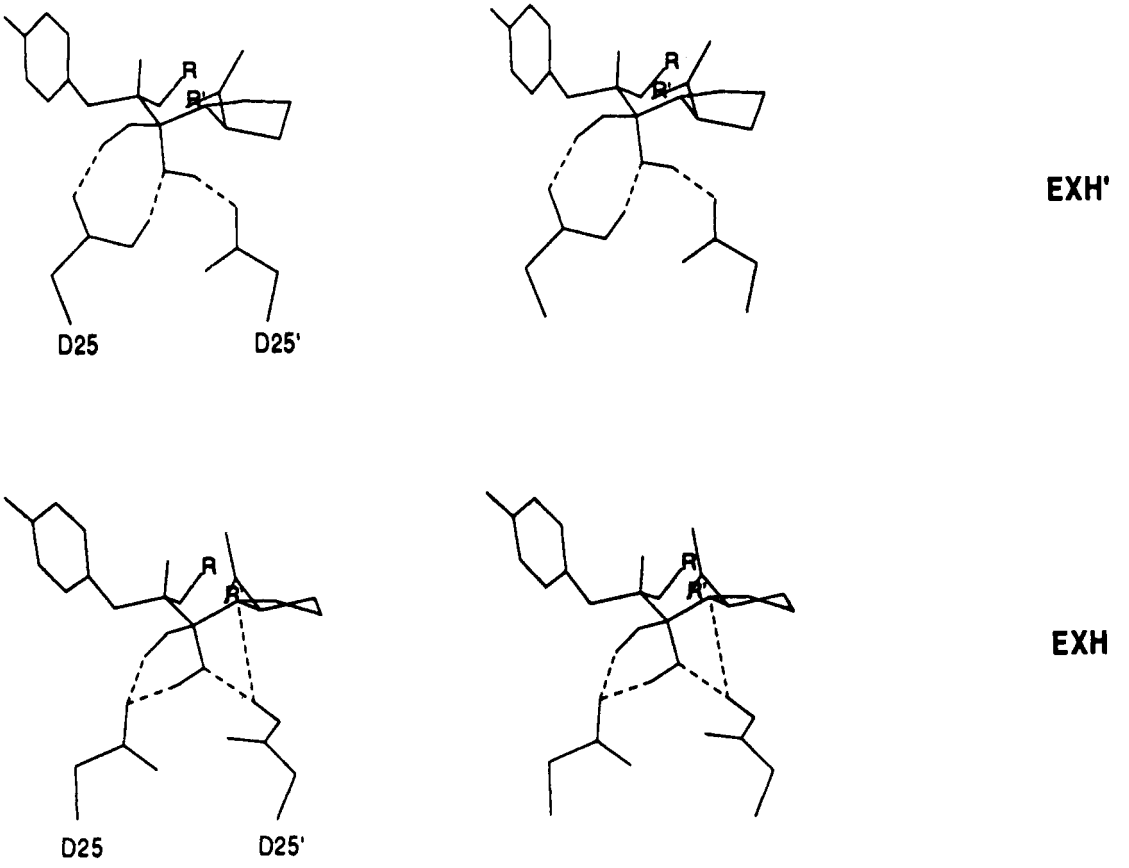


FIGURE 4: Stereodiagrams of energy-refined structural models for the enzyme-bound amide hydrate intermediates EXH and EXH′ as shown in Figure 3. The models of the amide hydrates of the Tyr-Pro dipeptide were constructed from crystallographic data as described under Experimental Procedures. D25 and D25′ correspond to Asp-25 and Asp-25′ in Figure 3. Dotted lines depict interatomic distances of (from left to right) 1.7, 1.8, and 1.7 Å for EXH′ and 1.7, 1.7, 1.8, and 3.1 Å for EXH.

EXH′ complex is chemically equivalent to EXH, its reversal to form the $^{18}O$-bearing substrate is likely to proceed with rate constants identical with those of the upper pathway. Assuming that the proton transfer in pathway b is rapid, the partitioning

ratio $v_{cat}/v_{ex}$ should provide a good estimate of $c_f$ for the catalytic pathway (eq 5).

In order to explore the energetics of the proposed enzyme–intermediate complex, we have constructed two structural models of the putative enzyme-bound amide hydrate intermediate of AcSQNYPVV-$NH_2$ (Figure 4) from the X-ray crystallographic structure of the complex of HIV-1 protease and a peptide analogue inhibitor containing a Phe-Pro hydroxyethylamine isostere (Swain et al., 1990). These two models are shown in Figure 4; in the EXH′ model the proton on Asp-25 (D25) was initially hydrogen bonded to the β-carboxylate of Asp-25′ (D25′), while in the EXH model the proton on Asp-25′ was initially placed in a conformation that would result immediately following its transfer from the lytic $H_2O$ molecule upon the formation of the amide hydrate intermediate. The two stereodiagrams complement the complexes EXH′ and EXH, respectively, in Figure 3. The final molecular mechanics energies are similar for the two systems, that is, the calculations indicate that EXH and EXH′ are isoenergetic.

The proton on Asp-25 of the EXH′ model is more closely associated with the hydroxyl group of the amide hydrate intermediate that resulted from the lytic water (1.8 Å) than either oxygen atom of Asp-25′. Rotation about the Cβ–Cγ bond of Asp-25 would allow protonation of the other hydroxyl group of the intermediate as detailed in Figure 3. The source of the proton for the departing proline amine is not clear from the EXH′ structure. For a similar structure, Bott et al. (1982) have suggested that the unprotonated aspartyl residue could assist in the transfer of the proton from the hydroxyl group of the amide hydrate (within 1.7 Å of Asp-25′ in Figure 4) to the proline. However, the proton inventory of such a mechanism would implicate the transfer of a single proton, contrary to results reported in the accompanying paper (Hyland et al., 1991). James and Sielecki (1985) have suggested that protonation of the amine in such a complex could be accomplished by the solvent.

For the structure EXH in Figure 4, the proton on Asp-25′ is within 3.1 Å of the proline nitrogen, while an oxygen atom of Asp-25 is within 1.7 Å of both hydroxyl hydrogen atoms of the amide hydrate. Collapse of this intermediate is proposed to occur via two simultaneous proton transfers: one from Asp-25′ to the proline nitrogen and the other from a hydroxyl group to Asp-25 (Figure 3). It is presumed that the substitution of the amide carbonyl with a hydroxyl group would enhance the basicity of the proline nitrogen due to loss of resonance, thereby facilitating transfer of this proton from Asp-25′ to the proline nitrogen during scission of the C–N bond. While the proton on Asp-25′ is not close enough to the proline nitrogen to form a strong hydrogen bond in the model of the intermediate, movement in the transition state may bring them into proximity, possibly upon pyramidal inversion of the proline nitrogen. Asp-25 is in an excellent position to assist the scission of the C–N bond by the concomitant deprotonation of one of the hydroxyl groups of the amide hydrate intermediate. The fact that both hydroxyl groups of the amide hydrate are held in a hydrogen-bonding arrangement by Asp-25 in the energy-refined model of EXH, such that both O–H bonds are antiperiplanar to the C–N bond, allows for developing lone-pair orbitals of either oxygen atom to remain antiperiplanar to the C–N bond, upon its deprotonation, in accord with the concept of stereoelectronic control for the breakdown of a tetrahedral intermediate (Deslongchamps, 1975).

## ACKNOWLEDGMENTS

We thank Dr. Walter Holl for performing p*K* determinations, Professor Paul F. Cook for critical reading of the manuscript and helpful suggestions, Professor Frank M. Raushel for useful suggestions, and Dr. Brian W. Metcalf for support of this research. We gratefully acknowledge Dr. Alexander Wlodawer for providing structural coordinates (and the corresponding manuscript) of an HIV-1 protease–inhibitor complex prior to publication.

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