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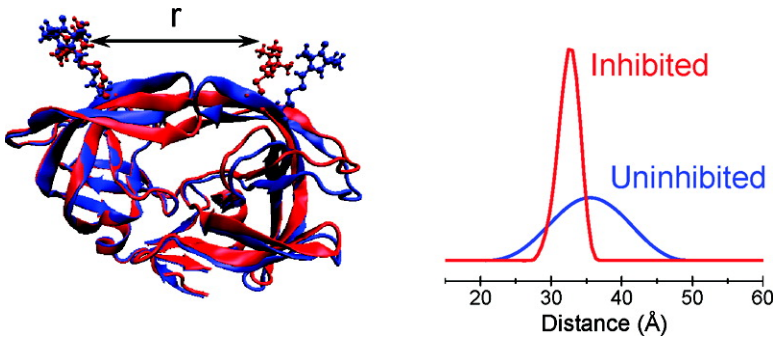

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Interflap Distances in HIV-1 Protease Determined by Pulsed EPR Measurements

Luis Galiano,[§] Marco Bonora,[†] and Gail E. Fanucci*,[§]

Department of Chemistry, University of Florida, P.O. Box 117200, Gainesville, Florida 32611, and Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306

Received June 5, 2007; E-mail: fanucci@chem.ufl.edu

Human immunodeficiency virus type 1 (HIV-1) protease plays an essential role in the processing of viral proteins encoded in the HIV genome and represents a major target of AIDS antiviral therapy. Specifically, this protease is responsible for the cleavage of the viral polyproteins *gag* and *gag-pol*. Inhibition of its activity prevents viral maturation, thus producing immature non-infectious viruses.[1] HIV-1 protease is a 99 amino acid aspartic protease homodimer with the active site formed by the contribution of one aspartic acid from each monomer.[2] The flap region in the protein is comprised of two β-sheets per monomer that have been shown to cover the active site (Figure 1), with substrate access to the active site cavity occurring via a conformational change. Motion/dynamics of the flaps in HIV-1 protease have been widely investigated by numerous biophysical techniques that include nuclear magnetic resonance (NMR),[3−5] isothermal calorimetry (ITC),[6−8] and molecular dynamics simulations.[9] However, an experimental characterization of the uninhibited open conformation is lacking. Here we report results from site-directed spin labeling (SDSL) double electron−electron resonance (DEER) studies of the conformations of the flaps of HIV-1 and demonstrate that these techniques can distinguish conformations of the flaps in the inhibited and uninhibited forms. Specifically, the distances between two spin labeled (SL) sites (K55SL−K55SL′) in HIV-1 protease as a function of two different spin labels, with and without inhibitor (Ritonavir),[10,11] are reported and compared. Upon addition of inhibitor, the distance between spin labels shortens by ∼3−4 Å (regardless of SL choice), indicating a closing of the flaps. More importantly, our results characterize the range of flap conformation in an uninhibited form, showing distances that span 26−48 Å, indicative of a broad range of conformations in this uninhibited state.

Distance measurements by SDSL pulsed EPR are based on the magnitude of the magnetic dipolar coupling of the unpaired nitroxide electrons, which scales as $1/r^3$, where r is distance between nitroxide spin labels. DEER has been used to study the structure and conformational changes in a variety of biomolecules.[12] This methodology determines the dipolar coupling between spins in the form of a modulation of the spin echo amplitude, with greatest sensitivity in the range of 15−60 Å, achieving a precision of 0.3 Å for the lower end of this range.[13,14]

For these studies, the HIV-1 protease LAI consensus sequence with mutations Q7K/L33I/L63I, which are known to stabilize the dimer, was used. We also introduced the mutations C67A and C95A to remove the two native cysteine residues (PMPR, penta-mutated protease). In order to minimize protein self-proteolysis, our initial experiments utilized the D25N active site mutation. This mutation has been shown by X-ray crystallographic models not to perturb the wild-type (WT) structure. Because many mutations in HIV-1 protease are implicated in multi-drug resistance, special care was taken when choosing a residue in the flap region as our reporter site for spin labeling (Figure 1).

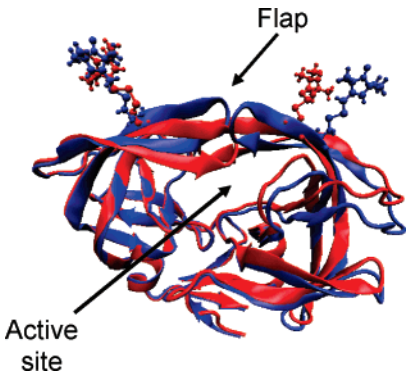

Figure 1. Model structures of HIV-1 (X-ray coordinates) showing the position of spin label attachment at position K55C (MTSL shown, appended with VMD[15] software). The two structures represent the protease in a closed (red, PDBID: 1HVI) and semi-open (blue, PDBID: 1HHP) conformation.

On the basis of reported results from saturation mutagenesis studies, K55 is one of the few available sites within the flap region tolerant of amino acid substitution while retaining WT activity.[16] Hence, K55C was chosen as the site to incorporate the nitroxide spin label moiety.

DEER experiments were performed on samples chemically modified at the cysteine in position 55 with two different nitroxide spin labels (Figure 2a,b), namely, 3-(2-iodoacetamido)-PROXYL (IAP) and (1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl) methanethiosulfonate (MTSL). These spin labels differ in the length and chemistry of attachment to the engineered cysteine, forming a disulfide bond in the case of MTSL and a carbon−sulfur bond for IAP. CD spectroscopy and enzyme inhibition studies show that the incorporation of the spin label does not significantly alter the structure or function of the active protein (Supporting Information).

Protein samples (70 ± 20 μM) containing glycerol were loaded into 4 mm EPR tubes and frozen in liquid N_2. The experiments were performed at 65 K using a Bruker E580/E680 X-band pulsed spectrometer operating near 9.7 GHz. For inhibited samples, a 1:4 molar ratio of protein/Ritonavir was used.[3] Nearly identical distance distribution profiles were obtained from data analysis either by direct inversion of the time domain dipolar spectra (Tikhonov regularization, TKR[17]) as implemented in DeerAnalysis2006[18] or by Monte Carlo (MC) fitting of the dipolar spectra.[19] Both techniques have advantages and disadvantages. Namely, TKR does not assume a given distribution function profile for distance distribution shape, but a regularization parameter to ensure a smooth solution is needed. On the other hand, MC fitting requires an assumed distance distribution shape (in this case, a set of Gaussian functions) but is less affected by experimental noise. Table 1 reports the results obtained for each different spin labeled sample. For each sample,

[§] University of Florida.
[†] Florida State University.

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Table 1. EPR Distance Measurements for K55SL-K55SL′ HIV Protease

	distance (Å)		Δ distance (Å)	fwhm (Å)		Δ fwhm (Å)
label	uninhibited	Ritonavir	uninhibited – Ritonavir	uninhibited	Ritonavir	uninhibited – Ritonavir
MTSL	35.5 (35.5)	32.6 (32.8)	2.9 (2.7)	10.4 (9.3)	3.0 (2.7)	7.4 (6.6)
IAP	35.4 (35.3)	32.4 (31.8)	3.0 (3.5)	6.8 (6.3)	4.5 (4.5)	2.3 (1.8)

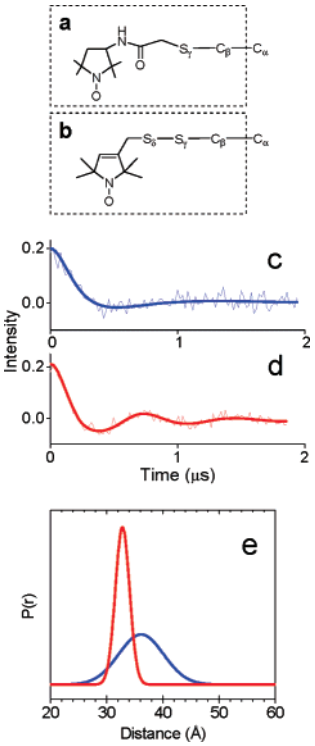

Figure 2. (a, b) Spin labels used in this study: (a) IAP; (b) MTSL. (c, d) Background-subtracted DEER dipolar echo evolution curves for K55C−K55C′ MTSL in (c) the absence and (d) the presence of Ritonavir (inhibitor). The solid lines overlaid on the experimental data represent the regenerated echo curves from Tikhonov regularization (TKR). (e) Normalized distance distribution profiles from TKR analysis of the dipolar echo curves in the absence (blue line) and presence (red line) of Ritonavir.

two distances and distribution profiles are given, as determined from the TKR and MC methods, respectively, with distances determined from the MC approach in parentheses. Example time domain dipolar spectra for PMPR+D25N+K55MTSL are shown in Figure 2c,d. Additional experimental echo curves, distance distribution profiles, data analysis, and L-curves can be found in the Supporting Information.

A Gaussian-shaped distance distribution profile with an average distance of 35.5 Å and a width of 10 Å is obtained for the PMPR+D25N+K55MTSL flap mutant in the absence of inhibitor. Upon addition of Ritonavir, a commonly used tight-binding protease inhibitor, the average distance shortens by 3 Å (to 32.6 Å) and the distribution is narrowed to 3.0 Å. In this inhibited state, the distribution breadth is interpreted to reflect spin label disorder rather than backbone disorder, as it is known that large amplitude backbone motions are restricted in the inhibitor-bound state.[20] Therefore, we conclude that the increased breadth of the MTSL distance distribution profile (absence of inhibitor) reflects an ensemble of conformations of the flaps that are "frozen out", thus reflecting a continuous range of motion during flap opening and closing.

The same trend is observed for the IAP spin label. However, the distribution width change is not as pronounced (only 2.3 Å), which likely results from enhanced spin label disorder due to the longer tether length of IAP compared to that of MTSL. It is also noteworthy that both spin labels reported maximum distances of 44−48 Å, providing an indication of the degree of flap opening in the uninhibited state.

The work presented here demonstrates the validity of SDSL and pulsed EPR methodologies applied to HIV-1 protease, and it establishes a framework for studying drug-resistant mutants thought to have modified conformational heterogeneity of the flaps.

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Supporting Information Available: Further experimental details, time domain dipolar spectra, distance distributions, circular dichroism spectra, and kinetic analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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