Use of Nitrogen-15 Kinetic Isotope Effects To Elucidate Details of the Chemical Mechanism of Human Immunodeficiency Virus 1 Protease

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ABSTRACT: We have used ^{15}N kinetic isotope effects of the HIV-1 protease-catalyzed peptidolysis of Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 to characterize the chemical mechanism of this enzyme. In addition, the multiple isotope effects have been determined by measuring the ^{15}N kinetic isotope effects in both H_2O and D_2O. The isotope effects, measured on values of V/K, were determined by the incorporation of a radiolabel (tritium and ^{14}C in peptides bearing the heavy and light isotopes, respectively) at a position remote from the isotopically labeled scissile peptide bond, such that the isotope effect was determined by measurement of the change in the $^{14}C/^{3}H$ ratio in recovered substrates at various fractions of reaction. At pH = 6.0 (37 °C), the nitrogen isotope effects were slightly, but significantly, inverse in both solvents: $^{15}(V/K)_{H_2O} = 0.995 \pm 0.002$, and $^{15}(V/K)_{D_2O} = 0.992 \pm 0.003$. The observation of an inverse nitrogen kinetic isotope effect implies that bonding to the nitrogen atom is becoming stiffened in a reaction transition state, and since this inverse isotope effect is enhanced in D_2O, this isotope effect likely arises from protonation of the proline nitrogen atom. We have used these data to formulate a chemical mechanism for HIV-1 protease with the following reaction sequence: (a) establishment of a low-barrier hydrogen bond from an active site aspartyl residue to the carbonyl oxygen of the scissile amide bond to form an "*O*-protonated amide" intermediate, (b) attack of water to produce the amide hydrate intermediate, (c) protonation of the proline nitrogen and deprotonation of a gem-diol hydroxyl group to form a zwitterion intermediate, and (d) breakdown of the zwitterion intermediate to yield reaction products.

Retroviruses encode a unique, homodimeric, aspartyl protease which processes to maturation the essential enzymes and internal structural proteins of the virion (Pearl, 1990). The retroviral protease of the human immunodeficiency virus, type 1 (HIV-1),[1] consists of two 99 amino acid polypeptides, each of which contains one of the two active site aspartyl groups which are found in the bottom of a deep cleft into which substrates bind (Wlodawer et al., 1989; Miller et al., 1989). Considerable evidence now exists that the inhibition of HIV-1 protease during its maturation within infected human T-lymphocytes will render the resulting virions noninfectious and replication-incompetent (Meek et al., 1990; Roberts et al., 1990). As a result, HIV-1 protease has been the subject of intense research since its initial characterization, particularly with respect to the discovery of specific inhibitors of the enzyme (Meek, 1992).

As an important adjuvant to inhibitor design, our laboratory sought a thorough characterization of the chemical mechanism of HIV-1 protease. With the oligopeptide substrate Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2, the protease was studied in terms of its pH rate dependence, solvent kinetic isotope effects, nonequilibrium isotope exchange, and isotope partitioning of $H_2{}^{18}O$ into both product and recovered substrate (Hyland et al., 1991a,b). We showed that (a) the two active site aspartyl groups, in opposite states of protonation, constitute a general acid and general base catalyst for the reaction; (b) the formation of an enzyme-bound "amide hydrate" reaction intermediate is reversible and precedes slower reaction steps which include C–N bond cleavage; and (c) C–N bond cleavage is not readily reversible by the enzyme-bound products of this substrate. These findings place tight constraints on the chemical mechanisms available to the protease. For example, the involvement of a covalent enzyme–peptide intermediate was shown to be inconsistent with the isotope partitioning data, and the apparent irreversibility of C–N bond cleavage on the enzyme indicates that the reaction steps that limit the value of the kinetic parameter V/K may be different from those that limit V. In addition, we measured the solvent isotope effects for this substrate (Hyland et al., 1991b).

However, by themselves, solvent isotope effects provide limited information about the relative rates of the prototropic steps since one cannot be certain which steps are or are not solvent isotope sensitive (D_2O sensitive) or which intrinsic isotope effects are likely to be expressed on the proton transfer of a given reaction step. In the current report, we use nitrogen kinetic isotope effect studies of the previously studied peptide substrate of HIV-1 protease, Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2. These studies were performed in conjunction with the use of the multiple isotope effects method (Hermes et al., 1982; Cleland, 1991). In this case, the ^{15}N kinetic isotope effects were measured in both H_2O and D_2O to determine if these isotope-sensitive steps involved proton transfers which could be slowed down by substitution with deuterium.

The low value of k_{cat}/K_m (V/KE_t) for this substrate (5700 M^{-1} s^{-1}; Hyland et al., 1991a) suggested that its catalytic commitment factor[2] is sufficiently low to allow the expression of the intrinsic isotope effects on the observed isotope effects.

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[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HIV-1, human immunodeficiency virus type 1; HPLC, high-performance liquid chromatography; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

[2] The catalytic commitment is the ratio of the forward rate constant for the isotope-sensitive step to the net rate constant for release of substrate from the enzyme (Northrop, 1977; Cleland, 1982).

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The results of these studies allowed us to evaluate the catalytic commitment factors for the reaction and to demonstrate that proton transfers occur within the reaction steps that give rise to the isotope effects on nitrogen. In addition, we were able to describe in part from the ^{15}N kinetic isotope effects the chemistry of the cleavage of the carbon–nitrogen bond of the enzyme-catalyzed peptidolysis reaction.

EXPERIMENTAL PROCEDURES

Enzyme and Chemicals. Recombinant HIV-1 protease as expressed in *Escherichia coli* (Debouck et al., 1987) was purified from bacterial lysates to apparent homogeneity according to the procedure of Grant et al. (1991). Protease solutions were stored at −80 °C in 50 mM sodium acetate (pH 5.0), 1 mM dithiothreitol, 1 mM EDTA, 0.35 M NaCl, and 20% glycerol at concentrations of 15–400 μg/mL. Protein concentrations were determined by use of the Bio-Rad protein assay method with bovine serum albumen as a protein standard or by the chromatographic method described by Grant et al. (1991).

Deuterium oxide (99.8–99.9 atom % D) was obtained from either MSD Isotopes or Aldrich Chemicals (Gold Label). Sodium [^{3}H]acetate (500 mCi/mmol) and sodium [1-^{14}C]-acetate (16 mCi/mmol) were obtained from Dupont-NEN. Dowex AG1-X8 anion-exchange resin (100–200 mesh, formate form) was obtained from Bio-Rad Laboratories. All other reagents were of the highest purity available.

Oligopeptides. The oligopeptide substrates Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 and Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 were obtained commercially (Bachem Inc., Torrance, CA, or Peninsula Laboratories, Belmont, CA). All peptides were homogeneous by reversed-phase HPLC (>99%), and their structures were confirmed by amino acid analysis and fast atom bombardment mass spectrometry. L-[^{15}N]Proline (>95 atom % ^{15}N) was incorporated into Ser-Gln-Asn-Tyr-L-[^{15}N]Pro-Val-Val-NH_2. Mass spectrometric analysis of the product Ser-Gln-Asn-Tyr-L-[^{15}N]Pro-Val-Val-NH_2 yielded a molecular weight of 806.2 for the MH^+ species (peaks at m/e 805 were at background levels), and the corresponding fragmentation patterns were consistent with the correct isotopic substitution.

Preparation of Remote Radiolabeled Isotopically Substituted Peptide Substrates. Commercial preparations of sodium [^{3}H]acetate and sodium [1-^{14}C]acetate were each diluted in glacial acetic acid to give, respectively, 3.6 and 1.2 mCi/mmol. *O*-Acetylation of *N*-hydroxysuccinimide by [^{3}H]-acetic acid and [1-^{14}C]acetic acid was performed by dicyclohexylcarbodiimide coupling with *N*-hydroxysuccinimide (Rappoport et al., 1974; Hartman et al., 1977). The products were recrystallized from 2-propanol and dissolved in DMF. Obtained were *O*-[^{3}H]acetyl-*N*-hydroxysuccinimide (3.6 mCi/mmol, 0.28 M) and *O*-[1-^{14}C]acetyl-*N*-hydroxysuccinimide (1.2 mCi/mmol, 0.48 M). The amino acid residue designated as [^{14}N]Pro contained natural-abundance enrichments of the indicated isotope, while [^{15}N]Pro represents isotope enrichments of >95%. Ser-Gln-Asn-Tyr-[^{15}N]Pro-Val-Val-NH_2 (10 mg, 0.0124 mmol) was separately dissolved in 1.6 mL of DMF/0.4 mL of 250 mM potassium phosphate buffer (pH 7.8), to which *O*-[^{3}H]acetyl-*N*-hydroxysuccinimide (0.0124 mmol) was added. The solution was allowed to stir at room temperature for 1 h, at which time ammonium chloride was added to quench the reaction. The mixture was lyophilyzed to dryness and resuspended in glacial acetic acid, and the product was purified by HPLC on a semipreparative Beckman C-18 column (10 × 250 mm, 5 mm) using a mobile phase consisting of 11% CH_3CN/0.05% trifluoroacetic acid (32 min), followed by a linear gradient of 11–18% CH_3CN/0.05% trifluoroacetic acid for 10 min and 18% CH_3CN/0.05% trifluoroacetic acid for 13 min (1.5 mL/min; detection at 220 nm). Obtained was [^{3}H]Ac-Ser-Gln-Asn-Tyr-[^{15}N]Pro-Val-Val-NH_2 (3.6 mCi/mmol). [^{3}H]Ac-Ser-Gln-Asn-Tyr-[^{14}N]-Pro-Val-Val-NH_2 was prepared in an identical fashion. [1-^{14}C]Ac-Ser-Gln-Asn-Tyr-[^{14}N]Pro-Val-Val-NH_2 (1.2 mCi/mmol) was similarly prepared from 10 mg of Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 and 0.0124 mmol *O*-[1-^{14}C]acetyl-*N*-hydroxysuccinimide. Radiolabeled peptide substrates were judged to be homogenous upon purification by reversed-phase HPLC as described above. The radiolabeled substrates could be quantitatively (>96%) converted to products by incubation with a high concentration of HIV-1 protease.

Determination of Kinetic Isotope Effects. ^{15}N kinetic isotope effects of the peptidolytic reaction of HIV-1 protease were determined using the competitive method in which the isotopically substituted peptide substrates contained remote radiolabels, similar to the methods of Parkin and Schramm (1987) and Harrison *et al.* (1990). The peptide substrate containing the heavy isotopic probe, [^{15}N]Pro, contained tritium in its N-terminal acetyl group, while the peptide bearing the light isotopic probes contained ^{14}C in their N-terminal acetyl groups. The isotope effects were measured by determination of the $^{14}C/^3H$ ratio found in the remaining substrate from a mixture of the heavy and light isotopically substituted peptide substrates at various fractions of total reaction (f) ranging from 0 to 0.9. Isotope effects were measured at 37 °C in H_2O and 80% D_2O (final solvent isotopic enrichment) using GAMT-NEDT buffer [50 mM each of glycine, sodium acetate, Mes, Tris-HCl, 1 mM dithiothreitol, 1 mM EDTA, 0.2 M NaCl, and 0.1% (v/v) Triton X-100; pH(D) = 4–6].

To measure the ^{15}N isotope effects, 360-μL reaction mixtures contained buffer, [1-^{14}C]Ac-Ser-Gln-Asn-Tyr-[^{14}N]-Pro-Val-Val-NH_2, and [^{3}H]Ac-Ser-Gln-Asn-Tyr-[^{15}N]Pro-Val-Val-NH_2. The ^{3}H- and ^{14}C-containing peptides were held at a ~1:1 molar ratio (0.25 mM each) such that $^3H/^{14}C$ = 2.7. Typically, each reaction mixture contained 0.5 mM total labeled peptide substrate (0.36 μCi of ^{3}H and 0.12 μCi of ^{14}C), and each sample removed for scintillation counting contained about 200 000 dpm ^{3}H and 75 000 dpm ^{14}C. The reactions were initiated by the addition of 0.08 mL of HIV-1 protease (160 nM) to the thermally equilibrated reaction mixture. At various times, 50-μL aliquots were removed from the reaction mixture and quenched with an equal volume of 0.16 N NaOH or 3% trifluoroacetic acid. For each experiment, a series of samples were quenched with either NaOH solution or trifluoroacetic acid prior to the addition of enzyme to serve as "zero-time" samples.

Separation of unreacted substrates from products ([^{14}C]-Ac-Ser-Gln-Asn-Tyr-CO_2H and [^{3}H]Ac-Ser-Gln-Asn-Tyr-CO_2H) was achieved by anion-exchange chromatography on a series of minicolumns of Dowex AG1-X8 (formate) (for samples quenched with base) as described by Hyland et al. (1990) or by reversed-phase HPLC (for acid-quenched samples) (Hyland et al., 1991a). Both chromatographic methods have proven to be quantitative in both recovery of products and separation of the Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 substrate from its Ac-Ser-Gln-Asn-Tyr-CO_2H product. For separation by ion-exchange chromatography, the quenched reaction mixtures were further diluted 3-fold with water immediately before application to the resin. The ^{3}H and ^{14}C content in the recovered substrates and products was measured on a Beckman Model LS-5801 liquid scintil-

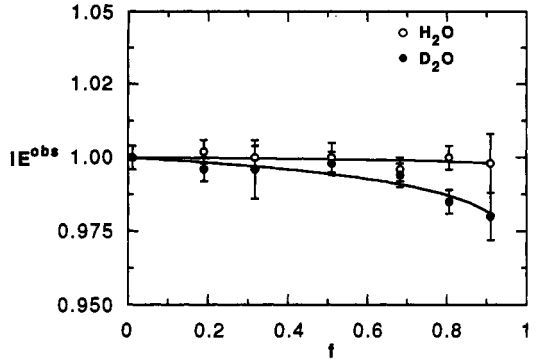


FIGURE 1: Measurement of the ^{15}N kinetic isotope effects of HIV-1 protease-catalyzed peptidolysis of Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 in both H_2O (○) and D_2O (●) (pH(D) = 6.0; 37 °C). IE_{obs} is the observed isotope effect, measured as the $(^{14}C/^{3}H)_0/(^{14}C/^{3}H)_f$ ratio of the chromatographically separated, residual substrates, as a function of the extent of reaction (f) by fitting data to eq 1 as described in the text. The data represent the averages of triplicate determinations, and the error bars represent the standard deviation of each value.

lation counter. Samples were counted six times for 10 min each, and the average of the six readings was obtained for each sample. The fraction of reaction was calculated from the ratio of recovered radioactivity, dpm product/(dpm product + dpm substrate). The isotope effects were determined from the $^{14}C/^{3}H$ ratio of the chromatographically separated, residual substrates, as a function of the extent of reaction, by fitting data to eq 1 (Harrison et al. (1990)), in which $(^{14}C/^{3}H)_0$ and $(^{14}C/^{3}H)_f$ are the radioisotopic ratio of the recovered peptide substrates at $t = 0$ and at the fraction of reaction, f, and α is the reciprocal of the isotope effect, k_{14}/k_{15}.

$$(^{14}C/^{3}H)_0/(^{14}C/^{3}H)_f = (1-f)^{(\alpha-1)} \quad (1)$$

The competitive method used here results in the determination of the isotope effect on the kinetic parameter V/K (Northrop, 1982), obtained from the reciprocal of α. In a single experiment, values of α, and thus the isotope effect on V/K, were obtained by fitting data to eq 1 (using the Kaleidagraph software of Synergy Software, Reading, PA) using values of $(^{14}C/^{3}H)_0/(^{14}C/^{3}H)_f$ measured at six or more values of f (0–0.9). The standard deviation of each value of $(^{14}C/^{3}H)_0/(^{14}C/^{3}H)_f$ was determined from the multiple rounds of scintillation counting, and this error was propagated into the solution of values of α as described by Parkin (1992). The reported values are the average of at least triplicate measurements for a single experiment in which a single experiment comprised a minimum of six determinations of the observed isotope effect, $(^{14}C/^{3}H)_0/(^{14}C/^{3}H)_f$.

Nomenclature. The nomenclature used to describe isotope effects is that of Northrop (1977) and Cook and Cleland (1981). The leading superscript on the kinetic parameter V/K indicates the type of isotope effect being measured, while the trailing subscript indicates the second isotope in a multiple isotope effects experiment (Cleland, 1991). For example, $^{D}V/K$ is the solvent isotope effect on V/K, while $^{15}(V/K)_{D_2O}$ and $^{15}(V/K)_{H_2O}$ are the ^{15}N isotope effects on V/K obtained in water and in D_2O and water, respectively.

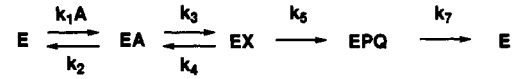
RESULTS AND DISCUSSION

Primary ^{15}N Kinetic Isotope Effects. A representative plot of the primary ^{15}N kinetic isotope effects for the HIV-1 protease-catalyzed cleavage of Ac-Ser-Gln-Asn-Tyr-[^{15}N]Pro-Val-Val-NH_2 in H_2O and 80% D_2O is shown in Figure 1 [pH-(D) = 6.0]. Isotope effects obtained by fitting of the data in Figure 1 to eq 1 were $^{15}(V/K)_{H_2O} = 0.995 \pm 0.002$ and $^{15}(V/K)_{D_2O} = 0.992 \pm 0.003$.[3] These isotope effects were significantly different from that measured in an identically performed control experiment using [^{3}H]acetyl- and [^{14}C]-acetyl-containing forms of Ac-Ser-Gln-Asn-Tyr-[^{14}N]Pro-Val-Val-NH_2, in which the measured isotope effect was, within experimental error, essentially unity [1.004 ± 0.004 (duplicate determinations); data not shown].[4] In the present case, the expression of the intrinsic isotope effect was more pronounced in $^{15}(V/K)$ for the experiment conducted in D_2O, indicating that the catalytic step in which C–N bond breaking or bond making occurs also involves a change in the fractionation factor of a proton. In contrast, O'Leary and co-workers have reported normal ^{15}N isotope effects (1.006–1.024) for the proteases papain (O'Leary et al., 1974) and chymotrypsin (O'Leary & Kluetz, 1972) and have suggested that C–N bond breaking is at least partially rate-limiting for these reactions. For HIV-1 protease, the inverse ^{15}N effect reflects the presence of a step (or steps) on the reaction pathway which has a large energy barrier and in which bonding to the nitrogen is becoming more stiffened, such as that which occurs upon protonation of a nitrogen atom (Weiss et al., 1987).

Analysis of Isotope Effects. We previously reported solvent isotope effects for the HIV-1 protease-catalyzed peptidolysis of Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 [pH(D) = 3.0–7.0]: $^{D}V/K = 1.05 \pm 0.05$ [pH(D)-independent value] and $^{D}V = 2.2 \pm 0.9$ at pH(D) = 6.0 (Hyland et al. (1991b). We also showed that following C–N bond cleavage the substrate Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 could not be re-formed from its enzyme-bound products, while the formation of the tetrahedral adduct of peptide and H_2O is reversible, as evidenced by the enzyme-catalyzed exchange of ^{18}O from $H_2{}^{18}O$ into the scissile carbonyl oxygen of the substrate (Hyland *et al.*, 1991a).

A mechanistic scheme for the HIV-1 protease-catalyzed cleavage of Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 is shown in Scheme I. EA is the Michaelis complex, which undergoes formation of a tetrahedral adduct of the scissile carbonyl group and substrate water, which we presume is followed by the protonation of the nitrogen of the proline residue to form EX. Such a mechanism would contribute to the measurement of an inverse ^{15}N isotope effect which is enhanced in D_2O. In the k_5 step, the C–N bond is broken to form the carboxylic acid and amine products (EPQ), which are presumably then released from the enzyme. While several discrete chemical steps are included in the k_3 step, this scheme is sufficient to describe the solvent and ^{15}N isotope effects. Primary solvent kinetic isotope effects are anticipated on the k_3 step in which the tetrahedral adduct of peptide and water is formed and the departing nitrogen atom is ultimately protonated, while a secondary solvent isotope effect of about 1.2 (Weiss et al., 1987) is predicted to occur on the k_5 step, in which cleavage of the C–$NH(D)^+$ bond occurs. ^{15}N isotope effects are expected on both the k_3 (protonation of the proline nitrogen) and k_5 steps. For the mechanism in Scheme I, expressions for

Scheme I



[3] Analysis of the significance of the differences in the $^{15}(V/K)_{H_2O}$ and $^{15}(V/K)_{D_2O}$ values by Student's t-test for the data shown in Figure 1 yielded a t-value of 1.83 for 33 total data points ($p < 0.1$).

[4] Values of $^{15}(V/K)$ were not corrected for this measured effect.

the solvent isotope effect on V/K and the ^{15}N isotope effects in both H_2O and D_2O are given by eqs 2–4.

$$^{D}V/K = [^{D}k_5{}^{D}K_{eq_3} + (k_5/k_4)(^{D}k_3 + k_3/k_2)]/ [1 + (k_5/k_4)(1 + k_3/k_2)] \quad (2)$$

$$^{15}(V/K)_{H_2O} = [^{15}k_5{}^{15}K_{eq_3} + (k_5/k_4)(^{15}k_3 + k_3/k_2)]/ [1 + (k_5/k_4)(1 + k_3/k_2)] \quad (3)$$

$$^{15}(V/K)_{D_2O} = [^{15}k_5{}^{15}K_{eq_3} + (^{D}k_3/^{D}k_5{}^{D}K_{eq_3})(k_5/k_4)(^{15}k_3 + k_3/k_2{}^{D}k_3)]/[1 + (^{D}k_3/^{D}k_5{}^{D}K_{eq_3})(k_5/k_4)(1 + k_3/k_2{}^{D}k_3)] \quad (4)$$

From an analysis of fractionation factors, $^{D}K_{eq_3}$ is approximately equal to 0.827, based on the ratio of fractionation factors of $(1.0)^2(0.92)/(1.12)(1.08)(0.92)$, in which the values 1.0, 0.92, 1.12, and 1.08 are the respective solvent fractionation factors (Quinn & Sutton, 1991) for water, a carboxylic acid (active site aspartyl residues), the OH of an alcohol (zwitterionic tetrahedral adduct) (Rolston & Gale, 1984), and a protonated amine. By analogy to the minimal mechanism used to analyze the solvent and ^{15}N isotope effects for the adenosine deaminase reaction (Weiss et al., 1987), and since we expect both $^{D}K_{eq_3}$ and $^{15}K_{eq_3}$ to be <1, we will assume a value of 1.0 for both $^{D}k_3$ and $^{15}k_3$, such that $^{D}k_4$ and $^{15}k_4$ reflect the equilibrium isotope effect in the reverse direction. Equations 2–4 then become eqs 5–7, in which a, the forward catalytic commitment factor, is $k_5/k_4(1 + k_3/k_2)$:

$$1.05 \pm 0.05 = (0.827^{D}k_5 + a)/(1 + a) \quad (5)$$

$$0.995 \pm 0.002 = (^{15}k_5{}^{15}K_{eq_{3H}} + a)/(1 + a) \quad (6)$$

$$0.992 \pm 0.003 = [(^{15}k_5{}^{15}K_{eq_{3H}})/(1.0041) + a/(0.827^{D}k_5)]/ [1 + a/(0.827^{D}k_5)] \quad (7)$$

As determined for a similar chemical step in the mechanism of aspartyl transcarbamoylase (Parmentier et al., 1992), the value of $^{15}K_{eq_3}$ in D_2O should be more inverse than that in H_2O ($^{15}K_{eq_{3H}}$) by a factor of 1.0041. Simultaneous solution of eqs 5–7 yielded values of $^{D}k_5 = 1.3 \pm 0.5$, $a = k_5/k_4(1 + k_3/k_2) = 0.4 \pm 0.2$, and $^{15}k_5{}^{15}K_{eq_{3H}} = 0.993 \pm 0.002$.[5] The low value of the catalytic commitment factor, $k_5/k_4(1 + k_3/k_2)$, suggests that the formation of the EX complex is highly reversible, and in fact, the enzyme-bound formation of the *N*-protonated amide hydrate intermediate may reach equilibrium with the substrate. This supposition is also inherent in our previous observation that ^{18}O is exchangeable from solvent into substrate via this intermediate in the EX complex.

Solutions of eqs 5–7 suggest that Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 is a "nonsticky" substrate.[6] This suggestion is in conflict with results of our previous $H_2{}^{18}O$ isotope partitioning data in which we estimated a catalytic commitment factor >12 (Hyland et al., 1991a). However, since the incorporation of ^{18}O into substrate re-formed from the amide hydrate intermediate occurs via a reaction pathway which is distinct from the direct desorption of substrate from the EX complex, these two reverse reactions need not proceed with similar rate constants.

The present kinetic model must also be compatible with the measured value ^{D}V for the substrate Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2, equal to 2.2 ± 0.9 at pH(D) = 6.0 (Hyland et al. 1991b). The nonunity value of ^{D}V indicates that a solvent isotope effect is expressed on at least one reaction step beyond the scission of the C–N bond (the first irreversible reaction step; k_5 in Scheme I). Therefore, a prototropic reaction step which does not appear in the V/K expression apparently contributes to the observed solvent isotope effect on V. Rebholz and Northrop (1991) have observed similar solvent isotope effects on $^{D}V/K$ and ^{D}V for the aspartic protease porcine pepsin and attributed the solvent isotope effect on V and the lack of one on V/K to a proton transfer after products have been released. While our data with HIV-1 protease are consistent with this explanation, an iso mechanism does not need to be invoked to explain the differences in expression of solvent isotope effects on $^{D}V/K$ and ^{D}V because, unlike those for pepsin, these kinetic parameters for HIV-1 protease are "uncoupled" by the apparent irreversibility of the enzyme-bound chemical steps for the substrate used in these studies.

Chemical Mechanism. In Figure 2 is a chemical mechanism for HIV-1 protease which is consistent with crystallographic data of the enzyme, our previous kinetic data (Hyland et al., 1991a,b), and the current isotope effects data. It is likely that, during the binding of the substrate, Asp-25 establishes a hydrogen bond to the carbonyl oxygen of the substrate (EA). We propose that this weak hydrogen bond then becomes a strong or "low-barrier" hydrogen bond (Kreevoy & Liang, 1980; Cleland, 1992) upon formation of the enzyme–substrate intermediate EA^+, in which the nitrogen becomes fully charged by virtue of the donation of its lone pair of electrons to the now electron deficient carbonyl carbon. Additional indirect evidence for the presence of this *O*-protonated amide species in EA^+ comes from the pH dependence of inhibition by a cationic, reduced amide inhibitor of HIV-1 protease (containing a TyrΨ[CH_2NH^+]Pro isostere) which may only bind to a form of the protease in which both aspartyl residues are unprotonated, as is approximated by the putative EA^+ complex (Hyland et al., 1991b). The carbonyl carbon atom of the *O*-protonated amide species in EA^+ is now suitably electrophilic to promote attack of the substrate water molecule to form the amide hydrate species in EX. Again, we depict for EX in Figure 2 a low-barrier hydrogen bond for the O–H–O bond of the (former) carbonyl–aspartate pair. Next, two discrete proton transfers lead to the formation of the zwitterionic species in EX^+, which subsequently collapses to form the amine and carboxylic acid products of the reaction in EPQ.

The establishment of low-barrier hydrogen bonds has been predicted to occur in enzyme–substrate complexes when the heavy atoms involved in the hydrogen bond have similar pK values[7] and the distances separating them is extremely short (<2.5 Å) (Cleland, 1992). Cleland and Kreevoy have proposed

[5] Error limits on the solved kinetic parameters a, $^{D}k_3$, and $^{15}k_5{}^{15}K_{eq_3}$ were calculated by solution of eqs 5–7 using the experimental upper and lower error limits of the experimental values of $^{D}V/K$, $^{15}(V/K)_{H_2O}$, and $^{15}(V/K)_{D_2O}$. For the case in which $^{D}k_3 > 1$, reasonable solutions for eqs 5–7 in which $^{D}k_5 = 1.15$ and in which $k_3/k_2 < 1$ were $a = k_5/k_4 = 0.07$; $^{D}k_3 = 2.5$; and $^{15}k_5{}^{15}K_{eq_3} = 0.995$.

[6] A "sticky" substrate is one which upon binding to an enzyme is converted to product at least as fast as it desorbs from the enzyme surface (Cleland, 1982).

[7] The pK_a of the protonated Asp-25 in the free enzyme is 5–6 (Hyland et al., 1991b), where it is likely to be strongly hydrogen bonded to the anionic Asp-25′. This pK_a may drop to <3 upon binding of substrate and the disruption of this hydrogen bond. The protonated, enol tautomer of the amide should have a pK_a < 0. In the highly hydrophobic environment of the substrate-bound active site of HIV-1 protease the pK_a values of the *O*-protonated amide and the carboxylic acid are likely to differ by 3–4 units, but may become matched in the transition state that leads to the EX^+ complex.

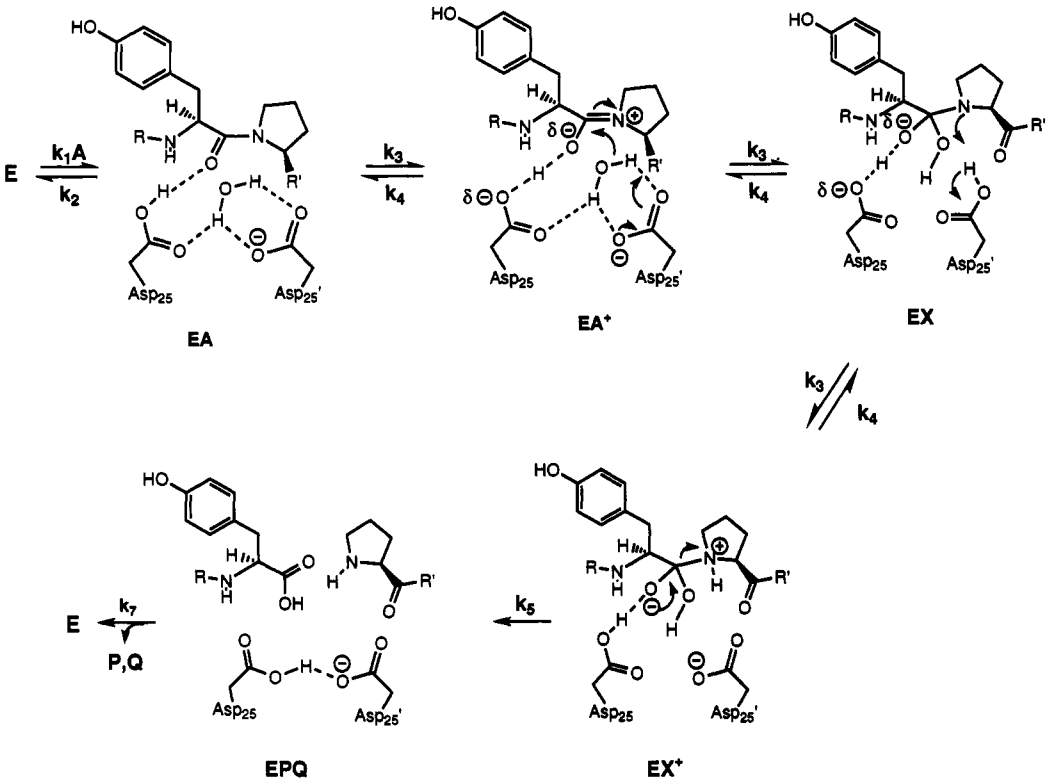


FIGURE 2: Proposed chemical mechanism for HIV-1 protease-catalyzed peptidolysis of Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2 based on crystallographic data, previous kinetic results, and the present isotope effects data. The negative charge is shown to be shared equally by the two oxygen atoms involved in the low-barrier hydrogen bond in the EA^+ complex.

that the energy accrued from the formation of such strong hydrogen bonds from weak ones in the conversion of enzyme–substrate to enzyme–intermediate complexes contributes significantly to enzymatic catalysis (W. W. Cleland and M. M. Kreevoy, personal communication). From recent crystallographic data of complexes of phosphinate-containing peptide analogue inhibitors of both penicillopepsin (Fraser et al., 1992) and HIV-1 protease (Abdel-Meguid et al., 1993), it is evident that hydrogen bonds between the active site aspartyl groups and the two oxygen atoms of this isosteric surrogate of the tetrahedral reaction intermediate are very short (<2.5 Å). By analogy, such short, low- barrier hydrogen bonds probably occur in the formation of the two enzyme–intermediate complexes of EA^+, EX, and possibly EX^+ in Figure 2; indeed, molecular modeling of the amide hydrate reaction intermediate of EX also indicates short hydrogen bonds (Hyland *et al.*, 1991a).

The bonding at the nitrogen atom in the two partial reactions that lead to the enzyme-bound species in EA^+ and EX is reminiscent of the glutamate dehydrogenase reaction, that is, formation of an amino acid imine followed by its hydration to form a carbinolamine intermediate. Weiss et al. (1988) have determined the ^{15}N fractionation factors of the unprotonated amino acid, the amino acid imine, and the carbinolamine relative to aqueous ammonia as 1.1063, >1.0192, and >1.0326, respectively. Using the appropriate ratios of these fractionation factors, we obtain values of $^{15}K_{eqEA+} = 0.9972$ and $^{15}K_{eqEX} < 0.987$. However, since the species in EX^+ is not a fully formed *O*-protonated amide, and since its nitrogen, while charged, is not protonated (as in the glutamate dehydrogenase case), these $^{15}K_{eq}$ values are likely to be less inverse. We estimate the product of the two equilibrium constants for the enzyme-bound species of complexes EA^+ and EX as $^{15}K_{eqEA+}{}^{15}K_{eqEX} < 0.99$.

In the next step, two proton transfers lead to the zwitterionic tetrahedral intermediate in EX^+, which should give rise to an additional inverse nitrogen isotope effect [$^{15}K_{eqEX+} = 0.9836$ or 0.980 in H_2O or D_2O, respectively (Weiss et al., 1987; Parmentier et al., 1992)]. Estimates of $^{15}K_{eq3H}$ and $^{15}K_{eq3D}$ from the multiplicative product $^{15}K_{eqEA+}{}^{15}K_{eqEX}{}^{15}K_{eqEX+}$ are then 0.974 and 0.970, respectively. From our experimentally determined value of $^{15}k_5{}^{15}K_{eq3H} = 0.993 \pm 0.002$, we may use the estimated value of $^{15}K_{eq3H}$ to calculate the intrinsic ^{15}N effect on the C–N bond cleavage: $^{15}k_5 = 1.020$. This intrinsic effect is similar to that measured for cleavage of a C–NH^+ bond in phenylalanine ammonia-lyase reaction (1.021; Hermes et al., 1985), but is a low value for an intrinsic isotope effect in which the nitrogen atom is attached to a carbon atom which is bound to (an) oxygen atom(s). The scission of the C–N bond in the adenosine deaminase reaction gave rise to the considerably larger intrinsic nitrogen isotope effect of 1.026 (Weiss et al., 1987). However, that a secondary amine is released in the HIV-1 protease reaction may account for the small intrinsic effect as the nitrogen atom remains stiffly bonded to two carbon atoms, opposed to hydrogen atoms in the ammonia product.

In the kinetic model of Scheme I and Figure 2, the estimated intrinsic solvent isotope effect of $^Dk_3 = 1.0$ includes proton-transfer reactions for formation of the EA^+, EX, and EX^+ complexes. This signifies that the outcome of all proton-transfer reactions of the proposed mechanism of HIV-1 protease yields no solvent isotope effect. The transfer of protons between oxygen atoms involved in low-barrier hydrogen bonds proceeds with inverse deuterium isotope effects (Cleland, 1992) (thus, $^Dk_{EA} = {}^Dk_{EA+} < 1$). However, the transfer of the proton to the proline nitrogen, $^Dk_{EX}$, is likely to express a normal solvent isotope effect since it is probably not involved in a low-barrier hydrogen bond. A combination of inverse and normal solvent isotope effects for the proton-transfer steps in Figure 2 could give rise to the observed value of $^DV/K = 1.05$ and the observed ^{15}N kinetic isotope effects. If one assigns discrete and separate reaction steps for the

formation of EA^+, EX, and EX^+ in Figure 2 and writes out the resulting kinetic equations of the form of eqs 2–4 for which $a = 0.4$, ${}^{D}k_{EA} = 0.6$, ${}^{D}k_{EA+} = 0.4$, and ${}^{D}k_{EX} = 3.2$ and for which ${}^{15}K_{eqEA+}$, ${}^{15}K_{eqEX}$, and ${}^{15}K_{eqEX+}$ are as described above, solutions for ${}^{D}V/K$, ${}^{15}(V/K)_{H_2O}$, and ${}^{15}(V/K)_{D_2O}$ can be shown to be 1.05, 0.994, and 0.991, respectively.

The mechanism of HIV-1 protease shown in Figure 2 is chemically reasonable and is completely consistent with available kinetic and crystallographic data. While our present analysis by no means constitutes a unique solution of the available data, all other attempts to fit these data to another mechanism have failed to yield a better fit. One satisfying aspect of the mechanism in Figure 2 is that the protonated and unprotonated active site aspartyl residues constitute respectively a general acid and a general base catalyst, owing to the establishment of strong hydrogen bonds between these residues and the two substrates. Therefore, the peptidolytic mechanism of HIV-1 protease contains simultaneously the key catalytic features of acid- and base-catalyzed hydrolysis of amides: activation of the amide carbonyl by protonation and formation of the hydroxide ion. Bennet et al. (1990) have similarly concluded from solvent isotope effects of unity and from ^{18}O exchange reactions that the mechanism of the acid-catalyzed hydrolysis of anilides involves sequentially the formation of an *O*-protonated amide and an amide hydrate intermediate and protonation of the departing amine prior to the collapse of the amide hydrate intermediate. One could then propose from our current findings that the chemical mechanism of HIV-1 protease utilizes more elements of classical acid-catalyzed amide hydrolysis than base-catalyzed catalysis. An appreciation of the mechanistic importance of this catalyst has implications for the design of mechanism-based inhibitors, which could be envisioned to elaborate highly electrophilic moieties upon their protonation, which would then result in the formation of a stable covalent bond with the unprotonated aspartyl residue.

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