Dissection of Binding Energy with Native and Ligand-Bound Protein Stabilities: Determining the Affinity of Ultratight-Binding Inhibitors of HIV-1 Protease and Its Drug-Resistance Mutants†

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The accurate determination of equilibrium binding constants is essential in studying all molecular interactions. The use of traditional titration methods, such as Scatchard analysis, requires that the concentration of macromolecule be in the range of the equilibrium dissociation constant. For high-affinity associations, however, the concentration of the macromolecule needs to be so low that the measurement of bound versus unbound form of reactants becomes practically impossible. This limitation imposes an obstacle for designing potent drug molecules for many important biological targets. For example, the K_i values of all clinically approved HIV protease inhibitors are in the picomolar range.[1] When carrying out titration experiments at the lowest feasible protein and inhibitor concentrations, many factors combine to hinder precise measurement. These include low signal-to-noise ratios, slow off-rates that prevent equilibrium from being established during the time of measurement, dissociation of protease dimer into inactive monomer at low protein concentration, and surface absorption of drug molecules. Limitations on the potency and bioavailability of current antivirals used in the treatment of AIDS combined with the rapid emergence of drug-resistant strains of HIV require the development of second-generation inhibitors that ideally are more potent and active against drug-resistant viral strains. The accurate determination of binding affinities for these inhibitors becomes an important issue for optimizing drug potency, and for establishing accurate biochemical resistance-profiles.

An alternative method for determining ligand-binding affinities is based on the folding and binding linked equilibrium (FABLE).[2] This concept is based on the phenomenon that when a macromolecule is in equilibrium between its folded and unfolded state, the addition of a ligand that preferentially binds to the folded state will stabilize the folded structure. FABLE has been pioneered to quantitatively determine binding constants using differential scanning calorimetry (DSC).[3] A disadvantage of the DSC method is that the binding parameters are determined at temperatures where the proteins are thermally denatured. These temperatures are usually much higher than either physiological temperatures or room temperature where most biological assays are performed. HIV-1 protease, for example, exhibits a T_m of 59 °C at pH 3.4.[4]

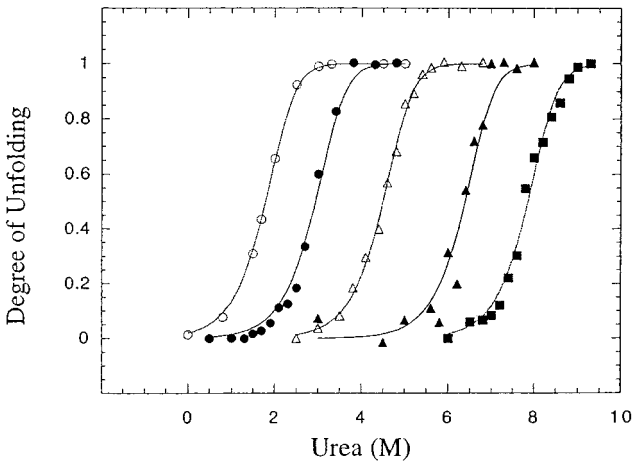

Figure 1. Urea denaturation of wild-type HIV-1 protease.[8] The data for different inhibitors are represented as follows: open circles, uninhibited enzyme; closed circles, pepstatin A; open triangles, RS500B (Randad et al., unpublished results); closed triangles, ritonavir; and closed squares, SBP99. The solid lines represent the best fit using the two-state model. For the uninhibited enzyme, the *m* value was determined to be 2400 kcal mol^{-1} $[urea]^{-1}$. This value was kept constant for all fittings. The unfolding free energy for enzyme/inhibitor complex and K_d for inhibitor binding determined from the two-state analysis are listed in Table 1.

Extrapolation of folding thermodynamic parameters over a wide temperature range is prone to large errors because the temperature dependency of ΔC_p is often difficult to measure. Another disadvantage of using DSC to monitor unfolding is that the thermal denaturation of most proteins is not reversible, whereas equilibrium unfolding is required by the FABLE analysis.

In this work, based on a urea-denaturation assay,[5] we utilized the FABLE method and determined K_d values down to 0.4 fM for HIV-1 protease (HIV PR) inhibitors, thus extending by at least 3 orders of magnitude the limit to which accurate K_d values can be measured using conventional enzyme assays. It was shown earlier that at increasing urea concentrations, wild-type HIV-1 protease (WT) underwent reversible denaturation concomitant with a decrease of its intrinsic fluorescence intensity.[6] In the present study, the stability of WT enzyme was determined in the absence and presence of four different inhibitors, pepstatin A, RS500B, ritonavir, and SBP99 (Figure 1).[7] The stability of uninhibited WT enzyme was determined at three different protease concentrations (data not shown). A global analysis using the two-state model yielded a ΔG of 12.6 ± 0.2 kcal/mol at pH 4.5 and 25 °C, or a dimer dissociation constant of 0.65 nM, in agreement with earlier reports.[4,6] The effect of inhibitor binding on protease stability can be represented as follows:

$$\Delta G' = \Delta G + RT \ln(1 + I/K_d) \qquad (1)$$

where $\Delta G'$ and ΔG are the free energy changes for unfolding of inhibitor-bound and uninhibited enzymes, R is the gas constant, K_d is the equilibrium dissociation constant for inhibitor, and I is the unbound inhibitor concentration. Under our experimental conditions, the inhibitor concentration (15 μM in all cases) was in molar excess to the protein dimer concentration (0.45 μM).

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(7) Wild-type HIV-1 protease and its V82F/I84V mutant were cloned, expressed, and purified as previously described.[1] Pepstatin A was purchased from Sigma (St. Louis, MO). RS500B and SBP99 were synthesized in our laboratory. Ritonavir was kindly donated by Abbott Laboratories.

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Table 1. Dimer Stability and Affinities to Protease Inhibitors of Wild-type HIV-1 Protease and Its V82F/I84V Mutant

inhibitor	$\Delta G'$ (kcal/mol)	K_d	K_i	K_d (ITC)
WT	12.59 ± 0.02			
WT/Pepstatin A	15.35 ± 0.07	0.15 ± 0.02 μM	0.12 μM	0.19 μM
WT/RS500B	19.09 ± 0.03	0.28 ± 0.01 nM	25.6 nM	
WT/Ritonavir	23.6 ± 0.1	0.14 ± 0.02 pM		
WT/SBP99	27.15 ± 0.06	0.38 ± 0.04 fM		
V82F/I84V (MUT)	13.7 ± 0.1			
MUT/Ritonavir	18.0 ± 0.1	11.0 ± 1.8 nM		
MUT/SBP99	27.0 ± 0.1	3.0 ± 0.5 fM		

Hence, I can be approximated using the total inhibitor concentration. Since equal inhibitor concentrations were used, differences in urea stability of the complexes reflected different binding affinities (Figure 1). Using eq 1, K_d values were determined from the differential stability of WT in the presence and absence of an inhibitor represented by $(\Delta G' - \Delta G)$ (Table 1). The binding energetics of pepstatin A was verified by two other independent techniques. Measurement using isothermal titration calorimetry yielded a ΔH of 4.8 kcal/mol and a K_d of 0.19 μM at 25 °C, consistent with previous results.[9] A K_i value of 0.12 μM was determined by enzyme kinetic assay using a fluorogenic substrate as described.[10] These results are in good agreement with the value of 0.15 μM measured by the FABLE assay. Previously we reported a K_i value of 8.1 pM for ritonavir.[1] A direct comparison with the K_d value obtained in this study is not possible since the pH and salt concentrations are significantly different. For the RS500B inhibitor, however, the K_i value is 2 orders of magnitude higher than the K_d determined by the FABLE assay. The discrepancy was possibly the result of the linkage of monomer−dimer equilibrium and inhibitor binding in the kinetic assay. To determine a K_d of 0.1 nM, a dimer concentration of 2.0 nM was used in the kinetic measurement. Since the dimer dissociation constant is 0.65 nM under these conditions, a significant population of protease was in the monomeric form before binding with inhibitor. As we reported earlier,[11] the linkage of dimerization upon inhibitor binding would result in a decrease of the apparent inhibitor affinity. SBP99 is a second-generation protease inhibitor being developed in our laboratory. Its affinity to WT, 0.38 fM, is well beyond the measurement range of enzymatic assays, and explains its high antiviral activity relative to other potent protease inhibitors, such as ritonavir.[12]

The V82F/I84V mutation (MUT) in HIV protease is commonly observed in drug resistant strains of HIV. This double mutant showed high resistance against all clinically approved protease inhibitors.[13] In comparison with WT, the stability of the mutant enzyme was determined in its free form and in complex with ritonavir and SBP99 (Figure 2). Interestingly, the uninhibited mutant showed higher dimer stability than WT by 1.1 kcal/mol.

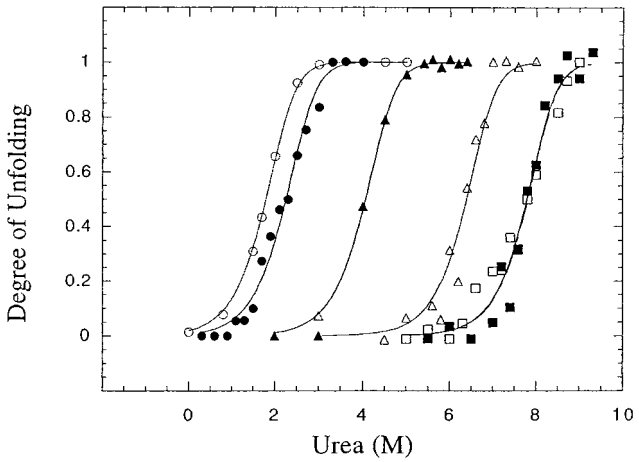

Figure 2. The stability of wild-type HIV-1 protease and its V82F/I84V mutant in complex with two protease inhibitors. The experiments were carried out under the same conditions as those in Figure 1. Open circles, open triangles, and open squares represent the data for uninhibited wild-type protease, wild-type/ritonavir, and wild-type/SBP99, respectively. The data for uninhibited mutant protease, mutant/ritonavir, and mutant/SBP99 are represented by the corresponding closed symbols, respectively. The solid lines represent the best fit using the two-state model. The determined K_d for SBP99 was 0.38 and 3.0 fM against the wild-type protein and the V82F/I84V mutant, while K_d for ritonavir was 0.14 pM and 11.0 nM, respectively.

The greater stability of MUT results in a nonspecific decrease of affinity for all protease inhibitors relative to WT. Increased dimer stability at acidic pH was also observed for the I84V mutant of HIV-1 protease.[14] In contrast to the unliganded enzymes, the WT/ritonavir complex is 5.6 kcal/mol more stable than the MUT/ritonavir complex (Table 1). Most of the 78571-fold increase in K_d, when ritonavir is bound to the V82F/I84V mutant relative to WT, can be accounted for by the significant destabilization of the mutant/inhibitor complex. On the contrary, complexes of SBP99 with both WT and the double mutant exhibited similar stabilities. The slight decrease in affinity of SBP99 to the mutant can be attributed entirely to the mutant's higher dimer stability.

HIV protease undergoes conformational changes upon binding to inhibitors.[15] The higher dimer stability of the I84V and V82F/I84V mutants relative to WT indicates that the mutants are more resistant to structural changes required for inhibitor binding. This provides a nonspecific obstacle for binding of inhibitors to the mutant enzyme, and suggests that the design of resistance repellent inhibitors ($K_{i,MUT}/K_{i,WT} < 1$) will be difficult, if not impossible in practice for these mutants.

An advantage of FABLE analysis is that binding free energy change is dissected into stabilities of the free and complexed forms of the enzyme (or receptor). As our results showed, this allows partitioning of the effects of mutations on native protein structure and on protein−ligand interactions. This approach is helpful in understanding the drug-resistance mechanism caused by mutations. In all, FABLE is a general assay method for the direct and quantitative measurement of equilibrium binding constants over a wide dynamic range. Lower values of K_d could be measured by using stronger denaturants. It is particularly well-suited for use with tight-binding ligands and does not require a priori design of specific biological assays.

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(8) The degree of unfolding was calculated by measuring the intrinsic emission fluorescence at 347 nm with an excitation wavelength of 280 nm. The excitation and emission slit width were 4 and 8 nm, respectively. The buffer was 20 mM sodium acetate, pH 4.5, 2 mM DTT. All experiments were performed at 25 °C. As described,[5] the unfolding free energy was obtained using the equation $\Delta G = \Delta G_o - m$[urea], where ΔG_o is the dimer stability in the absence of urea. Since the inhibitor is much smaller than the enzyme, all denaturation data were analyzed with a constant m value. This value was determined from the denaturation curve of uninhibited WT enzyme.

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