Conformational Selectivity of HIV-1 Protease Cleavage of X-Pro Peptide Bonds and Its Implications*  

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Kinetic measurements on a fluorescent peptide analog of the p17/p24 cleavage site of the Gag polyprotein demonstrate the conformational selectivity of human immunodeficiency virus, type 1 protease for the trans conformation of the Tyr-Pro bond. A mean cis/trans ratio of 0.3, and a cis → trans isomerization rate constant of 0.022 s⁻¹ are determined at T = 22 °C. This rate is in excellent agreement with that predicted by ¹⁹F NMR studies of structurally analogous peptides containing a fluorine/hydroxyl substitution on the tyrosyl residue. Addition of recombinant human cyclophilin resulted in a significant enhancement of this rate, and it is proposed that this enzyme, which has been shown to be associated with the Gag protein, functions as an auxiliary enzyme for the protease during cleavage in the virion.

Three of the eight consensus sequences cleaved by HIV-1 protease involve Araa-Pro bonds, where Araa corresponds to the aromatic amino acids tyrosine or phenylalanine (1). Since imide bonds are known to exhibit conformational cis-trans heterogeneity, the existence of such cleavage sites leads to questions concerning the conformational specificity of the protease. It has been demonstrated that cleavage of Xaa-Pro imide bonds by prolidase, aminopeptidase P, and carboxypeptidase P is specific for the trans conformation of Xaa-Pro dipeptides (2–5) and further that proteases can be conformationally selective even for nearby, non-scissile peptide bonds (6–9). Indeed, this conformational specificity of chymotryptic cleavage for the P₃–P₄ bond has provided the basis for assays used to demonstrate the existence of peptidyl proline isomerases (10). Several lines of evidence suggest that HIV protease might be specific for the trans imide bond conformation: 1) crystallographic studies of inhibitors containing proline (11) or thiazolidine (12) indicate a trans conformation; 2) the ability of the protease to cleave ordinary amide bonds as well as imide bonds suggests a capability for cleaving trans imide bonds (1). It is important to emphasize, however, that neither of these observations provides unambiguous proof. For example, it is well established that inhibitors that exhibit close structural relationships to natural substrates can nevertheless bind to enzymes in dramatically different conformations (13).

There are several motivations for understanding the conformational selectivity of HIV protease. 1) Many of the test peptides used to assay the protease contain imide Xaa-Pro bonds. In the event of conformational selectivity, the overall kinetic characterization of these assays should be generalized to include a combination of cleavage and isomerization rates, with the specific mix determined by the physical conditions such as temperature, buffer, etc. 2) Conformational selectivity by the protease could have significant implications for the kinetics of the cleavage process in vitro, closely analogous to the effect of cis-trans imide bond isomerism on protein folding and unfolding. The recent discovery of a close association between the peptidyl proline isomerase cyclophilin and the Gag polypeptide of HIV-1 (14–16) underlines the potential significance of this proteolytic selectivity.

The kinetic behavior of a trans-selective protease considered to cleave a trans bond irreversibly has been discussed by Lin and Brandts (2) and can be described by the following kinetic scheme,

\[
\text{cis peptide} \quad k_1 | k_2 \quad k_3 \quad k_4 \quad k_5 \\
\text{trans peptide} + E \xrightleftharpoons{k_{\text{c}}} E - S \xrightleftharpoons{k_{\text{t}}} E + \text{product peptides} \\
\]

Scheme 1

where \(k_1\) and \(k_2\) are the rate constants corresponding to the cis → trans and trans → cis interconversions, respectively, related by \(f_{\text{cis}} = f_{\text{trans}}\), where \(f_{\text{cis}}\) and \(f_{\text{trans}}\) are the fractional cis and trans concentrations, and the other rate constants are defined as indicated above. The corresponding differential equations describing the hydrolysis reaction can be solved using various numerical packages, and we have utilized the program Mathematica. These simulations indicate that conformational selectivity of a proteolytic enzyme can be demonstrated by kinetic measurements performed with protease concentrations high enough to allow separation of hydrolysis and isomerization. In the limit in which free and enzyme-complexed trans peptide are in equilibrium, i.e. \(k_1 \gg k_2\) and \(k_3 \gg k_4\), such that the system has an effective Michaelis constant: \(K_m = (k_2 + k_4)k_3 / k_1\), the hydrolysis will exhibit simple biphasic kinetic behavior if the substrate concentration \(S_0 < K_m\). Under such conditions, numerical solutions predict a time-dependent product formation characterized by a biphasic curve: an initial fast phase dominated by the trans rate of hydrolysis, followed by a slow phase dominated by the cis → trans isomerization rate. In this limit, the observed fast and slow rate constants approach \(k_{\text{c}}k_{\text{t}}E/K_m^2\) and \(k_2\), respectively, while the ratio of the pre-exponential weighting factors approaches the equilibrium \(k_{\text{c}}/k_{\text{t}}\) ratio.

EXPERIMENTAL PROCEDURES

Materials—The fluorescent peptide substrate, Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg, was obtained from...
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Fluorescence intensity measurements (circles) and kinetics simulation (lines) of the fluorescent peptide (Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg) (Molecular Probes) as a function of time in the presence of HIV-1 protease. The assay contained 1 μM fluorescent peptide and 1.9 μM HIV-1 protease (Bachem Bioscience, Inc.). Assay conditions were 1.0 M NaCl, 0.2% polyethylene glycol, and 0.1 M MES, pH 5.5, at room temperature (22 °C) in 200 μL total volume. Data were obtained every 0.5 s for 3 min using a Fluoroskan Ascent plate reader (LabSystems). Excitation and emission wavelengths were 355 and 485 nm, respectively. The theoretical curves show the normalized product, cis, and trans concentrations calculated by numerical solution of the kinetic scheme shown in the text using the DSolve command of the program Mathematica. Parameters used for the simulation were: S = 1 μM, E = 1.9 μM, k1 = 0.022 s−1, ftrans = 0.73, k1 = 3 × 104 M−1 s−1, cis = 0.3 × 104 M−1 s−1, ftrans = 0.73, k1 = 3 × 104 M−1 s−1. The values of k1, k2, f, and ktrans are somewhat arbitrary and are chosen to be significantly greater than the other rate constants involved (Michaelis approximation). Note that the ratio (k1/k2) corresponds to a Michaelis constant Kc = (k1 + k2)k3/2k4. The k1 values were determined independently by NMR, using an analogous, peptide in 1 M NaCl, 50 mM sodium acetate-d3, pH = 5.0, and 100% D2O. 19F NMR data obtained using magnetization transfer methods at high temperatures (50–80 °C) using a 5 mM sample of the fluorine-labeled peptide in 1 M NaCl, 50 mM sodium acetate-d3, pH = 5.0 (uncorrected) to give a final peptide concentration of ~12 mM. Fluorescence data point obtained from the slow rate portion of the biphasic product formation curve of Fig. 1, where the cleavage of the fluorescent peptide by HIV protease is observed to be rate-limited by cis → trans isomerization. An activation energy ΔG° = 17.4 kcal/mol is determined by linear regression of the NMR data. The 19F NMR data point (○) corresponds to magnetization transfer measurements performed at T = 25 °C on a 1 mM fluorine-labeled peptide in the presence of 10 μM recombinant human cyclophilin (Sigma) and is discussed in the text and in Fig. 3.

RESULTS AND DISCUSSION

Fig. 1 presents fluorescence data from studies performed on the fluorescent peptide substrate (Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg) (Molecular Probes), the sequence of which is derived from the Pr55ag p17/p24 cleavage site (22). Also shown are the product, cis, and trans normalized concentrations simulated using the kinetic model given above. As predicted, the time-dependent fluorescence intensity exhibits clear evidence of two distinct phases. Furthermore, other data acquired over a range of protease concentrations (1.9–3.2 μM) also exhibit a slow kinetic phase despite the high protease concentration. The slow rate constant for the assay conditions shown in Fig. 1 is determined to be 0.022 s−1 by a least squares fit of this data to a bi-exponential function. Similar values (0.022–0.023 s−1) were obtained at several protease concentrations and at two different concentrations of NaCl (1.0 and 0.4 M). Whereas the activity of the protease increased approximately 3-fold at the higher salt conditions, the observed value of the slow rate constant remained essentially constant. Thus, the slow rate constant observed in kinetic studies such as that shown in Fig. 1 does not correspond to a slower hydrolysis of the cis conformer, but rather to an enzyme-independent cis → trans isomerization.

Cis → trans isomerization rates at various temperatures were determined independently by NMR, using an analogous, fluorine-labeled test peptide also derived from the p17/p24 cleavage site, in which fluorine is substituted for the tyrosyl hydroxyl group: Ser-Gln-Asn-FPhe-Pro-Ile-Val-Gln (FPhe = 4-fluoro-l-phenylalanine). The 19F NMR spectrum of this peptide exhibits two well-separated resonances (Δν = 0.6 ppm) at an intensity ratio of cis/trans = 0.3, which can be shown to correspond to a single, interconverting species by magnetization transfer NMR methods. Values for the cis → trans rate constant were obtained at high temperatures (50–80 °C) using magnetization transfer techniques analogous to those described previously in 19F NMR studies of 4-fluoroPhe-labeled bradykinin (18). Alternatively, rate constants at lower temperatures (0–10 °C) were obtained by initially dissolving the peptide in a solvent (0.4 M LiCl/trifluoroethanol), which has been found to augment the cis/trans ratios of other peptides (21), followed by dilution into a 1 M NaCl buffer. The results determined from these studies are given in Fig. 2 as an Arrhenius plot, which shows the expected dependence on 1000/T with ΔG° = 17.4 kcal/mol. The cis → trans isomerization rate con-

![Fig. 1. Fluorescence intensity measurements (circles) and kinetics simulation (lines) of the fluorescent peptide (Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg) (Molecular Probes) as a function of time in the presence of HIV-1 protease. The assay contained 1 μM fluorescent peptide and 1.9 μM HIV-1 protease (Bachem Bioscience, Inc.). Assay conditions were 1.0 M NaCl, 0.2% polyethylene glycol, and 0.1 M MES, pH 5.5, at room temperature (22 °C) in 200 μL total volume. Data were obtained every 0.5 s for 3 min using a Fluoroskan Ascent plate reader (LabSystems).](image1)

![Fig. 2. Temperature dependence of the cis → trans isomerization rates, ktrans, of two peptides derived from the Pr55ag p17p24 cleavage site for HIV protease. Presented here as an Arrhenius plot depicting the relation ktrans = ktrans,uncorr, the isomerization rates were measured using methods specific to three temperature ranges: a. 19F NMR data obtained using magnetization transfer methods at high temperatures (50–80 °C) using a 5 mM sample of the fluorine-labeled peptide in 1 M NaCl, 50 mM sodium acetate-d3, pH = 5.0, and 100% D2O. b. 19F NMR data obtained by diluting the fluorine-labeled peptide from an initial 0.4 M LiCl/trifluoroethanol solution into 1 M NaCl, 50 mM sodium acetate-d3 in D2O, pH = 5.0 (uncorrected) to give a final peptide concentration of ~12 mM; c. fluorescence data point obtained from the slow rate portion of the biphasic product formation curve of Fig. 1, where the cleavage of the fluorescent peptide by HIV protease is observed to be rate-limited by cis → trans isomerization. An activation energy ΔG° = 17.4 kcal/mol is determined by linear regression of the NMR data. The 19F NMR data point (○) corresponds to magnetization transfer measurements performed at T = 25 °C on a 1 mM fluorine-labeled peptide in the presence of 10 μM recombinant human cyclophilin (Sigma) and is discussed in the text and in Fig. 3.](image2)
HIV-1 Protease Cleavage of X-Pro Bonds

The development of protease inhibitors is based on HIV protease assays, which typically, although not always, involve cleavage of Xaa-Pro peptide bonds (22–30). In these studies and the many reported kinetic studies using this approach, it is in general not clear how the cis-trans isomerization behavior has been dealt with. The results of such assays generally depend on the enzyme and substrate concentrations and on the physical parameters utilized in the studies. Simulations performed using the trans-selective hydrolysis model given above indicate that at protease concentrations 10 times less than those used to model the data of Fig. 1, product production curves are obtained that are qualitatively close to those predicted by simple, non-selective Michaelis-Menten kinetic schemes. However, for the non-selective model, an effective catalytic rate constant must be invoked such that $k_{cat} = k_{trans} [E] / [S_0]$, where $f_{trans}$ is the fractional concentration of the peptide with trans Xaa-Pro conformation. In this limit, the formation of product peptides is also fairly well approximated by a single exponential time course according to the following equation,

$$\text{Prod}[t] = S_0 - S_0 e^{-k_{app}t}$$

(Eq. 1)

where $k_{app} = k_{cat} \frac{E_o}{[S_0] + (k_{-1} + k_{1H})/k_1} = k_{cat} E_o / K_m$, $E_o$ and $S_0$ are the total enzyme and substrate concentrations.

As discussed below, the association of cyclophilin, an enzyme with peptidyl-proline cis-trans isomerase activity, with the Gag substrate of HIV protease suggests that it may function as an auxiliary enzyme to facilitate conversion of the non-cleavable, cis peptide conformation into active trans substrate for the protease. The cis-trans isomerization kinetics of the fluorinated peptide in the presence of recombinant human cyclophilin were determined at $T = 25^\circ C$ using the magnetization transfer NMR method. Typical NMR spectra observed in the absence (panels 1) and presence (panels 2) of cyclophilin are presented in Fig. 3. For clarity, cis (A) and trans (B) peaks are scaled to equal heights at equilibrium. Although isomerization of the peptide in solution at 25 $^\circ C$ is normally too slow to measure using this technique, the interconversion is readily observed in the presence of 10 $\mu M$ cyclophilin. In Fig. 3A (panel 2), the increased isomerization rate is manifest as a decreased net magnetization (peak height) of the cis peak as trans conformers with inverted spins rapidly convert to cis conformers and, conversely, cis conformers with non-inverted spins rapidly convert to the trans form. In Fig. 3B (panel 2) the initially inverted magnetization of the trans peak returns to equilibrium noticeably faster than in Fig. 3B (panel 1), where the interconversion rate is slow relative to the spin-lattice relaxation rate. Analysis of these spectra indicate that the cyclophilin-enhanced cis $\rightarrow$ trans rate, seen in Fig. 2 (●) is significantly faster than that predicted by regressing the peptide isomerization rates measured in the absence of cyclophilin.

Thus, we have found that cleavage by HIV-1 protease of a peptide related to the Pr55$^{gag}$ p17/p24 junction is selective for the trans conformation of the Tyr-Pro bond, and second, that a tyrosyl OH $\rightarrow$ F peptide analog is a substrate for recombinant human cyclophilin. Taken together, these observations suggest that the presence of cyclophilin as an auxiliary enzyme for the protease in vitro studies of the homodimeric enzyme show it to be fairly unstable at pH 7 (31), and, in particular, the active dimeric enzyme is stabilized in the presence of either inhibitors (32) or substrates (33). Once dissociated, the monomeric unit unfolds and becomes an excellent substrate for the active dimeric protease (34). To the extent that peptide cleavage and protease inactivation due to dimer dissociation are competing processes in vivo, cyclophilin could act to convert the Pr55$^{gag}$ p17/p24 cleavage site, as well as the other Phe-Pro sites, from protease-inactive cis conformations to protease-active trans conformations, thereby optimizing cleavage in the presence of an unstable protease. The association of cyclophilin with the Gag protein would appear to be consistent with this function. Beyond the question of protease stability, the presence of cis Xaa-Pro conformations could also alter the order of cleavage of the different sites by the protease. The order of cleavage of the Gag-Pol scissile bonds may also be important to achieve normal viral structure and function (35), and the presence of cyclophilin would reduce or eliminate perturbations of the normal order of cleavage resulting from the presence of a mixture of cis and trans Xaa-Pro bonds. Finally, the presence of cis conformations of target bonds could favor proteolytic cleavage of less susceptible alternate bonds, for example the Leu$^5$-Trp$^6$ bond of the protease itself (34, 36).

Navia et al. (37) have proposed that the dimeric structure of the protease may play a role in the timing of virus maturation, so that the protease is optimally active after the nascent virus particles have budded from the cell membrane, at which point the high concentration of intra-virion protease will favor the active dimer over the inactive monomer. Based on studies of the viral life cycle, Braat et al. (17) also suggest that cyclophilin A functions within the virion, but only after proteolysis and assembly are completed. They conclude that there is some (unknown) effect of cyclophilin that is important early in the viral life cycle and that the absence or inactivation of cyclophilin leads to no biochemically detectable assembly defects. This could indicate either that cyclophilin serves another purpose in addition to a role as an auxiliary enzyme for the protease or...
that some very subtle structural features resulting from the presence of incompletely or incorrectly cleaved Gag-Pol become significant at a time point prior to viral-directed DNA synthesis. Further studies will be required to sort out these possibilities.

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