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Monitoring Inhibitor-Induced Conformational Population Shifts in HIV-1 Protease by Pulsed EPR Spectroscopy†

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ABSTRACT: Double electron−electron resonance (DEER), a pulsed electron paramagnetic resonance (EPR) spectroscopy technique, was utilized to characterize conformational population shifts in HIV-1 protease (HIV-1PR) upon interaction with various inhibitors. Distances between spin-labeled sites in the flap region of HIV-1PR were determined, and detailed analyses provide population percentages for the ensemble flap conformations upon interaction with inhibitor or substrate. Comparisons are made between the percentage of the closed conformer seen with DEER and enzymatic inhibition constants, thermodynamic dissociation constants, and the number of hydrogen bonds identified in crystallographic complexes.

The experimental characterization of conformational changes, population shifts, dynamics, and allostery in biological macromolecules has recently received great attention (*1*, *2*). Site-directed spin labeling (SDSL) is a method well suited to the study of the structure, flexibility and mobility, and conformational changes in a wide range of macromolecular systems, including soluble proteins, membrane proteins, natively unstructured proteins, and nucleic acids (*3*−*5*). Because the method does not require fast isotropic tumbling in solution, there is no upper size limit to the types of complex assemblies to which this method can be applied. However, SDSL does require an EPR active probe at a desired site in the macromolecule.

The pulsed EPR method of DEER, also called PELDOR, has been growing in popularity and maturing in its applications (*3*, *6*). This experiment measures the strength of the dipolar coupling between spins, which is proportional to $1/r^3$, where r is the interspin distance. Hence, via measurement of the strength of the dipolar interaction, distances of 20−60 Å between spin labels can readily be experimentally determined. The most commonly used pulsed EPR experiment cited in the literature for protein samples is four-pulse DEER (*7*, *8*).

We have been applying the DEER method to characterize conformations of the β-hairpin turns (also known as *the flaps*), which cover the active site of HIV-1PR (*9*−*11*). HIV-1PR is an aspartic protease that processes the *gag-pol* polypeptide of the HIV-1 virus. Inhibition of the enzymatic activity of HIV-1PR results in the budding of immature noninfectious virus particles (*12*), and several antiretroviral inhibitor cocktails target this enzyme.

Investigations into the conformational sampling of HIV-1PR have appeared in the literature, and of particular interest are results from molecular dynamics (MD) simulations that propose that HIV-1PR samples a series of conformations in solution consisting of "closed", "semi-open", and "wide open" states (*13*, *14*). Via SDSL DEER measurements with a spin label incorporated at an aqueous solvent-exposed site in the flap region (Figure 1A), distances consistent with the presence of all three of these proposed conformers have been obtained (*9*, *15*) and MD simulations have accurately regenerated the experimentally measured distance profiles (*16*). However, our earlier DEER data for apo HIV-1PR (*9*) lacked a sufficiently high signal-to-noise ratio (SNR) for discerning the presence of distinct populations of conformations within the distance profile. Figure 1B compares our earlier and newer time domain data for apo HIV-1PR. The solid lines through the experimental data are the best solutions using Tikhonov regularization (TKR) analysis (*17*), and Figure 1C plots the corresponding distance profiles. Note the greater detail in the shape of the distribution profile when data are collected with a higher SNR (24:1) compared to the featureless broad distance profile (gray curve) which results from a low SNR (8:1).

The TKR method, a priori, does not assume a given shape for the distance distribution profiles (*18*). Here, we have reconstructed the TKR distance profile for apo HIV-1PR using a series of Gaussian distance populations (shown in Figure 1D). The four populations shown were combined to regenerate the TKR distance profile, and the average distance of each population corresponds well with a given structural conformation predicted from MD simulations or seen with X-ray crystallography. The average distance and relative percentage for each population are 24.7 Å and 4%, 33 Å and 3%, 36.4 Å and 86%, and 41 Å and 7% and assigned to conformations where the flaps are curled (*19*), closed, semi-open, and wide open, respectively (details of sample preparation, amino acid sequence, data collection and analysis, and error analysis are given as Supporting Information).

Most often, the DEER method is used to obtain values of distances to confirm that a conformational change has occurred. Often, these distances are also used as constraints in structural models. Here, we are showing that with detailed data analysis of experimental DEER echo curves with a sufficiently high SNR, along with results from MD simulations and X-ray models, the shape of the profile can be scrutinized to reveal populations of conformations contained within the ensemble. Given the numerous crystallographic structures available for HIV-1PR and results from MD and SDSL DEER, we describe the interaction of inhibitors with HIV-1PR as inducing a population shift among the conformers that the enzyme samples.

Furthermore, we find that the DEER method can be used to discriminate the mode or strength of interaction that the various

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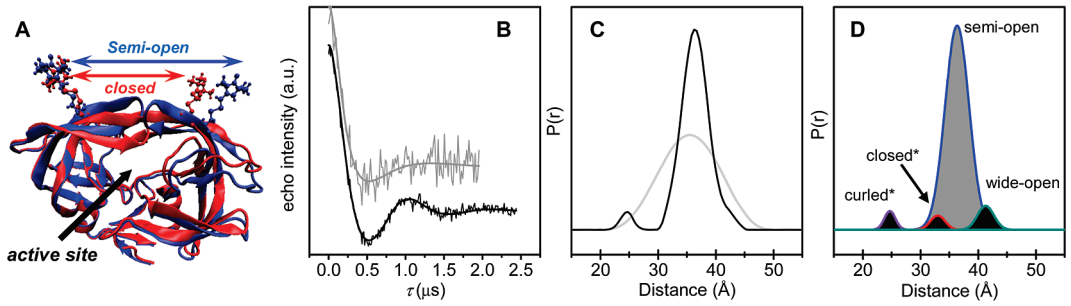

FIGURE 1: (A) Ribbon diagram of the HIV-1PR homodimer with spin labels at sites K55C and K55C′. Arrows show the expected distance change between spin labels in the closed and semi-open conformations. (B) Background-subtracted dipolar modulation echo curves for spin-labeled apo HIV-1PR. Newer data are shown in black with older data in gray. Solid lines are TKR regenerated echo curves using DEER Analysis 2008 (*17*). The older data are offset vertically for the sake of easy comparison. (C) Distance profiles of time domain data in panel B showing how the SNR impacts the shape of the distance profile. (D) Reconstruction of the distance profiles in panel C using a series of Gaussian-shaped population curves. Each population is assigned to a given structural conformation, discussed in this paper. The asterisks indicate populations questionable within the SNR. Details of data analyses, including error analyses, are given as Supporting Information.

inhibitors and substrate analogues make with HIV-1PR. Panels A and B of Figure 2 plot distance profiles determined for HIV-1PR with nine FDA-approved inhibitors (see Table 1 for names and abbreviations) (*20*). Data are also shown for interaction with a nonhydrolyzable peptide substrate mimic, Ca-P2. The DEER distance profiles differ among the various inhibitors. The data are plotted into two groups; those shown in Figure 2A are characterized as "strongly" interacting and close the flaps (DRV, TPV, LPV, RTV, SQV, APV, and Ca-P2). The inhibitors plotted in Figure 2B (ATV, NFV, and IDV) have "moderate" to "weak" interactions with the enzyme with the flaps remaining mostly semi-open. Definitions of "strong", "moderate", and "weak" are defined by how much the inhibitors shift the conformational population to the closed state. Just as the TKR distance profile for the apoenzyme was regenerated using a series of Gaussian-shaped populations, the same analyses were performed for data in panels A and B of Figure 2. Inhibitors defined as having strong interactions have ≥70% of the conformers in the closed flap conformation. For the remaining inhibitors, moderate interactions are seen for ATV, where ~40% of the population has shifted to the closed state, whereas IDV and NFV weakly interact with HIV-1PR, with <20% of the closed conformation found.

Table 1 compares the relative percentage of the closed conformation determined for each inhibitor with published values of enzyme inhibition constants, K_i (*21*, *22*), and of thermodynamic dissociation constants, K_D, determined from isothermal titration calorimetry (ITC) measurements (*22*, *23*). Also listed is the number of non-water-mediated hydrogen bonds each inhibitor makes with protease, excluding those to the active site aspartic acid residues, as determined from structures of enzyme−inhibitor complexes (*24*−*27*).

No strong correlation is seen between the percentage of the closed conformation and values of K_i. A moderate correlation is obtained for K_D values; when $K_D \leq 300$ pM, the inhibitors strongly shift the population ≥70% closed. A strong correlation is seen with the number of hydrogen bonds the inhibitors make with the protein. For IDV and NFV, defined as weakly interacting, two or three non-water-mediated hydrogen bond contacts are made. Typically, when four or more non-water-mediated hydrogen bonds occur, the DEER results show strong interactions with ≥70% of the population in the closed state.

Note, the construct used for DEER measurements does not contain the active site aspartic acid residue. The mutation to asparagine (D25N) was made to prevent autoproteolysis, which

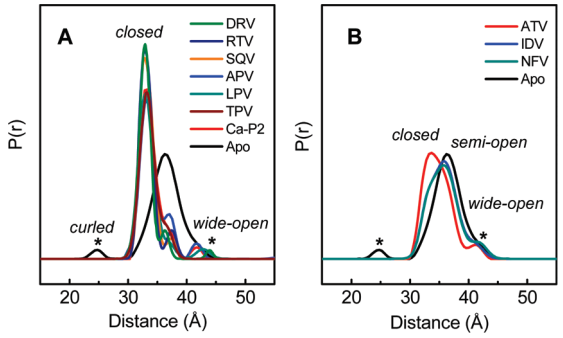

FIGURE 2: DEER distance profiles for HIV-1PR in the apo state and upon interaction with inhibitors. Those in panel A have strong interactions and are seen to induce >50% closed conformation, whereas in panel B, the inhibitors interact more weakly, with <50% of the conformers in the closed state. The asterisks indicate populations questionable within the SNR.

can affect the quality of the data and ease of data collection. This mutation is important to consider when making the previous comparisons. With the active enzyme, all of the inhibitors have subnanomolar dissociation constants (K_D values reported in Table 1 were collected for the active enzyme). The D25N mutation is likely to alter the values of K_D but not the rank order trend. Hence, given our construct has the D25N mutation, it is not surprising that the best correlation is obtained between the population shifts seen with DEER and the number of non-water-mediated hydrogen bonds, excluding those to the active site aspartic acid residues, identified in crystallographic protease−inhibitor complexes.

This work is significant because it demonstrates the sensitivity of the SDSL DEER method in interrogating population shifts and possible allostery in macromolecular complexes. It also demonstrates that careful analysis of the shape of the distance profile can provide detailed information about conformational sampling of the ensemble structures, which is significantly more information than either just a value of the most probable distance or confirmation of distance changes upon protein−small molecule interactions.

These findings also address a concern previously expressed about the DEER method. The pulsed EPR data are collected at cryogenic temperatures, because at room temperature, the spin memory time is too short for detection of the spin echo. Often, the question of whether the conformations trapped at 40−80 K accurately represent the thermodynamic conformations sampled

Table 1: Comparison of Percentage Closed Populations for Each FDA-Approved Inhibitor Obtained from DEER Analysis to Published Values of K_i, K_D, and the Number of Non-Water-Mediated Hydrogen Bonds Observed in Crystallographic Complexes

inhibitor	abbreviation	percentage closed (±5%)	K_i[a,b] (nM)	K_D[b,c] (pM)	# hydrogen bonds[d,e]
Nelfinavir	NFV	14	1.2[a]	670	2[d]
Indinavir	IDV	14	3.9[a]	590	3[d]
Atazanavir	ATV	41	0.48[a]	NA[h]	3[b]
Lopinavir	LPV	84	0.05[a]	36	3[f]
Amprenavir	APV	76	0.17[a]	220	5[d]
Darunavir	DRV	87	0.010[b]	10[b]	6[d]
Tipranavir	TPV	91	0.019[b]	19[b]	6[g]
Ritonavir	RTV	90	0.7[a]	100	7[e]
Saquinavir	SQV	93	1.3[a]	280	7[d]

[a]Data taken from ref *21*. [b]Data taken from ref *22*. [c]Data taken from ref *23*. [d]Data taken from ref *24*. [e]Data taken from ref *27*. [f]Data taken from ref *25*. [g]Data taken from ref *26*. [h]Data not available.

under physiological temperatures arises. Given the correlation observed for the relative trends of interactions of inhibitor with HIV-1PR by DEER analysis to values obtained for thermodynamics parameters measured with solution ITC, it is likely that the conformations detected for HIV-1PR under cryogenic conditions adequately represent the solution ensemble for this particular protein system. Finally, the DEER results show a trend for the relative ability of the inhibitors to close the flaps of HIV-1PR that strongly correlates with non-water-mediated hydrogen bonding of the inhibitor with the protease.

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NOTE ADDED AFTER ASAP PUBLICATION

After this paper was published ASAP August 24, 2009, a correction was made to the first paragraph on the second page; the corrected version was reposted August 25, 2009.

SUPPORTING INFORMATION AVAILABLE

Experimental details, sample preparation, time domain echo curves, and data analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Smock, R. G., and Gierasch, L. M. (2009) *Science 324*, 198–203.
2. Clore, G. M. (2008) *Mol. Biosyst. 4*, 1058–1069.
3. Fanucci, G. E., and Cafiso, D. S. (2006) *Curr. Opin. Struct. Biol. 16*, 644–653.
4. Stone, K. M., Townsend, J. E., Sarver, J., Sapienza, P. J., Saxena, S., and Jen-Jacobson, L. (2008) *Angew. Chem., Int. Ed. 47*, 10192–10194.
5. Sicoli, G., Mathis, G., Delalande, O., Boulard, Y., Gasparutto, D., and Gambarelli, S. (2008) *Angew. Chem., Int. Ed. 47*, 735–737.
6. Ghimire, H., McCarrick, R. M., Budil, D. E., and Lorigan, G. A. (2009) *Biochemistry 48*, 5782–5784.
7. Jeschke, G., and Polyhach, Y. (2007) *Phys. Chem. Chem. Phys. 9*, 1895–1910.
8. Pannier, M., Veit, S., Godt, A., Jeschke, G., and Spiess, H. W. (2000) *J. Magn. Reson. 142*, 331–340.
9. Galiano, L., Bonora, M., and Fanucci, G. E. (2007) *J. Am. Chem. Soc. 129*, 11004–11005.
10. Galiano, L., Ding, F., Veloro, A. M., Blackburn, M. E., Simmerling, C., and Fanucci, G. E. (2009) *J. Am. Chem. Soc. 131*, 430–431.
11. Galiano, L., Blackburn, M. E., Veloro, A. M., Bonora, M., and Fanucci, G. E. (2009) *J. Phys. Chem. B 113*, 1673–1680.
12. Ashorn, P., McQuade, T. J., Thaisrivongs, S., Tomasselli, A. G., Tarpley, W. G., and Moss, B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7472–7476.
13. Hornak, V., Okur, A., Rizzo, R. C., and Simmerling, C. (2006) *Proc. Natl. Acad. Sci. U.S.A. 103*, 915–920.
14. Hornak, V., Okur, A., Rizzo, R. C., and Simmerling, C. (2006) *J. Am. Chem. Soc. 128*, 2812–2813.
15. Torbeev, V. Y., Raghuraman, H., Mandal, K., Senapati, S., Perozo, E., and Kent, S. B. (2009) *J. Am. Chem. Soc. 131*, 884–885.
16. Ding, F., Layten, M., and Simmerling, C. (2008) *J. Am. Chem. Soc. 130*, 7184–7185.
17. Jeschke, G., Chechik, V., Ionita, P., Godt, A., Zimmermann, H., Banham, J., Timmel, C. R., Hilger, D., and Jung, H. (2006) *Appl. Magn. Reson. 30*, 473–498.
18. Chiang, Y. W., Borbat, P. P., and Freed, J. H. (2005) *J. Magn. Reson. 172*, 279–295.
19. Heaslet, H., Rosenfeld, R., Giffin, M., Lin, Y. C., Tam, K., Torbett, B. E., Elder, J. H., McRee, D. E., and Stout, C. D. (2007) *Acta Crystallogr. D63*, 866–875.
20. Hou, T. J., Zhang, W., Wang, J., and Wang, W. (2009) *Proteins 74*, 837–846.
21. Clemente, J. C., Moose, R. E., Hemrajani, R., Whitford, L. R., Govindasamy, L., Reutzel, R., McKenna, R., Agbandje-McKenna, M., Goodenow, M. M., and Dunn, B. M. (2004) *Biochemistry 43*, 12141–12151.
22. Muzammil, S., Armstrong, A. A., Kang, L. W., Jakalian, A., Bonneau, P. R., Schmelmer, V., Amzel, L. M., and Freire, E. (2007) *J. Virol. 81*, 5144–5154.
23. Yanchunas, J., Jr., Langley, D. R., Tao, L., Rose, R. E., Friborg, J., Colonno, R. J., and Doyle, M. L. (2005) *Antimicrob. Agents Chemother. 49*, 3825–3832.
24. Prabu-Jeyabalan, M., King, N. M., Nalivaika, E. A., Heilek-Snyder, G., Cammack, N., and Schiffer, C. A. (2006) *Antimicrob. Agents Chemother. 50*, 1518–1521.
25. Reddy, G. S. K. K., Ali, A., Nalam, M. N. L., Anjum, S. G., Cao, H., Nathans, R. S., Schiffer, C. A., and Rana, T. M. (2007) *J. Med. Chem. 50*, 4316–4328.
26. Nalam, M. N. L., Peeters, A., Jonckers, T. H. M., Dierynck, I., and Schiffer, C. A. (2007) *J. Virol. 81*, 9512–9518.
27. Prabu-Jeyabalan, M., Nalivaika, E. A., King, N. M., and Schiffer, C. A. (2003) *J. Virol. 77*, 1306–1315.