Determination of the Rate of Monomer Interchange in a Ligand-Bound Homodimeric Protein from NOESY Cross Peaks: Application to the HIV Protease/KNI-529 Complex

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The HIV protease is a viral enzyme whose function is essential in the life cycle of the AIDS virus.[1,2] As such, it is one of the prime targets of antiviral agents directed against AIDS.[3] The many inhibitors of the HIV protease that have been developed fall into two general classes, (1) 2-fold symmetric inhibitors and (2) asymmetric inhibitors. Crystal structures of the protease bound to symmetric inhibitors show that each monomer of the protease homodimer interacts in nearly identical fashion with each symmetry-related component of the inhibitor.[4,5] In crystal structures, a local asymmetric conformation is often observed at the tips of the protease flaps. However, when the protease is bound to a symmetric inhibitor in solution, a local conformational exchange of the tips of the flaps results in an average symmetric protease conformation, and the chemical shifts of the two monomers are identical.[6,7] In contrast, when the protease is bound to an asymmetric inhibitor, crystal structures show that each protease monomer interacts in a distinctly different way as seen, for example, in the case of the potent inhibitors KNI-529 and KNI-272.[8] Furthermore, the chemical shifts of the protease monomers are different when the protease is bound to either of these asymmetric inhibitors.[9,10] This latter point is illustrated in Figure 1, which shows strips obtained from a 3D NOESY $^{1}H-^{15}N$ HSQC spectrum of the protease bound to the asymmetric inhibitor KNI-529, for residues A28/128 and T31/131. Figure 1 reveals (1) that residues at the equivalent positions in the amino acid sequence (numbered i and $i + 100$) of each monomer have different amide chemical shifts and (2) that there are cross-peaks between these amides. Such cross-peaks cannot arise from NOEs (dipolar interactions) between the amide protons because the X-ray structure of the protease/KNI-529 complex shows that the amide proton internuclear distances are 7.8 and 22.7 Å in the cases of A28/128 and T31/131, respectively. The most plausible interpretation of these observations is that the cross-peaks arise from conformational exchange[11−13] of the two monomers, as a consequence of a reversal in the orientation of the bound inhibitor. To verify this hypothesis, cross-peak and diagonal peak intensities were measured for residues L24/124, A28/128, D30/130, G48/148, I54/154, T31/131, and V82/182 at temperatures varying from 25 to 45 °C. These residues were chosen for analysis because they have well resolved signals and because the internuclear distance of each pair of amide protons is greater than 7.8 Å in the X-ray structure. The ratio of the intensities of the diagonal and cross-peaks is given by

$$I_c/(I_d + I_N/3) = \{1 - \exp(-2kt)\}/\{(1 + \exp(-2kt)\} \quad (1)$$

where k is the rate of monomer interchange, I_c and I_N are the

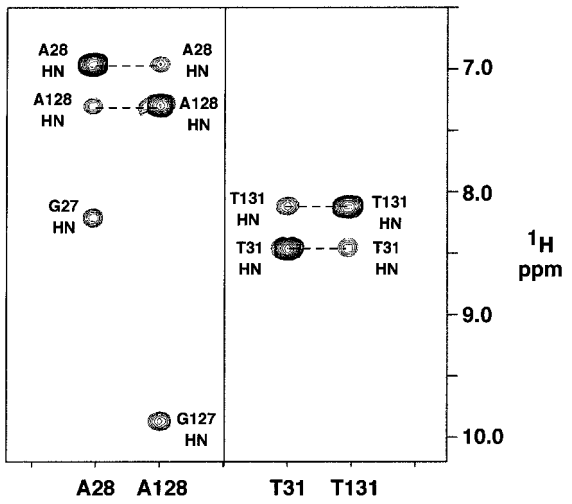

Figure 1. A composite of strips taken from a 3D ^{15}N-separated 750 MHz NOESY spectrum of the HIV protease/KNI-529 complex at the chemical shifts of A28, A128, T31, and T131. The spectrum was taken on a solution containing 50 mM NaAc, 0.6 mM protein dimer at pH 5.2 and 34 °C, with a mixing time of 250 ms. The $^{2}H/^{15}N$-labeled protease sample was prepared as described previously.[15] Note that monomer interchange is slow on the chemical shift time scale, enabling signals of residues i and $i + 100$ to be distinguished, but is sufficiently rapid to yield exchange cross-peaks between these residues. Sequential NH−NH NOE-type cross-peaks are observed between the amides of G27−A28 and G127−A128, because the internuclear distances between these pairs of amides are less than 2.8 Å. In contrast, the internuclear distances between the amide protons of A28/A128 and T31/T131 are 7.8 and 22.7 Å, respectively.

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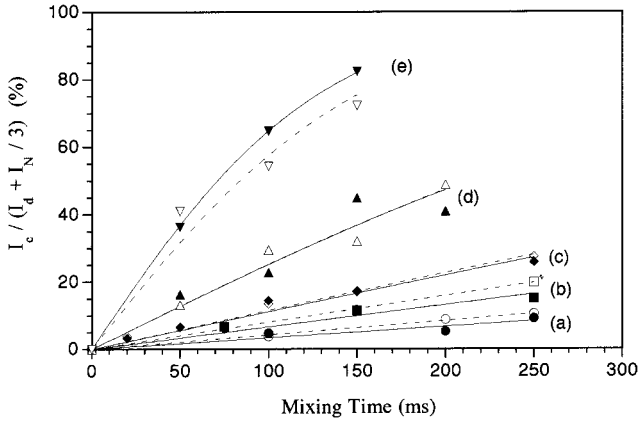
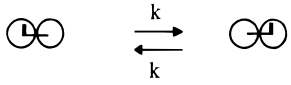
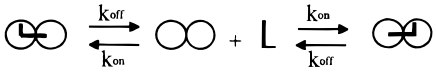


Figure 2. The ratio $I_c/(I_d+I_N/3)$ derived from NOESY spectra of the HIV protease/KNI-529 complex for residues T31 (filled symbol) and T131 (open symbol), plotted as a function of mixing time and temperature: (a) 25 °C; (b) 31 °C; (c) 34 °C; (d) 40 °C; (e) 45 °C. The curves are the best fits to the experimental data using eq 1 as discussed in the text. We note that the correction to I_d is $I_N/2$ when I_c/I_d and I_N/I_d are $\ll 1$. However, numerical simulations show that in this case the use of $I_N/3$ introduces negligible error because the correction itself is very small. Furthermore, $I_N/3$ is a significantly better correction when the cross-peak intensities are comparable to that of the diagonal.

Scheme 1



Scheme 2



intensities of the exchange and NOE cross-peaks respectively, and I_d is the intensity of the diagonal peak. Equation 1 differs from the exact equation for two-site exchange[11] by inclusion of the term $I_N/3$ to correct for the depletion of the diagonal peak by the NOE cross-peaks. Typically this was a small correction because our protein is perdeuterated and there are few NOE-type cross-peaks. Numerical simulations show that accurate values of k (errors <5%) are be obtained using eq 1 provided that (a) $I_N < I_d/2$ and (b) $I_c/I_d < 0.9$. Hence, only data that satisfied conditions (a) and (b) were fit using eq 1. The fits obtained for residues T31/131 are shown in Figure 2. When the data for all seven residues were compared, it was found that at each temperature, approximately the same value of k was obtained. This shows that the cross-peaks result from a global process, which affects the exchange of each pair of residues in the same manner. In addition, the exchange rate was found to be strongly dependent upon temperature, increasing from ca. 0.2 to 6 s^{-1} as the temperature increased from 25 to 45 °C.

Representing each protease monomer as an oval and the inhibitor by the symbol L, we consider two simple kinetic schemes for the monomer exchange reaction. In Scheme 1, we assume that the inhibitor flips without dissociating from the complex and find that a best fit of k vs $1/T$ using the equation $k = C \exp(-\Delta H^{\ddagger}/RT)$ yields an activation enthalpy of 32 kcal M^{-1}. Alternatively, in Scheme 2, we assume that the inhibitor must dissociate from the complex in order to flip. It has been shown that $K_i \approx 10^{-9}$ M for KNI-529[8] (note that in ref 8, KNI-529 is called epi-KNI-272). Assuming that $K_d = K_i$, one finds that $k_{on} \approx 10^{+9}\, k_{off}$. Applying this result and the steady-state approximation (d[OO]/dt = 0) in the kinetics equations for Scheme 2, one finds that $k = k_{off}/2$ (because there is a 50% chance that the inhibitor rebinds in its original orientation) and $k_{on} = k_{off}/2K_d$. Our measurements show that $k_{off} = 0.4\ s^{-1}$ at 25 °C, which implies that $k_{on} \approx 2 \times 10^8\ M^{-1}\ s^{-1}$, indicating that k_{on} is diffusion-controlled.[14] In a diffusion-controlled reaction $k_{on} \propto D_I$, where D_I is the diffusion coefficient of the inhibitor.[14] The equations $K_d \propto \exp(\Delta H/RT)$ and $k_{off}/k_{on} \propto K_d$ then imply that $k/D_T = A \exp(\Delta H/RT)$, where A is a constant and D_T is the ratio of the diffusion constants at temperatures T and 298 K. Using the Stokes/Einstein relationship, $D_I \propto T/\eta$, one finds that $D_T = (T/298)(\eta_{298}/\eta_j)$, where η_j is the viscosity of water at temperature j. The slope of plot of $\ln(k/D_T)$ vs inverse temperature, Figure 3, yields a favorable binding enthalpy of −28 kcal M^{-1}. This result is inconsistent with recent data[16] on the energies of the binding of acetyl pepstatin to the protease. Therefore it appears that KNI-529 flips without dissociating from the complex (Scheme 1). It is hoped that these results will simulate realistic calculations of the monomer interchange process, as such work should increase our understanding of the interactions between the protease and potent inhibitors.

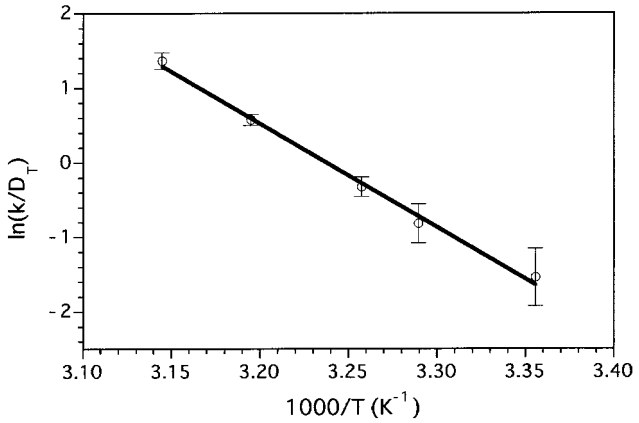


Figure 3. The plot of $\ln(k/D_T)$ in the HIV protease/KNI-529 complex as a function of inverse temperature; from the slope it is found that $\Delta H = -28$ kcal M^{-1}. The data points and their errors were derived from the averages and standard deviations of the rates obtained for all residues at a given temperature.

We note that neither structural information nor sequential assignments are needed to distinguish dipolar from exchange cross-peaks. The two types of cross-peaks can be identified (1) by the opposite temperature dependence of their NOESY build-up rates and (2) by the opposite signs of their ROESY signals. Hence, our approach can be used in an efficient manner to measure monomer exchange rates of a homodimer bound to a series of inhibitors.

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