Dissociation and Association of the HIV-1 Protease Dimer Subunits: Equilibria and Rates

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ABSTRACT: The kinetics and equilibrium properties were investigated for the interconversion between the active dimer of human immunodeficiency virus 1 (HIV-1) protease and its inactive monomeric subunits. The equilibrium dissociation constant (K_d) of the dimeric protease as well as the monomer association rate were obtained by monitoring the fluorescence change of an active-site-directed fluorescent probe (L-737244) upon its binding to the protease. The K_d of the HIV-1 protease is strongly pH dependent. At pH 5.5 where the enzyme is most active catalytically, the extrapolated values of K_d are 0.75 and 3.4 nM at 30 and 37 °C, respectively. The rate constant for HIV-1 monomer association, $\sim 4 \times 10^5$ M^{-1} s^{-1}, is within the range commonly observed for protein–protein interactions. Dimer dissociation was further scrutinized in the presence of an inactive, point mutant form of the enzyme. As a result of subunit exchange between the native and mutant enzymes and the formation of an inactive heterodimer, there was a time-dependent decrease in the activity of the native protease. Enzyme activity could be reinstated with the addition of an active-site-directed inhibitor (L-365862) which selectively binds active dimers. The rate of dimer dissociation was found to also decrease with pH. At pH 5.5 and 30 °C, the half-life for subunit dissociation is about 0.5 h. The slow dissociation, coupled with the high stability for dimer association, attests to the importance of allowing sufficient time for dimer–monomer equilibration in kinetic assays in order to avoid reaching erroneous conclusions in studies of dimer dissociation.

HIV-1 protease has been demonstrated to be essential for HIV replication in cell culture, both by genetic means (Kohl et al., 1988; Peng et al., 1989) and through the use of active-site-directed inhibitors of the enzyme (Ashorn et al., 1990; Meek et al., 1990; Roberts et al., 1990; Vacca et al., 1991).[1] One of the properties of the HIV protease that might be exploited for inhibition is the stability of the dimeric form of the enzyme. The active site of the enzyme is composed of equal contributions from each of the two identical, 99-residue subunits. Dissociation of the active, dimeric form of the enzyme results in complete loss of activity. Thus, a compound that binds preferentially to the monomeric subunit to prevent dimer formation would be inhibitory and potentially useful for therapeutic purposes. This dissociative scheme has been explored with *in vitro* enzyme assays using short peptides (Zhang et al., 1991; Babé et al., 1992) and a truncated form of the enzyme (Babé et al., 1991) as dissociative inhibitors. Peptides thought to be dissociative inhibitors have been tested as inhibitors of viral replication in cell culture (Schramm et al., 1991).

Attempts to identify potential dissociative inhibitors of the HIV-1 protease notwithstanding, a thorough determination of the critical parameters of the dimer–monomer equilibrium for this enzyme has not been done. In fact, there is currently a confusing array of data concerning these parameters, collected under a variety of conditions. The stability constant, K_d, of the HIV-1 protease dimer was first suggested to be 3.6 nM at pH 5.0 (Zhang et al., 1991) and 50 nM at pH 7.0 (Cheng et al., 1990). These K_d values have been reevaluated leading to much lower estimates of less than 80 pM at pH 5.0 and 5.5 (Jordan et al., 1992) and 39 pM at pH 6.0 (Grant et al., 1992). The rate of subunit exchange for the HIV-1 dimer has also not been examined, although both rapid and slow equilibrium treatments of the dimer–monomer conversion have been proposed (Cheng et al., 1990; Zhang et al., 1991; Babé et al., 1991; Kuzmic, 1993).

Given our recent findings that specific activity as a function of HIV-1 protease concentration cannot be reconciled with a significant concentration of monomer in rapid equilibrium with dimer at pH 5.0 or 5.5 (Jordan et al., 1992), we question the assumption of rapid monomer–dimer equilibration in both the collection and treatment of dissociative inhibition kinetic data. We also question whether significant, reversible dissociation of the dimeric protease to a discrete monomeric species occurs at all under common kinetic assay conditions. In this report, we describe nondenaturing conditions under which the HIV-1 protease monomer and dimer concentrations may be measured and reversible subunit exchange between the native enzyme and an inactive form of the enzyme can be demonstrated.

MATERIALS AND METHODS

Inhibitors. The substrate analog Ser-Gln-Asn-Phe-[Ψ-CH_2N]-Pro-Ile-Val-Gln, L-365862, K_i = 1 μM, was obtained as previously described (Heimbach et al., 1989). Preparation of the fluorescent active-site inhibitor, L-737244, will be described elsewhere (R. W. Hungate, manuscript submitted). The active-site-directed inhibitor L-689502, K_i = 0.3 nM, was prepared as described by Thompson et al. (1992).

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[1] Abbreviations: BSA, bovine serum albumin; HIV-1 and HIV-2, human immunodeficiency virus types 1 and 2, respectively; K_d, equilibrium constant for dissociation of the HIV protease dimer; K_i, inhibition constant; ACES, *N*-(2-acetamido)-2-aminoethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; PEG-8000, poly(ethylene glycol) 8000; D25N, point mutant of the HIV-1 protease in which the essential active-site residue Asp-25 has been altered genetically to an Asn; D126N, a tethered, single-chain form of HIV-1 protease in which the essential active-site residue Asp-126 has been genetically altered to an Asn.

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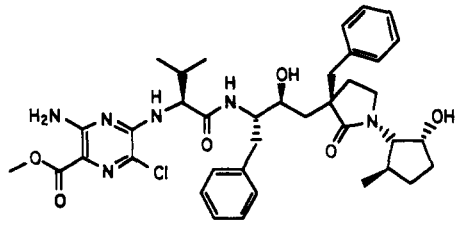

The fluorescent probe, L-737,244

Buffers. The following buffers were used: 50 mM sodium ACES, 50 mM sodium acetate, 10% glycerol, 0.1% PEG-8000, 1 mM DTT, and 1 mM EDTA, pH 3.5–7.5, pH adjusted with HCl (buffer A); buffer A with sodium chloride added to a final ionic strength of 0.4 (buffer B); 50 mM sodium acetate, pH 5.5, 10% glycerol, 0.1% PEG-8000, 1 mM EDTA, and 1 mM DTT (buffer C); 50 mM sodium acetate, pH 5.5, with 0.1% BSA (assay buffer); 50 mM MES, pH 7.0, 10% glycerol, 0.1% PEG-8000, 1 M NaCl, 1 mM EDTA, and 1 mM DTT (NaMES buffer).

Enzyme Preparations. HIV-1 protease was expressed in *Escherichia coli* using the expression system described and generously provided to us by Dr. J. Tang (Ido et al., 1991). Protein purifications were performed following dissolution of the expressed, insoluble inclusion bodies in 6 M guanidine HCl according to the procedure of Hirel et al. (1990). Purity was greater than 95% as judged with Coomassie brilliant blue staining of samples examined on 16% polyacrylamide gels (sodium dodecyl sulfate). Enzyme activity was determined using the substrate VSQN-(β-naphthylalanine)-PIV at pH 5.5, 30 °C, and HPLC analysis of the products as previously described (Heimbach et al., 1989). The active-site concentration found by titration with L-737244 was 89% of the value expected based upon total protein content of the sample (see below). Construction and purification of the active-site mutant D25N have been described (Darke et al., 1989). Previous work with native gel electrophoresis demonstrated the dimeric nature of D25N (Dilanni et al., 1990). For this study, equilibrium sedimentation measurements confirmed that the purified D25N was dimeric.

Fluorescence Measurements. Fluorescence data were collected with a Spex Fluorolog spectrometer using right angle detection. Samples were held in temperature-controlled, 3-mL quartz cuvettes with continuous stirring (Spectrocell, teflon paddle). Additions to the stirred samples were made with gas-tight syringes during data collection, so that closure of the sample compartment tripped the shutter open for immediate data collection. The excitation (360 nm) and emission (420 nm) wavelengths used were the spectral maxima for L-737244. The emission spectrum of L-737244 was found to be invariant in the pH range 3.5–7.5.

Equilibrium and Binding Rate Constants of L-737244. A 30-μL DMSO solution of various concentrations of L-737244 was injected into the cuvette containing the appropriate buffer (3 mL). The intrinsic fluorescence of the probe was recorded, 23 μL of enzyme was added to the stirred cell, and the fluorescence signal was recorded for approximately 20 s. Mixing of injected samples was determined to be 99% complete in 3 s. For calculation of K_i^{FP}, fluorescence intensity at exactly 5 s following introduction of the enzyme was used. All data collected for a given solvent condition (three different protein concentrations, 11 probe concentrations each) were curve-fit simultaneously to eq 1 which describes the expected change in fluorescence intensity for probe binding to a single class of sites. Parameters f, $[I]_t$, and $[E]_t$ represent the fluorescence

$$\Delta\text{fluorescence intensity} = \frac{f}{2}\{([I]_t + [E]_t + K_i^{FP}) - [([I]_t + [E]_t + K_i^{FP})^2 - 4[E]_t[I]_t]^{0.5}\} \quad (1)$$

enhancement factor, total probe concentration, and total enzyme concentration, respectively. In addition to K_i^{FP}, $[E]_t$ and f were allowed to be adjustable parameters during curve fitting (SigmaPlot software, Jandel Scientific) thus determining the active-site concentration of the enzyme stock solution.

The rate constant for probe dissociation from enzyme, k_{-2}, was determined by (1) addition of probe (20 nM) to an enzyme solution (30 nM), (2) observation of fluorescence until the signal was steady, (3) injection of an excess of active-site directed, nonfluorescent inhibitor, L-689502, and (4) collection of signal intensity data at 0.1-s intervals until the signal was equal to that of probe in the absence of enzyme. Loss of fluorescence intensity as a function of time was completely described by a single-exponential decay and was identical with different L-689502 final concentrations (500 nM, two runs and 1000 nM, four runs). Data were fit to

$$\text{fluorescence intensity} = \text{final intensity} + \text{total intensity change} \times \exp[-k_{-2}t] \quad (2)$$

Association rate constants (k_2) for L-737244 binding to dimeric enzyme were determined using pseudo-first-order conditions (probe concentration = $10[E]_t$) in a stopped-flow spectrofluorometer (Applied Photosystems). Data collection for 0.2 s after mixing of enzyme and probe yielded fluorescence increases completely described by a single exponential for all buffer conditions. Plots of the k_{obs} as a function of probe concentration (200–2000 nM) were linear with the slope equal to k_2.

HIV-1 Protease Dimer and Monomer Concentrations. Aliquots from an HIV-1 protease stock solution (pH 6.0, 1–26 μM) were added to a cuvette and incubated for various times prior to the addition of the fluorescence probe L-737244 ($[I]_t$ = 200 nM). The fluorescence signal intensity was then recorded for 200 s. For each experimental condition, control assays in which L-737244 was added to the cuvette just prior to the enzyme were also done. The fluorescence increase upon enzyme addition to excess L-737244 (control) was taken to be proportional to total enzyme (E_t) added, allowing conversion of subsequent time course data into DI (dimer–inhibitor) concentrations. Dimer ($[D]_o$) and monomer ($[M]_o$) concentrations at zero time and K_d values were calculated as follows: (1) The total amount of dimer $[D]_t$ includes a small amount not bound to inhibitor and was calculated from the K_i^{FP} and [DI] according to

$$[D]_t = [DI] + \frac{K_i^{FP}[DI]}{[I]_t - [DI]} \quad (3)$$

(2) Subsequent to the fast phase (~5 s) of the binding reaction, the next 100 s of data for the slowly rising fluorescence signal were fit to (see Appendix for derivation and limitations)

$$[D]_t = [D]_o + \frac{[M]_o}{2}\left[1 - \frac{1}{tk_1[M]_o + 1}\right] \quad (4)$$

where k_1, $[D]_o$, and $[M]_o$ were adjustable parameters (Kaleidagraph, Abelbeck Software, Inc.). This fit was performed in order to obtain $[DI]_o$ at zero time with a short extrapolation of 5 s. (3) $[M]_o$ was then calculated with

$$[M]_o = 2\{[E]_t - ([DI]_o + [D]_o)\} \quad (5)$$

Table 1: Equilibrium and Rate Constants for the Association Reaction of L-737244 with HIV-1 Protease

pH	buffer	temp (°C)	k_2[a] (M^{-1} s^{-1})	k_{-2}[a] (s^{-1})	K_i^{FP} (nM) (calculated)[b]	K_i^{FP} (nM) (measured)[c]
6.7	A	30	3.0×10^7	0.32	10.6	
7.0	A	30	(2.7×10^7)[d]	0.33	11.9	
7.3	A	30	(2.4×10^7)[d]	0.33	13.8	
7.5	A	30	2.2×10^7	0.34	15.2	
6.0	B	37	6.5×10^7	0.35	5.3	5.7
6.5	B	37	6.1×10^7	0.37	5.9	
7.0	B	37	2.6×10^7	0.38	14.7	
7.5	B	37	1.9×10^7	0.38	19.7	
7.0	NaMES	37	(5.5×10^7)[e]	0.19		3.4

[a] The inhibitor association and dissociation rate constants as in Scheme 1. [b] K_i^{FP} is equal to the ratio k_{-2}/k_2. [c] Steady-state measurements of K_i^{FP}. [d] Interpolated from the measurements at pH 6.7 and 7.5. [e] Calculated from k_{-2} and K_i^{FP}.

(4) K_d for the monomer–dimer equilibrium is given by

$$K_d = \frac{[M]_o^2}{[D]_t} \quad (6)$$

For experiments in NaMES buffer (Figure 1B), calculated monomer concentrations (eq 5) were also used to calculate K_d for all of the experiments done at different E_t with

$$[M]_o = \frac{1}{2}\left[-\frac{K_d}{2} + \left(\frac{K_d^2}{4} + 4K_d \cdot [E]_t\right)^{0.5}\right] \quad (7)$$

where K_d is the only adjustable parameter.

The rate constant for monomer association (k_1) was obtained by curve fit of the first 25–50 s of slow phase data to eq 4, using the $[M]_o$ and $[D]_o$ values determined above and k_1 as the only adjustable parameter.

Subunit Exchange Experiments. For studies in which the ratio of D25N to native protease was varied, incubations were in either buffer B (pH 5.1) or assay buffer, 37 °C with 100 nM protease. At specified times, aliquots were diluted 100-fold into an assay solution and incubated at 30 °C. Product formation in the assays was shown to be linear for 10 min in the absence or presence of D25N. Incubations with the genetically linked, inactive heterodimer, D126N, were performed in assay buffer at 30 °C with 102 nM protease and 1.1 μM or 22 μM D126N.

Kinetic Simulations. The Runge–Kutta method of kinetic simulation using defined differential equations was used to generate the theoretical curves shown in Figure 3B and 4A. The differential equations used are given in the Appendix.

RESULTS

Inhibition and Rate Constants for L-737244. Binding of L-737244 to HIV-1 protease produced a 230% enhancement of the inhibitor fluorescence emission. The fluorescence emission spectra, both bound and free, were insensitive to pH in the range 3.5–7.5. The inhibition constant of L-737244, K_i^{FP}, was measured at 37 °C by following the fluorescence enhancement at 420 nm in titrations. The results are given in Table 1. The related association and dissociation rate constants for L-737244 in its reaction with the HIV-1 protease are also given in Table 1. Since the rate constant measurements are relatively insensitive to protein concentration and absolute magnitude of the fluorescence enhancement, they were used to calculate K_i^{FP} for most conditions. Nevertheless, they confirmed the steady-state measurements of K_i^{FP} at pH 6.0 (eq 1 and Table 1) and at pH 5.5 (data not shown). From the data in Table 1, the half-life for the reaction of 200 nM probe with 20 nM enzyme was calculated to be <0.2 s for all solvent conditions shown so that formation of the enzyme–probe complex is complete within the mixing time of the reaction (<3 s).

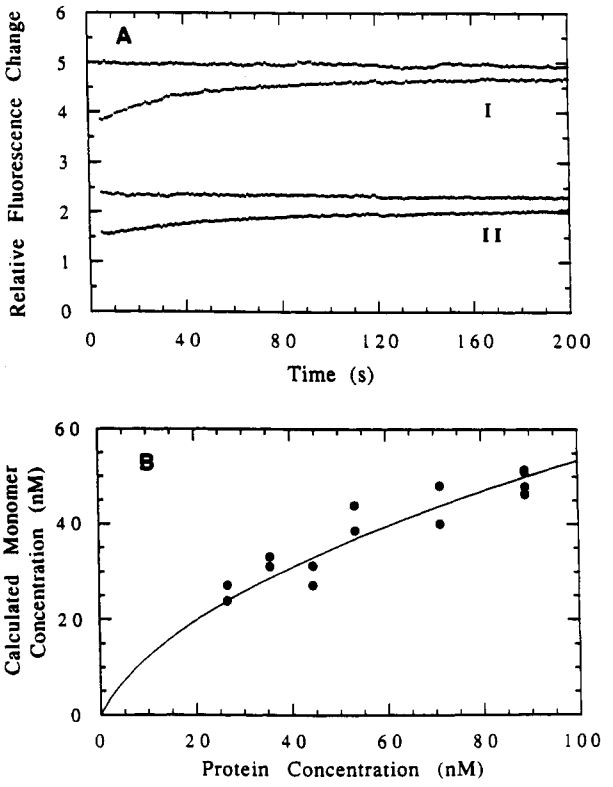

FIGURE 1: Time course of fluorescence signal of L-737244 after addition to HIV-1 protease at pH 7.0 and 37 °C in NaMES buffer. (A) The increase in fluorescence of L-737244 upon binding to HIV-1 protease. The pairs of lines labeled I and II correspond to different concentrations of enzyme in separate experiments, 89 and 36 nM, respectively. Within each pair of lines, the upper, horizontal line is the fluorescence increase found when enzyme was added to the cuvette already containing L-737244. The lower, curved line is the fluorescence increase obtained when enzyme was first diluted into and incubated in the cuvette for 5 min prior to addition of L-737244. The curves shown are representative of the change in curvature and the magnitude of the slow phase of fluorescence enhancement as a function of enzyme concentration. (B) Calculated monomer concentration as a function of total concentration of HIV-1 protease at pH 7.0. Monomer concentrations were calculated from the extrapolation of progress curves such as those in panel A to zero time (see Materials and Methods). Monomer concentrations obtained as a function of total concentration of enzyme were fit to eq 7 giving a K_d of 40 nM (solid line).

Dimer–Monomer Equilibria. The time course and extent of fluorescence enhancement as a function of protein concentration were first examined at pH 7 and 37 °C. When L-737244 was injected into a stirred solution *immediately* after injection of HIV-1 protease into the solution, the fluorescence increase upon binding of the probe to the enzyme was immediate and subsequently remained constant (Figure 1A, horizontal traces). The magnitude of the rapid increase was proportional to the concentration of enzyme injected. The same pattern was observed when enzyme was injected into the reaction mixture after L-737244 injection. On the other hand, when the enzyme was allowed to equilibrate in the buffer for 5 min prior to the addition of probe, the enhancement in fluorescence over time was biphasic, with an initial fast phase followed by a second, slow phase of fluorescence increase (Figure 1A, curved traces). The final amplitude of fluorescence changes was similar for a given total concentration of enzyme regardless of whether reactions were carried out with

Scheme 1

preincubation $M + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} D$

inhibitor binding $M + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} D \underset{k_{-2}}{\overset{k_2 I}{\rightleftharpoons}} DI$

or without preincubation of enzyme. These results suggested that the dimeric enzyme was dissociating into monomers in dilute solutions during preincubation. Indeed, when a form of the enzyme which cannot dissociate into subunits (DiIanni et al., 1990a) was substituted for the wild-type enzyme in the experiments described above, the fluorescence changes were not affected by preincubation of the enzyme. Preincubation of this single polypeptide form of the HIV-1 protease produced fluorescence changes the same as those shown in Figure 1A for the no-preincubation case (data not shown).

The reactions involving preincubation of the enzyme and subsequent binding of the fluorescent probe may be described by Scheme 1.

According to Scheme 1 the initial burst in fluorescence intensity of the probe observed upon binding was proportional to the initial concentration of dimer present, and the subsequent slow rise in fluorescence was due to a shift in the monomer–dimer equilibrium toward the dimer. Scheme 1 dictates that, following enzyme dilution, the amplitude of the burst phase should decrease as preincubation time increases and should be diminished to a final level dictated by K_d. Indeed, following enzyme dilution, fluorescence intensity of the burst phase decreased with increasing preincubation times up to a period of about 3 min after which it did not change. By allowing the diluted enzyme solution to reach equilibrium prior to addition of probe, the concentrations of monomer and dimer in the preincubated solution, and hence the K_d, were determined for pairs of curves such as those in Figure 1A (see Materials and Methods). In experiments conducted at six different protein concentrations, the K_d obtained did not vary with protein concentration, and the average value obtained was 39 ± 4 nM (NaMES buffer, pH 7.0, 37 °C). Data from all six experiments at different enzyme concentrations were also treated collectively as shown in Figure 1B by fitting calculated monomer concentrations to eq 7 (see Materials and Methods); the resultant K_d was 40 nM. At pH 7.0 and 30 °C in the NaMES buffer, a single determination yielded a lower K_d of 15.9 nM.

At low pHs (<6.5 at 37 °C and <6.0 at 30 °C), no discernable fluorescence difference between control (no enzyme preincubation) and preincubated samples could be observed so that monomer concentration could not be determined. To estimate the protease dimer stability at pH 5.5 (a typical pH used in assays), the value of K_d as a function of pH was determined for a range of pH values using the constant ionic strength buffers A and B. Linear extrapolation of the trends found in the experimental data to pH 5.5 yielded K_d values of 0.75 and 3.4 nM at 30 and 37 °C, respectively. The results are shown in Figure 2.

Subunit Association and Dissociation Rates. Scheme 1 also dictates that the rate of fluorescence increase in the slow phase of probe binding assays, i.e., the process of dimerization, is a function of monomer concentration. Although the rate of approach to a new equilibrium is a function of the four rate constants shown in Scheme 1, the initial approach to equilibrium subsequent to inhibitor binding is dominated by k_1 when [I] ≫ [D] and k_2[I] ≫ k_1, $2k_{-1}$, k_{-2} (see Appendix). The data points in the first 25–50 s for each of the slow phase

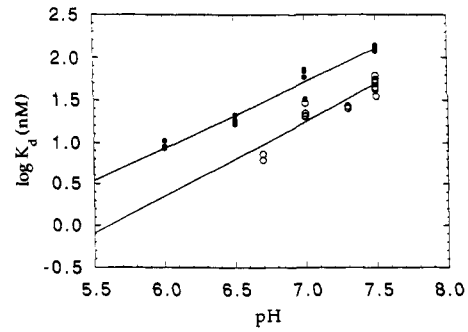

FIGURE 2: Effect of pH on the stability constant, K_d, of HIV-1 protease dimer at 30 and 37 °C. Equilibrium dissociation constants were determined with fluorescence recovery experiments as shown in Figure 1A using the constant ionic strength buffers A at 30 °C (○) and B at 37 °C (●).

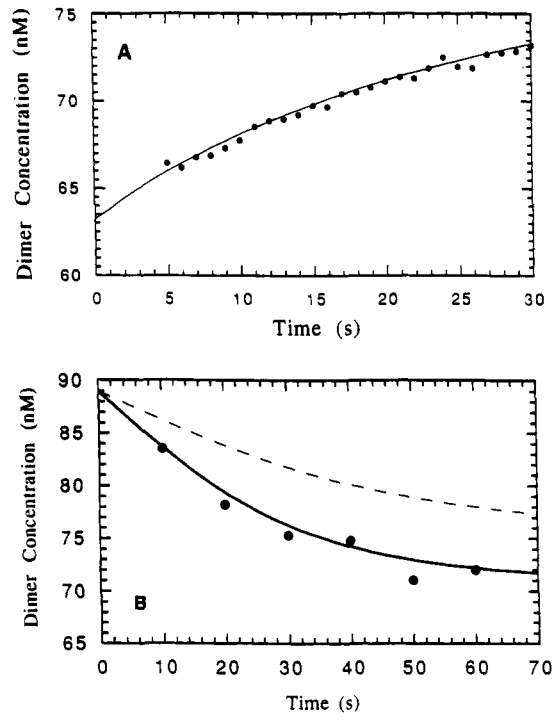

FIGURE 3: HIV-1 protease subunit association and dissociation rates at pH 7.0 and 37 °C in NaMES buffer. (A) Dimer concentration as a function of time after probe addition to preincubated enzyme. Fluorescence intensities for curves such as those in Figure 1, panel A, were converted to the corresponding dimer concentrations (Materials and Methods). The first 25 s of data were fit to eq 3 for the probe induced formation of dimers to obtain the rate constant for monomer association, k_1. The solid line is the fit of the data for one run, total enzyme concentration of 89 nM, obtaining k_1 = 7.0 × 10^5 M^{-1} s^{-1}. (B) Dimer concentration as a function of time following dilution of enzyme. Enzyme was diluted into the cuvette and preincubated for the times shown prior to the addition of L-737244. Dimer concentration, $[D]_o$, at the moment of probe addition was determined by extrapolation of the fluorescence progress curves to zero time, as described under Materials and Methods. Total concentration of enzyme added was 89 nM. The solid line depicts a computer simulation of dimer reequilibration upon dilution using the k_1 determined as in panel A (7.0 × 10^5 M^{-1} s^{-1}) and the k_{-1} value (0.014 s^{-1}) calculated from K_d and k_1 (Materials and Methods). For comparison, the dashed line shows a simulation with k_{-1} set at $^1/_2$ the actual value (k_1 the same).

curves were fit to eq 4. Figure 3A illustrates an example of such a fit. In NaMES buffer at 37 °C, k_1 was found to be (7.0 ± 1.7) × 10^5 M^{-1} s^{-1} and was independent of protein and probe concentrations. The values of k_1 collected under the various conditions used are shown in Table 2, together with the rate constants for subunit dissociation, k_{-1}, calculated from k_{-1} = ($^1/_2$)[k_1K_d] (see Appendix).

Table 2: Equilibrium and Rate Constants for the Monomer–Dimer Reaction of HIV-1 Protease

pH	buffer	temp (°C)	K_d (nM)	k_1[a] (μM^{-1} s^{-1})	k_{-1}[a] (s^{-1})	number of determinations
6.7	A	30	6.8 ± 0.8	0.44 ± 0.14	0.0015	2
7.0	A	30	23.1 ± 4.0	0.22 ± 0.03	0.0025	4
7.3	A	30	26.0 ± 1.2	0.21 ± 0.02	0.0027	2
7.5	A	30	48.0 ± 9.6	0.23 ± 0.02	0.0055	6
6.0	B	37	9.2 ± 0.8	0.73 ± 0.06	0.0034	4
6.5	B	37	18 ± 2.0	0.49 ± 0.13	0.0045	4
7.0	B	37	58 ± 18	0.58 ± 0.34	0.017	4
7.5	B	37	130 ± 9.2	0.38 ± 0.05	0.025	4
7.0	NaMES	37	39.0 ± 4.6	0.70 ± 0.05	0.014	14
7.0	NaMES	30	15.9	0.56 ± 0.12	0.0045	2

[a] The monomer association and dimer dissociation rate constants as defined in the Appendix, determined as described under Materials and Methods. The k_{-1} values listed were calculated as equal to the ratio $(K_d k_1)/2$. Error limits shown are standard deviations.

To assure that the overall treatment of the fluorescence data was consistent with Scheme 1, the dissociation rate constant, k_{-1}, was also determined directly at pH 7.0 and 37 °C. At a given time following dilution of concentrated enzyme, the fluorescent probe was added to the enzyme solution to measure the dimer concentration. By repeating this procedure, dimer concentration as a function of time was determined in NaMES buffer. The results are shown in Figure 3B. The anticipated time course of the approach to dimer–monomer equilibrium at pH 7.0 upon enzyme dilution was also computer simulated using the determined k_1 value and the calculated k_{-1} value obtained above. The simulated decrease in dimer concentration as a function of enzyme preincubation time is in excellent agreement with the experimentally determined values. For comparison, another simulation using a value of k_{-1} half of that determined above is also shown in Figure 3B as a dashed line.

Inactivation of HIV-1 Protease by Subunit Exchange. When active HIV-1 protease and the inactive D25N mutant were mixed, a time-dependent loss of enzyme activity was observed. Examples of these inactivation reactions are shown in Figure 4A. The inactivation data could be fit to a good approximation to first-order exponential decay (fits not shown). The apparent rate constant of inactivation, k_{inact}, decreased with increasing concentration of D25N, yielding a limiting rate constant k_{inact} of 1.68 ± 0.06 h^{-1} at pH 5.1 and 37 °C. The k_{inact} values obtained with various pH and D25N concentrations are compiled in Table 3. Inactivation rates were also observed to decrease with pH, such that at pH 3.5 or 4.5 no inactivation was discernable, while more rapid inactivation occurred at higher pH values (Table 3). As controls, incubation of the active protease with other proteins not expected to participate in specific subunit exchange, such as lysozyme, had no effect on activity. Also, the single-chain D126N form of the protease (DiIanni et al., 1990a), which cannot dissociate to monomers, also had no effect on the activity (data not shown).

The effect of the active-site directed inhibitor, L-365862, upon the rate and extent of inactivation by D25N is shown in Figure 4B. The inclusion of L365862, a substrate analog, slowed the inactivation process and a higher final level of activity was obtained. The slowing of the inactivation reaction was anticipated from the binding interactions known from X-ray crystallographic studies, wherein both subunits of the dimer interact equally with the inhibitor (Fitzgerald et al., 1991). In addition, affinity chromatography experiments have shown that this inhibitor binds much more tightly to the active

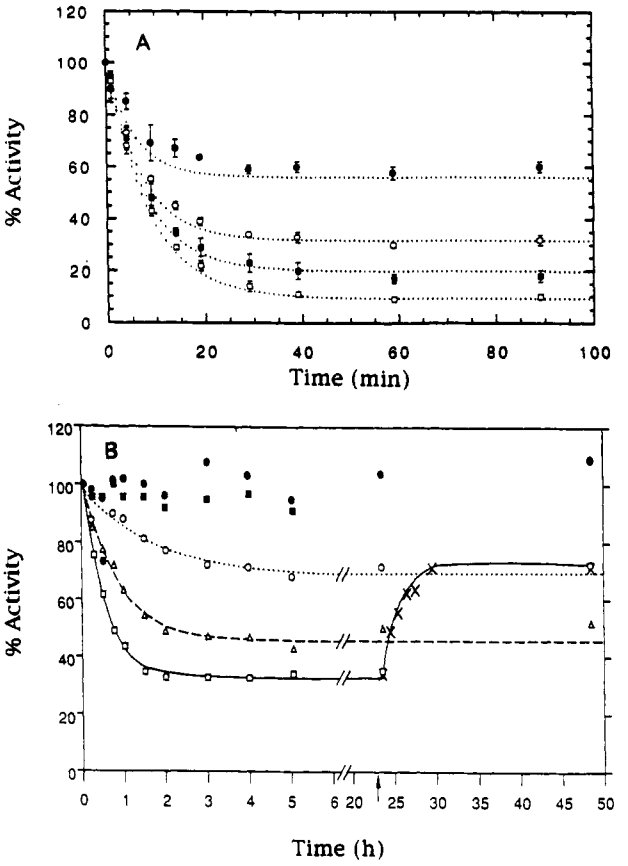

FIGURE 4: Time-dependent inactivation of HIV-1 protease in the presence of D25N. HIV-1 protease was incubated with D25N at pH 5.5, 30 °C in assay buffer. At the times indicated, an aliquot of the reaction mixtures was withdrawn and diluted into activity assays. (A) Effect of D25N concentration upon activity. Native enzyme (100 nM) was combined with various concentrations of D25N: (●) 151, (○) 600, (■) 1290, (□) 3500 nM. The dotted lines represent a computer simulation of Scheme 2, using the rate constants determined for k_1 and k_{-1} from fluorescence recovery experiments. The other rate constants for the D25N homodimer and heterodimer were arbitrarily set to $2k_1$ for k_2 and k_3 and $0.5k_{-1}$ for k_{-2} and k_{-3}. (B) Time-dependent inactivation and reactivation of HIV-1 protease in the presence of D25N and the inhibitor, L-365862. The activity remaining was monitored for (●) protease only, (■) protease + 22 μM L-365862, (□) protease + 2 μM D25N, (△) protease + 2 μM D25N + 4.4 μM L-365862, (○) protease + 2 μM D25N + 22 μM L-365862. The activity decreases to 35%, 52%, and 72% of the initial activity at concentrations of 0, 4.4, and 22 μM inhibitor, respectively. Nonlinear least-squares fit of the data to a first-order exponential yielded activity decay rates of 2.0 ± 0.1, 1.2 ± 0.1, and 0.65 ± 0.08 h^{-1} for 0, 4.4, and 22 μM inhibitor, respectively. After 23.5 h (arrow), 22 μM L-365862 was added to the protease + 2 μM D25N sample (□) and the percentage activity (based on the initial activity) was monitored (×).

form of the enzyme than to the D25N mutant (Heimbach et al., 1989; DiIanni et al., 1990b). Addition of L-365862 to an equilibrated mixture of native and D25N enzymes partially reinstated the activity of the protease (Figure 4B), consistent with the notion of selective stabilization of the active homodimer and demonstrating the reversibility of the reaction. These phenomena were independent of the structural class of inhibitor used, as an inhibitor with a hydroxyethylene isostere peptide replacement also produced the same effect.[2]

The results in Figure 4 and Table 3 may be described by Scheme 2 in which active homodimers (D) dissociate to

[2] The inhibitor used was *N*-(phenylmethyl)-5(*S*)-[(1,1-dimethylethoxycarbonyl)amino]-4(*S*)-hydroxy-6-(phenyl)-2(*R*)-(2-pyridylmethyl)hexaneamide, a compound similar in structure to those previously described (Vacca et al., 1991) but with lower inhibitory potency (IC_{50} = 4.2 μM).

Table 3: Inactivation of HIV-1 Protease after Mixing with the Active-Site Mutant, D25N[a]

buffer	additive	pH	temp (°C)	D25N concentration (nM)	residual activity (%)	k_{inact} (h^{-1})
assay	BSA	5.1	37	0	100	
assay	BSA	5.1	37	123	59	3.54
assay	BSA	5.1	37	490	31	2.16
assay	BSA	5.1	37	1060	14	1.68
assay	BSA	5.1	37	2860	6	1.68
A		3.5	30	400	100	<0.2
A		4.5	30	400	100	<0.2
A		5.5	30	400	44	1.13
A		6.5	30	400	34	3.35
A		7.5	30	400	5	9.02

[a] Native enzyme starting concentratino was 100 nM in all samples.

Scheme 2

$$M + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} D \qquad M' + M' \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} D'' \qquad M' + M \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} D'$$

monomers (M), which then reassociate with mutant monomers (M') produced by dissociation of the mutant homodimer (D''). The extent of heterodimer (D') formation would then determine the extent of activity loss.

Analysis of the rate of inactivation in terms of the rate constants in Scheme 2 is not straightforward since all six rate constants involved contribute to k_{inact}. However, when an infinite concentration of D25N is available, the situation is simplified in that k_{inact} becomes the same as k_{-1} (Scheme 2). As seen in Table 3, a limiting value of k_{inact} is obtained (1.68 h^{-1}) at high concentration ratios of D25N to wild-type enzyme such that the k_{inact} is comparable to the extrapolated k_{-1} determined from the fluorescence recovery experiments. In addition, the rate of inactivation was observed to be much less sensitive to absolute concentrations than to the ratios of the enzyme forms in the reaction (data not shown). This observation is consistent with Scheme 2 where the overall inactivation rate is governed by the formation of monomers.

DISCUSSION

We have applied two methods in this study, fluorescent probe binding and subunit exchange, to reveal the nature of the monomer–dimer transition for the HIV-1 protease. Together, these experiments have yielded a self-consistent body of evidence that can only be described by a slow monomer–dimer interconversion of the HIV-1 protease in solution.

Scheme 1 depicts the mechanism of binding of the fluorescent probe (L-737244) to the HIV-1 protease dimer which is in equilibrium with its monomeric counterpart. The possibility that the protease is in slow equilibrium with a dimeric conformational state which is unable to bind probe can be ruled out since the extent of the slow phase reaction with probe is protein concentration dependent (Figure 2) and the single polypeptide form of the enzyme does not exhibit the slow phase behavior. The possibility of L-737244 binding slowly to the monomeric protease can be ruled out since the value of k_1 determined from the slow phase of the fluorescence enhancement assays is insensitive to the probe concentration (data not shown).

We have previously shown that changing one of the two active-site Asp-25 residues to an Asn is sufficient for total inactivation of the HIV-1 protease, through use of a single polypeptide form of the enzyme, D126N, in which the two subunits are covalently linked (DiIanni et al., 1990a). The loss of activity upon mixing of the active enzyme and D25N mutant is then reasonably interpreted as the formation of an inactive heterodimer according to Scheme 2. The rates of dimer dissociation obtained in fluorescence experiments (k_{-1} in Table 2) as a function of pH are also consistent with slower monomer formation being the source of lower k_{inact} values in the subunit mixing experiments at lower pHs (Table 3). Finally, by applying Scheme 2 and the rate constants k_1 and k_{-1} determined from the fluorescence probe assays, the time course for subunit exchange, and thereby protease inactivation, was simulated with the Runge–Kutta method. The other rate constants in Scheme 2 are unknown but are expected to be similar to k_1 and k_{-1}. It can be seen in Figure 4A that, by setting k_2 and k_3 equal to $2k_1$, and k_{-2} and k_{-3} equal to $0.5k_{-1}$, the subunit exchange data are closely predicted by Scheme 2. The concordance of the measurements from two different experimental approaches to account for HIV-1 protease dimer dissociation lends credence to their validity and interpretations.

The monomer association rate constants obtained [k_1 = (2–7) × 10^5 M^{-1} s^{-1}] are within the range of other protein–protein association rates reported. For example, the melittin dimer association rate constant is about 10^5 M^{-1} s^{-1} (Schwarz & Beschiaschvili, 1988), and epidermal growth factor associates with its receptor with a rate constant of 1.2 × 10^6 M^{-1} s^{-1} (Mayo et al., 1989). Using the value determined for k_1 and K_d at pH 7 and 37 °C, the calculated value of k_{-1} is in excellent agreement with that obtained from direct measurements (Figure 3).

The stability of the HIV-1 protease dimer is strongly pH dependent, with the observed K_d ranging from 9 nM at pH 6.0 to 130 nM at pH 7.5 (37 °C). Extrapolation of the experimental K_d values at 30 °C to pH 5.5 yields a value of 0.75 nM (Figure 2). This value is consistent with our earlier report, which for pH 5.0 and 5.5 described *either* a slow subunit exchange process *or* a K_d less than 80 pM in the case of fast subunit exchange (Jordan et al., 1992). It can be seen from the dissociation rates found here that slow subunit exchange is the case which applies (Tables 2 and 3). Thus it is not possible to reconcile the data presented here with the report of Zhang et al. (1991), wherein an assumption of rapid equilibrium for the conversion of HIV-1 protease monomer and dimer was applied in the calculation of K_d to be 3.6 nM at pH 5.0 and 37 °C.

Higher temperatures promote dissociation, as can be seen from the increase in K_d values in Table 1. A 2–3-fold increase in K_d was observed with an increase in temperature from 30 to 37 °C. The K_d of 39 pM at 25 °C and pH 6.0 reported by Grant et al. (1992) is consistent with our findings when temperature differences are considered. Our K_d measurement of 15.9 nM at pH 7.0 and 30 °C in NaMES buffer is somewhat lower than the value of 50 nM obtained by Cheng et al. (1991) at 25 °C with the same buffer. In the study performed by Cheng et al. (1991), the concentration of the dimer was monitored by enzyme activity. In principle, this approach should give the same result as monitoring the dimer form with an active-site fluorescent inhibitor. The practical difficulty in this case, however, is that the dimer concentration was determined from the asymptotic slope of fluorescence intensity change as a function of time during catalytic turnover of a fluorescent substrate. The asymptotic slope is difficult to determine visually.[3] In the use of a fluorescent active-site

[3] An alternate approach in the use of continuous enzyme activity assays for the determination of stability constants of a dimeric enzyme has been proposed by Kuzmič (1993).

inhibitor, the dimer concentration is determined by an essentially instantaneous, absolute intensity readout which is likely to be more accurate.

The rate and equilibrium data presented here have implications for the proper kinetic analysis of catalysis and inhibition. If significant dissociation occurred during assay, erroneously high Michaelis and inhibition constants would obtain. On the other hand, competitive inhibitors evaluated at picomolar concentrations of enzyme should not be compromised so long as the incubation period with diluted enzyme is less than an hour at pH $\leq$ 5.5. The presence of substrate and inhibitor further stabilizes the enzyme and slows the dissociation process. Raising the pH toward neutrality, however, narrows the limits of incubation time and enzyme concentration within which enzyme dissociation can be ignored.

The pH of the reaction mixture and length of enzyme incubation need to be taken into consideration for the discovery of "dissociative" inhibitors. Sufficient time must be allowed for the protease dimer to dissociate to optimize the monomer binding by an inhibitor. The D25N subunit used in the present study may be regarded as the "perfect" peptide dissociative inhibitor, since it is in all aspects (except for the D25 $\rightarrow$ N substitution) indistinguishable from the native subunit. Even with this perfect dissociative inhibitor, at least 30 min is needed to obtain full inhibition at pH 5.5 and 30 °C (Figure 4A). A useful aspect of slow dimer dissociation is that an inhibitor which binds exclusively to monomer should exhibit a characteristic slow onset of inhibition when mixed with dimeric HIV-1 protease, under the solution conditions used in this study, thereby aiding in the identification of the inhibitor mechanism. Inhibition of the HIV-1 protease by putative dissociative inhibitors has recently been reported by both Zhang et al. (1991) and Babé et al. (1992). In both cases, the time of preincubation of the enzyme with the inhibitors at the pH values used in the studies would not have been sufficient to obtain full dimer dissociation and hence obtain accurate inhibition constants. In the case of the C-terminal peptide used by Zhang, the calculation of potency was complicated by the rapid equilibrium assumption for protease dissociation. In the study by Babé et al. (1992), it is possible that the short preincubation times used (5 min) resulted in underestimation of the potency of the peptides described.

Knowledge of the K_d values for dimer stability prompts the question of whether a dissociative inhibitor would have a chance to inhibit viral replication, particularly in comparison to the potent competitive inhibitors of replication currently being pursued. Aside from the timing considerations of slow dissociation, the binding of a dissociative inhibitor to a monomer must compete with the avid binding of the alternate subunit. In comparison, active-site-directed inhibitors compete only with substrate binding. Although binding constants for the protein substrates of HIV-1 protease have not been determined, the K_m values of peptide substrates representing the natural cleavage sites may be indicative of those affinities. The reported K_m values are on the order of 1 mM, suggesting that inhibition of protease function by a competitive inhibitor may be inherently easier to achieve. Finally, the dissociation equilibrium is shifted toward the dimer state by substrate stabilization, so that a dissociative inhibitor must compete with substrate as well as with the second protease subunit. Nevertheless, it may be that a dissociative inhibitor is most likely to succeed in preventing protease function by combining with a monomer (or precursor polyprotein) prior to its dimerization.

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APPENDIX

(A) Reassociation Kinetics of HIV-1 Monomers

Using the general equatitions to describe the appearance of dimer–inhibitor complex following addition of inhibitor, I,

$$M + M \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} D$$

$$D + I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} DI$$

the differential equations are

$$\frac{d[M]}{dt} = -k_1[M][M] + 2k_{-1}[D] \quad (1)$$

$$\frac{d[D]}{dt} = (1/2)k_1[M][M] - k_{-1}[D] - k_2[D][I] + k_{-2}[DI] \quad (2)$$

$$\frac{d[DI]}{dt} = k_2[D][I] - k_{-2}[DI] \quad (3)$$

To obtain the individual rate constants for the process, there are the following options: (1) Apply perturbation theory to solve the differential equations; this approach is inappropriate because the system in concern here is far from equilibrium. (2) Assume transient kinetics far from equilibrium; however, pseudo-first-order kinetics cannot be attained for the dimerization process. (3) Assume a steady-state for the attainment of [D]; this approximation is appropriate if all D is quenched by I (by introducing an excess amount of I in the assay reaction) and when $k_2 \gg k_1$, $2k_{-1}$, k_{-2}.

Thus, assuming

$$\frac{d[D]}{dt} = 0$$

then

$$(1/2)k_1[M][M] - k_{-1}[D] - k_2[D][I] + k_{-2}[DI] = 0$$

or

$$[D] = \frac{(1/2)k_1[M][M] + k_{-2}[DI]}{k_{-1} + k_2[I]}$$

From eq 3,

$$\frac{d[DI]}{dt} = \left\{k_2[I]\frac{(1/2)k_1[M][M] + k_{-2}[DI]}{(k_{-1} + k_2[I])}\right\} - k_{-2}[DI]$$

Setting [I] = i = constant, x = [DI] accumulated during slow phase of the reaction, and a_o = [M_o], at later times [M] = ($a_o - 2x$), so that

$$\frac{dx}{dt} = k_2 i\left\{\frac{(1/2)k_1(a_o - 2x)^2 + k_{-2}x}{k_{-1} + k_2 i}\right\} - k_{-2}x$$

or

$$\frac{dx}{dt} = \frac{k_2 i}{k_{-1} + k_2 i}\left\{(1/2)k_1(a_o - 2x)^2 + k_{-2}x - \frac{(k_{-1} + k_2 i)k_{-2}x}{k_2 i}\right\}$$

For $k_2i \gg k_{-1}$, $k_2i/(k_{-1} + k_2i) = 1$, so that

$$\frac{dx}{dt} = (1/2)k_1(a_o - 2x)^2$$

$$2\int \frac{dx}{k_1(a_o - 2x)^2} = \int dt$$

$$\frac{1}{k_1(a_o + 2x)} = t + C; \text{ at } t = 0,\ x = 0, \text{ and } C = \frac{1}{k_1 a_o}$$

$$\frac{1}{k_1(a_o - 2x)} = t + \frac{1}{k_1 a_o} \quad \text{or}$$

$$x = (1/2)a_o\left[1 - \frac{1}{t \cdot k_1 a_o + 1}\right]$$

Following the initial rapid, nearly complete, reaction of D with I, [DI] as a function of time is then

$$[DI] = [D]_o + (1/2)[M]_o\left[1 - \frac{1}{tk_1[M]_o + 1}\right]$$

Since $K_d = (2k_{-1}/k_1)$ for dimerization (see derivations in section B below), $k_{-1} = (1/2)K_d k_1$.

(B) Differential Equations for Kinetic Simulation of Subunit Exchange

For dimer dissociation, two monomers are produced per dimer so that

$$\frac{d[M]}{dt} = -k_1[M]^2 + 2k_{-1}[D]$$

when

$$\frac{d[M]}{dt} = 0,\ K_d = \frac{[M]^2}{[D]} = \frac{2k_{-1}}{k_1}$$

For a subunit exchange reaction of active dimer, D, with a homodimer mutant, D″, and its subunit, M′, in which a heterodimer, D′, is formed (Scheme 2 in Discussion), the differential equations are

$$\frac{d[M]}{dt} = -k_1[M]^2 + 2k_{-1}[D] - k_3[M][M'] + k_{-3}[D']$$

$$\frac{d[M']}{dt} = -k_2[M']^2 + 2k_{-2}[D''] - k_3[M][M'] + k_{-3}[D']$$

$$\frac{d[D]}{dt} = (1/2)k_1[M]^2 - k_{-1}[D]$$

$$\frac{d[D'']}{dt} = (1/2)k_2[M']^2 - k_{-2}[D'']$$

$$\frac{d[D']}{dt} = k_3[M][M']^2 - k_{-3}[D']$$

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