Substrate-Dependent Mechanisms in the Catalysis of Human Immunodeficiency Virus Protease†

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ABSTRACT: The most preferred residue in the substrates of human immunodeficiency virus (HIV-1) protease is glutamic acid in the P2′ position. The catalytic importance of this charged residue has been studied to obtain a detailed insight into the mechanism of action, which will promote drug design to combat the virus. To this end, we have synthesized Lys-Ala-Arg-Val-Leu*Phe(NO_2)-Glu-Ala-Nle (substrate E) and its counterpart containing the neutral Gln (substrate Q) in place of Glu. Kinetic analyses have shown that the specificity rate constants (k_{cat}/K_m) display bell-shaped pH dependencies for both substrates, but the pH-independent limiting value is 35–40-fold higher with substrate E than with substrate Q. In contrast to the pH–rate profiles of k_{cat}/K_m, there is a striking difference between the pH dependencies of K_m and k_{cat} for the two substrates. This indicates different ground state and transition state stabilizations in the two reactions. Solvent kinetic deuterium isotope effects show that the rate-limiting step for the hydrolysis of substrate E is a chemical step coupled with proton transfer whereas with substrate Q it is a physical step, presumably a conformational change. Accordingly, the charged residue in P2′ alters the rate-limiting step and the nature of the enzyme–substrate complex, resulting in different mechanisms for the two substrates.

The protease encoded by the human immunodeficiency virus type 1 (HIV-1)[1] is essential for specific processing of large viral polyproteins into individual structural proteins and enzymes. Hence, the HIV protease has received considerable attention as a potential therapeutic target of acquired immunodeficiency syndrome (AIDS), the disease caused by the virus (Huff, 1991; Johnston & Hoth, 1993). The enzyme is a member of the aspartic protease family and exists as a homodimer of identical polypeptide chains, each consisting of 99 amino acid residues. A long cleft formed at the interface of the dimer harbors the catalytically competent aspartyl residues, Asp25 and Asp25′, contributed by each subunit (Wlodawer et al., 1989). The cleft also contains eight subsites, S4, S3, S2, S1, S1′, S2′, S3′, and S4′, representing the corresponding binding sites of the P4, P3, P2, P1, P1′, P2′, P3′, and P4′ residues of an octapeptide substrate having an extended conformation. The substrate is cleaved between the P1 and P1′ residues. Close to the cleavage site are located some charged residues such as Asp29 and Asp30 at the Sn and Asp29′ and Asp30′ at the Sn′ side of the extended binding cleft. In the native enzyme, Asp29 forms ionic interactions with Arg8′ and Arg87 (Miller et al., 1989). This interaction may change or remains unchanged on ligand binding as found with a substrate-based inhibitor containing Arg as the P3′ residue. Specifically, in one of the monomers Arg8 is pushed away by the P3′ Arg of the inhibitor, which replaces it as the partner of Asp29′ in the salt bridge, whereas the corresponding charge interaction on the other (Sn) side (Asp29–Arg8′) is not affected (Miller et al., 1989). Because of the symmetric nature of the enzyme, the residues of the binding cleft are contributed from both monomers.

Many substrates of HIV protease contain Glu as the P2′ residue (Poorman et al., 1991; Chou, 1993). This residue is more conservative than P1 and P1′ of the scissile bond. Interestingly, the S2′ binding pocket only contains neutral residues, such as Val32′, Ile47′, Gly48′, and Ile50 (Miller et al., 1989). To study the importance of the charged P2′ Glu, we have synthesized the sensitive nonapeptide substrate KARVL*F(NO_2)EANle (Richards et al., 1990) and its counterpart KARVL*F(NO_2)QANle, containing the neutral glutamine in place of glutamic acid. Due to the chromogen F(NO_2), the hydrolysis of the substrates can be continuously monitored with a spectrophotometer, which provides more precise kinetic data than those obtained with the HPLC technique most frequently used. This permitted us to analyze readily the kinetic parameters of the reactions of the two substrates at different pH and ionic strength values and to demonstrate that the negative charge on the substrate remarkably affects both the efficacy and the mechanism of the catalysis.

EXPERIMENTAL PROCEDURES

Isolation of HIV Protease. (A) Preparation of Inclusion Bodies. Escherichia coli JM101 cells carrying the pPROLAC 6.5 recombinant plasmid were used to express the protease (Giam & Boros, 1988). Cells from 1000 mL of medium (4–5 g net weight) were stored at −18 °C. A 4 g portion of cell paste was suspended in 40 mL of 50 mM phosphate buffer, pH 7.5, containing 5 mM cysteine and 2 mM EDTA and disrupted by sonication over a salted ice bath with an MSE sonifier so that the absorbance at 550 nm decreased to about 30% of the initial value. Care was taken to keep the temperature of the suspension below 10 °C. The cell lysate was centrifuged at 10000*g* for 10 min, and the pellet was washed 2 times with short sonication using 10 mL of sonication buffer at each occasion. The precipitates (≈0.5

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[1] Abbreviations: HIV-1, human immunodeficiency virus, type 1; DTE, dithioerythritol; Phe(NO_2), 4-nitrophenylalanine; Nle, norleucine; EDTA, ethylenediaminetetraacetic acid.

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g) containing the inclusion bodies were stored at −18 °C or used immediately.

(B) Renaturation. Inclusion bodies corresponding to 1 g of cell paste were slowly stirred with a magnetic stirrer in 40 mL of 6 M guanidinium chloride containing 5 mM DTE (pH ≈4.8) at room temperature for 10 min. The almost clear solution was centrifuged at 12500*g* for 10 min at 4 °C and diluted 10-fold with cold 50 mM acetate buffer, pH 5.5, containing 10% glycerol, 2 mM cysteine, and 2 mM EDTA. The refolding mixture was kept at 4 °C for 10 min and then centrifuged on a Beckman J2-21 centrifuge at 4 °C, at 10000*g* for 10 min. To the clear supernatant (400 mL) was added ammonium sulfate to 50% saturation (313 g to 1000 mL). After incubation at 4 °C for 30 min, the precipitate was collected by centrifugation at 4 °C, at 10000*g* for 10 min. The resulting small precipitate in the large tubes was suspended in a few milliliters of ammonium sulfate solution saturated to 50% and centrifuged as above in a small tube.

(C) Extraction. The precipitate from the previous centrifugation was successively extracted with 2, 6, 6, and 2 mL of 50 mM acetate buffer, pH 4.0, containing 10% glycerol, 5 mM cysteine, and 2 mM EDTA. Each extraction was performed at room temperature with cold buffer while very slowly stirring the 2 and 6 mL samples for 5 and 10 min, respectively, with a magnetic stirrer. Each fraction was centrifuged at 4 °C, at 10000*g* for 5 min. The fractions containing the highest activities were combined and centrifuged at 4 °C, at 18000*g* for 10 min. The supernatant was concentrated with an Amicon PM 10 membrane so that the absorbance of the solution measured at 280 nm remained below 0.8 unit since at higher values the enzyme tends to precipitate. SDS gel electrophoresis displayed a main band at the expected position of HIV protease and a fainter, diffuse region corresponding to lower molecular mass proteins.

(D) Gel Chromatography. The final purification was achieved at 5 °C on a Sephacryl S100 HR column (Pharmacia) equilibrated with 50 mM phosphate buffer, pH 7.75, containing 5% glycerol, 0.1% poly(ethylene glycol) 4000, 0.1 M NaCl, 5 mM DTE, and 2 mM EDTA. A 4.5 mL sample was applied to the column (2.0 × 90 cm) and washed with the equilibration buffer at a flow rate of 16 mL/hr. Fractions of 4 mL were collected. The combined active fractions (16–20 mL) contained 0.6 mg of enzyme. The protein concentration was calculated from A_{280} = 1.18 for the 1 mg/mL solution calculated from the amino acid composition. A molecular mass of 21 584 Da was obtained for the dimer from the amino acid composition.

Substrates. Lys-Ala-Arg-Val-Leu-Phe(NO_2)-Glu-Ala-Nle (substrate E) and Lys-Ala-Arg-Val-Leu-Phe(NO_2)-Gln-Ala-Nle (substrate Q) were prepared by solid phase synthesis (ABI 431A), and purified by HPLC.

Activity Measurement. For routine assays, substrate E was dissolved in 1 mM HCl at a concentration of 2 mg/mL. The reaction was measured in 50 mM acetate buffer, pH 4.0, containing 1 mM EDTA, 1 mM DTE, 0.1 M NaCl, and 5% glycerol at 25 °C. The final volume of 800 μL contained 8 μL of substrate (18.3 μM) and an appropriate amount of enzyme (40–150 nM). The reaction was monitored by measuring the decrease in absorbance at 300 nm (Richards et al., 1990).

Active Site Titration. The C_2-symmetric inhibitor Q8024 [compound 3 in Jadhav and Woerner (1992)], which has a K_i of 0.16 nM, was dissolved in dimethyl sulfoxide (3.26 mM). This stock solution was first diluted 100-fold in dimethyl sulfoxide and then 70-fold in water (466 nM), and increasing concentrations of this solution (0.1–1.2 equiv) were added to the enzyme (about 45 nM) in 0.1 M acetate buffer, pH 4.9, containing 0.5 M NaCl, 1 mM EDTA, and 1 mM DTE. After 5 min incubation at 25 °C, the reactions were started with the addition of substrate E. The remaining activities calculated from the initial rates were plotted against the inhibitor concentration. The reuslting straight lines obtained at two substrate concentrations (18.3 and 36.6 μM) intersected the abscissa, within experimental error, at the same point. This inhibitor concentration was considered to be equivalent to the concentration of active enzyme.

Kinetics. When the K_m was sufficiently high, the rate constants were measured under first-order conditions, i.e., at substrate concentrations lower than K_m, and were calculated by nonlinear regression data analysis using the Grafit computer program (Sigma). The second-order rate constant (k_{cat}/K_m) as obtained by dividing the first-order rate constant by the active enzyme concentration present in the reaction mixture.

The k_{cat} and K_m values were determined from initial rate measurements. The data were calculated by fitting the experimental points to the Michaelis–Menten equation by using nonlinear regression analysis.

The pH dependence of the rate constants was measured in 50 mM acetate buffer, containing 1 mM EDTA, 1 mM DTE, and 5% glycerol, by using a Cary 1E spectrophotometer equipped with thermostatable cell holders. The ionic strength over the pH range was maintained by addition of NaCl to give identical conductivity values. At the end of the reaction, the pH of each sample was determined and found to be identical with the initial value. Theoretical curves for bell-shaped pH–rate profiles were calculated by nonlinear regression analysis, using the Grafit software, according to

$$k = k(\text{limit})[1/(1 + 10^{pK_1-pH} + 10^{pH-pK_2})] \quad (1)$$

where k may be either k_{cat} or k_{cat}/k_m, k(limit) stands for the pH-independent maximum rate constant, and K_1 and K_2 represent the acidic ionization constants of the acidic and basic forms, respectively, of the catalytically competent ionizing groups.

Additional ionization of substrate E was considered by using eq 2, where pK_E accounted for the dissociation of the glutamic acid. The pH–k_{cat} profile having a plateau at high and low pH was fitted to eq 3, where k_L and k_H gave the limiting values of k_{cat} at low and high pH, respectively, and pK_a reflected the ionization of a group in the enzyme–substrate complex.

$$k = k(\text{limit})[1/(1 + 10^{pK_1-pH} + 10^{pK_1+pK_E-2pH} + 10^{pH-pK_2})] \quad (2)$$

$$k = k_L/(1 + 10^{pH-pK_a}) + k_H/(1 + 10^{pK_a-pH}) \quad (3)$$

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^2H of deuterium oxide solutions can be obtained from pH meter readings according to the relationship p^2H = pH(meter reading) + 0.4 (Glasoe & Long, 1960).

RESULTS

The pH–rate profiles for the reactions of HIV protease with substrate E were measured at five ionic strengths. For the sake of clarity, only three curves are illustrated in Figure 1, but the parameters for all five curves are given in Table 1. All five data sets fit to bell-shaped curves, independent of whether the data were measured with first-order or Michaelis–

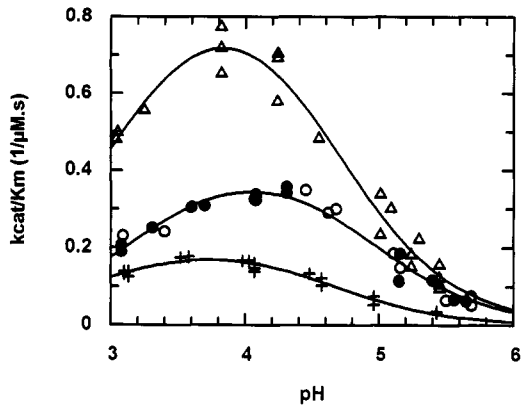

FIGURE 1: pH dependence of the specificity rate constant for the HIV protease reaction at different NaCl concentrations. The ionic strength was maintained constant over the pH range at each NaCl concentration. The reactions were measured at 25 °C in 50 mM acetate buffer containing 5% glycerol, 1 mM EDTA, and 1 mM DTE (+), with additional 0.2 M NaCl under first-order conditions (○) and with Michaelis–Menten kinetics (●), and with additional 1.0 M NaCl (△). The curves drawn through the experimental points were obtained from fitting to eq 1. The parameters of the curves are found in Table 1.

Table 1: Kinetic Parameters of HIV Protease Catalysis[a]

substrate	[NaCl] (M)	k_{cat}/K_m(limit) (mM^{-1} s^{-1})	pK_1	pK_2	condition[b]
E		210 ± 12	2.83 ± 0.09	4.62 ± 0.07	1
E	0.1	320 ± 11	2.85 ± 0.04	4.57 ± 0.04	1
E	0.2	434 ± 17	3.17 ± 0.06	4.93 ± 0.05	1,M
E	0.5	807 ± 68	3.14 ± 0.12	4.72 ± 0.08	1,M
E	1.0	932 ± 52	3.01 ± 0.09	4.65 ± 0.06	M
E	0.5/0.1	2.52			
Q	0.1	8.18 ± 0.61	2.96 ± 0.15	4.93 ± 0.11	1
Q	0.5	22.50 ± 1.97	3.23 ± 0.13	4.90 ± 0.11	1,M
Q	0.5/0.1	2.75			
E/Q	0.1	39.1			
E/Q	0.5	35.9			

[a] Measured at 25 °C and calculated from eq 1. [b] 1, first-order; M, Michaelis–Menten kinetics.

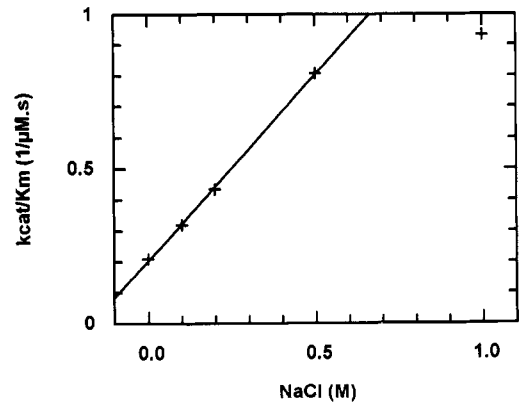

FIGURE 2: Effect of NaCl concentration on the limiting value of the specificity rate constant. The data were taken from Table 1. The straight line represents linear fit to the first four points.

Menten kinetics. As seen from Figure 1 and Table 1, there is a substantial rate enhancement with increasing salt concentration, while the pK_1 and pK_2 values change only slightly. A plot of the pH-independent limiting values of the rate constants against NaCl concentration exhibits linearity up to about 0.5 M salt (Figure 2).

The reaction conditions for measuring the rate constants were dependent on the K_m values which varied considerably with pH and ionic strength. For example, at low pH, in particular in the presence of high salt concentration, the low K_m (6 μM at pH 3.9 and 0.5 M NaCl) did not permit the use of first-order conditions. In contrast, first-order conditions were readily attained at high pH and low ionic strength. This is consistent with Figure 3a, which shows that after a maximum value $1/K_m$ decreases (K_m increases) with pH. It is important that the pK_1 and pK_2 values extracted from the plot of $1/K_m$ against pH are similar to the corresponding values obtained from the pH dependence of the specificity rate constants (Table 1). This is conceivable since both k_{cat}/K_m and $1/K_m$ follow the ionizations in the free enzyme and free substrate (Fersht, 1985). Whereas K_m considerably alters with pH (23-fold between pH 4 and 6, Figure 3a), k_{cat} changes to a smaller extent (1.8-fold in the same pH range, Figure 3b), indicating that it is the increase in K_m that primarily accounts for the decrease in k_{cat}/K_m at high pH.

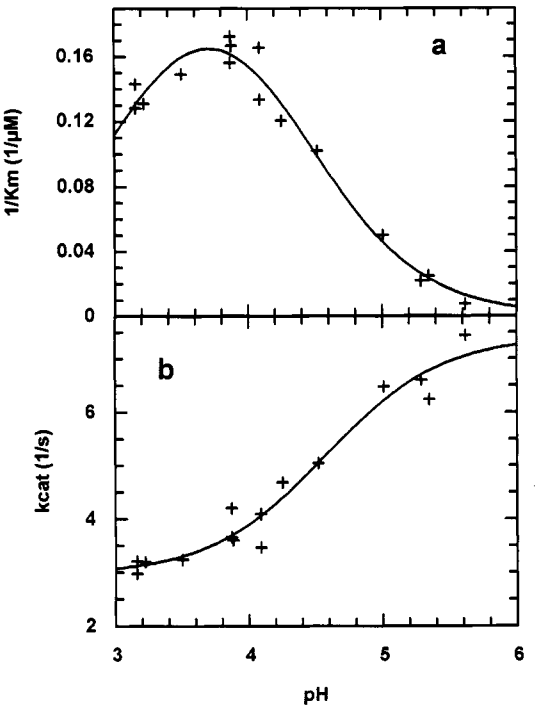

FIGURE 3: pH dependence of $1/K_m$ and k_{cat} for substrate E. The reactions were carried out in the presence of 0.5 M NaCl. (a) The points were fitted to an analogue of eq 1 containing $1/K_m$ in place of k. The following parameters were used: $1/K_m$(limit) = 0.212 μM^{-1}, pK_1 = 3.12, pK_2 = 4.76. (b) The points were fitted to eq 3, by using the parameters pK_a = 4.57, k_L = 2.95 s^{-1}, and k_H = 7.42 s^{-1}.

The specificity constant measured with substrate Q gave also bell-shaped pH dependencies at 0.1 and 0.5 M NaCl (Figure 4), but with limiting k_{cat}/K_m values 35–40 times lower compared with the corresponding parameters of substrate E (Table 1). However, the rate enhancements elicited by increasing ionic strength were similar with the two substrates (Table 1). The pH dependencies of K_m and k_{cat} for substrate Q were substantially different from those for substrate E. The K_m for substrate Q was 50 μM at pH 3.9 in the presence of 0.5 M NaCl, whereas the K_m for substrate E was 1 order of magnitude lower, 6 μM at pH 3.9. With increasing pH, the difference in K_m's gradually diminished, and the values became similar at about pH 5.5, as is apparent from a comparison of Figure 3a and Figure 5a. As for the pH dependencies of k_{cat}, a "wave-shaped" (Figure 3b) pH–k_{cat} profile was obtained for substrate E, whereas a bell-shaped one (Figure 5b) was observed for substrate Q.

General base-catalyzed reactions proceed in heavy water by a factor of 2–3 slower than in ordinary water (Bender &

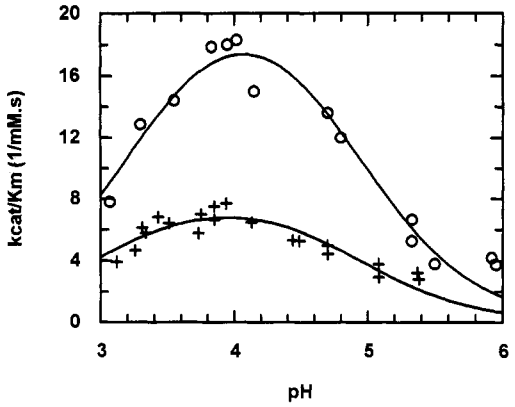

FIGURE 4: pH dependence of the reaction of HIV protease with substrate Q in the presence of 0.1 M (+) and 0.5 M (O) NaCl. The experimental conditions were the same as in Figure 1. The curves drawn through the experimental points were obtained from fitting to eq 1. The parameters of the curves are shown in Table 1.

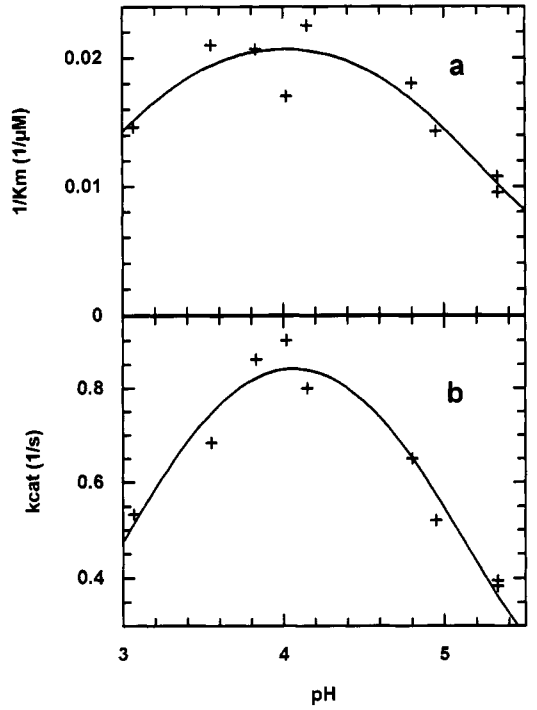

FIGURE 5: pH dependence of $1/K_m$ and k_{cat} for substrate Q. The reactions were carried out in the presence of 0.5 M NaCl. (a) The points were fitted to an analogue of eq 1 containing $1/K_m$ in place of k. The following parameters were used: $1/K_m$(limit) = 0.023 μM^{-1}, pK_1 = 2.79, pK_2 = 5.23. (b) The points were fitted to eq 1, by using the parameters k_{cat} = 1.00 s^{-1}, pK_1 = 3.04, and pK_2 = 5.09.

Kézdy, 1965). A solvent kinetic dueterium isotope effect characteristic of general base catalysis was found with substrate E, as is seen in Figure 6. In contrast, there was no significant effect with substrate Q. The parameters of the curves for both substrates are shown in Table 2, indicating that the shapes of the curves virtually do not change in heavy water. It can be seen from the ratios of the second-order rate constants that a chemical step is rate-limiting with substrate E, but not with substrate Q.

DISCUSSION

pH Dependence Studies. The HIV protease, similarly to other aspartic peptidases, contains a catalytic triad of two aspartic acids and a water molecule (Wlodawer & Erickson, 1993). This system is characterized by a bell-shaped pH dependence with respect to the specificity rate constant (k_{cat}/K_m), as has been observed for many aspartic peptidases [cf. Polgár (1989)], including the HIV protease (Ido et al., 1991; Hyland et al., 1991a). From a bell-shaped pH dependence curve can be extracted two pK_a values, which are characteristic of two catalytically competent ionizing groups. In the case of aspartic peptidases, there is a strong interaction between the two catalytic aspartic acids (Wlodawer et al., 1989; Wlodawer & Erickson, 1993), and this interaction substantially alters their pK_a values, one becoming lower and the other higher than that of an ordinary carboxy group (Polgár, 1989). The ionization of these two carboxy groups, which operate by a push–pull mechanism (Polgár, 1987a, 1989; Hyland et al., 1991a), may account for the bell-shaped pH–rate profiles since the assumption that ionization of the water molecule would occur in the free enzyme, rather than the ionization of the more acidic carboxy groups, is chemically unreasonable.

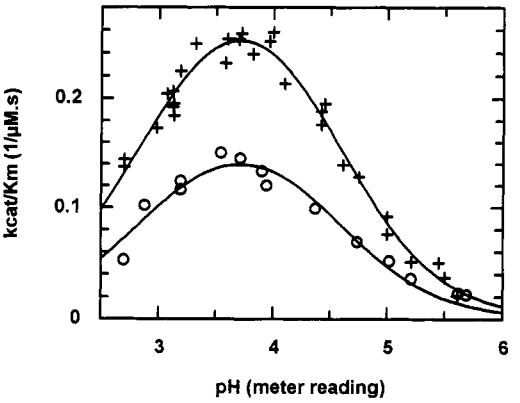

FIGURE 6: Kinetic deuterium isotope effects. The reactions were measured with substrate E in water (+) and heavy water (O) in the presence of 0.1 M NaCl.

Table 2: Kinetic Solvent Isotope Effects on the Reaction Parameters[a]

condition	k_{cat}/K_m(limit) (mM^{-1} s^{-1})	pK_1	pK_2
substrate E			
H_2O	320 ± 11	2.85 ± 0.04	4.57 ± 0.04
^2H_2O	181 ± 17	2.87 ± 0.12	4.55 ± 0.11
$H_2O/^2H_2O$	1.77		
substrate Q			
H_2O	8.18 ± 0.61	2.96 ± 0.15	4.93 ± 0.11
^2H_2O	7.50 ± 0.99	2.85 ± 0.18	4.88 ± 0.15
$H_2O/^2H_2O$	1.09		

[a] Measured at 25 °C in the presence of 0.1 M NaCl and calculated from eq 1.

The pH dependence of k_{cat}/K_m can also be affected by the ionizing groups of the substrate. Hence, the P2′ glutamic acid, the most conserved residue in the substrates of HIV protease, may distort the simple bell-shaped pH–rate profile. The 35–40-fold difference between the rate constants for substrates E and Q (Table 1) indicates that the charged Glu in P2′ is indeed important catalytically. It is, therefore, interesting that both substrate E and substrate Q have similar pH–k_{cat}/K_m profiles, so that the dissociation of the extra carboxy group of substrate E apparently does not modify the bell-shaped profile (Figure 1).

It can be assumed that the two forms of substrate E having ionized or unionized glutamic acid display different reactivities, the un-ionized form being presumably 35–40-fold less reactive, as is substrate Q. Whether or not the two forms of substrate E are hidden in an apparently bell-shaped curve was tested by fitting the data points to eq 2, which involves the contribution

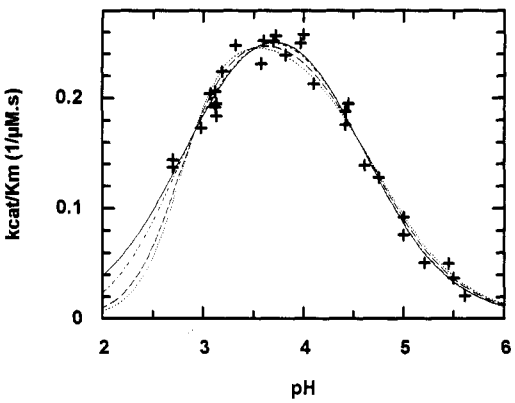

FIGURE 7: Demonstration that substrate ionization may not significantly perturb the pH dependence curve. The reactions were measured in the presence of 0.1 M NaCl. (—) Equation 1; (– · –) eq 2 with pK_E arbitrarily set to 2.0; (– –) eq 2 with pK_E set to 3.0; (···) eq 2 with pK_E = 4.5. The parameters of the curves are shown in Table 3.

Table 3: Variation of Kinetic Parameters at Arbitrarily Set pK_E Values[a]

parameters	bell-shaped	pK_E = 2.0	pK_E = 3.0	pK_E = 4.5
k_{cat}/K_m (mM^{-1} s^{-1})	320 ± 11	313 ± 10	288 ± 10	270 ± 9
pK_1	2.85 ± 0.04	2.78 ± 0.05	2.40 ± 0.06	1.08 ± 0.08
pK_2	4.57 ± 0.04	4.58 ± 0.04	4.66 ± 0.05	4.72 ± 0.06

[a] Measured in the presence of 0.1 M NaCl and calculated from eq 2.

of the ionized substrate, but neglects the contribution of the assumed 35–40-fold less reactivity of the un-ionized form (Figure 7). The interconversion of the two forms occurs with pK_E. The calculations were made at fixed pK_E values of 2.0, 3.0, and 4.5 (Table 3), allowing a wide range for the dissociation of the carboxy group. It is seen in Figure 7 that the data points fit to both eq 1 and eq 2 within experimental error. Significant deviation would only be expected well below pH 3, which is, however, not accessible to experimentation. Consequently, the observation of simple bell-shaped curves in Figure 1 neither verifies nor precludes the involvement of a third ionizing group, which could give an overlapping ionization with pK_1. However, the large difference in the k_{cat}/K_m values of substrates E and Q clearly demonstrates the importance of the charged glutamic acid compared with the neutral glutamine.

Our results shown in Figure 1 differ in some respects from those obtained with peptides similar to substrate E. These peptides contained norleucine amide or methionine (Richards et al., 1990) in place of the C-terminal norleucine of substrate E. The kinetic parameters determined at 37 °C and at 1 M ionic strength provided similar results with the two substrates containing the carboxy or carboxylic amide group at the C-terminus. This indicated that ionization of the C-terminal carboxy group did not affect the pH dependence of the reaction. However, they found a much higher pK_2 (5.7) compared with our result (4.65, Table 1, 1 M NaCl). The pK_1 was not observed in that study because the rate constants were not determined below pH 4.1. The pH dependence of k_{cat} was also different, inasmuch as Richards et al. (1990) found that the rate constant was independent of pH, whereas we observed a significant enhancement in k_{cat} with increasing pH (Figure 3b).

The pH dependencies of k_{cat} and k_{cat}/K_m were also examined in two other studies. One study used four substrates containing the Tyr–Pro and Leu–Phe scissile bonds and Val or Leu as the P2′ residue (Hyland et al., 1991a). In the presence of 0.2 M NaCl at 37 °C, the pK_1 and pK_2 values extracted from the pH–k_{cat}/K_m profile were found to be significantly higher (3.4–3.7 and 5.5–6.5, respectively) than those observed in the present study at 25 °C (3.18 and 4.97, respectively). The other study (Ido et al., 1991) employed AcSQNYPVVR substrate and found bell-shaped curves for both k_{cat}/K_m (pK_1 = 3.32 and pK_2 and 6.80) and k_{cat} (pK_1 = 5.10 and pK_2 = 6.92). Interestingly, the k_{cat} for substrate E conformed to a "wave-shaped" curve as in the study by Hyland et al. (1991), but for substrate Q, it gave a bell-shaped curve as found by Ido et al. (1991). It appears then that the pH dependence of k_{cat} is very much dependent on the substrate.

Scheme 1

$$E + S \rightleftharpoons ES \rightleftharpoons E'S \rightleftharpoons ET \rightleftharpoons E + P' + P''$$

Since the previous studies (Richards et al., 1991; Hyland et al., 1991a; Ido et al., 1991) were performed at 37 °C, we also measured the reactions at that temperature. It was found that the limiting value of the rate constant increased about 2.8-fold with a concomitant decrease in K_m, and the pK_2 did not shift toward higher values (not shown). Thus, the effects of temperature cannot account for the difference between the previous (Richards et al., 1990) and present results. The reason for the difference is not known.

The effect of ionic strength on HIV protease catalysis has already been demonstrated (Hyland et al., 1991b; Cheng et al., 1990; Richards et al., 1990), but its dependence on pH has not been determined. Our results have shown that, because of the changes in pK_a's with ionic strength, comparison of the rate constants at a single pH, particularly outside the optimum, may not be meaningful. Therefore, determination of the effect of ionic strength at a single pH may be of limited value. The reactions measured with AcAKILFLDG-NH_2 or AcRA-SQNYPVV-NH_2 at pH 6.0 far from the pH optimum (Hyland et al., 1991b) differ from that shown in Figure 2, inasmuch as the specificity rate constants were increased in a nonlinear (concave up) fashion. It is not clear whether the different characteristics of the substrate, the higher temperature value, the distinct pH above pK_2, or the combination of these factors, could account for the different results.

The Rate-Limiting Step. The study of kinetic deuterium isotope effects has proved to be rewarding in the study of the mechanism of action of proteases. Such studies have shown that the catalysis proceeds slower in heavy water by a factor of 2–3, indicating that the rate-limiting step of the reaction involves general base/acid catalysis (Polgár, 1987b). General base and general acid catalyses, respectively, promote the formation and decomposition of the tetrahedral intermediate, an obligatory species on the reaction pathways of peptidases, including the aspartic enzymes.

There is only one study concerning the kinetic deuterium isotope effects on the reactions of HIV protease (Hylands et al., 1991a). Using two substrates, both possessing the Tyr–Pro scissile bond and Val at position P2′, this study demonstrated the lack of kinetic deuterium isotope effects on the pH-independent specificity rate constant and explained the result in terms of rate-limiting isomerization of the enzyme–substrate complex (ES ⇌ E′S) as shown in Scheme 1, which represents the minimal mechanism of the hydrolysis of substrate (S) to products (P′ and P″). Indeed, a conformational change on the binding of substrate is consistent with X-ray crystallographic studies, which have shown that a glycine-rich loop, known as the "flap", moves considerably on substrate binding to cover the peptide embedded in the cleft (Miller et al., 1989). Our result with substrate Q is similar

to that obtained by Hyland et al. (1991a). On the other hand, the reaction with substrate E exhibits a reasonable isotope effect (Table 2), indicating that the rate-limiting step with this peptide should involve proton transfer, which is associated with the formation (E′S ⇌ ET in Scheme 1) or the decomposition (ET ⇌ E + P′ + P″ in Scheme 1) of the tetrahedral intermediate. Thus, the active conformation of the enzyme–substrate complex can be attained much faster with the substrate bearing the ionized glutamic acid.

Nature of the Enzyme–Substrate Complex. The preference of HIV protease for Glu or, to a lesser extent, Gln in P2′ was explained in terms of hydrogen bonding of the side chain carboxy or carboxylic amide group to the backbone NH of residues 29′ and 30′ of the enzyme (Griffiths et al., 1992), based on the structure of an inhibitor containing the neutral Gln at the P2′ position (Miller et al., 1989). Although the substrate with the negatively charged Glu in P2′ may assume a different binding mode, recent X-ray crystallographic studies on a HIV-2 protease complex with reduced peptide inhibitor [Phe-Val-Phe(CH_2-NH)Leu-Glu-Ile-NH_2] have shown that the glutamate side chain of the inhibitor does form hydrogen bonds with the main chain amido groups of residues 29′ and 30′ (Tong et al., 1993). Since the active sites of HIV-1 and HIV-2 proteases are practically identical, the HIV-1 protease complexes formed with substrate E and substrate Q may exhibit similar structures. However, it cannot be ruled out that the glutamic acid of a different substrate may assume a different position in the complex with another type of substrate, such as substrate E.

Even if their structures are similar, the complexes formed with substrate E and substrate Q must be different with respect to the strengths of the hydrogen bonds with the Glu and Gln residues. Specifically, the hydrogen bonds formed between uncharged partners contribute to the specificity of binding by about 0.5–1.5 kcal/mol, and this value increases up to 3–6 kcal/mol when one of the partners is a charged group (Fersht, 1987). Since 1.364 kcal/mol of energy is required for a 10-fold rate enhancement, the 37.5-fold average increase in k_{cat}/K_m (Table 1) with substrate E relative to substrate Q requires 5.1 kcal/mol, which is in the permitted range of the energy difference existing between hydrogen bonds involving neutral and charged residues. Thus, the gain in binding energy can alone account for the lower K_m and/or higher k_{cat} with substrate E.

The importance of Glu in the P2′ position is further supported by the following observation. In a poor substrate having a Tyr–Pro scissile bond (Lys-Ala-Arg-Val-Tyr*Pro-Glu-Ala-Nle-NH_2), the k_{cat} decreased more than 5000-fold when Glu was replaced by Gln (Griffiths et al., 1992). In this example, the Glu residue was able to govern the scissile bond to a position suitable for hydrolysis, whereas the other compound did not react at all, probably because it preferred a different, nonproductive binding mode.

It should be noted that K_m is not necessarily identical with K_s, the enzyme–substrate dissociation constant (Ido et al., 1991), but it usually approaches it in the case of aspartic peptidases (Medzihradszky et al., 1970). The difference in K_m for substrates E and Q is 1 order of magnitude at pH 3.9–4.0, but substantially changes with pH, and practically disappears around pH 5.5. It has previously been reported that major changes occur in k_{cat} rather than in K_m when substituting different residues for Glu in P2′ (Griffiths, 1992). However, this finding is only valid in the higher pH region where the experiment was performed (pH 4.72).

The present measurements carried out in a wide pH range (3–6) indicate that with substrate E there is a large variation in K_m, while k_{cat} increases to a small extent with increasing pH (Figure 3). On the other hand, with substrate Q the K_m changes only slightly, whereas k_{cat} fits to a bell-shaped curve (Figure 5), as found with k_{cat}/K_m, though the change with pH was less pronounced. Accordingly, the origin of the large differences between the specificity rate constants for substrate E and substrate Q depends on the pH. Near the maximum of the specificity rate constant, the change in K_m predominates, whereas with increasing pH the change in k_{cat} becomes more and more important, and the difference in K_m disappears at about pH 5.5. This indicates that at pH 5.5 stronger interactions are formed in the transition state of the substrate E reaction compared to the substrate Q reaction, having reduced k_{cat}, but similar K_m. On the other hand, at the optimum activities, the better ground state stabilization, and to some extent the better transition state stabilization as well, can account for the higher k_{cat}/K_m of the substrate E reaction.

The stronger binding with substrate E appears to be a fast step, which takes place more rapidly than the rate-limiting chemical step involving general base/acid catalysis. In contrast, the rate-limiting step with substrate Q is a conformational change presumably induced by substrate binding. Consequently, the additional negative charge on substrate E facilitates peptide binding and formation of the productive enzyme–substrate complex. This leads to the change in the rate-limiting step when compared to the catalytic mechanism of substrate Q possessing a neutral P2′ residue.

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