Rate-determining Steps in HIV-1 Protease Catalysis

THE HYDROLYSIS OF THE MOST SPECIFIC SUBSTRATE*

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The human immunodeficiency virus type-1 (HIV-1) encodes a protease which is essential for the production of infectious virus. The protease prefers substrates that contain glutamic acid or glutamine at the P2' position. The catalytic role of these residues has been studied by using a highly specific fluorogen substrate, 2-aminobenzoyl-Thr-Ile-Nle-Phe(NO2)-Gln-Arg (substrate QR), and its counterpart (substrate ER) containing Glu in place of Gln. The newly designed substrate ER that contains a pair of charged residues at P2' and P3' sites is the most specific substrate described so far for HIV-1 protease. The specificity rate constant (k_{cat}/K_m = 2.1 \times 10^8 \text{ M}^{-1} \text{s}^{-1}) approaches, but does not reach, the diffusion limit. This follows from the appreciable solvent kinetic deuterium isotope effects on the rate constants, indicating that, independent of the salt concentration, the rate-limiting step of the catalysis is a chemical process rather than a physical one. The reaction also has positive entropy of activation. On the other hand, the rate-limiting step for substrate QR changes with increasing salt concentration from a physical to chemical step, while the negative activation entropy becomes positive. The rate increase with substrate ER is 50-fold with respect to substrate QR in the presence of 0.1 M NaCl and diminishes to 3.5-fold at 2.0 M NaCl concentration, as a consequence of a considerable rate increase at high salt concentration with substrate QR but not with substrate ER. The K_m value is much lower for the substrate ER (0.8 \mu M) than for substrate QR (15 \mu M), indicating a more effective binding for substrate ER at 0.1 M NaCl. Unexpectedly, the strong binding appears to be achieved by the unionized form of Glu in P2', as follows from the remarkably different pH-rate profiles for substrates QR and ER. The effective binding elicited by the glutamic acid may be utilized in designing inhibitors for therapeutic purposes.

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The abbreviations used are: HIV-1, human immunodeficiency virus type 1; Phe(NO2), 4-nitrophenylalanine; Nle, norleucine; Mes, 4-morpholinoethanesulfonic acid.

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enzyme concentration. The values were calculated by fitting the data to the Michaelis-Menten equation by nonlinear regression analysis.

Because of the low $K_m$ for substrate ER, the kinetic parameters were usually determined from progress curves at substrate concentrations of about 6 $K_m$. The values were calculated with the linearized form of the integrated Michaelis-Menten equation (Equation 1) where $(1/t)\ln([S]_t - [P])/([S]_0 - [P]) t$ is plotted versus $[P]/t$. The slope of the curve is $-1/K_m$, and the intercept on the ordinate is $V/K_m$ (7).

$$\frac{(1/t)\ln([S]_t - [P])/([S]_0 - [P])}{t} = V/K_m - \left(\frac{1}{K_m}\right)[P]/t$$  \hspace{1cm} (Eq. 1)

where $[S]$ and $[P]$ are the initial substrate concentration and the product concentration, respectively. When it was possible, the constants were checked at first order conditions, both methods giving similar results.

Theoretical curves for bell-shaped pH-rate profiles were calculated by nonlinear regression analysis using Equation 2.

$$k = k_{\text{limit}} \times \left(\frac{1}{1 + 10^{pK_a - pH} + 10^{pK_b - pH}}\right)$$  \hspace{1cm} (Eq. 2)

where $k$ is the parameter examined, $k_{\text{limit}}$ stands for the maximum value of the parameter, and $K_a$ and $K_b$ represent the acidic ionization constants of the ionized and unionized forms, respectively, of the catalytically competent groups.

Rate-limiting general base/acid catalysis was tested in heavy water (99.9%). The deuterium oxide content of the reaction mixture was at least 95%. The $p$H of deuterium oxide solutions can be obtained from pH meter readings according to the relationship $p$H = pH (meter reading) + 0.4 (8).

Temperature dependence of the rate constants were determined in thermostated cell holders. The reactions were started after the thermal equilibrium had been reached in the cell, which was controlled with a Digi-Sense thermistor thermometer (Cole-Palmer). Activation parameters were calculated from the linear plots of $\ln(k/T)$ versus $1/T$ (Equation 3), where $k$ is the rate constant, $R$ is the gas constant (8.314 J/mol/K), $T$ is the absolute temperature, $N_A$ is the Avogadro number, $h$ is the Planck constant, the enthalpy of activation $\Delta H^*$ = -slope 8.314 J/mol, the entropy of activation $\Delta S^*$ = intercept - 23.76 8.314 J/mol/K. The free energy of activation, $\Delta G^*$, was calculated from Equation 4.

$$\ln(k/T) = \ln(R/N_Ah) + \Delta S^*/R - \Delta H^*/RT$$  \hspace{1cm} (Eq. 3)

$$\Delta G^* = \Delta H^* - T\Delta S^*$$  \hspace{1cm} (Eq. 4)

RESULTS

The $p$H dependence of the reaction of substrate QR with HIV-1 protease has been measured at varying NaCl concentrations. As reported in Fig. 1, the $k_{\text{cat}}/K_m$ values conform to bell-shaped pH-rate profiles and increase markedly with the ionic strength. Fig. 2 shows that the rate increase exhibits a concave up fashion. The $pK_a$ values do not change very much with the ionic strength, with the exception of $pK_1$ at 2.0 M NaCl (Table I). The increase of $k_{\text{cat}}/K_m$ with NaCl concentration is the consequence of both an increase in $k_{\text{cat}}$ and a decrease in $K_m$ (Table II).

Compared with substrate QR, substrate ER reacts at a much higher rate, $k_{\text{cat}}/K_m$ being of about 50-fold at 0.1 M NaCl but only of 3.5-fold at 2.0 M salt concentration (Fig. 3 and Table I). Furthermore, the rate constant for substrate ER does not increase with the NaCl concentration, and the active pH range is narrower. The effects of NaCl concentration on $k_{\text{cat}}$, $K_m$, and $k_{\text{cat}}/K_m$ are reported in Table II. A slight decrease with increasing ionic strength can be observed for both $k_{\text{cat}}$ and $K_m$, which results in a virtually unchanged specificity rate constant. It may be noticed that $k_{\text{cat}}$ decreases with the salt concentration for substrate ER, while for substrate QR it increases.

General base-catalyzed reactions proceed slower by a factor of 2–3 in heavy water than in ordinary water (9). Solvent kinetic deuterium isotope effects characteristic of general base catalysis were found with substrate ER (Table III) in the presence of both 0.1 M and 2.0 M NaCl. The ratios of the rate constants were calculated at the maximum activities, where small changes in pH did not significantly interfere with the results. Surprisingly, with substrate QR an isotope effect characteristic of general base catalysis was only observed at high ionic strength, whereas at low salt concentration a significant isotope effect was not found (Table III).

The temperature dependences of $k_{\text{cat}}/K_m$ for substrates QR, ER, Q, and E have been examined. The temperature dependence and the related parameters of the pH-rate profile for substrate E are shown in Fig. 4 and Table IV, respectively. It is seen that $pK_a$ does not virtually change with temperature, while $pK_b$ decreases slightly. Table V shows that the substrates containing Glu at P2 display positive $\Delta S^*$, whereas those having Gln exhibit negative $\Delta S^*$. The difference between the two types of substrate is also illustrated by the Eyring plots of Fig. 5, showing much steeper slopes with substrate ER than...
with substrate QR. The two least square lines for substrate ER (0.1 M and 2.0 M NaCl) are seen to cross one another near 25 °C. In the case of substrate QR, the activation parameters are significantly dependent on the ionic strength (Fig. 5 and Table V). While $\Delta S^\circ$ values concerning substrates E and ER are positive with respect to $k_{cat}/K_m$, they are negative for $k_{cat}$.

**DISCUSSION**

**Un-ionized Glu in P2' Facilitates Substrate Binding**—The amino acid sequence around the scissile Met-Met bond of the virus polyprotein is one of the regions that is most readily hydrolyzed by the HIV-1 protease (10). The internally quenched fluorogen substrate QR is derived from this sequence by substituting norleucine and p-nitrophenylalanine for the methionine residues, and by adding a 2-aminobenzoyl group to the N terminus of the peptide (4).

In a study on substrates E and Q (3), it has previously been shown that substitution of Glu for Gln at P2' increases the specificity rate constant about 40-fold. Based on the structure of the enzyme complexed with the inhibitor N-acetyl-Thr-Ile-Asp-Nle-[CH$_2$-NH]-Nle-Gln-Arg-NH$_2$ (MVT-101) (5), it has been suggested that the P2' side chain carboxy or carboxamide group forms hydrogen bonds with the backbone NH groups of Asp29 and Asp30' (11). A similar conclusion has been drawn from the structure of HIV-2 protease complexed with a reduced peptide inhibitor containing Glu in P2' (12). In contrast, crystallographic studies with another inhibitor, which contains His in P3', have suggested that Glu in P2' forms hydrogen bonds with both the backbone amide and the side chain carboxy of Asp30' located at the substrate binding site (13).

The pH dependence of $k_{cat}/K_m$ reflects the dissociation of the catalytically competent ionizing groups of the free enzyme and free substrate (9) and may serve as a basis for distinguishing between the two binding modes discussed above. In the case of aspartic peptidases, there is a strong interaction between the two catalytic aspartic acids (1), and this interaction substantially alters their pK$_a$ values, one being lower and the other higher than that of an ordinary carboxy group (14). In the simplest case, when the substrate does not ionize in the pH range studied, pK$_a$ and pK$_b$ extracted from the bell-shaped pH-rate profile depend primarily on the ionizations of Asp-25 and Asp-25' although the interaction between the two carboxy groups of this catalytic dyad is presumably affected by the electric environment involving the ionization states of Asp-30 and Asp-30'. In addition, pK$_a$ is complicated by conformational changes which occur below pH 4 (15). In the case of substrate ER, the ionization state of Glu in P2' controls the rate and the pH-rate profile. The large downward shift in pK$_a$ suggests that it is the protonated glutamic acid that is responsible for the rate enhancement. While the glutamic acid proceeds from un-ionized to ionized state with increasing pH, the binding becomes less effective. This is demonstrated in Fig. 3b which shows that $K_m$ strongly increases (1/K$_m$ decreases) at the high pH values. In the case of substrate QR the change in $K_m$ is much less (not shown). It should be emphasized that these kinetic results only support the participation of the protonated form of Glu at the P2' position, but do not permit us to distinguish between the binding modes involving a backbone NH group or the carboxy group of Asp30'. In the latter case, however, it is again the un-ionized form that should be involved. The data are also consistent with the existence of different binding modes for the two substrates.

**The Effects of Ionic Strength Are Different in the Reactions of Substrates ER and QR**—The high $k_{cat}/K_m$ with substrate ER is due to the much lower $K_m$ value compared to the $K_m$ for substrate QR (Table II). This difference resembles the case of substrates E and Q (3) and indicates that substrate ER binds more strongly than does substrate QR, thereby increasing $k_{cat}/K_m$. Table II shows that the small decrease in $K_m$ for substrate ER at high ionic strength is accompanied by a similar decrease in $k_{cat}$, resulting in negligible changes in $k_{cat}/K_m$. In contrast, the $k_{cat}$ for substrate QR increases considerably with the ionic strength, accounting for a significant portion of the increase in $k_{cat}/K_m$. These data do not support the universal validity of the idea that the rate enhancement with ionic strength arises from the “salting-in” of substrate to the enzyme surface (16, 17). In the catalysis with substrate QR, the increasing salt concentration facilitates the chemical reaction ($k_{cat}$) probably by stabilizing the protein structure as has previously been demonstrated (15). Such effects are not apparent in the reaction of substrate ER where the stronger binding presumably elicits greater stabilization.

**The Rate-determining Step Is Dependent on Ionic Strength and the Nature of Substrate**—In the reactions of chymotrypsin and subtilisin, a kinetic deuterium isotope effect of 2–3 has indicated that the rate-limiting step of the catalytic process is the formation of the tetrahedral intermediate by general base catalysis or the decomposition of the resulting intermediate by general acid catalysis (9). Such an isotope effect is not evident in the reaction of substrate QR at 0.1 M NaCl, while a normal effect of about 2 is found at 2 M NaCl concentration (Table III). It is apparent from these results that the rate-limiting step alters with increasing ionic strength, changing from a physical step at low salt concentration to a chemical one at high salt concentration. The physical step is most likely an isomerization of the enzyme-substrate complex (3, 18).

The rate constant for substrate ER is much higher (2.1 × 10$^5$)

### Table I

| Substrate | [NaCl] | $k_{cat}/K_m$ (limit) | pK$_a$ | pK$_b$ |
|-----------|--------|----------------------|--------|--------|
| QR | 0.1 | 414 ± 12 | 3.30 ± 0.06 | 6.32 ± 0.07 |
| QR | 0.5 | 737 ± 22 | 3.53 ± 0.05 | 6.14 ± 0.06 |
| QR | 1.0 | 1317 ± 41 | 3.56 ± 0.05 | 6.17 ± 0.06 |
| QR | 2.0 | 4492 ± 159 | 4.01 ± 0.08 | 6.26 ± 0.08 |
| ER | 0.1 | 21032 ± 8327 | 4.21 ± 0.11 | 7.46 ± 0.11 |
| ER | 2.0 | 15645 ± 4020 | 4.72 ± 0.11 | 5.44 ± 0.11 |
| ER/QR | 0.1 | 50.8 | | |
| ER/QR | 2.0 | 3.5 | | |

### Table II

| Substrate | [NaCl] | pH | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|-----------|--------|----|-----------|-------|--------------|
| QR | 0.1 | 4.80 | 5.80 | 15.2 | 0.38 |
| QR | 1.0 | 4.90 | 9.25 | 8.73 | 1.06 |
| QR | 2.0 | 5.00 | 19.48 | 5.98 | 3.25 |
| ER | 0.1 | 4.52 | 5.46 | 0.84 | 6.5 |
| ER | 2.0 | 5.15 | 4.14 | 0.69 | 6.0 |
The rate-limiting step with substrate ER is not affected by the salt concentration and shows that at low ionic strength it is rate-limiting. Alternatively, the binding mode for substrate ER may require a less pronounced conformational change. In the case of substrate QR, however, isomerization of the enzyme-substrate complex speeds up with the salt concentration according to a concave up fashion (Fig. 2). Similar curves were also observed with other substrates (19), but kinetic isotope effects were not determined at high salt concentrations (18).

The particular role of Glu in P2 of substrate QR changes from a considerably negative value to slightly negative for substrates Q and QR although ΔS* for substrate QR changes from a considerably negative value to slightly above zero, while the salt concentration increases from 0.1 to 2 M NaCl. It is important that the reactions with positive ΔS* exhibit large positive ΔS* values—The particular role of Glu in P2 also manifests in the activation parameters of substrate hydrolysis. The activation entropy is a negative value for most enzymic reactions because the transition state is usually more ordered as is the ground state. It is seen in Table V that ΔS* is positive for both substrate E and substrate ER, whereas it is negative for substrates Q and QR although ΔS* for substrate QR changes from a considerably negative value to slightly above zero, while the salt concentration increases from 0.1 to 2 M NaCl. It is important that the reactions with positive ΔS* display significant kinetic deuterium isotope effects, referring to a rate-limiting chemical step in the reaction.

The positive ΔS* with substrates E and ER can be interpreted in terms of hydration of the reacting species which release the water molecules in the chemical transition state. This is particularly important at high ionic strength where...
hydration is heavier (15), which makes the entropy of activation more positive. In the case of $k_{cat}$, which does not involve a binding component, the $\Delta S^*$ is negative for substrates E and ER (Table V), as expected. The above results clearly illustrate that Glu in P2 endows the substrate of HIV-1 protease with the highest specificity ever found. The use of Glu in inhibitors may help design potent therapeutic agents for HIV-1 infection. The inhibitors that have entered clinical study are limited by the emergence of resistant viral variants. A particular benefit of drugs with Glu in P2 may be that mutation of Asp-29 or Asp-30 involved in the binding generates inactive or ineffective enzymes (20–22), which rules out the formation of resistant variants at these sites.

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**FIG. 5.** Eyring plots for the reactions of substrates QR and ER. Open and full signs stand for substrates QR and ER, respectively, in the presence of 0.1 M (●), 0.5 M (○), and 2.0 M (□) NaCl.