J|A|C|S COMMUNICATIONS

Published on Web 12/31/2008

Dynamics of "Flap" Structures in Three HIV-1 Protease/Inhibitor Complexes Probed by Total Chemical Synthesis and Pulse-EPR Spectroscopy

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Heterogeneity of the mobile "flap" structures in HIV-1 protease (residues 37−61 in each domain of the homodimeric protein) has recently attracted much attention.[1] Molecular dynamics simulations suggested an equilibrium between closed, semiopen, and fully open ensembles in the unliganded HIV-1 protease, with semiopen conformers being predominant;[1] this is also in agreement with experimental NMR solution studies.[2] This equilibrium is believed to play a role in catalysis by "fine-tuning" substrate binding and product release.[3] Also, it was shown that H-bonds donated by the amide −NH− moieties of Ile50 and Ile50′ to carbonyls of substrate or inhibitor through a well-ordered conserved water molecule ("water 301") are vital for the enzyme to be active. If both Ile50 and Ile50′ amides were replaced by esters, thus knocking out the possibility to form amide −NH− H-bonds, the enzyme had reduced proteolytic activity.[4]

An experimental approach that is very suitable for elucidating conformational states of the flap structures in solution is distance measurements by pulse-EPR spectroscopy in spin-labeled constructs and was realized for the first time by Fanucci and co-workers.[5] They found a very broad distance distribution for the unliganded form of HIV-1 protease and a much more narrow distribution for the complex with the inhibitor Ritonavir. Here, we report interflap distances determined by the Double Electron Electron Resonance (DEER) method[6] for complexes of HIV-1 protease with three different peptidomimetic inhibitors. Our data also suggest a refinement of the results previously obtained for the unliganded HIV-1 protease.

Both active HIV-1 protease and its inactive [D25N] analogue were prepared in nitroxide spin-labeled form by total chemical synthesis.[7] Active protease, despite having MTSSL-spin label at positions 55 and 55′, retained native-like enzymatic activity suggesting that such substitution does not significantly affect the conformational equilibrium in spin-labeled analogues. The three inhibitors studied here represent three different principal states on the reaction coordinate of enzyme-catalyzed peptide bond hydrolysis (Figure 1a).[4] The MVT-101 inhibitor (K_d 0.83 μM) is structurally similar to an "earlier" transition state, whereas the KVS-1 inhibitor (IC_{50} 6.3 nM) in hydrated gem diol form is a fully isosteric, nonhydrolyzable replacement for the tetrahedral intermediate in the reaction.[7] The JG-365 inhibitor (K_d ∼2 nM) has an additional methylene unit (−CH_2−) and thus mimicks a "later" transition state, where the substrate is undergoing fission at the gem-diol (−$C(OH)_2$−) and −NH_2^+− junction (Figure 1b).

Solutions of the spin-labeled enzymes complexed with inhibitors for DEER measurements were prepared at 40−50 μM for HIV-1 protease, and inhibitors were added at 30−100-fold molar excess to ensure saturation of the enzymes. Measurements were performed at 55 K with flash-frozen solutions using 4-pulse DEER, and data acquisitions took 24−48 h depending on the spin−echo modulation intensity for different complexes to achieve the best possible *S*/*N* ratio. Distance distribution profiles were determined using an optimal Tikhonov regularization parameter when fitting the spin−echo evolution curves.[8]

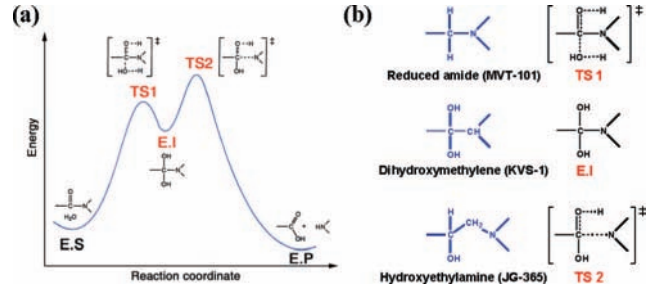

Figure 1. (a) Hypothetical reaction coordinate diagram for peptide bond hydrolysis catalyzed by HIV-1 protease adopted from ref 4. E.I = enzyme.intermediate. (b) The inhibitors studied were designed to mimick different species on the reaction coordinate.

Very sharp distance distributions were observed for the complexes with MVT-101 inhibitor, and only slightly wider distributions for the complex of active HIV-1 protease with KVS-1, indicating that the flaps are quite rigid in these two complexes. In contrast, when complexed with the JG-365 inhibitor the flaps gave a very broad distance distribution, but as in the two former complexes three well-developed maxima were observed (Figure 2). It should be noted that in the case of KVS-1 the results were different for the complex of active HIV-1 protease versus the inactive [D25N] chemical analogue. We recently demonstrated that the active HIV-1 protease enzyme converts the keto-group of KVS-1 into a gem-diol in analogy with the first step of the proteolytic reaction, whereas with the [D25N] inactive enzyme analogue the keto-group remains intact.[7] Interestingly, this difference in the chemical structure of the active site region appeared to be reflected in the interflap distance profiles.

Previously a very broad interflap distance distribution obtained by the DEER method has been reported for the unliganded HIV-1 protease; the data were fitted with a single broad Gaussian.[5] With two spin-labels introduced at the same positions we also observed a rather broad distance distribution by the DEER method for unliganded HIV-1 protease; however, we observed three distinct maxima (Figure 2e). We were able to reproduce this result three times for active HIV-1 protease and found an essentially identical profile for the inactive [D25N] chemical analogue of the enzyme,

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10.1021/ja806526z CCC: $40.75

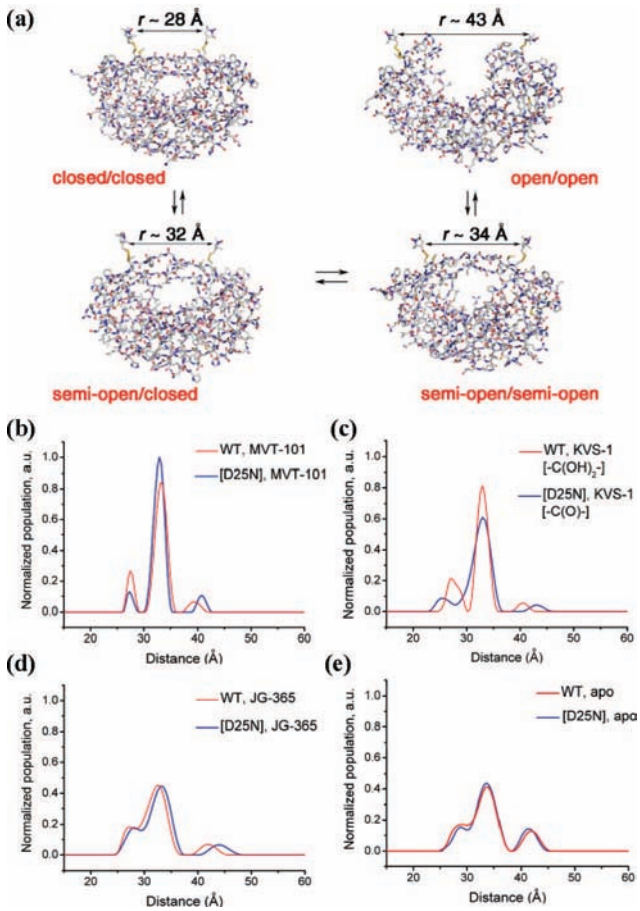

Figure 2. (a) Snapshots of the various conformational states of the HIV-1 protease "flaps". Mean nitroxide-to-nitroxide O···O distance is depicted for each state. (b−d) DEER distance profiles extracted using DeerAnalysis2008 software[8] for complexes with inhibitors and (e) for inhibitor-free HIV-1 protease. Red line = wild-type HIV-1 protease; blue line = [D25N] analogue. See Supporting Information for more details.

indicating that products of autoproteolysis (up to 5−10% by reversed-phase HPLC analysis) that appear when folding and handling samples of the active enzyme do not interfere with the measurements. Remarkably, recent full-atom explicit water MD simulations for the unliganded "apo" form of HIV-1 protease reproduced our experimental results, showing three maxima.[9] It is possible that insufficient *S/N* in the previous measurements hindered the authors in fitting their data for the unliganded enzyme with a profile containing three major conformers; however, this does not undermine the importance of their innovative work.

It is widely assumed that, in complexes with inhibitors, the HIV-1 protease flaps adopt a "closed/closed" conformation. This belief has its origin in the numerous crystal structures determined for a variety of HIV-1 protease/inhibitor complexes, in which the flaps are observed to be closed over inhibitor molecules. Our data, however, do not support such a view for the enzyme complexed with inhibitors when in solution. Using flash frozen samples that represent snapshots of the solution state, we observed three distinct conformations of the flaps. The major peak (at ~32.6 Å) does not fit well to the "closed/closed" model for the two flaps, where the average distance is much shorter (~25−29 Å). Indeed, such a 25−29 Å conformer was observed in all three complexes with inhibitors, albeit sparsely populated, with the third (minor) peak at >40 Å corresponding to an ensemble of "open/open" conformers. We thus interpret the major peak at ~32.6 Å in all three inhibitor complexes as an asymmetric "semiopen/closed" conformational state. Indeed, such a conformer has been observed by X-ray crystallography with the same crystallization conditions as those used to obtain crystal structures of the "closed/closed" conformer.[10] In the unliganded form of the HIV-1 protease, the major peak is at a distance of ~34 Å and corresponds to a symmetric "semiopen/semiopen" conformational ensemble.

The intrinsic asymmetry of the two domains of HIV-1 protease complexed with the peptidomimetic inhibitor KNI-272 has been observed previously by NMR; moreover, the two domains were found to have different dynamic properties.[11] However, these studies were aimed at the dynamics on a sub-ns time scale, which is not indicative of large-scale motions such as opening and closing of the flaps. In the unliganded enzyme, large scale flap opening−closing events occurring on the μs−ms time scale were experimentally elucidated by NMR;[2] such a long time scale explains why such events are rarely observed in explicit water MD simulations. When the HIV-1 protease is complexed with inhibitors, the large-scale dynamics should attain even slower rates, which makes it more difficult to study with MD methods in explicit solvent. Interestingly, however, in a recent MD simulation of the HIV-1 protease complexed with a urea-based inhibitor (i.e., lacking water 301), a dynamic ensemble of "closed/semiopen" conformers was observed, where on average only one flap is H-bonded to the inhibitor at any given moment.[12]

Overall, these results suggest that at the different stages of the catalytic reaction the flaps are likely to adopt different dynamic properties. In the "earlier" transition state and the tetrahedral intermediate, a rigid conformation with stable H-bond(s) is preferred, while in the "later" transition state the flaps become more flexible (similar to when without inhibitor), possibly assisting in product release. A dominant "closed/semiopen" conformer is in agreement with a previously suggested hypothesis and associated experimental data, where a mechanistic role of flap−substrate interactions is to stabilize a transition state via formation of H-bonds and where interaction with the backbone −NH− of Ile50/50′ of one flap only is needed to retain nearly wild-type enzyme catalytic activity.[13]

Acknowledgment. This research was supported by the Office of Science (BER), U.S. DOE, Grant No. DE-FG02-07ER64501.

Supporting Information Available: Synthetic procedures, analytical data, molecular modeling and MD simulations, details for pulse-EPR experiments and data analysis, and additional comments. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA806526Z