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KINETIC ASSAY FOR HIV PROTEINASE SUBUNIT DISSOCIATION

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The kinetics and thermodynamics of the monomer—dimer equilibrium for HIV-1 proteinase are investigated in a concentration jump experiment, at a concentration of the substrate that is substantially lower than the Michaelis constant. Under these conditions the substrate-induced stabilization of the active dimer is suppressed, and the integral rate equation can be obtained in a closed form. Both the monomer—dimer bimolecular association rate constant and the corresponding equilibrium dissociation constant are obtained directly by nonlinear regression analysis of the reaction time-course. In buffers of low ionic strength and in the absence of external ligands (substrates and inhibitors), the equilibrium dissociation constant at 37°C is 440 ± 52 nM, a value significantly higher than previous estimates obtained at a comparatively high concentration of substrates.

The proteinase encoded by the human immunodeficiency virus (HIV proteinase) is an obligatory homodimer. A quantitative kinetic and thermodynamic description of the monomer—dimer equilibrium has received considerable effort, but the results obtained thus far are contradictory. Different experimental methods provide widely varying estimates for the monomer—dimer dissociation constant K_d. Holzman et al. [1] studied the dimerization properties of HIV-2 proteinase by analytic ultracentrifugation, and reported K_d between 28 μM and 87 μM. Dissociative properties of the HIV-1 proteinase were studied by indirect kinetic methods, wherein the fractional population of the catalytically active enzyme dimer is estimated from the rate of substrate hydrolysis. By using a variety of experimental conditions, different laboratories reported K_d values ranging between 3.6 nM [2] and 50 nM [3]. From the results of a systematic study of the HIV-1 proteinase's stability and sensitivity to various buffer additives, Jordan et al. [4] concluded that the dissociation constant is much lower that 1 nM, probably in the picomolar range. Grant et al. [5], in a study of the HIV-1 unfolding mechanism, estimated K_d to be less than 100 nM. Babé et al. [6] observed a concentration dependence of specific activity for the HIV-1 proteinase which corresponds to K_d 5 nM.

ABBREVIATIONS: Abz - *ortho*-aminobenzoic acid; CHAPS - 3-[(3-cholamidopropyl)-dimethylamino]-1-propane sulfonate; DMSO - dimethylsulfoxide; DTT - dithiothreitol; EDTA - ethylenediamine tetraacetic acid, disodium salt; Hepes - N-[2-hydroxyethyl] piperazine-N'-[2-ethane sulfonate] ; HIV - human immunodeficiency virus; Phe(NO_2) - *para*-nitrophenylalanine, RSV - Rouss sarcoma virus.

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Some of the differences among conflicting estimates for K_d can be explained by physical effects, such as differences in ionic strength, pH, or the presence of organic co-solvents. In this study, an additional factor is considered that can influence the dissociative properties of HIV proteinase, that is, the binding of substrate. Substrate-induced stabilization of oligomeric enzymes has been observed in many cases (*vide infra*). Thus, in a kinetic experiment designed to monitor subunit dissociation, the apparent dimer dissociation constant might be influenced by the very presence of the monitoring ligand. An important thermodynamic quantity to consider in this case is the overall formation constant for the *termolecular* Michaelis complex. A method is presented herein that can be used to study the kinetics of subunit dissociation by following the time-course of substrate hydrolysis, and that can, at the same time, minimize the substrate-induced perturbation of the monomer—dimer equilibrium. The experimental condition required is that the substrate be present at an initial concentration much lower than the Michaelis constant. The important advantage in adhering to these conditions is that the integral rate equation for substrate hydrolysis can be obtained in a closed form, to give a mathematical model that is easily implemented in a variety of computing environments. Both the bimolecular rate constant for subunit association and the corresponding thermodynamic equilibrium constant are then simply obtained by nonlinear regression analysis of the reaction time course.

In buffers of low ionic strength and at a low concentration of the substrate ($S \leq 0.1\ K_m$), the dimer dissociation constant measured by the new method is 440 ± 52 nM. This value is significantly higher than previous estimates obtained at comparatively high concentration of synthetic substrates, and is closer to Holzman's [1] estimate for HIV-2 proteinase obtained by sedimentation analysis in the absence of ligands.

THE MODEL EQUATION

The mathematical model derived below describes the time course of substrate hydrolysis when, at the outset of the assay, the initial conditions are perturbed by a concentration jump. The enzyme is first equilibrated in a stock solution, at a total (analytic) monomer concentration E_0. From the definition of the equilibrium dissociation constant $K_d \equiv M^2 / D$, where M is the monomer and D the dimer concentration, and from the mass balance equation $E_0 = M + 2D$, the equilibrium concentration of the dimer in the stock solution is $D_0 = \frac{1}{8}[K_d + 4E_0 - \sqrt{(K_d^2 + 8\ K_d E_0)}]$. The quantity D_0 is required for expression of the lower limit in the analytic integration of differential equation (1) which describes the kinetics of subunit dissociation. In eq. (1), k_1 is the bimolecular subunit association rate constant ($M^{-1}sec^{-1}$), and k_2 is the dissociation rate constant (note that $K_d \equiv k_2 / k_1$). The differential equation (1) was solved analytically after separation of variables. At the initial time, an aliquot of the enzyme is diluted from the stock solution (concentration E_0) into the assay buffer (concentration E). At an arbitrary later time t, the concentration of the catalytically active dimer is calculated by using the integral rate equation (2), in which α, α_0, β, β_0, and γ are auxiliary constants.

The differential equation for the hydrolysis of substrate (3) also can be integrated analytically, after separation of variables. The closed-form solution is greatly simplified if we assume that the substrate is initially present at a concentration that is substantially lower (e.g., ten times) than the numerical value of the Michaelis constant. Under these conditions, the

integral rate law is represented by equation (4). Finally, the instrumental response F (fluorescence or absorbance) in a typical continuous concentration jump experiment is described by equation (5), where $\Delta\varepsilon$ is the difference between molar instrumental response coefficients of the substrate and the product, and F_0 is the instrumental signal at initial time.

$$\frac{dD}{dt} = k_1 M^2 - k_2 D \tag{1}$$

$$D = \frac{1}{8}\frac{(\beta-\alpha)\gamma\exp(k_1\alpha t) - (\beta+\alpha)}{\gamma\exp(k_1\alpha t)+1} \tag{2}$$

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

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

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

$$\frac{dS}{dt} = -k_{cat} D \frac{S}{S+K_m} \tag{3}$$

$$S = S_0 \exp\left(-\frac{\beta+\alpha}{8}\frac{k_{cat}}{K_m}t + \frac{1}{4k_1}\frac{k_{cat}}{K_m}\ln\frac{\gamma\exp(\alpha k_1 t)-1}{\gamma-1}\right) \tag{4}$$

$$F = F_0 + \Delta\varepsilon\,(S_0 - S) \tag{5}$$

EXPERIMENTAL

The HIV-1 proteinase 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). When the proteinase was received, the active site concentration was 0.90 moles per mole of dimeric protein, as determined by active site titration with a tight-binding competitive inhibitor ($K_i \approx 0.2$ nM) under experimental conditions that suppress the dissociation of the active dimer (saturating concentration of the substrate, preincubation of the enzyme and the inhibitor prior to assay). The proteinase showed unaltered specific activity for several months. Immediately before enzymatic assays, an aliquot (10 μl) was allowed to thaw at 4 °C, was diluted with 90 μl of the above assay buffer, and subsequently stored on ice. A solution (1.00 mM) of the fluorogenic peptide substrate Abz-Thr-Ile-Nle-Phe(NO_2)-Gln-Arg-NH_2 [7] was prepared in DMSO, and diluted with the assay buffer to a stock solution (100 μM) which was kept on ice. An aliquot of the substrate solution (typically 80 μl, final concentration 4.0 μM) was diluted with the assay buffer to a final volume of 1.99 ml in a 2.5 ml fluorometric cuvette. The concentration of DMSO (a partial denaturant [4]), in a typical enzyme assay was 0.4%. The substrate was allowed to equilibrate at 37°C in a thermostated cell holder for 10 minutes. An aliquot (10.0 μl, final concentration 14.3 nM) of the enzyme stock solution was added, the assay mixture was carefully stirred for 5 sec with a polyurethane stirring rod, and data collection immediately begun. Fluorescence intensity (excitation 337 nm, emission 410 nm) was recorded for 10 minutes on a modified Perkin-Elmer MPF-4 spectrofluorimeter, by using a commercial digital-analog data acquisition system (On Line Instrument Systems Inc., Jefferson, GA) driven by an Intel-80386 based personal computer. The raw data (600 data-points) were transferred to a Macintosh IIfx computer and statistically analyzed as described below.

RESULTS

A typical progress curve from the hydrolysis of the fluorogenic peptide substrate Abz-Thr-Ile-Nle-Phe(NO_2)-Gln-Arg-NH_2 [7] is shown in Figure 1. The data were fitted to equation (5) by using a nonlinear least-squares optimization routine (Marquardt algorithm [8]). The optimal value of the equilibrium dissociation constant K_d and of the second-order association rate constant k_1 resulting from these data were 440 ± 52 nM and 0.066 ± 0.007 $\mu M^{-1}sec^{-1}$, respectively. From these values the dissociation rate constant $k_2 = k_1K_d$ is obtained as 0.029 sec^{-1}, which corresponds to a 24 sec half-time for reestablishing the new monomer—dimer equilibrium upon dilution. The uncertainty of fitting parameters was expressed as asymptotic standard deviations from the diagonal elements of the final curvature matrix, or as confidence intervals at 68% confidence level obtained by F-testing of non-optimal χ^2 [9]. All computations were performed, and the graphics presented in this work was produced, by using a program executable on the Apple Macintosh II family of computers with at least 4 megabytes of random-access memory. The program is available upon written request to the author.

Preliminary results indicate that both K_d and k_1 depend on the ionic strength, while the off-rate constant k_2 is less affected. The equilibrium dissociation constant decreased, and the bimolecular association rate constant increased with an increase in ionic strength. Unfortunately a systematic investigation was hampered by the limited solubility of the fluorogenic substrate in high-salt buffers.

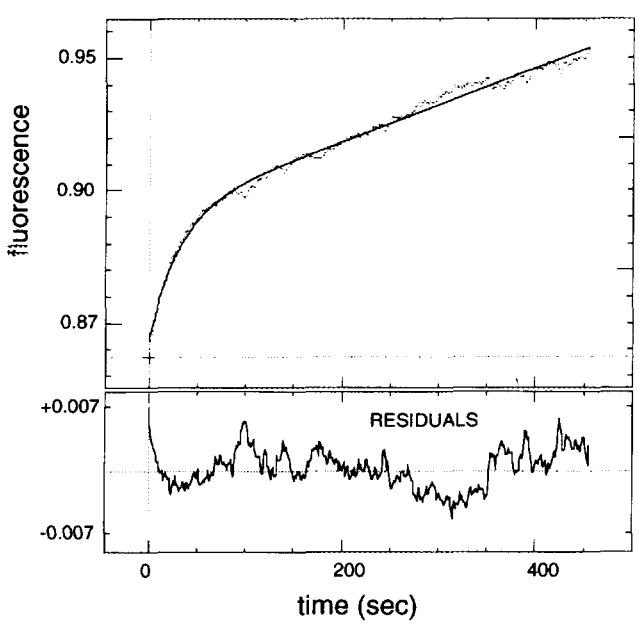

Figure 1. Hydrolysis of Abz-Thr-Ile-Nle-Phe(NO_2)-Gln-Arg-NH_2 [7] (4.0 μM) catalyzed by the HIV-1 proteinase (6.45 nM). In the nonlinear least-squares fit of data to equation (5), the constant parameters were E_0 = 1.29 μM, E = 6.45 nM, S_0 = 4.0 μM, and $\Delta\varepsilon = 1.19 \times 10^6$, while the optimized parameters were F_0 = 0.843 ± 0.001, k_{cat} / K_m = 0.036 ± 0.005 $\mu M^{-1}sec^{-1}$, K_d = 440 ± 52 nM, and k_1 = 0.066 ± 0.007 $\mu M^{-1}sec^{-1}$.

DISCUSSION

The concentration jump experiment was first used on HIV-1 proteinase by Cheng et al. [3], but the concentration of the substrate in this account was rather high (50 μM) compared to the Michaelis constant (110 μM). Approximately one third of the enzyme dimer is present as the Michaelis complex at this concentration. Under these conditions, the substrate-induced stabilization may influence the kinetics of monomer—dimer dissociation. Data analysis employed in this early study was based on an approximate graphical method developed by Seifert et al. [10] for tryptophan synthase. Tangents to the nonlinear progress curves are drawn by hand, and the slopes are analyzed by simple linear regression to obtain k_1 but not K_d. This method of analysis is highly sensitive to the slope obtained at equilibrium, a point which is arbitrarily declared by the investigator. The new kinetic assay represents an improvement over the earlier methods. The substrate concentration (typically 4.0 μM) is kept well below the Michaelis constant (reported value 40 μM [7]), so that the monomer—dimer equilibrium is not affected, and the corresponding mathematical model gives both k_1 and K_d directly from the primary experimental data by nonlinear regression. This method of statistical analysis provides reliable confidence intervals of estimated parameters, and is easily implemented in any computing environment which allows the user to specify the model equation. It is important to note that the bimolecular association rate constant (0.066 μM^{-1} sec^{-1}) agrees with the previous estimate (0.07 $\mu M^{-1}sec^{-1}$), despite the use of a different method and differences in physical conditions of the assay (Cheng et al. [3] used 50 mM Hepes buffer pH 7.0 with 1.0 M sodium chloride).

The stability of many dimeric and tetrameric enzymes is increased upon the binding of substrates. For example, phosphofructokinase is an obligatory tetramer. The bound substrate, fructose 6-phosphate, spans the inter-domain catalytic region, and makes contact with all four enzyme subunits. Deville-Bonne and Else [11] found that the dissociation of phosphofructokinase is markedly suppressed upon substrate binding. Similarly, Kashem and Hammes [12] found that the association of chicken liver fatty acid synthase, an obligatory dimer, is dramatically enhanced by the presence of NADPH and acetoacetyl-coenzyme A. The retroviral integrase from RSV [13] appears to function as an obligatory dimer, as does the HIV proteinase, and both enzymes also form a tetramer. The monomer—dimer dissociation constant of RSV integrase, in the absence of DNA as substrate, is 5.7 μM, and the apparent Michaelis constant for a duplex 15-mer substrate is 7.5 μM. Upon close inspection of the published substrate-saturation data [13], it appears that the initial velocity at 37.5 μM substrate deviates positively from the simple Michaelis-Menten model. This behavior would be expected if the enzyme were kinetically stabilized upon substrate binding. These examples suggest that, due to substrate-induced stabilization of the HIV-1 proteinase dimer, previous reports for K_d (3.5 nM [2], 50 nM [3]) obtained at comparatively high concentrations of substrates may be understimated.

An additional factor that may explain the comparatively high value of K_d (440 nM) is the ionic strength. Bowie and Sauer [14] determined that the stability of the dimeric Arc repressor is greatly increased by an increase in potassium chloride concentration. The ionic strength in the experiments reported here (0.01 M) is substantially lower than in the previous studies of monomer—dimer dissociation (typically 1.00 M). It is however close to the conditions used in the sedimentation study of HIV-2 proteinase (0.03 M), which also gave a

comparatively high estimate for K_d. In enumerating various experimental factors affecting the stability and dissociative properties of HIV-1 proteinase, autolysis does not rationalize the rapid loss (on the timescale of seconds or minutes) of specific activity observed in the concentration jump experiment. Jordan et al. [4] observed that the spontaneous proteolytic deactivation occurs on the time-scale of hours, and only then at comparatively high enzyme concentration, in the micromolar range.

In summary, a method is presented that can be used to measure the monomer—dimer dissociation kinetics for the HIV-1 proteinase, by direct nonlinear regression analysis of the time-course of substrate hydrolysis. The method is based on a simple mathematical model that applies to enzyme assays conducted at substrate concentrations substantially lower than the Michaelis constant. Under these conditions and in a low-salt buffer, the value of K_d is higher than previous reports indicate. Knowledge of the dissociative properties of HIV proteinase is important both in translating the results of *in vitro* inhibition studies into an *in vivo* model of the virion, and in understanding the process of HIV proteinase activation.

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