Two-Step Binding Mechanism for HIV Protease Inhibitors

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ABSTRACT: Rate constants for binding of five inhibitors of human immunodeficiency virus (HIV) protease were determined by stopped-flow spectrofluorometry. The two isomers of quinoline-2-carbonyl-Asn-PheΨ-[CH(OH)CH_2N]Pro-O-*t*-Bu (*R* diastereomer = **1R**; *S* diastereomer = **1S**) quenched the protein fluorescence of HIV protease and thus provided a spectrofluorometric method to determine their binding rate constants. The dissociation rate constants for acetyl-Thr-Ile-LeuΨ(CH_2NH)Leu-Gln-Arg-NH_2 (**2**), (carbobenzyloxy)-PheΨ[CH(OH)CH_2N]Pro-O-*t*-Bu (**3**), and pepstatin were determined by trapping free enzyme with **1R** as **2**, **3**, and pepstatin dissociated from the respective enzyme·inhibitor complex. Association rate constants of **1R**, **2**, and pepstatin were calculated from the time-dependent inhibition of protease-catalyzed hydrolysis of the fluorescent substrate (2-aminobenzoyl)-Thr-Ile-Nle-Phe(NO_2)-Gln-Arg-NH_2 (**4**). The kinetic data for binding of **1S** to the protease fit a two-step mechanism. K_d values for these inhibitors were calculated from the rate constants for binding and were similar to the respective steady-state K_i values.

Human immunodeficiency virus (HIV)[1] protease is a 22-kDa dimer of two identical 11-kDa subunits (Meek et al., 1989). The protease maturation from the *gag-pol* polyprotein gene product is autocatalytic (Debouck et al., 1987). Since the protease is required for maturation of infectious HIV particles (Kohl et al., 1988; Peng et al., 1989; Gottlinger et al., 1989), it is a potential target for chemotherapeutic treatment of AIDS. Numerous peptide-based inhibitors and nonpeptide inhibitors have been synthesized [recently reviewed by Huff (1991) and Norbeck and Kempf (1991)]. Some of these compounds inhibit maturation of viral particles in vitro (Huff, 1991; Norbeck & Kempf, 1991; Petteway et al., 1991).

Many inhibitors induce a significant conformational change in HIV protease, as demonstrated by comparing the three-dimensional structure of the enzyme (Navia et al., 1989; Lapatto et al., 1989; Wlodawer et al., 1989) with that of the enzyme·inhibitor complex (Swain et al., 1990; Fitzgerald et al., 1990; Jaskolski et al., 1991; Erickson et al., 1990). The inhibitor-induced conformational change is not highly dependent on inhibitor structure (Wlodawer et al., 1991; Erickson et al., 1990). Even though protease–inhibitor interactions in crystals have been analyzed thoroughly, the details of the kinetics for inhibitor binding have not been investigated. We report here the kinetic mechanism for the binding of five HIV protease inhibitors.

MATERIALS AND METHODS

Materials. HIV-1 protease was cloned and expressed in *Escherichia coli* and purified by methods to be described elsewhere (E. D'Souza, C. Blance, and D. Stammers, unpublished data). Acetyl-Thr-Ile-Leu(CH_2NH)Leu-Gln-Arg-NH_2 (**2**) was prepared as described (Sasaki et al., 1987; Toth et al., 1990). (2-Aminobenzoyl)-Thr-Ile-Nle-Phe(NO_2)-Gln-Arg-NH_2 (**4**) was from Cambridge Research Biochemicals; pepstatin was from Bachem Bioscience Inc. (Philadelphia, PA); MES, DTT, and Triton X-100 were from Sigma (St. Louis, MO).

Preparation of Quinoline-2-carbonyl-Asn-PheΨ[CH(OH)-CH_2N]Pro-O-t-Bu [R Diastereomer (1R) and S Diastereomer (1S)] and CBZ-PheΨ[CH(OH)CH_2N]Pro-O-t-Bu (Mixture of Epimeric Alcohol Isomers, 3). **1R** and **3** were synthesized previously (Roberts et al., 1990). Briefly, we prepared these compounds as follows. *N*-CBZ-L-phenylalanine chloromethyl ketone was reacted with a 2-fold excess of L-proline in 1,4-dioxane. The reaction product was reduced with $NaBH_4$ in EtOH at room temperature for 30 min and purified by silica gel. The NMR spectrum and FAB MS [469.3 $(M + H)^+$] of the product were consistent with a mixture of epimeric alcohols (**3**). The CBZ moiety was removed with 5% palladium on carbon and hydrogen in EtOH and the free amino terminus was acylated by CBZ-L-asparagine (4-nitrophenol) ester in 1,4-dioxane. The product was chromatographed on silica gel (EtOAc/MeOH, 9:1) to yield a purified colorless foam, FAB MS 583 $(M + H)^+$. The CBZ moiety was removed as described above, and the free amino terminus of the resulting product was acylated with quinoline-2-carboxylic acid as described above. The epimeric isomers (**1R** and **1S**) were separated by chromatography on silica gel (EtOAc/MeOH, 19:1), to give an NMR spectrum, FAB MS 604 $(M + H)^+$, and an optical spectrum (maxima for **1R** at 243 and 310 nm) that were consistent with the structure of the expected product. The stereochemistry of **1** at the carbon of the hydroxyethylamine that bears the hydroxyl was assigned on the basis of the relative potency of inhibition of the protease, as described by Roberts et al. (1990) and Rich et al. (1991).

Fluorometric Protease Assay. Protease (3–700 nM) was assayed fluorometrically at 25 °C with **4** at concentrations of 10–20 μM (Toth & Marshall, 1990) in 100 mM MES at pH 5.5 with 5% DMSO (buffer A). Fluorescence increase due to hydrolysis of the substrate was monitored either on a KON-

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[1] Abbreviations: FAB MS, fast atom bombardment mass spectrum; HIV, human immunodeficiency virus; MES, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HEA, hydroxyethylamine; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; CBZ, carbobenzyloxy; AIDS, acquired immunodeficiency syndrome; *t*-Bu, *tert*-butyl. Amino acids are designated by the standard three-letter code; Ψ indicates a reduced peptide bond (as designated inside the parentheses).

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TRON Model SFM 25 spectrofluorometer at an excitation wavelength of 325 nm and an emission wavelength of 420 nm, or on an Applied Photophysics stopped-flow spectrofluorometer at an excitation wavelength of 325 nm and emission scattering.

Enzyme Fluorescence Measurements. Protein fluorescence measurements were made in buffer A, at 25 °C, with an excitation wavelength of 280 nm. Typically, the protease concentration was 350 nM. Stopped-flow data were collected with an excitation wavelength of 280 nm and emission scattering.

Equations for Interpretation of Pre-Steady-State Kinetic Data. A one-step binding model (eq 1a) requires that the approach to equilibrium be a pseudo-first-order process when

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \tag{1a}$$

the concentration of inhibitor is much greater than the enzyme concentration (eq 1b). Alternatively, the approach to equi-

$$k_{obs} = k_1[I] + k_{-1} \tag{1b}$$

librium in a two-step process (eq 1c) can be biphasic. If the two steps are well separated kinetically and the concentration

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}I^* \tag{1c}$$

of inhibitor is much greater than the enzyme concentration, the approach to equilibrium is a biphasic process. The dependence of the pseudo-first-order rate constant on the concentration of inhibitor for the fast phase is given by eq 1d (Johnson, 1992). The complete expression for the inhibitor

$$k_{obs} \cong k_1[I] + k_{-1} + k_2 + k_{-2} \tag{1d}$$

concentration dependence of k_{obs} of the slow phase approximates a hyperbola as defined in eq 1e (Johnson, 1992). The

$$k_{obs} \cong \frac{k_1[I](k_2 + k_{-2}) + k_{-2}k_{-1}}{k_1[I] + k_{-1} + k_2 + k_{-2}} \tag{1e}$$

slope of a plot of k_{obs} vs [I] at low inhibitor concentrations approximates the limiting slope of the hyperbola and is given by eq 1f (Johnson, 1992).

$$\text{limiting slope} \cong \frac{k_1(k_2 + k_{-2})}{k_{-1} + k_2 + k_{-2}} \tag{1f}$$

Equation for Steady-State Kinetic Analysis. Initial velocity data from the inhibition of substrate hydrolysis were fitted to

$$V = V_{max}IC_{50}/([I] + IC_{50}) \tag{2}$$

to obtain IC_{50} values. K_i values[2] were calculated from IC_{50} values.

$$IC_{50} = [E]/2 + K_i\{1 + [S]/K_m\} \tag{3}$$

Titration of HIV Protease Active Sites. Enzymatic activity, with fluorometric substrate **4**, of a protease solution (protein absorbance of 0.005 at 280 nm) was monitored on the stopped-flow spectrofluorometer at pH 6.5. The data for titration of enzymatic activity with **1R** were fitted to the quadratic equation that gave E_t (260 ± 20 nM) and an apparent inhibition constant (8 ± 5 nM). This method was only used to estimate the enzyme concentration, not to estimate the IC_{50} or K_i for **1R**.

[2] Inhibition was assumed to be competitive [K_m for **4** was 60 ± 8 μM, similar to that of Toth and Marshall (1990) (40 μM)]. Typically, the substrate concentrations were 0.17–0.33 times K_m. Inhibitor concentrations were at least 3-fold greater than enzyme concentrations.

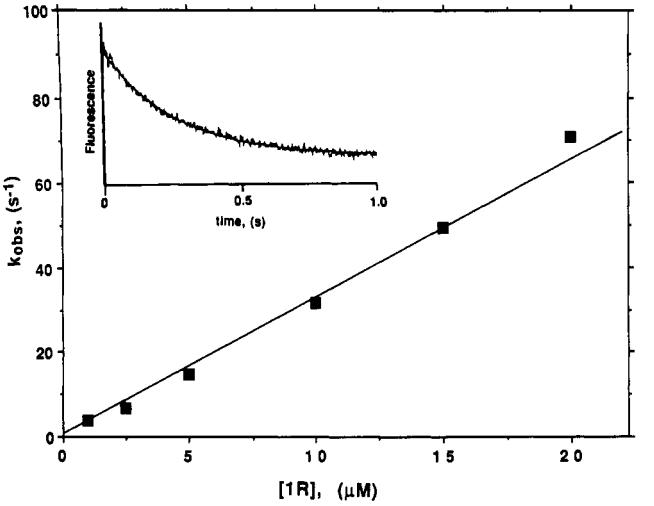

FIGURE 1: Association rate constant for HIV protease and **1R**. The dependence of the observed rate constants on the concentration of **1R** was determined from the time-dependent quenching of the protease fluorescence, as described in the text. Inset: Time course for fluorescence quenching of 0.35 μM protease by 1 μM **1R**.

Time Courses for Inhibitor Binding to the Protease. Single exponential time courses (400 data points) of changes in the intrinsic protein fluorescence were fitted with the software provided with the SF.17MV stopped-flow spectrofluorometer (Applied Photophysics Limited, Leatherhead, U.K.). The data for the approach to steady-state of substrate hydrolysis were fitted to an equation with a single exponential plus a linear term. The rate constants calculated from these data were fitted to eq 1b, 1d, or 1f.

Statistical Analysis. The constants defined by the linear equations were fit by standard linear regression analysis. The constants defined by the nonlinear equations were estimated by an iterative nonlinear least-squares fitting routine (Bevington, 1969). Error estimates were taken from the error matrix generated by the fitting routine. Error estimates for values calculated from fitted values (for example, the calculated K_d values) were determined by the propagation of error analysis (Bevington, 1969).

RESULTS

Enzyme Fluorescence Changes. The fluorescence emission spectrum of HIV protease had a maximum at 350 nm with an excitation wavelength of 280 nm. The fluorescence peak was decreased approximately 5%, by 1 μM **1R** (data not shown). A stopped-flow spectrofluorometer that detected emission scattering was used to study the time course for quenching. **1R** decreased the protein fluorescence by approximately 10% in a time-dependent manner (Figure 1,inset). Because the inhibitor had a fluorescence that was <1% of the enzyme fluorescence, the inhibitor had minimal absorbance at 280 nm (the maximum quenching due to an inner filter effect at this concentration of **1R** was 0.2%), and the protein fluorescence decrease was time-dependent, we concluded that the interaction of the inhibitor with the protease was responsible for the quenching of the protein fluorescence. Inhibitors **2**, **3**, and pepstatin did not quench the enzyme fluorescence.

Association and Dissociation Rate Constants for 1R. The time courses for the fluorescence quenching of HIV protease (0.35 μM) by **1R** (1 μM) were fitted to a first-order process with a pseudo-first-order rate constant of 3.9 ± 0.1 s^{-1} (Figure 1, inset). The pseudo-first-order rate constants were linearly dependent on inhibitor concentration (Figure 1). The slope of this linear plot yielded a bimolecular association rate constant of (3.1 ± 0.2) × 10^6 M^{-1} s^{-1} (eq 1b).

The dissociation rate constant for **1R** was determined directly by trapping free enzyme with pepstatin, a second inhibitor (Seelmeier et al., 1988). Since the fluorescence of the enzyme·pepstatin complex was similar to that of native enzyme and the enzyme·**1R** complex had reduced fluorescence, dissociation of the enzyme·**1R** complex was monitored by an increase in enzyme fluorescence. The rate constant for the time-dependent increase in fluorescence due to the release of **1R** from the protease·**1R** complex was independent of the pepstatin concentration between 25 μM (0.122 ± 0.001 s^{-1}) and 75 μM (0.124 ± 0.001 s^{-1}). The apparent dissociation rate constant for **1R** from the protease·**1R** complex was estimated from the average of these two values (0.123 ± 0.002 s^{-1}). The K_d calculated from the bimolecular association rate constant (3.1 ± 0.2) × 10^6 M^{-1} s^{-1} and the dissociation rate constant (0.123 ± 0.001 s^{-1}) was 40 ± 3 nM, which was similar to the experimentally determined steady-state K_i value of 33 ± 2 nM and the literature value of 23 nM (Roberts et al., 1990).

The amplitude of the fluorescence increase following the binding of pepstatin to protease released from the protease·**1R** complex (average of 230 mV) was slightly larger than the amplitude of the decrease caused by the binding of **1R** (average of 170 mV). Thus, the enzyme·pepstatin complex was more fluorescent than free enzyme. However, the fluorescence changes due to the binding of pepstatin were too small to determine binding rate constants for pepstatin.

Binding Rate Constants for 1S. **1S** and **1R** quenched the protein fluorescence to a similar extent. However, unlike **1R**, the time course for protease fluorescence quenching by 15 μM **1S** was a biphasic process (Figure 2A). This biphasic process suggested a two-step mechanism for **1S** binding to protease (eq 1c). The relative amplitude of the fast and slow phases varied with the concentration of **1S**.[3] At high **1S** concentrations (40–160 μM) the amplitude of the fast phase was dominant (Figure 2C). The k_{obs} value for this process was proportional to **1S** concentration (Figure 3A) with a slope of (1.1 ± 0.1) × 10^6 M^{-1} s^{-1}. The slope approximated k_1 in the two-step mechanism (eq 1d). The y-axis intercept was 29 ± 11 s^{-1} and approximated $k_{-1} + k_2$ (if $k_2 > k_{-2}$, as will be verified below) (Johnson, 1992). The slow phase was dominant at low concentrations of **1S** (1 – 7.5 μM) (Figure 2B). Ideally, the dependence of the slow-phase k_{obs} on inhibitor concentration approximates a hyperbola defined by eq 1e. However, because the amplitude of the slow phase decreased to unmeasurable levels at high inhibitor concentrations, this hyperbolic dependence could not be observed. Because of these limitations of the data, we used an alternative approach to estimate the rate constants that define this phase. At low concentrations of inhibitor, the amplitude of the slow phase was maximal and the concentration dependence of k_{obs} gave

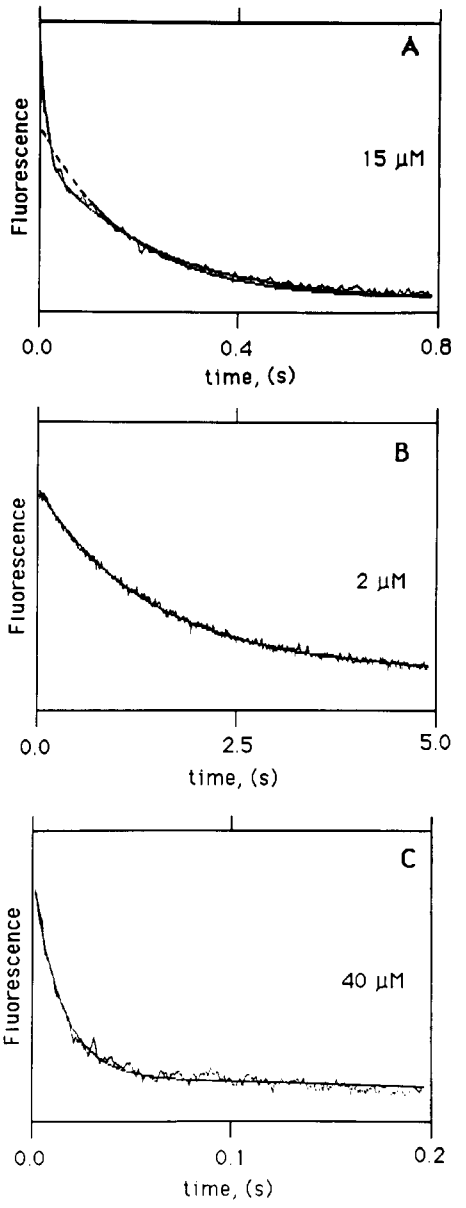

FIGURE 2: Protease fluorescence emission during association of protease and **1S**. Time courses for fluorescence quenching of the protease by binding of **1S**. Protease (0.35 μM) was mixed with **1S** at 15 μM (A), 2 μM (B), and 40 μM (C). The data of panel A were fitted to a double exponential (solid line) or a single exponential (dashed line).

an approximation for the limiting slope of the hyperbola (eq 1f). The slow-phase k_{obs} was proportional to **1S** concentration (Figure 3B) with a slope of (0.28 ± 0.02) × 10^6 M^{-1} s^{-1}. The slope approximated $(k_2k_1)/(k_{-1} + k_2)$, assuming $k_2 > k_{-2}$ (as will be verified below). The values for k_{-1} (20 ± 10 s^{-1}) and k_2 (7 ± 3 s^{-1}) were calculated from values and equations defining k_{obs} for the two reaction phases and the stated assumptions.

The apparent dissociation rate constant for **1S**-inhibited enzyme (0.070 ± 0.005 s^{-1}) was determined by trapping free enzyme with pepstatin, as described for **1R**. In the two-step model (eq 1c), the apparent dissociation rate constant is approximated by $k_{-2}[k_{-1}/(k_{-1} + k_2 + k_{-2})]$ (Johnson, 1992). k_{-2} was calculated to be 0.1 ± 0.1 s^{-1} from the apparent dissociation rate constant (0.070 ± 0.005 s^{-1}) and estimates of k_{-1} (20 ± 10 s^{-1}) and k_2 (7 ± 3 s^{-1}). This result verifies the previous assumption that k_{-1} and k_2 were much greater than k_{-2}. These rate constants for the **1S** binding to protease are summarized in Table I. The K_d was approximated by

[3] The maximum amplitude of the fast phase (observed at high inhibitor concentration) was similar to the maximum amplitude of the slow phase (observed at low inhibitor concentration). This suggested that the fluorescence of E·I was similar to that of E·I* (eq 1c). Thus, at higher concentrations of **1S**, the amplitude of the fluorescence change for the formation of E·I* became very small.

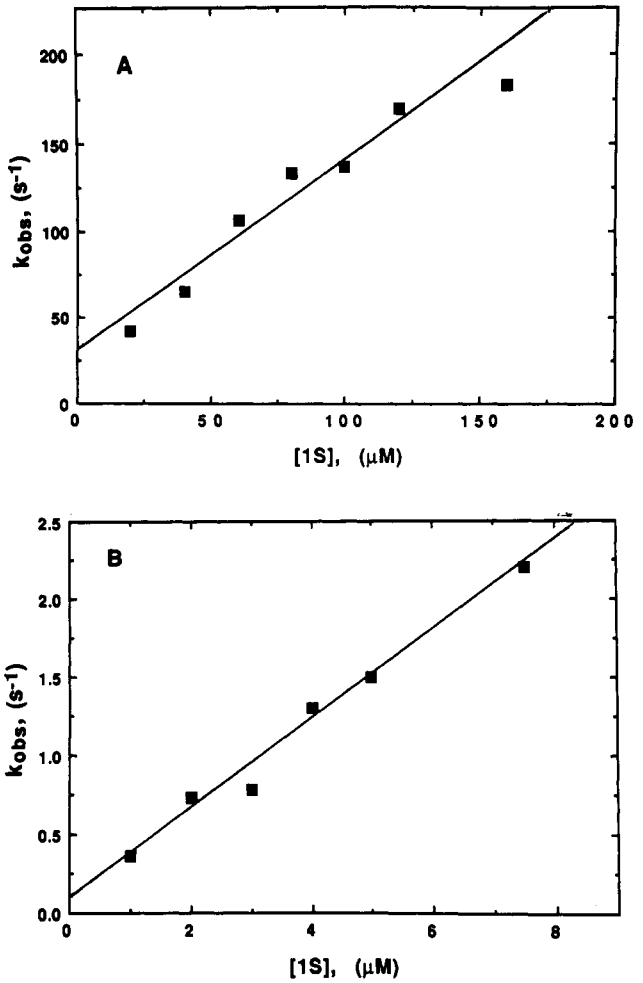

FIGURE 3: Dependence of the rate constants for the fast phase (A) and slow phase (B) on **1S** concentration. Fast-phase and slow-phase k_{obs} for protease binding **1S** was determined from the time-dependent quenching of the protease fluorescence. The data for 20 μM **1S** were included in the fit for the fast phase because the fast-phase amplitude was more than 70% of the total amplitude.

Table I: Kinetic Constants for Two-Step Binding of 1S[a]

compd	$k_1 \times 10^{-6}$ (M^{-1} s^{-1})	k_{-1} (s^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})	K_i (μM)	$K_{d\text{-}calc}$ (μM)
1S	1.1 ± 0.1	20 ± 10	7 ± 3	0.1 ± 0.1	0.14 ± 0.01	0.3 ± 0.3

[a] Rate constants for **1S** binding were determined experimentally as described in the text.

$(k_{-1}/k_1)(k_{-2}/k_2)$ (Johnson, 1992) and was calculated to be 300 ± 300 nM. The steady-state K_i value was 140 ± 10 nM. The error in the values for k_{-1}, k_2, and especially k_{-2} were large due to the propagation of the error in the value for the y-intercept of the fast-phase k_{obs} vs [**1S**] plot (29 ± 11 s^{-1}). Therefore, to confirm that these estimated rate constants for the two-step mechanism were reasonable, the kinetic scheme (eq 1c) was simulated by numerical integration[4] using rate constants similar to the estimated values. The experimental data superimposed on the simulated data.

[4] The kinetic scheme of eq 1c was simulated by numerical integration using a fourth-order Runge-Kutta method (Kreyszig, 1963) that was implemented on a Macintosh II personal computer. The time course for fluorescence quenching of the protease by 15 μM **1S** was simulated with the following parameters: the fluorescence of E·I was assumed to be quenched 1.3-fold more than E·I*; and k_1, k_{-1}, k_2, and k_{-2} were 1.4 × 10^6 M^{-1} s^{-1}, 22 s^{-1}, 8 s^{-1}, and 0.09 s^{-1}, respectively. The experimental time course was superimposable on the simulated time course for fluorescence quenching.

Table II: Kinetic Constants for Apparent One-Step Binding of HIV Protease Inhibitors

compd	$k_1 \times 10^{-6}$ (M^{-1} s^{-1})	k_{-1} (s^{-1})	K_i (μM)	$K_{d\text{-}calc}$ (μM)
1R	3.1 ± 0.2[a] 2.5 ± 0.5[b]	0.123 ± 0.001[d]	0.033 ± 0.002	0.040 ± 0.003
2	0.16 ± 0.01[b]	0.204 ± 0.003[e] 0.38 ± 0.07[b]	1.4 ± 0.1	1.3 ± 0.1
3	0.82 ± 0.08[c]	14 ± 1	17 ± 1	
pepstatin	0.80 ± 0.04[b]	0.65 ● 0.01[e] 0.9 ± 0.1[b]	0.62 ± 0.06	0.81 ● 0.04

[a] Determined by protein fluorescence quenching by **1R**. [b] Determined by analysis of the time-dependent onset of inhibition of **4** hydrolysis. [c] Calculated from K_i and k_{-1}. [d] Determined by trapping free enzyme with pepstatin. [e] Determined by trapping free enzyme with **1R**. Details are described in the text.

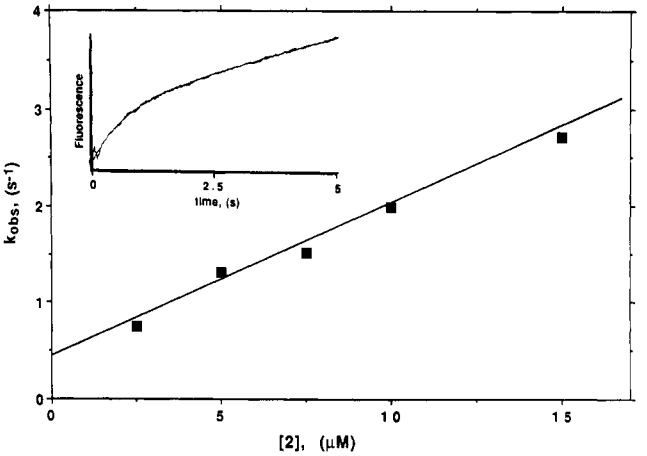

FIGURE 4: Association rate constant for HIV protease and **2**. Dependence of the observed rate constants for **2** binding on the concentration of inhibitor. The rate constants for the approach to steady state of HIV protease with fluorometric substrate **4** were determined as described in the text. Inset: Time course for the inhibition of 0.175 μM protease, by 7.5 μM **2**, in the presence of 10 μM **4**.

Dissociation Rate Constants for 2, 3, and Pepstatin. Dissociation rate constants for **2**, **3**, and pepstatin were determined by trapping the free enzyme formed from dissociation of the respective protease-inhibitor complex with excess **1R**. The reaction was monitored by the quenching of protein fluorescence of enzyme that was typically at a concentration of 350 nM. The initial concentrations of inhibitors were 2.5–3 times their respective K_i value and at least 4 times the total enzyme concentration. Concentrations of **1R** (typically 5–15 μM) were sufficient such that the observed dissociation rate constants were independent of **1R** concentration. The dissociation rate constants determined for **2**, **3**, and pepstatin were 0.204 ± 0.003, 14 ± 1, and 0.65 ± 0.01 s^{-1}, respectively (Table II). Interestingly, **2** and **1R** had similar dissociation rate constants (0.204 ± 0.003 s^{-1} vs 0.123 ± 0.001 s^{-1}) even though the K_i for **2** was 50-fold higher than the K_i for **1R**.

Association Rate Constants for 1R, 2, 3, and Pepstatin. Since no change in enzyme fluorescence occurred upon binding **2**, **3**, and pepstatin, the association rate constants for these inhibitors could not be determined by fluorescence quenching. However, the onset of inhibition of substrate **4** hydrolysis by **1R**, **2**, or pepstatin was monitored on the stopped-flow spectrofluorometer (Figure 4, inset). This progress curve was fitted to an initial exponential decay and a final steady-state rate of product formation. The pseudo-first-order rate constants for the approach to steady state were linearly dependent on the concentration of **2** (Figure 4). The bimolecular rate constant was (1.6 ± 0.1) × 10^5 M^{-1} s^{-1}, and the

dissociation rate constant was 0.38 ± 0.07 s^{-1} (eq 1b). This dissociation rate constant was similar to that obtained by analysis of enzyme fluorescence quenching (0.204 ± 0.003 s^{-1}). Since the latter dissociation rate constant contained less error, it was used in the calculation of the K_d for **1R**. The K_d calculated from the dissociation rate constant (0.204 ± 0.003 s^{-1}) and the bimolecular association rate constant [(1.6 ± 0.1) × 10^5 M^{-1} s^{-1}] was 1.3 ± 0.1 μM, which was similar to the steady-state K_i (1.5 ± 0.1 μM). The association rate constants for **1R** and pepstatin were determined as described for **2** (Table II). The association rate constant for **1R** determined by analysis of the onset of substrate inhibition [(2.5 ± 0.5) × 10^6 M^{-1} s^{-1}] was comparable to that determined from analysis of enzyme fluorescence quenching [(3.1 ± 0.1) × 10^6 M^{-1} s^{-1}]. K_d values, which were calculated from the association and dissociation rate constants, were similar to the respective steady-state K_i values (Table II). The association rate constant for **3** was not determined by this method because the approach to steady state was too rapid. However, the association rate constant for **3** [(0.82 ± 0.08) × 10^6 M^{-1} s^{-1}] was calculated from the steady-state K_i of 17 ± 1 μM and the k_{-1} of 14 ± 1 s^{-1}.

DISCUSSION

Structural studies suggest that HIV protease undergoes large conformational changes upon binding many inhibitors (Swain et al., 1990; Fitzgerald et al., 1990; Jaskolski et al., 1991; Erickson et al., 1990). These studies are consistent with a two-step inhibition mechanism with a relatively rapid formation of a collision complex, followed by a slower conformational change to a tighter complex.[5] In accord with this, binding of **1S** was biphasic. The association and dissociation rate constants (k_1 and k_{-1}) for formation of the collision complex were 1.1 × 10^6 M^{-1} s^{-1} and 20 s^{-1}, respectively. The rate constants k_2 and k_{-2}, for interconversion of the collision complexes to the tighter complex, were 7 s^{-1} and 0.1 s^{-1}, respectively. The association rate constant for formation of the collision complex was near the lower limit of typical enzyme–substrate association rate constants [10^6 M^{-1} s^{-1} – 10^8 M^{-1} s^{-1}; tabulated by Fersht (1985)], the upper limit of these rate constants being diffusion controlled. The magnitude of k_1 was less than that expected for a diffusion-controlled process. Finally, since k_{-1} was 3-fold larger than k_2, formation of the collision complex was more rapid than the conformational change.

The kinetics of **1S** binding provide evidence for a two-step mechanism with initial binding and a conformational change. Further, because the conformational change of the protease observed in enzyme-inhibitor complexes is not highgly dependent on inhibitor structure (Wlodawer et al., 1991; Erickson et al., 1990), and since this conformational change has been observed with inhibitors analogous to **1R**, **2**, and pepstatin (Jaskolski et al., 1991; Krohn et al., 1991; Miller et al., 1989; Fitzgerald et al., 1990), it is reasonable to assume that **1R**, **2**, and pepstatin are bound to a similar conformation of protease. Thus, a simple one-step binding mechanism would require that the enzyme in this altered conformation directly binds inhibitor and that the conformation of free enzyme is similar to the conformation of E·I complexes. Two problems make this mechanism unlikely. First, the solution structure of free enzyme most likely resembles the crystal structure of free enzyme rather than that of E·I complex. Second, access to the active site of free protease in the altered conformation is sterically prohibited (Gustchina & Weber, 1990). Together, these data suggest that **1R**, **2**, and pepstatin may have physically bound to the protease in a two-step process even though they were described kinetically by a one-step mechanism. Although the kinetics for **1R** binding to the protease appeared monophasic, a two-step mechanism would appear monophasic if most associations to form the collision complex resulted in the proposed conformational change (i.e., $k_2 > k_{-1}$). Rate constants were calculated for a two-step mechanism for **1R** binding with the following assumptions: (1) k_1 was 3.1 × 10^6 M^{-1} s^{-1}, which was equal to the observed association rate constant for **1R**; (2) k_{-1} for **1R** was similar to k_{-1} for **1S** (20 s^{-1}); (3) k_2 was 5 times larger than k_{-1}, such that formation of the collision complex was not in rapid equilibrium; and (4) k_{-2} was made 0.6 s^{-1} so that the overall dissociation rate constant for the enzyme (approximated by $k_{-2}[k_{-1}/(k_{-1} + k_2)]$) was equal to the experimentally determined apparent dissociation rate constant (0.1 s^{-1}). The K_d was calculated to be 39 nM from these rate constants and was similar to the observed K_i of 33 nM. Thus, these rate constants provide a possible (but not unique) example of how **1R** binding to protease may have occurred in two steps that appeared monophasic.

The dissociation rate constant for **3** of 14 s^{-1} was similar to the dissociation rate constant for **1S** from the collision complex with protease (k_{-1} = 20 s^{-1}). In addition, **1R**, **1S**, **2**, and pepstatin, which presumably all induce the enzyme conformational change, had overall dissociation rate constants less than 1 s^{-1}. Thus, **3** may not have had sufficient binding energy to induce the conformational change. Further, a dissociation rate constant of less than 1 s^{-1} may be indicative of inhibitors that induce a conformational change upon binding to protease.

Recent molecular modeling, enzyme kinetics, and structural studies suggest that the backbones of the *R* and *S* diastereomers of HEA inhibitors bind in similar orientations with conformational perturbations to accommodate the hydroxyl binding to the catalytic aspartate residues (Ferguson et al., 1991; Rich et al., 1991; Krohn et al., 1991). The differences in the rate constants, which define the binding of **1R** and **1S**, may result from differences in the conformation of the inhibitor when bound to the enzyme.

Since **2**, pepstatin, **1R**, and **1S** presumably induced similar conformational changes in the enzyme upon binding, it was interesting that only **1R** and **1S** quenched the protein fluorescence. Fluorescence quenching can occur if the emission energy is transferred to an absorbing chromophore. This energy transfer requires both proximity and correct orientation of the emitting and absorbing groups, as well as an overlap in the emission wavelength and the absorbing wavelength of the groups (Stryer, 1978). For example, the human serum albumin fluorescence emission spectrum has a maxima at 340 nm that is quenched by the addition of an unsaturated fatty acid that absorbs at the albumin emission maximum (Berde et al., 1979). Pepstatin and **2** do not contain aromatic chromophores. In contrast, compound **1R** had an absorbance maximum at 310 nm. Since the fluorescence emission maxima for HIV protease was 350 nm (Figure 1), the protease·**1R** complex may satisfy both the proximity and absorption/emission energy requirements for fluorescence quenching.

[5] Alternatively, the slow conformational change may occur prior to binding of inhibitor. This mechanism was unlikely for **1S**. If the conformational change occurred first, k_{obs} for the slow phase decreases as the inhibitor concentration was increased, whereas if the conformational change occurred second, k_{obs} for the slow phase increases with inhibitor concentration (as observed with **1S**) (Fersht, 1985).

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REFERENCES

Berde, C. B., Hudson, B. S., Simoni, R. D., & Sklar, L. A. (1979) *J. Biol. Chem. 254*, 391–400.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, pp 204–246, McGraw-Hill Book Co., New York.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.

Debouck, C., Gourniak, J. G., Strickler, J. E., Meek, T. D., Metcalf, B. W., & Rosenberg, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8903–8906.

Erickson, J., Neidhart, D. J., VanDrie, J., Kempf, D. J., Wang, X. C., Norbeck, D. W., Plattner, J. J., Rittenhouse, J. W., Turon, M, Wideburg, N., Kohlbrenner, W. E., Simmer, R., Helfrich, R., Paul, D. A., & Knigge, M. (1990) *Science 249*, 527–533.

Ferguson, D. M., Radmer, R. J., & Kollman, P. A. (1991) *J. Med. Chem. 34*, 2654–2659.

Fersht, A. (1985) in *Enzyme Structure and Mechanism*, 2nd ed., pp 121–154, W. H. Freeman and Co., New York.

Fitzgerald, P. M. D., McKeever, B. M., VanMiddlesworth, J. F., Springer, J. P., Heimbach, J. C., Leu, C.-T., Herber, W. K., Dixon, R. A. F., & Darke, P. L. (1990) *J. Biol. Chem. 265*, 14209–14219.

Gottlinger, H., Sodroski, J., & Haseltine, W. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5781–5785.

Gustchina, A., & Weber, I. T. (1990) *FEBS. Lett. 269*, 269–271.

Huff, J. R. (1991) *J. Med. Chem. 34*, 2305–2314.

Jaskolski, M., Tomasselli, A. G., Sawyer, T. K., Staples, D. G., Heinrikson, R. L., Schneider, J., Kent, S. B. H., & Wlodawer, A. (1991) *Biochemistry 30*, 1600–1609.

Johnson, K. A. (1992) in *The Enzymes* (Sigman, D. S., & Boyer, P. D., Eds.) Vol. 20, 3rd ed. Academic Press, Orlando, (in press).

Kohl, N. E., Emini, E. A., Schleif, W. A., Davis, L. J.; Heimbach, J. C., Dixon, R. A. F., Scholnick, E. M., & Sigal, I. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4686–4690.

Kreyszig, E. (1963) *Advanced Engineering Mathmatics*, p 96, Wiley and Sons, New York.

Krohn, A., Redshaw, S., Ritchie, J. C., Graves, B. J., & Hatada, M. H. (1991) *J. Med. Chem. 34*, 3340–3342.

Lapatto, R., Blundell, T., Hemmings, A., Overington, J., Wilderspin, A., Wood, S., Merson, J. R., Whittle, P. J., Danley, D. E., Geoghegan, K. F., Hawrylik, S. J., Lee, S. E., Scheld, K. G., & Hobart, P. M. (1989) *Nature (London) 342*, 299–302.

Meek, T. D., Dayton, B. D., Metcalf, B. W., Dreyer, G. B., Strickler, J. E., Gorniak, J. G., Rosenberg, M., Moore, M. L., Magaard, V. W., & Debouck, C. (1989) *Proc. Natl. Acad. Sci., U.S.A. 86*, 1841–1845.

Miller, M., Schneider, J., Sathyanarayana, B. K., Toth, M., Marshall, G. R., Clawson, L., Selk, L., Kent, S. B. H., & Wlodawer, A. (1989) *Science 246*, 1149–1152.

Navia, M. A., Fitzgerald, P. M. D., McKeever, B. M., Leu, C.-T., Heimbach, J. C., Herber, W. K., Sigal, I. S., Darke, P. L., & Springer, J. P. (1989) *Nature (London) 337*, 615–620.

Norbeck, D. W., & Kempf, D. J., (1991) in *Annual Reports in Medicinal Chemistry* (Bristol, J. A., Ed.) pp 141–150, Academic Press, San Diego.

Peng, C., Ho, B. K., Chang, T. W., & Chang, N. T. (1989) *J. Virol. 63*, 2550–2556.

Petteway, S. R., Jr., Lambert, D. M., & Metcalf, B. W. (1991) *Trends Pharmacol. Sci. 12*, 28–34.

Rich, D. H., Sun, C.-Q., Vara Prasad, J. V. N., Pathiasseril, A., Toth, M. V., Marshall, G. R., Clare, M., Mueller, R. A., & Houseman, K. (1991) *J. Med. Chem. 34*, 1222–1225.

Roberts, N. A., Martin, J. A., Kinchington, D., Broadhurst, A. V., Craig, J. C., Duncan, I. B., Galpin, S. A., Handa, B. K., Kay, J., Krohn, A., Lambert, R. W., Merrett, J. H., Mills, J. S., Parkes, K. E. B., Redshaw, S., Ritchie, A. J., Taylor, D. L., Thomas, G. J., & Machin, P. J. (1990) *Science 248*, 358–361.

Sasaki, Y., Murphy, W. A., Heiman, M. L., Lance, V. A., & Coy, D. H. (1987) *J. Med. Chem. 30*, 1162–1166.

Seelmeier, S., Schmidt, H., Turk, V., & von der Helm, K. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6612–6616.

Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819–846.

Swain, A. L., Miller, M. M., Green, J., Rich, D. H., Schneider, J., Kent, S. B. H., & Wlodawer, A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8805–8809.

Toth, M. V., & Marshall, G. R. (1990) *Int. J. Peptide Protein Res. 36*, 544–550.

Toth, M. V., Chiu, F., Glover, G., Kent, S. B. H., Ratner, L., Vander Heyden, N., Green, J., Rich, D. H., & Marshall, G. R. (1990) in *Peptides: Chemistry, Structure and Biology, Proceedings of the American Peptide Symposium, 11th, 1989* (Rivier, J. E., & Marshall, G. R., Eds.) pp 835–838, Escom Science Publishing, Leiden.

Wlodawer, A., Miller, M., Jaskolski, M., Sathyanarayana, B. K., Baldwin, E., Weber, I. T., Selk, L. M., Clawson, L., Schneider, J., & Kent, S. B. H. (1989) *Science 245*, 616–621.

Wlodawer, A., Miller, M., Swain, A. L., & Jaskolski, M. (1991) in *Methods in Protein Sequence Analysis* (Jornvall, H., Hoog, J.-O., Gustavson, A. M., Eds.) pp 215–221, Birkhauser Verlag, Basel.