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Mechanical Effects on the Kinetics of the HIV Proteinase Deactivation

Petr Kuzmič,[1] Anne G. Peranteau, Carlos García-Echeverría,[2] and Daniel H. Rich

School of Pharmacy and Department of Chemistry, University of Wisconsin, 1101 University Avenue, Madison Wisconsin 53706

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The proteinase from HIV undergoes rapid and irreversible deactivation caused by mild mechanical stirring. Both the free enzyme and the ternary Michaelis complex disappear in two separate first-order processes, with half-times of 3.0 and 0.8 minutes, respectively. Ignoring these deactivation steps distorts the results of kinetic analyses. © 1996 Academic Press, Inc.

The aspartic proteinase from HIV (HIV-Pr), an obligate homodimer, remains an attractive target for antiviral drug design [1,2]. Understanding the kinetic mechanisms of inhibition depends on the knowledge of physico-chemical factors which influence HIV-Pr stability [3]. It is known that HIV-Pr stability depends on the presence of polymeric additives and co-solvents [4], salts [5], substrates [6], and inhibitors [7]. Here we report that HIV-Pr is very sensitive to mild mechanical stirring, under the conditions that are typical for a standard magnetically stirred spectrophotometric cuvette compartment. After 10 minutes of stirring at 400–600 r.p.m., approximately 90% of the enzyme activity is lost owing to a first-order deactivation. The Michaelis complex is also destroyed in a first-order process, and the rate constant is even greater in this case (halftime 0.8 min). These results must be taken into account in quantitative kinetic analyses whenever the proteinase is subjected to mechanical stress.

METHODS

The fluorogenic substrate Lys-Ala-Arg-Val-Tyr-Phe(NO_2)-Glu-Ala-Nle-NH_2 [8] was prepared by the standard Fmoc-solid phase peptide synthesis, or, in later stages of this research, it was obtained from Bachem (Philadelphia, PA). The kinetic properties of both peptide preparations were identical. Stock solutions of the substrate (1.0 mM in deionized water) were kept on ice. A sample of the HIV-1 proteinase was received from Dr. Paul Darke (Merck Research Laboratories) as a solution in 100 mM sodium acetate pH 5.0 (0.31 mg/ml, 95% specific activity, 13.6 μM active sites), and was stored at −80°C. A 10μl aliquot of the concentrated enzyme was thawed by the addition of 90 μl of the assay buffer (100 mM sodium acetate pH 5.0, 4 mM EDTA, 5 mM DTE, 0.1% v/w PEG, 1.0 M NaCl) and the enzyme stock solution was subsequently kept on ice. Fluorimetric assays were conducted as described previously [9] on a Hitachi-3010 spectrofluorimeter (excitation at 275 nm, emission at 305 nm, excitation and emission slit width 5 mm, damping constant 0.5 sec).

The effect of mechanical stirring on the enzyme–substrate complex. The assay buffer (1450 μl) was thermally equilibrated at 25°C. An aliquot of the substrate stock was added (1 to 10 μl, final concentration 0.66 to 6.66μM), followed by the addition of the enzyme stock (10μl, final concentration 9 nM). The progress of the reaction was monitored for 3 minutes with or without mechanical stirring (500 r.p.m.), induced by a 1 × 5 mm Teflon-coated magnetical stirring rod.

The effect of mechanical stirring on the free enzyme. The assay buffer (1450μl) was thermally equilibrated at 25°C. An aliquot of the enzyme stock (5 μl, final concentration 4.5 nM) was added and incubated in the spectrofluorimetric cell for a given amount of time (0.5 to 13 min) with or without mechanical stirring. At the end of the incubation period, substrate was added (1.5μl, final concentration 1 μM) and data collection was commenced. Readings of fluorescence were collected at 0.1 or 0.5 sec intervals by using SpectraCalc (Galactic Software, Inc.), exported as text files, and analyzed by using the program DYNAFIT [10].

[1] To whom correspondence should be addressed: Fax: (608) 262-0381 E-mail:pkuzmic@facstaff.wisc.edu.

[2] Present address: Ciba-Geigy, Basel, Switzerland.

Abbreviations: DTE, dithioerythritol; EDTA, ethylenediamine tetraacetic acid, disodium salt; Fmoc, fluorenylmethyl carbonyl; HIV, human immunodeficiency virus; PEG, polyethylene glycol 8,000–12,000.

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RESULTS

The progress of HIV-1 protease-catalyzed hydrolysis of a fluorogenic peptide, with or without mechanical stirring under otherwise identical conditions, is shown in Figure 1. The simple Michaelis-Menten mechanism (Scheme 1, $k_{de} = k_{dc} = 0$) failed to fit the progress curves shown in Figure 1a. By trial and error we found that at least two additional reaction steps were required for close fit of data in Figure 1a, namely, the disappearance of the enzyme (rate constant k_{de}) and the disappearance of the ternary Michaelis complex (rate constant k_{dc}). Importantly, when the decay of the enzyme monomer was also incorporated into the kinetic mechanism, this extended model did not fit the data unless the monomer decay rate constants was extremely low. These results show that the enzyme dimer and the ternary Michaelis complex, but not the inactive enzyme monomer, undergo first-order irreversible deactivation (see Scheme 1).

			$k_{(\rightarrow)}$	$k_{(\leftarrow)}$
$M + M$	$\rightleftharpoons$	E	k_{md}	k_{dm}
$E + S$	$\rightleftharpoons$	ES	k_{on}	k_s
ES	$\longrightarrow$	$E + P$	k_r	
ES	$\longrightarrow$	FS	k_{dc}	
E	$\longrightarrow$	F	k_{de}	

SCHEME 1

In the least-squares regression analysis, the association rate constant k_{on} was held at a constant value of 100 $\mu M^{-1}sec^{-1}$. The best-fit values of deactivation rate constants were $k_{dc} = (0.0153 \pm 0.0003)$ sec^{-1} and $k_{de} = (0.0039 \pm 0.0011)$ sec^{-1}, which corresponds to deactivation half-times 0.8 and 2.9 minutes, respectively. The best fit values of rate constants for substrate hydrolysis were $k_s = (188 \pm 44)$ sec^{-1} and $k_r = (9.0 \pm 1.5)$ sec^{-1} (Michaelis constant $K_M = 1.97$ μM). In contrast, the progress curves shown in Figure 1b matched the simple Michaelis-Menten mechanism very well. The best fit values of rate constants for substrate hydrolysis were $k_s = (169 \pm 32)$ sec^{-1} and $k = (8.1 \pm 1.0)$ sec^{-1} (Michaelis constant $K_M = 1.77$ μM).

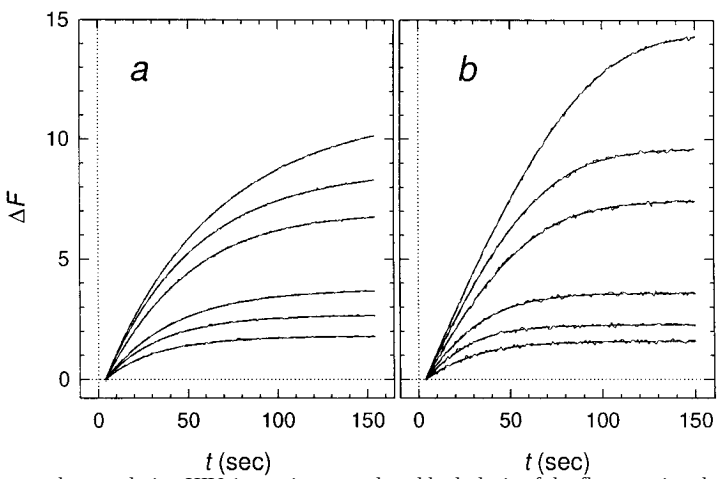

FIG. 1. Fluorescence changes during HIV-1 proteinase-catalyzed hydrolysis of the fluorogenic substrate (a) with or (b) without mechanical stirring. Progress curves (top to bottom) correspond to initial substrate concentration 6, 4, 3, 1.5, 1, and 0.66 μM.

Initial velocities from both sets of assays were fit to the Michaelis-Menten equation (Figure 2). The best-fit values of kinetic constants were $K_M = (1.71 \pm 0.28)\ \mu M$, and $V_{max} = (0.235 \pm 0.015)$ a.u./sec in the absence of stirring, and $K_M = (2.31 \pm 0.24)\ \mu M$, and $V_{max} = (0.279 \pm 0.012)$ a.u./sec with mechanical stirring. Thus the values of the Michaelis constant obtained by four different methods, by the analysis of progress curves or by the analysis of initial velocities with or without stirring, agree within 15% of the median. These results show that the analysis of progress curves is internally consistent, and therefore the estimate of deactivation rate constants are reliable.

To investigate the deactivation of the enzyme dimer, HIV-1 proteinase alone was incubated for a given amount of time in the stirred cuvette compartment, and the reaction was started by the addition of the substrate. Initial velocity of the ensuing reaction was determined by fitting each progress curve in Figure 3 separately to the kinetic model in Scheme 1. The plot of initial velocities against the pre-incubation time (Figure 4, filled circles) is a double exponential, $v = a_1 \exp(-k_1 t) + a_2 \exp(-k_2 t)$. The best-fit values of adjustable parameters were $a_1 = (0.0107 \pm 0.0019)$ a.u./sec, $a_2 = (0.0134 \pm 0.0020)$ a.u./sec, $k_1 = (0.0172 \pm 0.0033)\ sec^{-1}$, and $k_2 = (0.0033 \pm 0.0004)\ sec^{-1}$. Thus the smaller amplitude (30% of the total) corresponds to the faster decay (half-time 0.7 min), and the larger amplitude (70% of the total) corresponds to the slower decay (half-time 3.5 min). The value of k_2 agrees with the value of $k_{de} = (0.0039 \pm 0.0011)\ sec^{-1}$ (half-time 2.9 min), obtained independently by the analysis of progress curves in Figure 1a.

The apparent first-order rate constant k_2 probably originates in the reversible dimermonomer dissociation. The least-squares fit of the initial velocities in Figure 4 to a kinetic model represented by the first and the last step in Scheme 1 gave $k_{md} = (1.14 \pm 0.14)\ \mu M^{-1} sec^{-1}$, $k_{dm} = (0.0068 \pm 0.0013)\ sec^{-1}$, and $k_{dc} = (0.0054 \pm 0.0002)\ sec^{-1}$. These values, and the corresponding dissociation constant $K_D = K_{md}/k_{dm} = 6.0$ nM, are in good agreement with the values reported in the literature ($k_{md} = 0.92\ \mu M^{-1} sec^{-1}$, $K_D = 4.1$ nM [11]).

In a control experiment, we preincubated the enzyme for a given amount of time without stirring, and, as above, started each assay by the addition of the substrate. The progress curves observed in this experiment were virtually identical (Figure 3b). Within 15 minutes of pre-incubation, no significant loss of activity was observed. In fact a small increase in activity (approximately 5%) occurred after 0.5 minutes of incubation (Figure 4, open circles).

DISCUSSION

We found that the HIV-1 proteinase undergoes rapid irreversible first-order deactivation owing

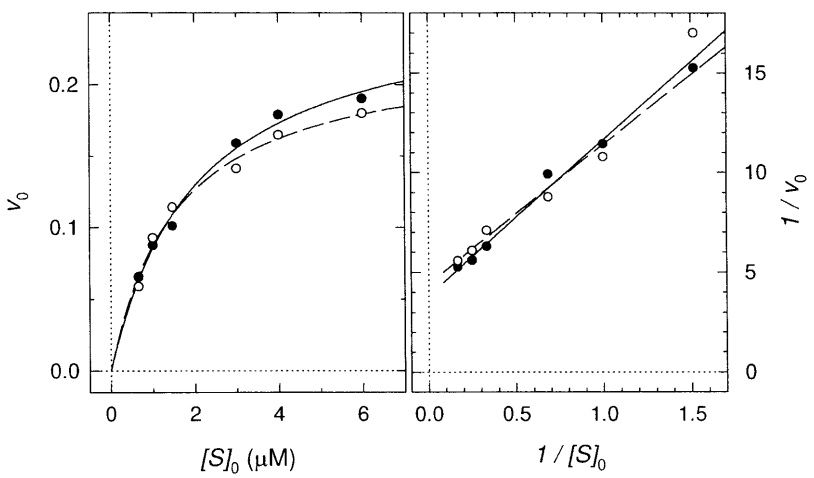

FIG. 2. Least-squares fit of initial velocities with (filled circles) and without (open circles) mechanical stirring to the hyperbolic Michaelis–Menten equation, and the corresponding Lineweaver–Burk plot.

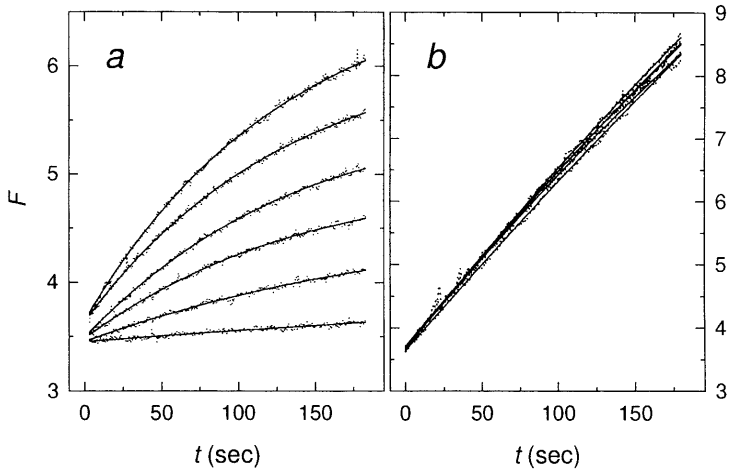

FIG. 3. Fluorescence changes during HIV-1 proteinase-catalyzed hydrolysis of the fluorogenic substrate (a) with or (b) without mechanical stirring. Progress curves (top to bottom) correspond to preincubation times 0.5, 1, 2, 5, 7.5, and 10 minutes, respectively.

to mechanical stirring. The first-order exponential decay is the standard mechanism for irreversible deactivation of many enzymes [12]. For example, a recent report shows that arginase [13] also undergoes first-order deactivation upon mechanical stirring. Here we report for the first time that the Michaelis complex also can decay via a first-order process. The marked instability is surprising for a polypeptide as small (22 kDa) as HIV-Pr. Shearing forces probably do not play a role, unless the protease in our experiment exists as a microscopic aggregate with much larger molecular weight. We experimented with various settings of the magnetic stirrer, and found that both rate constants of deactivation increased with the stirring rate. Coating the surface of the quartz fluorescent cuvettes with silanizing agents did not prevent deactivation.

The results of this study are of interest to those investigators who study the HIV proteinase by physico-chemical methods, such as stopped-flow spectrophotometry, rapid-quench kinetics, high-pressure effects, micro-calorimetry, or surface plasmon resonance (BIAcore). Typically in using such methods the HIV proteinase is subjected to continuous stirring or other forms of mechanical

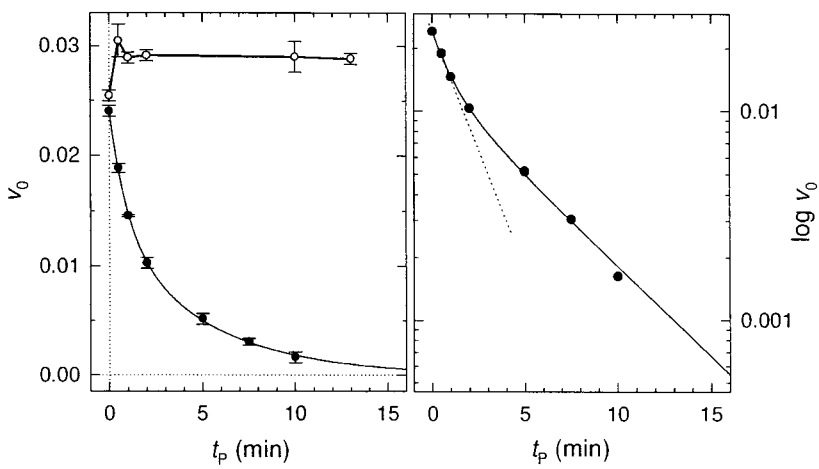

FIG. 4. Initial velocities from Figure 3 in dependence on the preincubation time t_p with (filled circles) or without (open circles) mechanical stirring. Left panel: least-squares fit to Scheme 1. Right panel: least-squares fit to a double exponential.

stress. Interpretation of the experimental data then depends on the proper accounting of deactivation phenomena reported in this paper.

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