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Inhibitor-induced Conformational Shifts and Ligand Exchange Dynamics for HIV-1 Protease Measured by Pulsed EPR and NMR Spectroscopy

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Abstract

Double electron-electron resonance (DEER) spectroscopy was utilized to investigate shifts in conformational sampling induced by nine FDA-approved protease inhibitors (PIs) and a non-hydrolyzable substrate mimic for human immunodeficiency virus type 1 protease (HIV-1 PR) subtype B, subtype C and CRF_01 A/E. The ligand-bound subtype C protease has broader DEER distance profiles but trends for inhibitor-induced conformational shifts are comparable to those previously reported for subtype B. Ritonavir, one of the strong-binding inhibitors for subtype B and C, induces less of the closed conformation in CRF_01 A/E. ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) spectra were acquired for each protease construct titrated with the same set of inhibitors. NMR ¹H-¹⁵N HSQC titration data show that inhibitor residence time in the protein binding pocket, inferred from resonance exchange broadening, shifting or splitting correlates with the degree of ligand-induced flap closure measured by DEER spectroscopy. These parallel results show that the ligand-induced conformational shifts resulting from protein-ligand interactions characterized by DEER spectroscopy of HIV-1 PR obtained at cryogenic temperature are consistent with more physiological solution protein-ligand interactions observed via solution NMR.

Keywords

HIV-1 protease; HSQC; SDSL-DEER; natural polymorphism; flap; PELDOR; EPR spectroscopy

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Supporting Information Further experimental details, protein sequences, data and error analyses, HSQC spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Human immunodeficiency virus type 1 protease (HIV-1 PR), a homodimeric aspartic protease consisting of 99 amino acids in each subunit, is an essential enzyme necessary for the generation of mature, infectious virus particles. Since 1995, HIV-1 PR has served as a successful target in therapeutic regimens that employ protease inhibitors (PIs). However, the emergence of amino acid substitutions as naturally evolving polymorphisms and drug-selected mutations have reduced the effectiveness of several inhibitors, leading to failure of Highly Active Antiretroviral Therapy (HAART).¹⁻⁴

HIV-1 is categorized into different groups, subtypes and circulating recombinant forms (CRF). The genomes of HIV-1 subtypes contain between 10% and 30% sequence variation, including variations in the protease gene.^{2,5} Among the 34 million people infected worldwide, over 23 million live in Africa.⁶ HIV-1 subtype C, the prevalent African variant, contains 30% genomic variation relative to subtype B,⁷ which is the predominant variant in Western Europe and North America. The prevalent subtype in south and south-east Asia, where 4 million people are living with HIV-1 infection, is CRF_01_A/E.^{8,9} Given that current FDA approved PIs are developed with subtype B as the therapeutic target, it is not surprising that naturally-occurring polymorphisms in other subtypes lead to lower inhibitor binding affinity by 2- to 7-fold.^{10,11} Even though this decrease is modest and does not necessarily result in drug resistance, certain polymorphisms have been shown to amplify the drug-resistant effects of subsequent mutations.¹⁰⁻¹² Given that access to the active site cavity is modulated by two β -hairpins, termed the flaps,¹³ many mutations confer drug resistance by altering HIV-1 PR flap heterogeneity¹⁴⁻¹⁶ and dynamics.^{17,18} Thus, an understanding of how the polymorphisms alter protein structure, flexibility and interaction with PIs may provide insights for the design of next-generation PIs to tailor-fit particular subtypes or circulating recombinant forms.

Site-directed spin labeling (SDSL) pulsed electron paramagnetic resonance (EPR); specifically double electron-electron resonance (DEER) spectroscopy, is a spectroscopic method that has been used to monitor conformational ensemble sampling in apo and inhibitor-bound HIV-1 PR.^{14,16,19,20,21} Because HIV-1 PR is a homodimer, substitution of lysine at position 55 with a cysteine, designated as K55C/K55C' in the flap of each monomer, generates sites for attachments of nitroxide radical labels. The spin labels at these sites serve as a pair of reporters for DEER distance measurements (Figure 1). The magnitude of the magnetic dipolar coupling of the two spins, which is inversely proportional to the cubic distance between the two spins,^{22,23} is obtained from Tikhonov regularization (TKR) methods of the background-subtracted DEER echo curves and subsequently utilized to produce distance profiles.²² By using site K55C/K55C' labeled with methanethiosulfonate (MTSL) as a reporter of HIV-1 protease conformational ensembles, we have developed a model that decomposes the distance distribution profiles into nominally four protease conformations; namely curled/tucked, closed, semi-open and wide-open conformations.^{14,16} Our previous work has also shown that amino acid sequence variations among subtypes,¹⁴ as well as drug-pressure selected substitutions,¹⁶ alter the conformational sampling ensemble. Additionally, the addition of inhibitors to subtype B and MDR769, a multidrug resistant sequence, were shown to induce shifts in the conformational ensemble to the closed conformation.^{19,21}

Although SDSL-DEER provides direct information about distances that may not readily be attained from other biophysical methods, the methodology is often criticized for lack of physiological relevant conditions. One of these is the requirement of cryogenic temperatures (20 – 80 K) to prolong phase memory relaxation time (T_m). Another is the usage of glassing agents for freezing, which may alter the thermodynamic properties of the system and

inadvertently act as osmolytes.²⁴ A third is the effect that the rate of freezing has on the thermodynamic equilibrium of conformational sampling distributions.²⁵ Consequently, questions are often raised regarding how and if an induced conformational shift in the presence of a ligand is altered by sample freezing and the presence of co-solutes, which are variations from physiological conditions.

With regards to HIV-1 PR, we have previously shown that various osmolytes do not alter the conformational sampling of apo HIV-1 PR.²⁶ Here, to address the effects of temperature on relating the effects of protein-ligand binding on conformational shifts determined with DEER and solution binding equilibria, we utilized solution NMR spectroscopy to examine protein-ligand interactions in solution under more physiologically relevant conditions. To this end, uniformly ¹⁵N labeled HIV-1 PR was titrated with inhibitors and the backbone chemical shift and peak intensity perturbations were monitored in the ¹H-¹⁵N-HSQC spectra. This method enables the approximation of inhibitor residence time and binding strength at room temperature; giving a relative estimate of the ligand exchange dynamics, which we then show are related to the induced conformational shifts detected with DEER spectroscopy.

In both the NMR and EPR investigations, the total protein concentrations were similar (*i.e.* ~ 50 μ M dimer). The HSQC titration data support the results of SDSL-DEER and suggest that pulsed EPR can be used to accurately characterize protein-inhibitor interactions in conformationally flexible enzyme systems such as HIV-1 PR. The results also indicate that the presence of the glycerol co-solute did not substantially perturb the trends in protein-ligand interactions. Additionally, in this study, parallel comparisons are made among subtype B, subtype C, CRF_01_A/E as well as MDR769 flap distance profiles using SDSL-DEER and protease-inhibitor interaction dynamics via ¹H-¹⁵N-HSQC.

MATERIAL AND METHOD

Cloning and Site-directed Mutagenesis

DNA that encodes *E. coli* codon-optimized subtype B, subtype C, CRF_01_A/E or MDR 769 HIV-1 PR were purchased from DNA 2.0 (Menlo Park, CA). Each construct was cloned into pET-23a vector (Novagen, Madison, WI) under the control of T7 promoter. Stabilized (Q7K, L33I, L63I) and inactive (D25N) construct of subtype B (B_{si}), subtype C (C_{si}), CRF01_AE (AE_{si}) and inactive MDR 769 (MDR_i), with and without incorporated labeling sites (K55C) were made using the site-directed mutagenesis kit (Stratagene). Note that this procedure renders all mutations symmetrically applied to both subunits of the homodimer. Moreover, natural cysteine residues (C67A and C95A) in these constructs are mutated to alanine to ensure site-specific labeling and prevent nonspecific disulfide bond formation. The fidelity of the HIV-1 PR genes was confirmed by Sanger DNA sequencing (ICBR Genomics Facility, University of Florida). The complete amino acid sequences of the variants utilized in this study are given in the Supporting Information.

Protein Expression, Purification, and Spin Labeling for DEER Experiments

Protein expression, purification, and spin-labeling were carried out as previously described^{14,27} with the following modification: the inclusion bodies resuspension buffer pH used for anion exchange depends upon the isoelectric point (pI) of a given construct. The buffer pH for B_{si} , C_{si} , AE_{si} and MDR_i 769 were adjusted to 9.30, 9.55, 9.20, 8.80; respectively. MTSL was added in three- to four-fold molar excess to 8 μ M HIV-1 PR homodimer in 10 mM Tris-HCl, pH 6.9, and the reaction is allowed to proceed in the dark for 12 hours at 25 °C, 150 rpm. Excess free spin label is removed by buffer exchange into 2mM NaOAc, pH=5.0 using HiPrep 26/10 desalting column.

DEER Experiments and Sample Preparation

Protein samples were made 50 μ M HIV-1 PR homodimer in 20 mM D₃/NaOAc/D₂O, pH 5.0, 30% D₈-glycerol. Inhibitor or substrate mimic was added at three-fold excess to HIV-1 PR and the solution is allowed to equilibrate at room temperature for 30-45 minutes. Samples were then transferred to a 4-mm quartz EPR tube and flash frozen in liquid nitrogen before inserting the tube into the resonator. All pulsed EPR data were collected with a Bruker EleXsys E580 spectrometer equipped with the ER 4118X-MD-5 dielectric ring resonator at 65 K using a four-pulse DEER sequence,²⁸ described in detail previously.¹⁶ The DEER dipolar modulation curves were background subtracted, high-pass filtered, and converted to distance distribution profiles *via* Tikhonov regularization (TKR) using DeerAnalysis2008 (<http://www.epr.ethz.ch/software/index>).^{29,30} The correct background subtraction level was determined using a self-consistent analysis procedure, where a series of Gaussian-shaped populations representing the nominal conformations of HIV-1 PR^{14,19} with estimated relative percentage, full width at half maximum (FWHM), and most probable distance were summed to reconstruct the distance profile *via* DeerSim. DeerSim is a Matlab based program that our lab developed and is available upon request. Using this software, the dipolar evolution curve is regenerated from the summed Gaussian profile for comparison to the experimental background-subtracted data and TKR fit.¹⁹ The optimal regularization parameter³¹ was selected to guarantee conversion accuracy from the dipolar modulation curve to a TKR distance profile as previously described.^{14,16,19} An example of full data analysis is provided in Supporting Information.

Protein Expression and NMR Sample Preparation

DNA encoding *E. coli* codon-optimized HIV-1 PR amino acid sequence B_{si}, C_{si}, AE_{si} and MDR_i lacking the K55C substitution (*i.e.*, K55) were cloned into pET-23a vector (Novagen, Madison, WI) under the control of T7 promoter. The vector was transformed in BL21*(DE3)pLysS *E. coli* cells (Invitrogen, Carlsbad, CA) and grown in modified minimal media with ¹⁵NH₄Cl (Sigma-Aldrich, St. Louis, MO) as the sole nitrogen source. Overexpression of HIV-1 PR was induced when optical density of the culture is 0.8 (measured as absorbance at 600 nm), by adding isopropyl- β -D-thiogalactoside (IPTG) to a final media concentration of 1 mM. Induction was allowed to proceed at 37 °C for 5 – 6 hours. HIV-1 PR was purified from inclusion bodies as described previously.^{14,19,26} [U-¹⁵N] HIV-1 PR in the NMR sample was prepared at 40 μ M homodimer in 2 mM D₃-NaOAc buffer at pH 5.0 with 10% D₂O and 0.1 mM DSS as internal reference.

¹H-¹⁵N HSQC Titration Experiments

Stepwise addition of inhibitors or substrate into 40-45 μ M HIV-1 PR to a final concentration of 1:1.5 protease dimer -to-inhibitor ratio for subtype B, subtype C and CRF_01_A/E was performed. This ratio is lowered to 1:1 for MDR 769. All ¹H-¹⁵N HSQC spectra for [U-¹⁵N] HIV-1 PR were acquired at 293 K using a Bruker Avance II spectrometer with a 5 mm TXI cryoprobe operating at 600 MHz (AMRIS Facility, University of Florida). Movements or disappearance of peaks were monitored by overlaying sequential ¹H-¹⁵N HSQC spectra. Protease dimer -to-inhibitor ratio at 1:1 was chosen for peak counting because significant amounts of free and bound protease are present under this condition, thereby giving a more observable peak pattern change. NMRPipe³² and Sparky (Goddard and Kneller, Sparky 3, UCSF, San Francisco) were used for processing and analysis of NMR data. Backbone chemical shift assignments were determined and reported in a previous publication.³³

RESULTS AND DISCUSSION

DEER Data Analysis

The effect of inhibitors on the conformational sampling ensemble of subtype B, subtype C and CRF_01 A/E, were investigated with DEER spectroscopy. Distance distribution profiles for the K55R1/K55'R1 (Figure 1) pair located in the solvent-exposed flap sites were determined in the presence and absence of inhibitors. Figure 2 shows select background-subtracted time-domain DEER modulation echo curves for subtype C, where the effects of inhibitor binding are readily apparent in the changes of the frequency of oscillations in the modulation curves. For example, addition of indinavir (IDV) causes almost no visual change in the DEER echo modulation curve when compared to that for the apo enzyme (Fig 2A). On the other hand, tipranavir (TPV) binding causes a strong change in the DEER signal compared to the apo state (Fig 2C). Specifically, TPV binding generates data with higher frequency oscillations, indicative of a narrower distance profile. Additionally, for TPV, the first minimum in the DEER echo curve occurs sooner, indicative of a shorter most probable distance. These changes are consistent with a shift in the conformational sampling to the closed conformation.^{19,34-36} In comparison, the data for nelfinavir (NFV) show a small but detectable change in the DEER signal (Fig 2B).

Deer echo curves are analyzed with software package DeerAnalysis (<http://www.epr.ethz.ch/software/index>)^{29,30}, where TKR methods are used to generate corresponding distance profiles. Figure 3 summarizes the resultant distance profiles obtained for subtype B,¹⁹ subtype C and CRF_01 A/E in the apo state and presence of 9 FDA inhibitors and the C_{Ap}2 non-hydrolyzable substrate mimic with sequence H-Arg-Val-Leu-r-Phe-Glu-Ala-Nle/NH₂ (r = reduced). All background subtracted time-domain modulation echo curves and complete data analyses can be found in the Supporting Information. The most probable distance for the apo constructs vary slightly. Distances of $36.2 \pm 0.2 \text{ \AA}$, $36.9 \pm 0.9 \text{ \AA}$, and $35.3 \pm 0.2 \text{ \AA}$ were obtained for subtype B, subtype C and CRF_01 A/E; respectively, providing an average most probable distance for the three constructs of $36.1 \pm 0.8 \text{ \AA}$ (shown as solid black line in Fig 3 and summarized in Table 1). The DEER results show an average flap conformation in apo subtype C that is more "open" than subtype B, whereas for CRF_01 A/E the flap conformation is more closed than observed in subtype B. These trends are also seen in X-ray structures.^{37,38} For subtype B, subtype C and CRF_01 A/E, the presence of numerous inhibitors shifts the probable distance to near $33 \text{ \AA}$, with specific values ranging between $32.8 \pm 0.2 \text{ \AA}$ - $33.8 \pm 0.2 \text{ \AA}$. For all three constructs with inhibitor IDV or NFV, the most probable distance decreases only slightly, if at all.

From modeling of X-ray structures and molecular dynamic simulations a distance of $36 \text{ \AA}$ is assigned to the semi-open population, whereas a distance of $33 \text{ \AA}$ is consistent with the closed conformation.^{14,34} In many cases, smaller peaks, located at $26 - 31 \text{ \AA}$ and $40 - 45 \text{ \AA}$, are also seen in the DEER distance profiles in the apo state and in the presence of inhibitors. We assign these distances to the curled/tucked³⁹ and wide-open conformations;⁴⁰⁻⁴² respectively. Our assignments of these intra-spin label distances of K55R1/K55'R1 to corresponding protein conformations is based on previous molecular dynamics simulation models and X-ray studies.^{34,40,43,44} Interestingly, the presence of the curled/tucked state (marked by asterisks in Figure 3) is more pronounced in the DEER distance profiles for CRF_01A/E compared to subtypes B or C. X-ray crystallography study has shown that the salt bridge that form between Glu35 and Arg57 in subtype B does not exist in CRF_01 A/E due to polymorphisms.^{11,45} This altered salt bridge pattern may provide a structural underpinning for why an asymmetric open-like conformation is stabilized relative to the wide-open conformation in CRF_01 A/E.

During data analysis of the DEER echo curves, it is important to carefully remove the effect that arises from intermolecular spin-spin interactions generating the background subtracted echo curves as shown in Figure 2.²⁹ To achieve this goal, we utilize a self-consistent method of background subtraction where we fit the TKR distance profiles from DeerAnalysis with a linear combination of Gaussian shaped functions, which are then used to regenerate an echo curve.¹⁹ The resultant curve is compared to our experimental data and the process is repeated until a “match” is obtained. The significance of each population that comprises < 20% of the total population is validated by suppressing that peak and comparing the theoretical curve to experimental data. By this means, we typically obtain certainty for distance peaks that comprise 5% of the total population when our signal to noise ratio is 25.¹⁹ In general, up to 4 functions, *i.e.*, populations, are necessary for adequate fitting of the TKR profile. These four populations we assign to the HIV-1 PR conformations mentioned above; namely curled/tucked, closed, semi-open, and wide-open.^{14,16}

The population analysis result for each construct is given in Figure 4, which shows the relative percentages, *i.e.*, the fractional occupancy, of each of these conformational states. These data show that, compared to subtype B, for the apo enzymes, subtype C contains a larger percentage of the wide-open state, whereas the conformational ensemble for CRF_01 A/E contains increased percentages of the closed and curled/tucked states with no wide-open conformation detectable within experimental error.¹⁴ These differences in conformational ensemble fractional occupancy combine together in a way that varies the most probable distances mentioned above, which again agrees with insights from X-ray structures of apo enzymes.^{37,38,46}

Figure 4 also shows the relative populations of each variant in the presence of inhibitors. For all three variants, addition of inhibitors shifts the distance profiles in a manner where the population of semi-open and wide-open states decreases with a concomitant increase in the closed state population. Interestingly, the curled/tucked population, the hypothesized asymmetric open-like state, remains unchanged within error throughout for all inhibitor binding in CRF_01 A/E. Furthermore, for CRF_01 A/E ATV binding appears to induce more of a closed state than in subtype B or subtype C. On the other hand, RTV induces less of the closed conformation in CRF_01 A/E than subtype B and C. CAP2 is a nonhydrolysable substrate mimic inhibitor and is used as a positive control as it is expected to cause a shift to the closed-state for each construct.

Based upon the results of the DEER population analysis data, we defined the effects of inhibitors to induce flap closure into three categories designated as “weak,” “moderate” or “strong,” depending on the degree to which a ligand shifts the fractional occupancy to the closed state. To better quantify this variable, we define the following parameter:

$$\Delta c = \%c(\text{inhibitor}) - \%c(\text{apo}) \quad (\text{Eq.1})$$

with %*c* designating the fractional occupancy of the closed state in the presence and absence of inhibitor. “Weak” inhibitors are categorized as those with $\Delta c < 20\%$, which also have a DEER distance profile with a most probable distance similar to that of the apo state (see Tables 1 and 2); indinavir (IDV) and nelfinavir (NFV) are considered weak for all three constructs. ATV is also considered “weak” for CRF_01 A/E. Inhibitors that have $\Delta c > 50\%$ are considered “strong”; saquinavir (SQV), lopinavir (LPV), darunavir (DRV), and tipranavir (TPV) are examples of inhibitors that are “strong” for all three constructs. For $20\% \leq \Delta c \leq 50\%$, inhibitors are classified as “moderate”. Figure 5 plots Δc values for each construct; the dashed and dotted lines in the figures show the cut-off values for the classifications of “weak”, “moderate” and “strong”. Table 2 summarizes the classification of inhibitors for each construct studied here and for MDR769, a multidrug resistant construct

that we recently investigated by DEER spectroscopy.²¹ Figure 5D shows that, in general, the inhibitors induce a similar or slightly smaller shift to the closed state for subtype C and CRF_01 A/E than for subtype B.

¹H-¹⁵N HSQC Titration Experiments

The exchange dynamics of inhibitor-protein interactions were interrogated via ¹H-¹⁵N HSQC NMR experiments, where increasing concentrations of each PI were titrated into each HIV-1 PR variants. Because amide backbone chemical shifts are sensitive to changes in the local environment, such as inhibitor binding to a protein, the exchange dynamics of the inhibitors with protease can be assessed by monitoring shifts and broadening of the HSQC resonances. The type of change in the HSQC resonances, *i.e.*, shifts or broadenings, is dictated by the exchange rate between unbound and bound species relative to differences in the chemical shifts of the bound and unbound state.⁴⁷ As illustrated in Figure 6, residues under slow exchange appear as two distinct resonances that represent the free and inhibitor bound states. Meanwhile, intermediate exchange with a ligand results in cross-peak broadening, and possible disappearance. Finally, residues under fast exchange have a resultant resonance that appears at a weighted average chemical shift; merging the free and bound states into a single HSQC cross-peak. One unique attribute of homodimeric proteins, such as HIV-1 PR, is the chemical shift degeneracy of residues for each monomer due to the C₂ symmetry in the apo enzyme. However, upon binding with an asymmetric ligand (all of the inhibitors utilized here are asymmetric) the residues for each monomer will sense disparate environments, leading to the observance of peak splitting for bound species in the HSQC spectra. In the situation of slow exchange coupled to the lost degeneracy for the bound protease, the HSQC cross-peak for the affected residue can be split into as many as three separate resonances (two for each bound monomer and a degeneracy signal for the free protease).

To assess the effect of ligand-binding to subtype B, subtype C, CRF_01 A/E, and MDR769, ¹H-¹⁵N HSQC titration experiments were performed using 9 FDA-approved inhibitors. Recently, we reported the backbone NMR chemical shift assignments for subtype C, CRF_01 A/E and MDR 769.³³ The backbone assignments for subtype B have been reported earlier by others.⁴⁸ Figure 7 shows representative ¹H-¹⁵N HSQC spectra for free and select inhibitor bound subtype C protease samples. Throughout the course of titrations, chemical shift perturbations and signal intensities were measured for the resonances. Similarly as with the DEER experiments, we find the results from the NMR experiments to be grouped into categories; however, here we only distinguish two; namely “fast/intermediate exchange” and “slow exchange”. The PIs in the former category are characterized as those inhibitors that result in resonance disappearances or limited changes in the HSQC spectra as inhibitor is titrated to a 1:1 protease dimer-inhibitor ratio. Meanwhile, ligands in the “slow” category are designated by inducing resonance shifts or splittings in the HSQC spectra. A gradual decrease in the signal intensity or complete disappearance of the resonance signal suggests that ligand/binding exchange rate in the protease-ligand (*i.e.*, PL) equilibrium (Eq 2) is in the intermediate exchange regime of NMR timescale or when $k_{ex} \approx |\Omega_f - \Omega_b|$, where Ω_b is the frequency of a resonance in the presence of a ligand, while Ω_f is the frequency in the free protein. On the other hand, resonance shifting and splitting corresponds to slow exchange ($k_{ex} \ll |\Omega_f - \Omega_b|$). The opposite case is described as fast exchange ($k_{ex} \gg |\Omega_f - \Omega_b|$), where the resonances shift and coalesce into a single signal.

Figure 7 shows select ^1H - ^{15}N HSQC spectra overlain for unbound subtype C HIV-1 PR (blue) and inhibitor-bound protease for each category described above, *i.e.*, with IDV, NFV and TPV (red); respectively. Subtype C bound to IDV shows minimal chemical shift perturbation compared to unbound protease, with only five resonance disappearances. These results suggest low binding affinity, which is consistent with fast exchange for most residues and translates to minimal perturbation of the HSQC resonances of the free protease. The lack of perturbations in the HSQC spectrum with IDV can also be understood by considering that when the binding affinity is low there are simply less protein molecules in the bound state that contribute to the total spectrum. When binding with 1:1 protease dimer-to-inhibitor ratio NFV, ~ 16 cross-peaks disappear; indicating intermediate exchange. Intermediate exchange was also observed for ATV and APV. The HSQC splitting pattern in Figure 7C shows that when subtype C is bound to TPV, cross peaks shift and split, indicating slow exchange for this inhibitor. Peak splitting and shifting was also observed for inhibitors LPV, RTV, SQV and DRV. Analogous HSQC spectra were acquired and analyzed for subtype B, CRF_01 A/E, and MDR769 with and without inhibitors. Representative HSQC spectra are provided in supporting information. The results for categorizing inhibitors into a fast/intermediate or slow exchange regime with each construct are summarized in Table 3.

Comparison of Pulsed EPR and NMR Results

The effects of inhibitors on flap closure and exchange dynamics for various HIV-1PR constructs are summarized in Table 2 and Table 3; respectively. As can readily be seen, the effects of inhibitors such as IDV, NFV, TPV, LPV and DRV are unchanged in both types of interactions. However, changes in inhibitor-protein interactions are observed for inhibitors such as ATV, APV, RTV and SQV. Specifically RTV is observed to change from slow exchange in Subtype B and C to fast/intermediate exchange with CRF_01 A/E and MDR769. The HSQC spectra for RTV bound HIV-1 PR constructs can be found in supporting information. SQV also changes from slow exchange to fast/intermediate exchange with MDR769. From the NMR perspective, these changes indicate weaker inhibitor interactions with the protein. This same “switch” can be seen among the DEER categories for inhibitor ability to induce flap closure. RTV and SQV are in the “strong” category for subtype B and C. RTV move to the “moderate” category for CRF_01 A/E, whereas both RTV and SQV move to the “weak” category for MDR769. The finding that RTV and SQV have weaker interactions with MDR769 is not surprising given that this drug resistance construct exhibits higher levels of resistance to most FDA-approved PIs, with the exception of DRV, TPV and LPV.^{49,50}

To correlate the relationship between flap closure and ligand dynamics, we plotted the number of HSQC cross-peaks against the fractional occupancy of the closed conformation from DEER analysis (Δc) for the various HIV-1 PR constructs (Figure 8). The grouping in the data shows the relationship between the ability of the inhibitors to induce the closed conformation and the ligand-protein exchange rate. In general, for the HIV-1 PR constructs investigated here, inhibitors that are classified as “weak” or “moderate” *via* DEER distance profiles consistently give HSQC spectra that contain little to no change or resonance disappearance, *i.e.*, they exhibit fast/intermediate exchange. The addition of the DEER induced conformational shift characterization of “weak,” “moderate” and “strong”, allows for a second dimension in the separation of protein-inhibitor interactions that are not discriminated within the NMR experiments. On the other hand, inhibitors described as “strong” for inducing a predominantly closed population, cause shifts or splitting in the HSQC resonances. This suggests that “weak” or “moderate” inhibitors, which render the protease flap conformation to be predominantly semi-open, have shorter residence time (*i.e.*, fast/intermediate exchange) in the active site pocket or occupy the pocket with increased

dynamic mobility but do not escape.^{51,52} Meanwhile, “strong” inhibitors, which promote flap closure, correspond to PIs that have less dynamics in the binding cleft (*i.e.*, slow exchange). The only exception to these trends appears to be APV with both subtype B and MDR769, where DEER results characterize the degree of flap closure as strong, but NMR experiments indicate that the inhibitor is experience intermediate/fast exchange. This discrepancy may originate from the smaller size of APV or the solutes may alter the interactions of this inhibitor.

Nevertheless, the relationship observed between induced conformational shifts observed with DEER spectroscopy and the ligand exchange dynamics inferred from solution NMR spectroscopy indicate that the presence of glycerol and the freezing of samples required for DEER do not artificially perturb protein-ligand interactions in HIV-1 PR. Additionally, the findings indicate that DEER spectroscopy is a suitable means for interrogating protein-ligand interactions, capable of producing results similar to those obtained from standard NMR methods. Although both NMR and DEER are suitable for the small water-soluble HIV-1 PR, DEER spectroscopy can be utilized on protein systems and macromolecular complexes that are too large or too heterogeneous for solution NMR investigations. In this way, the studies on HIV-1 PR presented here also represent a simple model system for investigating the limitations and capabilities of DEER spectroscopy.

Biological Significance: Relating Δc to K_i Values

Given that all of our DEER data, as well as NMR data, were collected on protease samples rendered inactive by the D25N mutation, we wanted to assess the relationship of these results to the inhibition constants determined with active HIV-1 PR enzymes. Because HIV-1 PR is a protease that will undergo autoproteolysis,⁴⁶ which can complicate data acquisition and interpretation in both EPR and NMR applications,⁴⁸ we chose the inactive enzyme for our spectroscopic studies due to enhanced protein stability. Although the catalytic aspartic acid residues contribute strongly to the overall inhibitor binding energy, this single mutation may not significantly alter the overall structural binding pattern of the inhibitors, which has been shown for DRV protein complexes with both active and D25N HIV-1 PR.⁵³ With active enzyme, the FDA-approved inhibitors have dissociation constants, K_d , in the nanomolar range.¹² Introduction of the D25N mutation is known to drop the dissociation constant to the micromolar range.⁵³ However, the micromolar binding range is perfect for these comparative NMR and EPR studies. If we were to use the active enzyme with inhibitor $K_d \sim$ nM, we would observe “strong” binding in each case given that the protease concentration in both NMR and EPR experiments is in the micromolar range. To our advantage, the lowered affinity due to the D25N mutation allows for interrogation of how other protein:ligand contacts impact ligand exchange dynamics and induced population shifts.

To investigate how our results obtained from pulsed EPR and NMR experiments compare to biologically relevant parameters, we plotted values of previously reported inhibition constants of various inhibitors against Δc for subtypes B, C⁵⁴ and CRF_01 A/E³⁸(Figure 9). The results are in general agreement with the nominal categories to which the inhibitors are assigned. The “weak” inhibitors cluster as one group whereas the “moderate” inhibitors bunch together with “strong” inhibitors. A similar clustering pattern was observed when HSQC peak number is plotted against K_i (Figure S-7). In this analysis, ATV and SQV are outliers for subtype B and C, while CRF_01 A/E only has SQV as exception. A possible explanation for this discrepancy can be that in contrast to the DRV HIV-1PR complexes, the D25N substitution may alter the manner in which these inhibitors bind to inactive compared to active enzyme.^{52,53} Nevertheless, there is a general similarity of the “groupings” observed suggesting that the NMR and EPR results with inactive enzyme provide trends that are

related to protein-inhibitor interactions in active enzyme, but where possible differences are observed with some inhibitors.

CONCLUSIONS

This combined EPR and NMR investigation shows that inhibitors that bind to HIV-1 PR and induce flap closure; *i.e.*, high Δc , undergo slow NMR exchange, indicated by resonance shifts or splitting in ^1H - ^{15}N HSQC titration experiments. This result is understood by considering that slow exchange correlates with a longer residence time of the inhibitor in the active site pocket, which can be “trapped” while freezing in the DEER experiment. Meanwhile, inhibitors that do not induce substantial flap closure; *i.e.*, low Δc , give HSQC spectra with resonance disappearance or no change; corresponding to intermediate to fast exchange in the NMR timescale or shorter residence time of the ligand in the binding cleft. Overall, the results demonstrate that the presence of glycerol and freezing of the sample does not significantly perturb protein-ligand interactions in HIV-1 PR, and provide a framework of how to relate differences in changes of induced conformational shifts among HIV-1 PR constructs with solution protein-ligand interactions as well as enzymatic inhibition constants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Ribbon diagram of CRF_01 A/E (PDB file 3LZS) protease in complex with Darunavir (colored in green). MTSL spin probes (K55R1) are incorporated *in silico* via MMM 2011.2 and rendered as gray sticks. The distance between spin labels is expected from modeling to be $\sim 33\text{\AA}$. Each monomer of HIV-1 PR is rendered in a different color for clarity.

Figure 2.

The comparison of background-subtracted time-domain dipolar modulation for subtype C apo (blue) and upon addition of inhibitors (red) (A) indinavir (IDV) (B) nelfinavir (NFV) and (C) tipranavir (TPV). The results are discussed within the text.

Figure 3.

Distance distribution profiles for (A) subtype B, (B) subtype C and (C) CRF_01 A/E in free (apo) and inhibitor-bound protease, where NFV=nelfinavir; ATV=atazanavir; IDV=indinavir; RTV=ritonavir; SQV=saqunavir; APV=amprenavir; CaP2=substrate mimic; LPV=lopinavir; DRV=darunavir; TPV=tipranavir. Distances of 33 Å and 36 Å are indicated by the dashed red line and solid black line, and these distances are assigned to the closed and semi-open conformational ensembles; respectively.

Figure 4. Relative percentage of each conformational population; *i.e.*, fractional occupancy, for (A) subtype B, (B) subtype C and (C) CRF_01 A/E in free (apo) and inhibitor-bound protease, with the following legend: closed (gray), semi-open (red striped), wide-open (blue) and curled/Tucked (green hatched).

Figure 5.

Change in the fractional occupancy of the closed state, Δc , for inhibitor-bound proteases (**A**) subtype B, (**B**) subtype C and (**C**) CRF_01 A/E, where $\Delta c = \% \text{closed (inhibitor)} - \% \text{closed (apo)}$. (**D**) Plot of the change in fractional occupancy of the close state, $\Delta\Delta c$, between variants and subtype B, where $\Delta\Delta c = \Delta c (\text{variants}) - \Delta c (\text{subtype B})$. The dotted and dashed lines mark the boundaries for the classification of weak, moderate and strong interactions taken as 20% and 50%; respectively.

Figure 6.

Simulated 1D NMR spectra of a two-site chemical exchange process. Ω_f and Ω_b are the chemical shifts at a ligand-free and ligand-bound protein site, respectively. k_{ex} is the exchange rate between the free and bound protein. To simplify, relaxation constants were set as $R_{2f} = R_{2b} = 10 \text{ s}^{-1}$ and the populations of free and bound protein were assumed to be equal ($p_f = p_b = 0.5$), with parameters $\Delta\omega = |\Omega_f - \Omega_b| = 120 \text{ Hz}$ and $k_{ex} = 0.1 - 1000 \text{ s}^{-1}$. Calculations and graphics were executed using *Mathematica* (Wolfram Research).

Figure 7.

Select ^1H - ^{15}N HSQC spectra of HIV-1 PR subtype C in the apo (red) state and bound to 1:1 inhibitor: protein dimer (blue). Spectra were collected in 2mM D_3 -NaOAc buffer acquired at pH=5 and 20°C. (A) Limited change was observed with addition of 1-fold IDV due to low binding affinity or fast exchange. (B) Titration of 1-fold Nelfinavir (NFV) causes at least 16 peaks to disappear due to fast/intermediate exchange. (C) Addition of one-fold TPV causes several peaks to move and/or split due to slow exchange. Total peak number is seen to increase. Direction of peak movement is shown by the green arrows. Similar ^1H - ^{15}N HSQC spectra were collected for subtype C, subtype B, CRF_01A/E and MDR769 with inhibitors; data are given in the Supporting Information.

Figure 8.

Comparison of number of ^1H - ^{15}N HSQC resonances to changes in fractional occupancy, Δc , induced by inhibitor binding for (A) subtype B, (B) subtype C, (C) CRF_01_A/E, and (D) MDR 769. Error bars are estimated at $\pm 7\%$ for Δc and at ± 3 for number of HSQC peaks. The horizontal dashed line marks the number of resonances for the apo protease, and serves as demarcation line between fast/intermediate and slow exchange groups; corresponding to the “weak/moderate” and “strong” interactions; respectively. The vertical lines indicate borders among “weak”, “moderate” and “strong” as determined by the DEER results. Data are discussed further within the text.

Figure 9.

Plots of $K_i^{38,54}$ versus Δc for (A) subtype B, (B) subtype C and (C) CRF_01 A/E. Here, the “moderate” binding inhibitors are grouped together with the “strong” inhibitors. SQV is an outlier for all subtypes, with ATV being an outlier for subtype B and C. Data are discussed within the text. Error bars are estimated at $\pm 7\%$ for Δc and taken from references (38,54) for K_i values.

Table 1

Summary of most probable distances from DEER distance profiles of HIV-1PR constructs

Most probable distance ($\pm 0.2\text{\AA}$)			
Inhibitors	Subtype B	Subtype C	CRF_01 A/E
Apo	36.2	36.9	35.3
IDV	35.8	37.3	34.8
NFV	35.8	36.5	35.6
ATV	33.8	35.8	33.7
APV	33.2	33.5	32.9
LPV	33.0	33.1	32.8
DRV	33.2	33.3	33.0
RTV	32.9	33.7	33.0
TPV	32.9	32.8	32.9
SQV	32.8	33.8	32.7
CAP2	33.0	33.5	33.1

Table 2

Summary of induce-flap-closure ability for inhibitors on different HIV-1 PR constructs based on the DEER percentage change in flap closed conformation

HIV-1 PR Constructs	Inhibitor Category from DEER data		
	Weak	Moderate	Strong
subtype B	IDV, NFV	ATV	APV, RTV, SQV, TPV, LPV, DRV
subtype C	IDV, NFV, ATV	APV	RTV, SQV, TPV, LPV, DRV
CRF_01 A/E	IDV, NFV	ATV, APV, RTV	SQV, TPV, LPV, DRV
MDR 769	IDV, NFV, ATV, APV, RTV, SQV,		TPV, LPV, DRV

Table 3

Summary of exchange dynamics assignments for different HIV-1 PR constructs in the presence of inhibitors based on their impact on HSQC resonance pattern

HIV-1 PR Constructs	Inhibitor Category based upon HSQC data	
	Fast/intermediate exchange	Slow exchange
subtype B	IDV, NFV, ATV, APV	RTV, SQV, TPV, LPV, DRV
subtype C	IDV, NFV, ATV, APV	RTV, SQV, TPV, LPV, DRV
CRF_01 A/E	IDV, NFV, ATV, APV, RTV	SQV, TPV, LPV, DRV
MDR 769	IDV, NFV, ATV, APV, RTV, SQV	TPV, LPV, DRV