

Flexibility and function in HIV-1 protease

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HIV protease is a homodimeric protein whose activity is essential to viral function. We have investigated the molecular dynamics of the HIV protease, thought to be important for proteinase function, bound to high affinity inhibitors using NMR techniques. Analysis of ¹⁵N spin relaxation parameters, of all but 13 backbone amide sites, reveals the presence of significant internal motions of the protein backbone. In particular, the flaps that cover the proteins active site of the protein have terminal loops that undergo large amplitude motions on the ps to ns time scale, while the tips of the flaps undergo a conformational exchange on the μ s time scale. This enforces the idea that the flaps of the proteinase are flexible structures that facilitate function by permitting substrate access to and product release from the active site of the enzyme.

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The aspartyl proteinase of the HIV-1 virus is a homodimeric enzyme that cleaves the polypeptide products of the *gag* and *pol* viral genes, yielding structural proteins and enzymes that are essential to the life cycle of the virus. Inhibition of this proteinase leads to the production of non-infectious viral particles^{1,2}. This enzyme has thus become a primary target for drug design based on numerous X-ray structures of the free proteinase and the proteinase complexed with a variety of inhibitors³. Structural differences between the viral and cellular proteinases have been exploited to obtain inhibitors specific for the viral enzyme. One significant structural difference between the viral/cellular proteinases is the presence/absence of a structural water coordinated to the tips of the proteinase's flaps and the inhibitor. The cyclic diol inhibitor, DMP323 (Fig. 1a)⁴ contains a strategically-placed urea oxygen that mimics the oxygen of the key structural water, thereby excluding the water molecule^{4,5} and imparting specificity for the viral proteinase. The linear diol inhibitor, P9941 (Fig. 1b)⁶ is a less specific inhibitor, because it lacks a urea oxygen, and the structural water is retained in the proteinase/P9941 complex⁵. The higher affinity of the DMP323 inhibitor is ascribed to its design, which preorganizes proteinase-binding substituents on a rigid, seven membered ring.

Structural information obtained from X-ray diffraction studies³ and molecular dynamics simulations^{7,8} have indicated that proteinase flexibility plays a significant role in function. Heteronuclear ¹H-¹⁵N NMR is a powerful approach for elucidating the motions that occur within the protein backbone, and provides information about the amplitudes and rates of motions of individual bonds.

Motions over a wide range of time scales, ranging from ms to ps, can be probed. We have employed ¹⁵N NMR relaxation techniques to investigate the flexibility of the proteinase bound to the DMP323 and P9941 inhibitors, in order to gain information about proteinase function and inhibitor binding.

Spin relaxation and molecular motion

Nuclear spin relaxation and molecular motion are closely related⁹. This is because molecular reorientation produces fluctuating magnetic fields that cause transitions between nuclear spin states. In proteins, reorientation of the NH bond axis is the motion responsible for relaxation of the amide ¹⁵N spin. This motion is characterized by the spectral density function, $J(\omega)$. Physically, $J(\omega)$ is proportional to the amplitude of the fluctuating magnetic field at the frequency ω , and is directly related to three ¹⁵N spin relaxation parameters^{9,10}: the ¹⁵N longitudinal relaxation time, T_1 ; the transverse relaxation time, T_2 ; and the nuclear Overhauser enhancement, NOE. These parameters can be measured for nearly every amide site in isotopically labelled proteins using sensitive ¹H detected two-dimensional experiments¹⁰⁻¹⁵.

In order to obtain useful information about protein dynamics from relaxation data one must express the spectral density function in terms of quantities that are related to the amplitudes and correlation times (both overall and internal) of molecular motions. In early studies of protein dynamics, the spectral density function was generated using a specific model for protein internal motion, and the suitability of a particular model was judged by comparing measured relaxation parameters

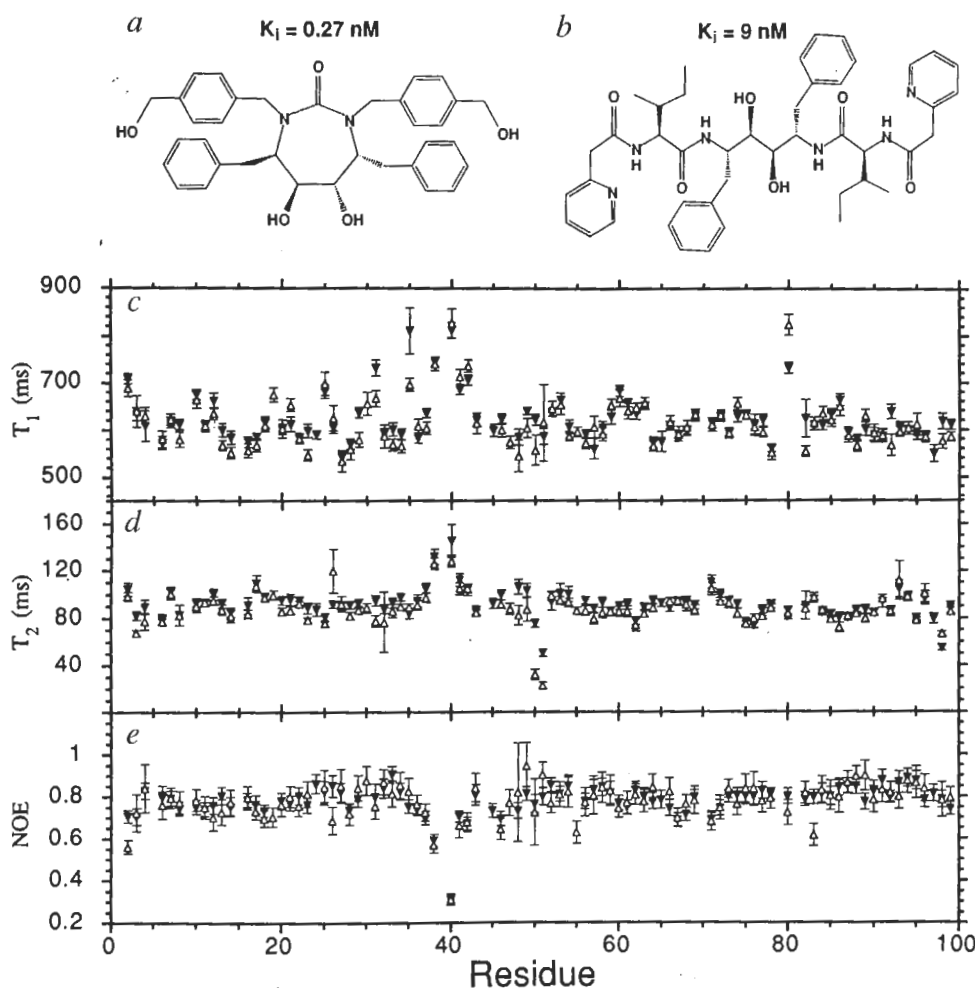


Fig. 1 Inhibitors *a*, DMP323 and *b*, P9941, their respective inhibition constants, K_i , and experimental relaxation parameters *c*, T_1 ; *d*, T_2 ; and *e*, NOE values of HIV-1 protease/inhibitor complexes, DMP323 (▼) and P9941 (Δ).

with those calculated using the model. More recently a model-free formalism^{16,17} has been developed and is widely used^{10,18–24} to extract information about molecular motion from the spectral density function. According to this formalism, when overall motion is isotropic and not correlated with internal motions,

$$J(\omega) = S^2\tau_m / (1 + \omega^2\tau_m^2) + (1 - S^2)\tau_c / (1 + \omega^2\tau_c^2) \quad (1)$$

where τ_m is the overall correlation time that characterizes the rigid body rotation of the entire protein, $1/\tau = 1/\tau_m + 1/\tau_c$, τ_c is an effective correlation time characterizing the rapid internal motions (that is, those for which $\tau_c \ll \tau_m$) of the NH bond, and S is the generalized order parameter. This expression is exact when $(\omega\tau_c)^2 \ll 1$, which corresponds to $\tau_c < 100$ ps at 500 MHz proton Larmor frequency. The order parameter provides information about the amplitude of the internal motion of the NH bond at each site in the protein, provided that the internal motion is faster than the overall reorientation of the protein, that is $\tau_c \ll \tau_m$. Although the order parameter is a model independent quantity, it can be

readily calculated for any model of NH bond reorientation^{16,17}. For example, if the NH bond diffuses in a cone of semi angle θ , S^2 is given by:

$$S^2 = \cos^2\theta^2 (1 + \cos\theta)^2/4 \quad (2)$$

This equation illustrates that $S^2=1$ in the absence of motion ($\theta=0^\circ$) and $S=0$ when motion is isotropic ($\theta=180^\circ$), results which apply for all types of internal motion. Finally, we note that the equation for $J(\omega)$ has been extended¹⁹ to the case where the internal motion takes place on two fast, but significantly different time scales (that is characterized by correlation times τ_1 and τ_2 with $\tau_1 \ll \tau_2 < \tau_m$).

Chemical exchange

While all three NMR parameters are sensitive to rapid internal motions (on the ps to ns time scale), T_2 is also affected by chemical exchange, on the ms to μ s time scale, provided that the chemical exchange affects the ^{15}N chemical shift. In the simplest case, an exchange process involving two states that have equal populations in fast exchange ($\tau_{ex} < T_{CP}/5$, $(\Delta\omega\tau_{ex})^2 \ll 1$), the exchange contribution, R_{ex} , to $1/T_2$ is given by²⁵:

$$R_{ex} = (\Delta\omega/2)^2 \tau_{ex} ((1 - (2T_{CP}/T_{CP})) \quad (3)$$

where τ_{ex} is $2/k$, k is the rate constant for the exchange, $\Delta\omega$ is the difference in ^{15}N chemical shifts in the two states (in rad s^{-1}), and T_{CP} is the interval between 180° pulses used in the T_2 pulse sequence¹², ~ 1 ms in the measurements reported here. Because R_{ex} is proportional to $(\Delta\omega)^2$, R_{ex} increases as the square of the external magnetic field, and T_2 measurements at two fields can be used to confirm the presence of conformational exchange. However, such measurements do not yield a value for τ_{ex} unless $\Delta\omega$ is known or assumed. It is sometimes possible to determine $\Delta\omega$ and τ_{ex} from measurements of $1/T_{1\rho}$, the relaxation rate of spin locked ^{15}N transverse magnetization. For two equally populated sites, the exchange contribution to $1/T_{1\rho}$ is given by^{25,26}

$$R_{ex} = (\Delta\omega/2)^2 \tau_{ex} / (1 + \omega_1^2 \tau_{ex}^2) \quad (4)$$

where ω_1 is the spin lock field strength. Provided that $\omega_1 \tau_{ex} \sim 0.5$, measurements of R_{ex} as a function of ω_1 yield both τ_{ex} and $\Delta\omega$.

It is perhaps worth noting that, because $\Delta\omega$ is typically $\leq 2 \times 10^3$, R_{ex} is insignificant unless τ_{ex} is $> 10^{-6}$. Even when we speak of the fast limit when discussing chemical exchange, therefore, the fast chemical exchange

time scale is several orders of magnitude slower than the time scale of internal motions (τ_c is in the range ns to ps) that affect the order parameter. In other words, the order parameter is only sensitive to motions on a time scale that is much shorter than the overall correlation time of the protein ($\tau_c \ll \tau_m$). It is not sensitive to motions on the chemical exchange time scale because $\tau_{ex} \gg \tau_m$.

Measurements and analyses

The HIV-1 protease contains two identical monomers (1-99/1'-99'). For both complexes residues X and X' have equivalent chemical shifts, demonstrating that the average dimer conformation is symmetric when bound to each symmetric inhibitor. All backbone amide signals have been assigned^{5,27} except for Leu 5, and relaxation parameters were obtained for 86 assigned ¹⁵N sites in each complex. Data were not obtained for the six proline residues, for six residues in each complex whose signals were poorly resolved, and Leu 5. The measurement and model-free analysis^{13,16,17,19,24} of ¹⁵N relaxation parameters, T_1 , T_2 and NOE (Fig. 1) yielded the overall correlation time, τ_m , as well as the residue specific order parameters, S^2 , internal effective correlation times, τ_c , and rates due to chemical exchange, R_{ex} , for each complex (Fig. 2). As expected on the basis of their nearly identical molecular weights, the overall correlation times for the two complexes were found to be essentially the same, 9.2 ns (DMP323) and 9.3 ns (P9941). These correlation times are in accord with overall correlation times reported^{14,21,24,28,29} for proteins having a variety of molecular weights.

Even though the K_i values of the two inhibitors differ by 30-fold, the two complexes have nearly identical average order parameters ($\langle S^2 \rangle = 0.85$, DMP323; $\langle S^2 \rangle = 0.87$, P9941). In addition the values of S^2 for individual residues in the DMP323 and P9941 complexes are closely correlated (Fig. 2a). Omitting Gly 48, the difference between S^2 values for the two complexes has a root-mean-square value of 0.042, in accord with the average standard errors in S^2 : 0.031 for each complex. Structural studies have shown that Gly 48 is hydrogen-bonded to the P3 carbonyl group of linear inhibitors closely analogous to P9941 (ref. 3). DMP323 does not contain a P3 site⁴, and the Gly 48 NH does not form a hydrogen-bond in the crystal structure of the proteinase/DMP323 complex. The absence of a Gly 48 hydrogen-bond acceptor in the DMP323 inhibitor is consistent with the significantly smaller value of S^2 observed for Gly 48 in the DMP323 complex.

These observations indicate that, with the exception of Gly 48, the flexibility of the proteinase backbone is essentially the same in the two complexes. One possible interpretation of this result is that the NMR order parameters are not sensitive to the differences in interactions between the proteinase and the two different ligands. A more intriguing interpretation is that, in the bound state, the proteinase interacts equally well with each of the two ligands, in spite of their differences in K_i and structure.

Large amplitudes fast motions

Internal motions on the ns-ps time scale result in values of S^2 that are less than unity. While most residues in both complexes have $S^2 > 0.8$ (Fig. 2a), indicating a well ordered backbone structure and restricted internal motion, twelve residues in the DMP323 complex (Fig. 3a) and nine residues in the P9941 complex have S^2 values of 0.5-0.76. Order parameters in this range correspond to NH bond reorientation of 40°-70° (using the cone model^{16,17} to interpret S^2). Most residues executing large amplitude motions are in loops of the proteinase (Fig. 3a). Residues Leu 38, Gly 40, Arg 41 and Trp 42 (residue 39 is a proline) form the largest cluster of residues in this class and are located in the loops (Fig. 3a) at the end of the flaps that fold over the active site. These four residues also have $S^2 < 0.76$ in the P9941 complex, and have elevated B-factors in the proteinase/DMP323 crystal structure. Crystallographic studies have indicated that flexibility of the flaps allows substrates and inhibitors access to the active site of the proteinase³. Furthermore, proteinase activity is diminished by non conservative, amino-acid substitutions at Leu 38

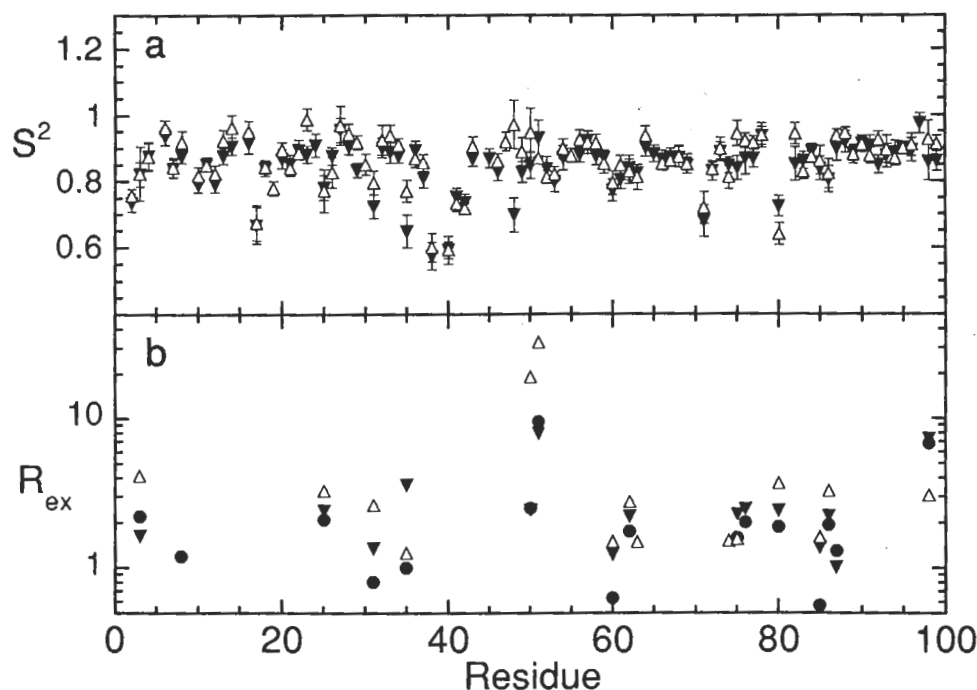


Fig. 2 a, Generalized order parameters (S^2) and b, the chemical exchange contribution to $1/T_2$ (R_{ex}), plotted as a function of residue number for the two complexes (▼) DMP323 using three relaxation parameters, T_1 , T_2 at 500 MHz and NOE at 600 MHz; (●) DMP323 using four relaxation parameters, T_1 , T_2 at 500 MHz and T_2 , NOE at 600 MHz; and (Δ) P9941 using three relaxation parameters, T_1 , T_2 at 500 MHz and NOE at 600 MHz.

and Gly 40, indicating that conformational characteristics of the loop are important for function³⁰. Taken together, these results suggest that loop flexibility facilitates substrate binding and product release.

Sites affected by chemical exchange

Our analysis of the relaxation data showed that fifteen residues in the proteinase/DMP323 complex (Fig. 3*b*) were affected by chemical exchange on the ms- μ s time scale (Figs 2*b*, 3*b*). Virtually the same residues experience exchange in each of the complexes studied (Fig. 3*b*). Most of these are either involved in inter-monomer contacts or interact with the inhibitor, although a cluster extends across the β -sheet core (residues Asp 60, Ile 62, Val 75 and Leu 76). Based on NMR and X-ray data, it has been proposed³¹ that catalytic Asp 25/25' side chains and the diol groups of DMP323 form two hydrogen-bonding networks in dynamic equilibrium. Exchange between these two networks may be responsible for the exchange broadening observed for the amides of Asp 25 and of neighbouring residues Arg 8, Ile 85, Gly 86 and Arg 87 (Fig 3*b*).

Residues at the tips of the flaps (Ile 50 and Gly 51) and in the amino and carboxy termini (Val 3 and Asn 98) are involved in inter-monomer interactions. These residues experience chemical exchange which can be attributed to conformational changes that appear to be related to function. In nearly all proteinase crystal structures, asymmetric β I/ β II hydrogen-bonded turn conformations (Fig. 4) are observed at the tips of the flaps³, however, in the crystal structure of the proteinase/DMP323 complex, symmetric β II/ β II non-hydrogen-bonded turn conformations (Fig. 4) are observed. Analysis of a 3D ¹⁵N separated NOESY spectrum of the proteinase/DMP323 complex indicates that, in solution, the conformation of the tips of the flaps is not simply a β II

turn, but is a mixture of β I and β II turns. Although the asymmetric hydrogen-bonded conformations are indicated by most of the structural data and are consistent with the NMR relaxation and NOESY data, β II/ β II symmetric conformation in solution is not excluded. The structural data, taken together with the analysis of the Ile 50/Gly 51 relaxation data, suggest a dynamic model in which the three flap conformations depicted in Fig. 4 undergo exchange.

It should be noted that the conformational transition depicted in Fig. 4 is highly localized, and can be thought of as an approximate 180° flip of the Ile 50/Gly 51 peptide bond. This results in a large change in orientation of the Gly 51 NH bond, which explains the large value of R_{ex} observed for Gly 51 in both the DMP323 and the P9941 complex. In contrast with the orientation of the NH bond of Gly 51, the orientation of the NH bond of Ile 50 is only slightly changed by the proposed flap conformational change. This is a consequence of the position of Ile 50 (residue 2) and Gly 51 (residue 3) in the β -turn and is consistent with X-ray structural data which show that the Ile 50 NH donates a hydrogen bond to an internal water molecule³ or to the cyclic urea oxygen⁴, regardless of whether a β I or β II turn is present. Hydrogen-deuterium exchange experiments show protection factors³² of at least 100-fold for the Ile 50 NH in both the DMP323 and P9941 complexes, indicating that the Ile 50 NH forms hydrogen bonds in solution. We therefore conclude that the Ile 50 NH bond is not highly flexible, and that the exchange broadening experienced by the Ile 50 amide proton is due to fluctuations in its magnetic environment caused primarily by the large conformational change of neighbouring atoms in the Ile 50/Gly 51 peptide moiety.

An estimate of the correlation time, τ_{ex} , of the flap conformational change was obtained in the following manner. The $T_{1\rho}$ values of the Ile 50 and Gly 51 ¹⁵N spins

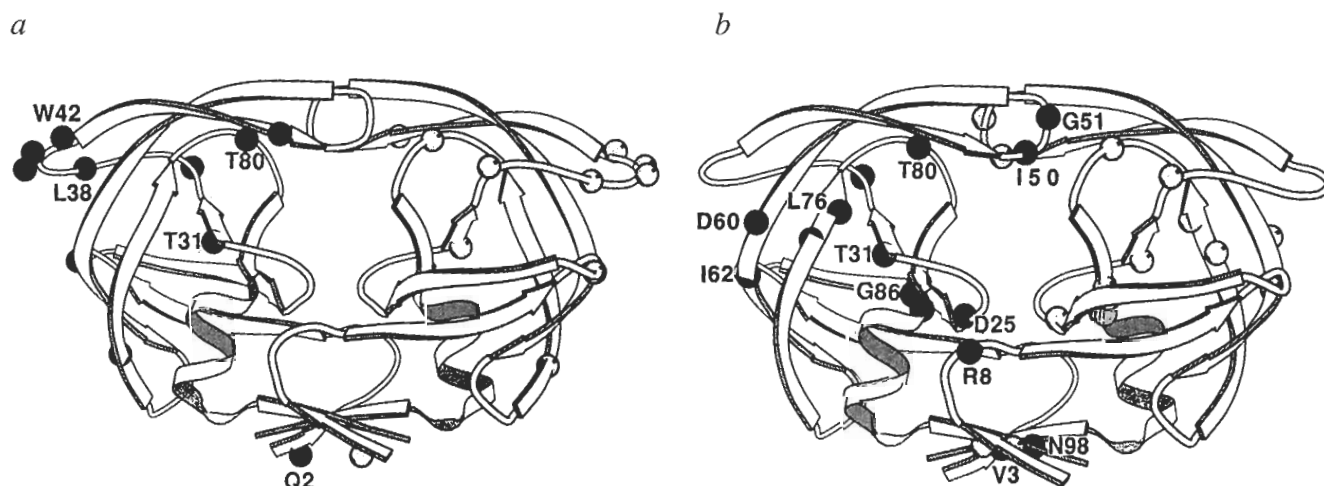


Fig. 3 Proteinase amide sites in the DMP323 complex *a*, undergoing rapid large amplitude motions ($S^2 < 0.76$, three standard deviations less than the average value of S^2); *b*, affected by chemical exchange. Black and stippled spheres identify and distinguish equivalent sites in the two monomer units. The NH bonds of the residues depicted in (a) reorient internally on the time scale of ~ 1–100 ps. In the P9941 complex, all sites identified in (a) with exception of residues 31, 35 and 48, have $S^2 < 0.76$. The amide sites depicted in (b) are affected by chemical exchange processes on the time scale μ s–ms. These residues, and essentially the same residues in the P9941 complex, required the inclusion of an exchange term, R_{ex} , to fit the relaxation data. Residues were mapped onto the crystal structure of the proteinase/DMP323 complex⁴ using Molscript⁴².

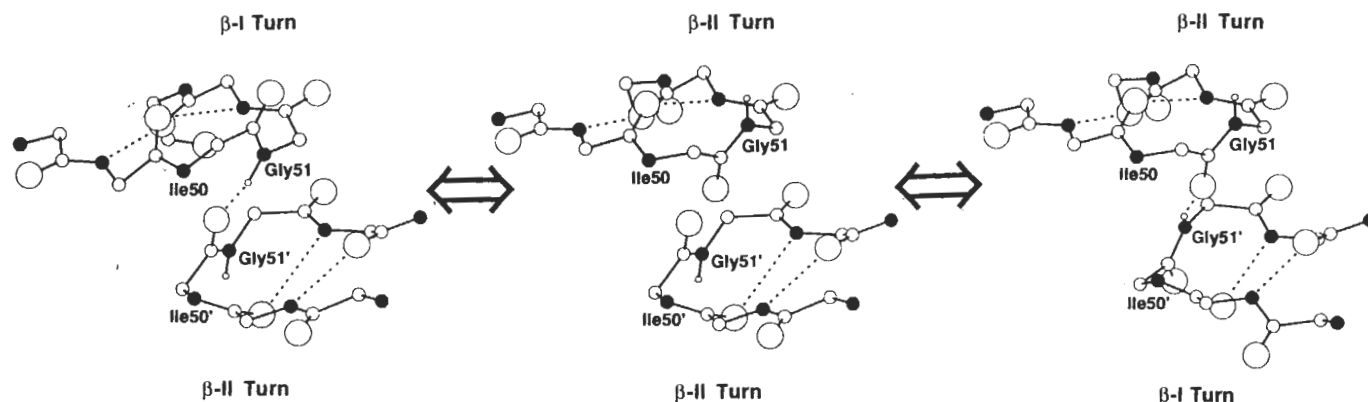


Fig. 4 Proposed model of internal motion at the tips of the proteinase flaps. A conformational switch involving residues Ile (50,50') and Gly (51,51'). In order to form the hydrogen bond between the Ile 50 carbonyl and the Gly 51' NH group observed in most crystal structures of proteinase/inhibitor complexes³, the Ile 50/Gly 51 peptide plane in one monomer must be oriented in opposite directions from the Ile 50'/Gly 51' plane in the other monomer. Only the asymmetric conformations will result in the formation of the Ile 50/Gly 51' and Ile 50'/Gly 51 hydrogen bonds. Even though the hydrogen-bonded proteinase conformations are asymmetric, they are isoenergetic and their rapid exchange results in a symmetric NMR spectrum reflecting the dynamic averaging of the homodimer bound to a symmetric inhibitor. The chemical exchange reduces the T_2 values of the Ile 50 and Gly 51 ^{15}N nuclei, demanding the inclusion of a chemical exchange term in the data analysis.

were measured using spin lock fields in the range 1.0–4.5 kHz. At all spin lock fields used, $T_{1\rho}$ was found to be equal (within 10 %) to T_2 for each residue, indicating that chemical exchange is in the fast limit. An upper limit estimate of τ_{ex} was obtained by assuming that the two hydrogen-bonded conformations depicted in Fig. 4 have relative populations of ~ 0.5 . Eqs. (3) and (4) show that if $\omega_1/2\pi \leq 4.5$ kHz, $T_{1\rho}$ and T_2 will be nearly equal provided that $\tau_{\text{ex}} \leq 10$ μs . Furthermore, eq. (3), combined with the observation that $R_{\text{ex}} \geq 10$ for Ile 50 and Gly 51 (Fig. 2) and the estimate that $\Delta\omega \sim 2 \times 10^3$, shows that $\tau_{\text{ex}} \sim 10$ μs . We therefore conclude that the flap conformational exchange takes place on the time scale of ~ 10 μs .

Residues Ile 50 and Gly 51 are conserved in retroviral proteinase primary sequences, and non-conservative substitutions of these residues strongly diminish proteinase activity³⁰, indicating that the structure and dynamics at the tips of the flaps are related to function. We suggest that the various structures formed at the tips of the flaps as observed by crystallography and NMR stabilize the proteinase/substrate complex, yet have the flexibility to facilitate product release following catalysis.

Two additional residues at the dimer interface, Val 3 and its neighbour, Asn 98 (Fig. 3b) also experience exchange broadening. All available crystal structures show that these residues have amide protons that are hydrogen bonded and are located in highly ordered β -sheets. ^{15}N and ^{13}C separated NOESY spectra and hydrogen-deuterium exchange protection factors (at least 100-fold for Val 3 and Asn 98 amide hydrogens) confirm that these structural features are also present in solution. In addition, we have seen no evidence for exchange broadening in the ^1H , ^{13}C or ^{15}N signals of the Glu 2 or Asn 98 side chains. It is very unlikely, therefore, that these residues are flexible. The chemical exchange experienced by Val 3 and Asn 98 (and possibly Arg 8) is ascribed to the backbone flexibility of nearby residues Thr 4 and Leu 5, which

are in a solvent exposed loop. This statement is supported by the observations that the backbone amide signal of Thr 4 is very weak because it has a very large proton linewidth; and that the amide signal of Leu 5 has not been observed, presumably because it is severely exchange broadened²⁷. It is noteworthy that the primary autolysis site in HIV-1 protease is the Leu 5–Trp peptide bond³³, and it has been suggested³³ that the rate of Leu 5–Trp bond cleavage may regulate the activity of the proteinase *in vivo*. If this is the case, the conformational dynamics of residues Thr 4 and Leu 5 could significantly affect the viral life cycle.

Methods

Recombinant HIV-1 protease was expressed in *Escherichia coli* strain BL21(DE3) using a T7 expression system³⁴. The protein sequence is that of the HXB2 isolate with an Ala to Cys substitution at position 95. Uniform enrichment with ^{15}N was achieved by growing the bacteria in M9 minimal medium containing $^{15}\text{NH}_4\text{Cl}$ (Cambridge Isotopes, Cambridge, MA, USA). NMR spectra were recorded on solutions containing the HIV-1 protease/inhibitor complex (0.75 mM) in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (95%/5%) containing sodium acetate (50 mM, pH 5.2) and dithiothreitol (5 mM). Sample volumes were either 430 μl in Wilmad tubes (Wilmad Glass Company, Buena, NJ, USA) or 220 μl in Shigemi NMR tubes (Shigemi, Inc., Allison Park, PA, USA). NMR tubes were purged with either argon or nitrogen and the Wilmad tubes sealed with a septum cap or by flame, while the Shigemi tubes were tightly wrapped with Teflon tape and parafilm.

T_1 and T_2 values were measured on a Bruker AM 500 spectrometer as described elsewhere¹², with 6–7 relaxation delay points defining each decay curve. T_1 values were determined from two independent sets of relaxation measurements for each complex (data available on request). A numerical fitting approach²² was used to determine the T_1 values and standard errors (as given by the error bars in Fig. 1) using conjugate gradient minimization and Monte Carlo simulation. T_2 values at 500 MHz were determined from two or three sets of relaxation data sets independently measured for P9941 and DMP323 complexes, respectively. In addition, one set of T_2 values was measured for the DMP323 complex on a Bruker AMX 600 spectrometer (data

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not shown). The T_2 values and associated standard errors were determined in the same manner as described for the T_1 data. NOE values were measured on a Bruker AMX 600 spectrometer using inverse detection and a water flip back pulse scheme³⁵. Each NOE value was determined from the ratio of signal intensities recorded in the presence or absence of proton saturation. A small correction factor of a few percent was applied to the intensity ratio, to compensate for incomplete magnetization recovery during the recycle delay in the NOE experiment³⁵. Standard errors were determined from the r.m.s. noise in the two-dimensional baseplane³⁵. The $T_{1\rho}$ data were acquired on a Bruker AM500 spectrometer using continuous ^{15}N spin locking³⁶ with RF fields of 1.0, 2.0, 3.0 and 4.5 kHz.

To derive τ_m , the ratio T_1/T_2 was calculated for each residue and the average value and standard deviation of the ratio were determined for each complex. Then the relaxation parameters of residues having (a) T_1/T_2 values within one standard deviation of the mean value and (b) NOE values greater than 0.65 were fit assuming a fixed value of τ_m , with model-free parameters S^2 and τ_e allowed to vary until the residual, χ^2 ,

$$\chi^2 = \sum (T_1^{\text{exp}} - T_1^{\text{calc}})^2 / \sigma_{T_1}^2 + \sum (T_2^{\text{exp}} - T_2^{\text{calc}})^2 / \sigma_{T_2}^2 + \sum (\text{NOE}^{\text{exp}} - \text{NOE}^{\text{calc}})^2 / \sigma_N^2 \quad (5)$$

was minimized³⁷. The equation defines the global χ^2 target function, where the summation extends over the amino-acid residues, the superscripts indicate either experimental or calculated values, and σ is the standard error of the parameter, $i=1,2,3$. The global χ^2 target function was calculated for values of τ_m in the range from 4.0–14.0 ns in steps of 0.1 ns yielding the value of τ_m for which χ^2 was a minimum. With τ_m fixed at this value, residues were selected for which the residue specific values of χ^2 (the χ^2 function calculated for each residue) were less than 4, indicating that they were well fitted by the two parameter model-free formulation. The values of τ_m that minimized the global χ^2 for this set of residues were then determined to be 9.16 ± 0.06 and 9.25 ± 0.06 ns for the DMP323 and P9941 complexes respectively. The uncertainties in the τ_m values represent the 90 % confidence limits in the values of τ_m as determined by the $\Delta\chi^2 \leq 2.71$ criterion³⁸, and should be regarded as underestimates of the true uncertainties which we estimate to be 2–3 % due to systematic errors (as determined by the reproducibility of relaxation measurements) in the relaxation measurements.

We have assumed that the proteinase reorients isotropically, like a spherical particle. However, the X-ray coordinates⁴ show that the protease/cyclic urea inhibitor complex approximates the shape of an oblate ellipsoid of revolution. Including a 3 Å hydration shell³⁹, the ratio of semi-major axes is calculated to be 1/1.6. Calculations, based on Woessner's equations⁴⁰, show that neglect of anisotropic motion for such an oblate ellipsoid introduces r.m.s. errors of 2.3 % in the calculated values of the NMR relaxation parameters. As these errors are smaller than the 2.5–4.5 % r.m.s. errors in the measured relaxation parameters, neglect of anisotropic reorientation will not significantly affect the analysis of the relaxation data. Using overall correlation times of 9.2 and 9.3 ns for the DMP323 and P9941 complexes respectively, the

relaxation data for each residue were fitted with the three following formulations of the model-free approach: (1) using two model-free parameters, S^2 and τ_e , where τ_e is an effective correlation time characterizing rapid internal motions of NH bonds on the ps time scale, and S^2 is the generalized order parameter that provides information about the angular amplitude of rapid NH bond reorientation; (2) using S^2 , τ_e and a contribution, R_{ex} , (in the equation for $1/T_2$) to account for chemical exchange^{10,41}; and (3) using three model-free parameters S_1^2 , S_2^2 and τ_e , where $S_1^2=S_2^2$, S_1^2 and S_2^2 refer to rapid internal motions characterized by different time scales^{19,24}. The two parameter formulation provided satisfactory fits for all but about twenty residues. Of these, fifteen residues in each complex required inclusion of an exchange contribution, R_{ex} , which is plotted as a function of residue number in Fig. 2b. Residues 17, 38, 48 and 71 in the DMP323 complex (17, 38 and 71 in the P9941 complex) required three model-free parameters to fit the relaxation data. Uncertainties in the model-free parameters were determined using a Monte Carlo procedure¹⁴. Values of τ_e were typically in the range of 1–100 ps, but are not listed individually because their uncertainties were large, usually > 40 %. A particular model-free formulation was considered to provide an adequate fit to the data provided that the residue specific value of χ^2 was less than 6.8. The probability, Q , that a correct model will, by chance, have a residue specific value of $\chi^2 > 6.8$ is 1 %³⁸. We prefer accepting a few fitted data sets with low Q s, rather than improving a fit with more parameters that have questionable meaning. In addition, as noted above, our calculated relaxation parameters contain small errors due to anisotropic overall motion and systematic sources. As a consequence of these non-Gaussian sources of error, an acceptable model will have some outlier points with large χ^2 values³⁸. Finally, for a few residues having $\chi^2 > 6.8$ it was observed that the error in the (residue specific) relaxation rate was less than the average error in the rate (for all residues). In these cases, the final value of χ^2 was determined using the average error. Because the chemical exchange contribution to T_2 increases as the square of the field strength, a set of T_2 values was also measured at 600 MHz, for the DMP323 complex, and the data analysis was repeated using four sets of relaxation parameters. The same fifteen residues that required an R_{ex} contribution to fit the three relaxation parameters also required an R_{ex} term to best fit the four parameter data, and the values of R_{ex} (at 500 MHz) are plotted in Fig. 2b. All fifteen residues had best fit values of χ^2 less than 6.8, except for Gly 35 and Ile 85, suggesting that the motion of these residues must be analyzed using a more complex model or that the errors in their relaxation parameters are underestimated.

Hydrogen exchange rates of backbone amide protons were determined by recording ^1H - ^{15}N heteronuclear single quantum spectra as a function of time, ranging from 5 min to 24 hr, after dissolving each complex in D_2O (DMP323 at pD=6.5 and P9941 at pD=5.2, uncorrected meter reading) at 34 °C. Protection factors against exchange were calculated as recently reported³².

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