The p*Ka* Shift of the Catalytic Aspartyl Dyad in the HIV-1 Protease Complexed with Hydroxyethylene Inhibitors

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The protease of human immunodeficiency virus 1 (HIV-1) plays an essential role in maturing infectious HIV particles.[1,2] Its structure and function have been well characterized to develop potent inhibitors as the therapeutic agent for AIDS.[3,4] The HIV-1 protease belongs to the class of aspartic proteases, and contains a pair of catalytic Asp residues. In the active homodimeric HIV-1 protease, the catalytic aspartyl dyad (Asp25 and Asp125) is located at the subunit-subunit interfacal region, which is at the base of the substrate binding site.[5,6] It is known that the HIV-1 protease is active in such conditions as one of the aspartic residues is protonated, which implies that the protonation state of the Asp dyad is the key to understand the catalysis mechanism.[7] Upon binding a substrate or an inhibitor into the active site, the water molecules at the active site are displaced and the dyad carboxyl groups may make contacts with the bound molecule. As a consequence, pK_as of the dyad would be altered.

A number of theoretical investigations focused on the protonation states of the dyad. Molecular dynamcis simulations were indirectly used to study the protonation states either by following the carboxyl hydrogen trajectory in relation to electronegative atoms located around the hydrogen or by examining structural amenability with an assumed protonated state.[8] Free energy simulations were performed to investigate feasible hydrogen positions by reproducing experimentally determined binding constant values.[9,10]

A direct approach to evaluate pK_as of ionizable groups embedded in a protein is to use continuum electrostatic models. A modified Tanford-Kirkwood model was used to predict the pK_as of the aspartyl dyad to be 4.0 and 4.1 in the HIV-1 protease.[11] The Poisson-Boltzmann (PB) method provides more detailed treatment of molecular structure than the modified Tanford-Kirkwood model. Trylska *et al.* used the PB model to examine the protonation states of the Asp dyad in the free HIV-1 protease and in inhibitor complexes.[12] They tested a variation of the PB models on MVT-101 (a basic secondary amine), KNI-272 (a peptiomemetic transition state analog), DMP-323 and XK-263 (cyclic ureas).

In this work, we utilize a version of the PB method[13] to calculate the pK_a shifts of the Asp dyad in the HIV-1 protease complexed with the transition state mimic hydroxyethylene inhibitors. The pK_a shift is interpreted as the extent of stabilization of a protonation state of the Asp dyad, which would provide a lead in understanding the cleavage mechanism of the protease. We have selected the hydroxyethylene inhibitor and HIV-1 protease complexes of which the X-ray crystallographic coordinates are available on Protein Data Bank (PDB). The coordinate set are for the apoenzyme (1G6L)[14] and complexes of AAF-Ψ[CHOHCH$_2$]-AAV-OMe (1AAQ),[15] Ac-SLNF-Ψ[CHOHCH$_2$]-PIV-OMe (7HVP)[16] and VSQLN-Ψ[CHOHCH$_2$]-VIV (8HVP).[17]

The hydrogen bonding feasibility of titrable residues (His, Glu, Asp, and Lys) is examined by positioning hydrogens wherever possible into the heavy atom structure of 1G6L.[10] 12 Lys residues are set to have ε-NH_3^+ and two histidine residues, His69 and His169, are assumed to take the δ-N hydrogenated form. All Glu and Asp residues are set to be carboxylate except the catalytic Asp dyad. Four possible mono-protonated states of the dyad are considered in the pK_a shift calculations. After all residue types are determined, CHARMM HBUILD function is used to place hydrogen atoms into the heavy atom coordinates from PDB. With coordinates of heavy atoms fixed, the Adopted Basis Newton-Raphson minimization method is applied to optimized hydrogen atoms into the most stable structure. The energy minimization is performed until the root mean square of energy gradients reaches 0.005 kcal/molÅ.

The pK_a shift is the relative pK_a value to pK_a of the aqueous solution and is proportional to the difference between the free energy change of the protonated and unprotonated states in protein and that in solution.

$$\Delta pK_a = -\frac{\Delta\Delta G}{2.303RT} \quad (1)$$

R is the gas constant and T is the absolute temperature. We assumed the room temperature, 298.15 K. $\Delta\Delta G$ is evaluated as the difference in the electrostatic component of free energy changes upon protonation in protein and in solution.[13]

$$\Delta\Delta G = (\Delta G^{p}_{protein} - \Delta G^{u}_{protein}) - (\Delta G^{p}_{solution} - \Delta G^{u}_{solution}) \quad (2)$$

The superscript "p" designates the protonated state and "u" does the unprotonated state.

The PBEQ module of CHARMM is used to solve the PB equation for each protonation state of the catalytic Asp dyad. The HIV-1 protease is put in a 60 × 60 × 60 cubic grid box with the grid placed every 0.5. The electrostatic potential is calculated at each grid point. We employ the dielectric constant of 80 for the solvent water and 4 for the interior of protein. The atomic radii are set to the values derived from

the average radial charge distribution function by Roux *et al.*[18] and the atomic partial charges are taken from the all-hydrogen parameter set of CHARMM.[19]

The pK_a shifts of all possible mono-protonated states of the Asp dyad in HIV-1 proteases including an apoenzyme and three hydroxyethylene binding complexes are calculated with the PB method and shown in Table 1. pK_a of the Asp residues is increased as much as 5.24 in the free HIV-1 protease (1G6L). The shift is obtained when the O_1 atom of Asp25 is protonated. It means that stability of the protonated Asp over the unprotonated Asp is increased by 7.2 kcal/mol as compared to that in aqueous solution. In our calculation, Asp25 and Asp125 show a little difference in the pK_a shift and the equivalent carboxyl oxygen atoms of an Asp residue. O_1 and O_2, seem to be placed in unequal environment. The breakage of symmetric pK_a shifts is due to asymmetric orientation of water and/or inhibitor molecules in the active site of the protease.

The active site water in 1G6L and the hydroxyl group of the hydroxyethylene moiety in the HIV-1 protease complexes function as the hydrogen bond donor and/or acceptor. The hydrogen bonding interactions enhance the stability of the mono-protonated Asp dyad and result in positive pK_a shifts. The tightly bound inhibitors position the hydroxyethylene hydroxyl group closer to the carboxyl dyad and generate stronger electrostatic interactions to the dyad than the loosly bound water molecule does. As a consequence, we observe larger pK_a shifts in the complexes than in the apoenzyme.

In a series of controlled calculations, pK_a shifts are evaluated for the HIV-1 proteases with an active site water (1G6L) and the inhibitor (1AAQ, 7HVP and 8HVP) removed from the structure. The results are collected in Table 2 to be compared to the values in Table 1.

Similar pK_a shifts are obtained for all "free" HIV-1 proteases, ranging from 2.80 to 4.59. Our results suggest that the catalytic Asp is more basic than Asp in an aqueous solution by more than three units in the pK_a scale. It is likely that one of the dyad Asps is protonated at the physiological pH. We also find that the transition state minic hydroxyethylene inhibitors enhances the basicity of the Asp dyad greater extent than the water molecule does at the active site.

Table 1. The pK_a shifts of the catalytic Asp dyad in HIV-1 proteases

Protonated Site	1G6L	1AAQ	7HVP	8HVP
Asp25 $O_{\delta 1}$	5.24	11.38	9.99	13.03
Asp25 $O_{\delta 2}$	2.47	9.91	7.79	11.07
Asp125 $O_{\delta 1}$	4.02	9.71	11.13	11.98
Asp125 $O_{\delta 2}$	1.96	6.43	8.29	9.88

Table 2. The pK_a shifts of the catalytic Asp dyad in "free" HIV-1 proteases[a]

Protonated Site	1G6L	1AAQ	7HVP	8HVP
Asp25 $O_{\delta 1}$	4.33	4.43	3.89	3.28
Asp25 $O_{\delta 2}$	4.09	3.77	3.29	2.81
Asp125 $O_{\delta 1}$	4.35	4.59	3.35	3.31
Asp125 $O_{\delta 2}$	4.12	3.98	2.99	2.98

[a]The active site crystallographic water of 1G6L and inhibitors of other HIV-1 proteases are removed from the structures used in the PB method.

While the current set of methodological parameters, *i.e.*, the dielectric constants, atomic radii and the molecular mechanical potential, would not be accurate enough to yield the absolute pK_a value of a titratable residue, the PB method is useful to evaluate the stability of a protonation state on a semi-quantitative scale. Considering the limited accuracy of molecular mechanical parameters and assumptions entailed in the PB method, we would not be able to determine the preferable protonation site of the Asp dyad, although we see the difference in pK_a shifts. However, our results warrant the following thermodynamic arguments. The Asp dyad of the apoenzyme is likely in a mono-protonated state and hydrogen donor-acceptor ability is enhanced in a transition/intermediate state of the proteolytic cleavage mechanism of the HIV-1 protease. There have been lengthy elaboration on the mechanism by which the catalytic Asp dyad cleave peptide bonds. Our result is strongly correlated and supports the general acid - general base mechanism.[8] A kinetic appreciation and more quantitative assessment of intermediate states in the protease catalysis process would be possible in a reaction path calculation using the combined quantum and molecular mechanical potentials.[20]

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