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STABILIZATION OF HIV PROTEINASE DIMER BY BOUND SUBSTRATE

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Upon the binding of a synthetic nonapeptide substrate, the catalytically active dimeric form of HIV proteinase is strongly stabilized against dissociation into inactive subunits. The dissociation of the ternary Michaelis complex into protein monomers is immeasurably low (apparent dissociation constant in the picomolar range), while the dimer-to-monomer equilibrium dissociation constant at pH 4.7 and at ionic strength 1.0 M is 30.4 ± 1.6 nM. Consequently, the apparent activity of HIV proteinase depends on the order in which the enzyme and the substrate are added to *in vitro* assays. Substrate–induced stabilization should be carefully considered in designing kinetic studies of all dissociative retroviral enzymes, the proteinase, the integrase, and the reverse transcriptase.

The proteinase encoded by the human immunodeficiency virus (HIV proteinase) is an obligatory homodimer. We have recently proposed [1] that the vast differences between various estimates for the dissociation constant, from less than 1 pM [2] to 87 μM [3], could be explained among other factors by a stabilizing effect of the bound substrate. In this report we show that a strong stabilization of the dimeric active form of the HIV proteinase does occur upon the binding of a synthetic nonapeptide. The dissociation of the *ternary* Michaelis complex into protein subunits could not be detected, while the dissociation constant for the proteinase dimer alone is in the nanomolar range. This finding is important for proper design and interpretation of *in vitro* kinetic assays. When the enzyme is incubated before the addition of the substrate, it partially dissociates into inactive subunits. The equilibrium between the active and the inactive forms is achieved typically in five minutes. On the other hand, when the enzyme is the *last* added component, and when the substrate is present at a concentration that exceeds the Michaelis constant, the ternary Michaelis complex forms rapidly and the dissociation into inactive monomer

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ABBREVIATIONS: CHAPS – 3-[(3-cholamidopropyl)-dimethylamino]-1-propane sulfonate; DTT – dithiothreitol; EDTA – ethylenediamine tetraacetic acid, disodium salt; HIV – human immunodeficiency virus; HPLC – high-pressure liquid chromatography ; FAB-MS – fast-atom-bombardment mass spectroscopy; ODE – ordinary differential equation; Phe(NO_2) – *para*-nitrophenylalanine.

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subunits is prevented. Thus measurements of the enzyme active-site concentration, the turnover number of substrates, and the inhibition constants of tightly bound inhibitors depend on the order in which the enzyme and the substrate are mixed.

EXPERIMENTAL

The chromogenic substrate Lys-Ala-Arg-Val-Tyr-Phe(NO_2)-Glu-Ala-Nle-NH_2, first reported by Richards et al. [4], was prepared by solid phase methods [5], purified to homogeneity by reversed-phase HPLC on Vydac-C18, and characterized by amino acid analysis and high-resolution FAB-MS. The Michaelis constant under the assay conditions described below was K_m = 3.2 ± 1.0 μM. The HIV-1 proteinase was received as a gift from Dr. R. A. Mueller (G. D. Searle & Co., Skokie, IL). The enzyme was stored at –80°C as a stock solution (0.31 mg/ml, 14.3 μM) in 20 mM sodium phosphate buffer (pH 6.4, 1 mM EDTA, 1 mM DTT, 0.1 mM CHAPS, 20% v/v ethylene glycol). The active-site concentration was determined by Ms. Kathryn Houseman (G. D. Searle & Co.) under the conditions described earlier [1]. Kinetic experiments were performed in 100 mM sodium acetate (pH 4.7, 4 mM EDTA, 5 mM DTT, ionic strength adjusted to 1.0 M by the addition of sodium chloride). Immediately before enzymatic assays, an aliquot of the proteinase stock (10 μl) was allowed to thaw at 4°C, was diluted with 90 μl of chilled (0°C) assay buffer, and subsequently stored on ice. A solution (1.00 mM) of the above substrate was prepared in deionized water, and was stored on ice. The assay buffer (0.96 ml) was equilibrated for 10 minutes at 37°C in the thermostated cuvette compartment of a Cary-15 spectrophotometer. An aliquot (10 μl) of the enzyme solution was introduced as a droplet on a polyethylene stirring rod, the solution was stirred gently for 3 sec, and the timing device was started. After predetermined time intervals (15 sec, 30 sec, 1 min, 2 min, etc.), the substrate solution (30 μM, final concentration 30 μM) was added by using the plastic stirring rod, and absorbance changes at 295 nM were monitored for 5 minutes. 300 Datapoints were collected in each assay by using a commercial data acquisition software package (On Line Instrument Systems Inc., Jefferson, GA). The time (t) vs. absorbance (A) data were fitted to a polynomial equation [6] $A = a_0 + a_1 (t - t_0) + a_2 (t - t_0)^2$, where t_0 is the mixing-delay time (3 sec). Analysis was restricted to first or second-order terms of the polynomial, according to Knowles [7]. Initial velocities v_0 were calculated in molar units (M/sec) as $a_1 \times \Delta\varepsilon$, where $\Delta\varepsilon$ is the difference molar absorption coefficient. The initial velocities were analyzed by nonlinear least-squares fit [8] to equation (1), which can be derived from the integral rate equation for the dissociation of the active HIV proteinase dimer (equation (4) in ref. [1]). The uncertainties of fitting parameters were calculated as reported previously [1].

$$v_0 = \frac{S_0}{S_0 + K_m} \frac{k_{cat}}{8} \frac{(\beta - \alpha)\gamma \exp(k_1 \alpha t) - (\beta + \alpha)}{\gamma \exp(k_1 \alpha t) + 1} \qquad (1)$$

$$\alpha = \sqrt{K_d}\sqrt{K_d + 8E} \qquad \beta = K_d + 4E$$

$$\alpha_0 = \sqrt{K_d}\sqrt{K_d + 8E_0} \qquad \beta_0 = K_d + 4E_0$$

$$\gamma = \frac{E(\beta_0 - \alpha_0) - E_0(\beta + \alpha)}{E(\beta_0 - \alpha_0) - E_0(\beta - \alpha)}$$

Figure 1 shows the results of a modified concentration jump experiment. Unlike in the traditional experimental design [1], the substrate was added in saturating quantities ($S_0 \gg K_m$) after different enzyme pre-incubation times. During the incubation time, the enzyme partially

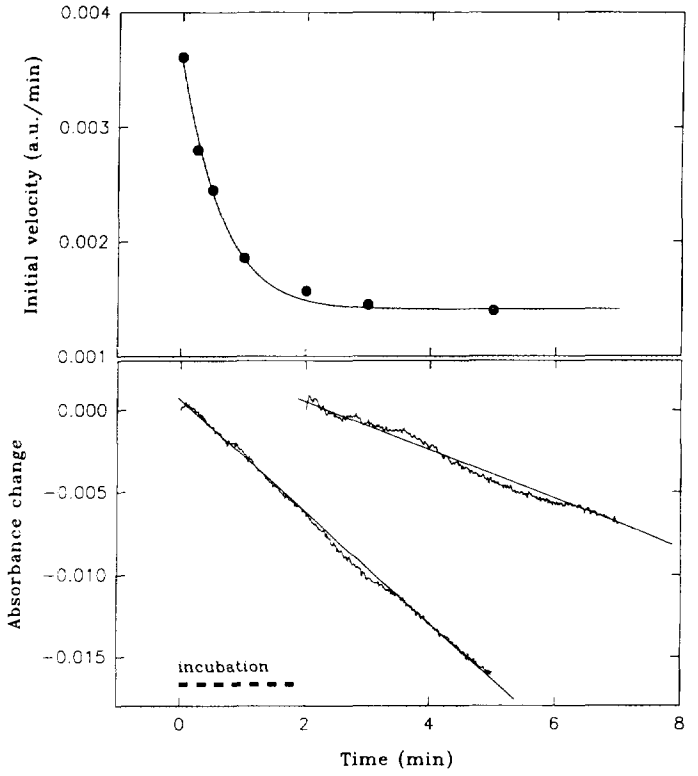

Figure 1. Hydrolysis of the chromogenic susbtrate Lys-Ala-Arg-Val-Tyr-Phe(NO_2)-Glu-Ala-Nle-NH_2 [4] (30 μM, K_m = 3.1 ± 1.0 μM) catalyzed by the HIV proteinase (6.5 nM) at pH 4.7 (100 mM sodium acetate, 37°C, 1.0 M NaCl). *Lower panel:* Reaction progress. The thick dashed line indicates the preincubation time that elapsed before the start of a representative assay (right trace). The thin solid lines drawn through experimental data show the linear least-squares fit to obtain reaction velocities. *Upper panel:* Reaction velocities (circles), and the best-fit curve obtained by nonlinear least-squares regression to equation (1).

dissociated into inactive subunits. The equilibrium between the active dimer and the inactive monomer was established approximately in five minutes. *Within the first two minutes of pre-incubation, the HIV proteinase lost more than 50% of activity due to dissociation* (Fig. 1, upper panel). However, in the presence of the substrate no loss of activity was observed two minutes after the start of the reaction (Fig. 1, lower panel, left trace). *The slope of the progress curve at time two minutes was identical to the slope at time zero*, which means that the concentration of the active enzyme species did not change within that time. Moreover, when the substrate was added after the enzyme had already partially dissociated, further decomposition into inactive subunits was arrested (Fig. 1, lower panel, right trace). These results show that the only observable dissociation pathway for the ternary Michaelis complex is $E_2S \rightarrow E_2 + S$. The alternate dissociation pathway $E_2S \rightarrow E + ES$ is not involved. We used the computer program VODE [9] (*V*ariable Coefficient *ODE* Solver) to simulate the dissociation of the ternary complex, and found that the corresponding equilibrium dissociation constant is lower than 1 pM.

From the principle of microscopic reversibility, it seems that the substrate has little or no affinity for the inactive monomer subunit of HIV proteinase. Certain preliminary observations however suggest that interactions between the proteinase monomer and the substrate cannot be excluded. When the substrate was added after a prolonged incubation of the enzyme (more then 10 minutes), the residual enzymatic activity emerged only gradually, after a distinct lag period of about 30 sec (data not shown). This transient phase could be explained either as a conformational change in the HIV proteinase dimer, induced by the binding of the substrate, or as a substrate–induced dimer assembly. Examples of substrate–induced stabilization of oligomeric enzymes are given in reference [1].

Nonlinear least-squares fit of residual HIV proteinase activity *vs.* pre-incubation time to equation (1) provided estimates of kinetic constants for the reaction $E_2 \rightarrow E + E$. The best-fit estimate for the equilibrium dissociation constant K_d is 30.4 ± 1.6 nM, in a close agreement with the value observed by Cheng et al. [10] under comparable experimental conditions. The best-fit estimate for the association rate constant k_1 is 0.47 ± 0.03 $\mu M^{-1}sec^{-1}$. The dissociation rate constant can be calculated as $K_d \times k_1 = 0.014$ sec^{-1}. It is interesting to compare these values with the results of measurements conducted at low ionic strength [1]. An increase in the ionic strength from 0.01 M to 1.00 M causes a 7-fold increase in the association rate constant, but only 2-fold decrease in the dissociation rate constant. This suggests a possible involvement of nonspecific intermolecular interactions ("salting-in" effect) in the assembly of the active HIV dimer.

In this study we have proved that the active dimeric form of HIV proteinase is markedly stabilized upon the binding of a synthetic nonapeptide substrate. Within detection limits, the dissociation of the ternary Michaelis complex into protein subunits could not be observed, while the enzyme dimer in the absence of substrate dissociates readily. This may explain why laboratories that employed different protocols for enzymatic assays also reported very different values for the apparent dimer-to-monomer dissociation constant. When the substrate is the last added component in a kinetic assay, the enzyme will have partially dissociated during the pre-incubation period. On the other hand, when the enzyme is added to a highly saturating substrate solution, little or no dissociation will be observed.

Substrate–induced assembly of the HIV protease dimer may play a regulatory role in the HIV replication cycle. When protein precursors of structural polypeptides are processed, and the mature virion is thus formed, the protease may dissociate into inactive subunits and further proteolysis stops. It is interesting that all retroviral enzymes, the integrase [11], the reverse transcriptase [12], and the proteinase, are dissociative dimers. In quantitative kinetic studies of these important therapeutic targets, substrate–induced stabilization ought to be carefully considered.

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