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# Quantized Water Access to the HIV-1 Protease Active Site as a Proposed Mechanism for Cooperative Mutations in Drug Affinity

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Supporting Information

**ABSTRACT:** The development of resistance to different drugs remains a major problem for a wide range of infections. In particular, combinations of specific mutations, which individually demonstrate no effect, exhibit significant cooperativity. Here we show that changes to the energy of ligand binding in different resistant HIV-1 proteases are correlated with the creation of water binding sites in the active site. This correlation is conserved across two drugs (ritonavir and lopinavir). We propose that individual mutations induce changes in flap packing that are insufficient to allow water binding but in combination allow access, leading to the observed cooperative resistance.

The human immunodeficiency virus (HIV-1) protease (PR) has been exploited extensively as a drug target, with a large number of PR inhibitors approved by the Federal Drug Administration (for reviews, see refs 1 and 2). The usefulness of PR inhibitors is limited by the emergence of drug resistant variants. These mutations occur at a wide range of different positions, both proximal and distal to the ligand/drug binding site, and several mutations have been shown to behave cooperatively. Specifically, Ohtaka et al. determined that pair mutations in the flap region and dimerization surface individually induce negligible alterations to the binding of different drugs but in combination with a pair mutation at the active site significantly reduce the level of binding.[3] The mechanism of this cooperation is obscured by the nature of the mutations, which exclusively swap hydrophobic residues for other hydrophobic residues, and would not be expected to alter the net or surface charge. The closely related inhibitors ritonavir (RTV) and lopinavir (LPV) (Figure S1 of the Supporting Information) occupy similar binding orientations in the enzyme active site. Here we describe the analysis of simulations of lopinavir that reproduce correct binding energies (including cooperative behavior), revealing the creation of specific water pockets, which induce realistic changes in interaction strength as determined by a coarse grained representation of the free energy of binding, based on molecular mechanics Poisson−Boltzmann solvent accessible surface area calculations (MMPBSA[4]). We extend our approach to simulations of RTV and find that energies of binding are not accurately reproduced if the structure does not deform to allow water access. We exploit the similarities of the LPV and RTV backbones to generate new RTV-bound systems that have undergone appropriate changes in structure and run short molecular dynamics (MD) simulations. These systems do not revert to the water-excluded wild-type (WT) state and show sensitivity to mutants, demonstrating a correlation between the experimental binding energies and water access. This presents a testable hypothesis, which may be applied to other mutants. Our results show the importance of specific water binding and suggest a common mechanism of cooperative resistance for these related drugs. Furthermore, our findings are in good agreement with the proposed substrate−envelope hypothesis of drug binding in HIV, where specific mutations alter the shape of the binding pocket leading to the drug molecules fitting less well and binding less strongly.[5,6]

Homology models of six HIV-1 PR variants were built and simulated using MD approaches, following the resistant forms characterized by Ohtaka et al.[3] Simulations were performed with NAMD2[7] and the AMBER ff03 force field[8] (see the Supporting Information for methodological details). The sequences considered were WT HXB2 and various combinations of pair mutations in different specific regions: active site (AS, V82A/I84V), flap region (FL, M46I/I54V), and dimerization interface (DM, L10I/L90M) (Figure S4 of the Supporting Information). Isothermal calorimetry experiments determined that in isolation DM and FL mutations had negligible effects on the free energy of binding of a range of drugs, while AS mutations had a small effect. In contrast, a four-point mutation (QM, consisting of AS and FL mutations) demonstrated cooperativity between the pair mutations leading to a superadditive change in the free energy of binding. A six-point mutation (HM, with all pair mutations from AS, DM, and FL) further demonstrated this cooperativity, despite the distance between the mutations in space. Simulations of these mutations revealed that the predicted free energies of interaction reproduced this cooperativity in PR−LPV complexes.[9] Here we propose an experimentally testable mechanism for this superadditive behavior and test this mechanism by extending the simulations to include RTV.

Visualization of the PR−LPV simulations of these complexes reveals that structural rearrangements in the PR over the course of the simulation lead to the creation of new specific water binding sites in the ligand binding cleft. VolMap analyses in

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VMD[10] after fitting to the drug diaminoalcohol backbone show that new water pockets develop with high occupancy (≥80%) in individual simulations, after fitting to the drug backbone atoms. In addition to the conserved "flap water"[11] included in this simulation, pockets of tightly bound water could be identified in the HM PR neighboring the two secondary amine groups of the drug [labeled HAD and HAC (Figure 1)], as well as deep penetrating waters interacting with the central hydroxyl group and catalytic aspartic acid residues [labeled O4 (Figure 1), adjacent to AS mutants]. Water pockets are present but less common in QM simulations, and absent from WT simulations. Water entry occurs from bulk to pockets that interact with carbonyl oxygen atoms from glycine (HAD/HAC) and catalytic aspartate residues [O4/HAD (Figure S3 of the Supporting Information)]. To quantify this analysis, hydrogen bonding patterns of the two amine groups and hydroxyl group and neighboring water molecules were measured (Tables S1 and S3 of the Supporting Information). These reveal that the free energy of binding corresponds closely with water access to the backbone amine and alcohol groups (Figure 1), and specifically, enhanced access to the HAD amine group and adjacent backbone hydroxyl is correlated with larger changes in free energy. We suggest that the DM and FL mutations cause insufficient perturbation to the structure to allow water access individually, but the AS mutation overcomes this energy barrier. Once this barrier is overcome (i.e., in the presence of the AS mutant), the mutations increase water access and thus reduce the level of drug binding.

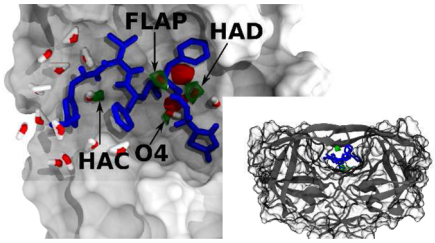


**Figure 1.** Water access to the drug binding cleft, with high-occupancy water pockets rendered as green isosurfaces overlaid onto a snapshot of the protein surface (white, excluding flaps, with catalytic aspartate residues colored red), drug (blue), and specific water molecules [red/white licorice; labeled individual water binding sites (HAD, HAC, O4, and FLAP) are defined in text] (protein topology inset).

It has been previously suggested that some resistance mutations induce alterations in the free energy of ligand binding by small motions that subtly repack the hydrophobic core through isoenergetic slippage, leading to changes in flap behavior.[12] Similarly, flap tip dynamics has been suggested as a mechanism of drug resistance,[13] and we may expect specific water binding to influence motions of the flap regions. Fitting the trajectories to the $\alpha$-carbon backbone reveals that all mutant PRs undergo small flap rearrangements relative to WT. These small alterations of the packing of the flaps around the drug indicate that individual DM and FL mutations fail to undergo changes that are sufficient to allow water access to the drug. In contrast, AS, QM, and HM mutations allow differential access to the hydrophobic core by opening the flaps to varying degrees, allowing individual water molecules to access different water sites defined by the drug and the protein surface.

Experimentally, the cooperative effects of these pair mutations have been found in RTV and LPV binding. The drugs share a common diaminealcohol backbone, and thus, it would seem likely that the mechanism of cooperative resistance for both drugs would be conserved. To test this, we applied the same approach to simulations of RTV complexes as previously applied to LPV complexes (50 × 6 ns simulations).[9]

The ensemble protocol failed to reproduce the experimentally determined changes in the free energy of binding of RTV between the WT and HM forms (Table S2 and Figure S5 of the Supporting Information). Further examination revealed that there are no rearrangements in flap location, or increased water access, which had been observed in the simulations of the PR–LPV complex. This unexpected result supports our hypothesis that the changes in the free energy of binding due to resistance mutations arise from flap rearrangement and specific water access, in that in these simulations a failure to undergo conformational changes in the flaps has prevented water entry and any changes in the energies of binding. Extension of individual simulations to 20 ns (for the HM system) fails to allow water entry. It does raise the question of whether the entry of water into the RTV binding cleft would be sufficient to account for cooperative resistance. To test this, we built a new set of conformations of RTV in complex with WT and HM HIV-1 PR, based on the conformation of each PR–LPV complex after 3 ns. In these "swap" simulations, RTV was exchanged for LPV based on shared diaminoalcohol backbone atoms with clashing water molecules removed. After a short energy minimization, 2 ns MD simulations were performed on the new WT, FL, DM, AS, QM, and HM complexes and the resultant data were analyzed. To demonstrate the independence of simulations from their starting conformations, we also reverted AS and HM forms to WT. In each case, the level of water binding at the HAD site was reduced to WT levels.

MMPBSA calculations performed on the trajectories reveal that in the new simulation systems the WT energies remain unchanged in the swap simulations compared to the standard methodology (Table S2 of the Supporting Information). In contrast, the MMPBSA energies calculated over the AS/QM/HM swap simulations show a large decrease in binding energy compared to the WT energies and HM simulations performed using the standard methodology. While the correlation of the predicted binding energies with experimental results for mutants is good, the absolute values are different, preventing cross drug comparison. In contrast with this, both RTV and LPV simulations show strong correlation between the water hydrogen bond frequency (defined here as the number of water hydrogen bonds per frame) at the HAD site and the energies of binding, which generate linear regressions with nearly identical slopes (Figure 2). Exploiting this similarity, we combined the RTV and LPV hydrogen bond data by adding a correction term to the measured energies based on the differences in the intercept of the regression. This gave a single data set showing high ($R$ = 0.94) correlation with experimental energies and confirms that the mechanism of resistance through quantized water access and contribution of water hydrogen bonding to the binding free energy is shared between the two drugs. In both LPV and RTV swap simulations, we observe exchange of water from binding sites to the bulk.

Our results demonstrate the role of water binding in the mechanism of drug resistance in the HIV-1 PR. The role of water molecules in drug binding has been previously characterized in a wide range of other systems, but the superadditive effect of mutants observed here is novel and in agreement with experimental observations on highly resistant

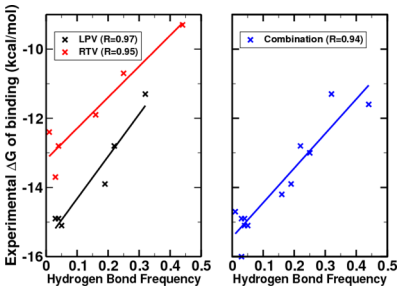


**Figure 2.** Correlation of HAD hydrogen bond frequency (number of water hydrogen bonds per frame, with a standard deviation of bootstrapped mean values of <0.01 in all cases) with the experimentally determined free energy of binding.

mutant forms of the PR that show an increased level of binding cleft opening and flap mobility, which may reflect increased water access.[14,15] It is further supported by recent experimental−theoretical approaches that indicate multidrug resistant forms of the protease have higher desolvation energies.[16] We expect that increased water access preferentially alters binding selectivity over following the substrate envelope hypothesis. Explicit solvent MD-based approaches are uniquely able to offer novel insights into such structural and dynamic behavior through our ability to observe the stochastic entry of single water molecules into the gaps between the inhibitor and active site created by mutation. These results additionally highlight new issues concerning MD-based approaches for drug design. The efficiency of sampling allowed by the ensemble approach taken here is required for identification of the individual water binding sites because of the stochastic nature of water entry; individual simulations even in the HM systems do not uniformly display water entry, which could prevent observation in a single simulation. Additionally, the predicted energies of binding are highly dependent on the equilibration of the system, allowing the possibility of water entry. Finally, structurally defined metrics are sufficient for predicting drug binding energies relative to different resistance mutations and to one another, while the energies calculated by MMPBSA are too inaccurate to be useful. The data demonstrate the importance of water in drug resistance and offer new insights into how point mutations behave cooperatively in drug resistance.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Tables of water binding frequencies and calculated energies, drug structure, and detailed methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

B.A.H. and D.W.W. contributed equally to this work.

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### Notes

The authors declare no competing financial interest.

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