# Relative Binding Free Energies of Peptide Inhibitors of HIV-1 Protease: The Influence of the Active Site Protonation State

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Hydrogen bonding plays an important role in the stabilization of complexes between HIV-1 protease (HIV-1 PR) and its inhibitors. The adequate treatment of the protease active site protonation state is important for accurate molecular simulations of the protease-inhibitor complexes. We have applied the free energy simulation/thermodynamic cycle approach to evaluate the relative binding affinities of the *S* vs *R* isomers of the U85548E inhibitor of the protease. Several mono- and diprotonation states of the catalytic aspartic acid residues of the protease active site were considered in the course of molecular simulations. The calculated difference in binding free energy of the *S* vs *R* isomers strongly depended on the location of proton(s), but in all cases the binding free energy of the *S* inhibitor was higher. On the basis of our calculations, we propose that in the HIV-1 PR–inhibitor complex only one catalytic aspartic acid residue is protonated and that the binding free energy of the *S* isomer is ca. 2.8 kcal/mol higher than that of the *R* isomer. The accuracy of these predictions shall be evaluated when binding affinities of both isomers become available.

## Introduction

HIV-1 protease (HIV-1 PR) catalyzes the conversion of a polyprotein precursor encoded by *gag* and *pol* genes to mature proteins needed for the production of infectious HIV particles.[1] Inhibition of this process is regarded as a promising approach for the treatment of AIDS. The structure of the HIV-1 PR was recently resolved by X-ray crystallographic methods.[2] The protease is a symmetrical dimer, and each monomer consists of 99 residues. The active site includes two catalytic aspartic acid residues, one from each chain (designated as Asp25 and Asp125, according to the nomenclature of Jaskolski et al.[3]). It is believed that during catalysis the catalytic water molecule attacks the carbonyl carbon of the peptide bond of a substrate while the carbonyl oxygen accepts the proton from one of the catalytic aspartic acid residues.[4] This mechanism leads to the formation of the tetrahedral transition state of the peptide substrate. Catalytic studies also suggested that in the transition state one of the catalytic aspartic acid residues exists in the neutral form whereas another residue is negatively charged.[4]

The transition-state-mimetic approach has produced numerous highly potent peptide inhibitors of HIV-1 PR in recent years.[5-8] Many of these compounds incorporate a hydroxyethylamine moiety that mimics the transition state of peptide substrates of the protease and interacts with the carboxyl groups of the catalytic aspartic acid residues of the protease. The hydroxyethylamine moiety also introduces a new chiral center into the polypeptide backbone of the inhibitors, and the configuration of this active center may substantially influence the activity of the inhibitors.[8,9]

The presence of the two aspartic acid residues in the protease active site and the existence of the unusual chiral center in the backbone of protease inhibitors raises two important questions that shall be addressed in the course of rational inhibitor design: what is the protonation state of the catalytic aspartates upon binding the inhibitor, and how strongly does the configuration of the chiral center influence the binding affinity of the inhibitors? In principle, both questions may be addressed experimentally. Neutron diffraction experiments should be able to determine the location of a proton on one of the carbonyl oxygen atoms of the aspartic acid side chains; however, this has not been done yet. The role of chirality in the binding affinity of the inhibitors can be studied by stereospecific synthesis of the inhibitors followed by experimental evaluation of binding constants. However, relative binding constants of stereoisomers of only a few inhibitors have been reported so far.[6,8]

Molecular simulations may provide more detailed information about the nature of the protease–inhibitor interactions. When Swain et al.[10] first solved the X-ray crystal structure of the complex of the protease with the JG365 inhibitor, the relative binding constants of the two isomers of the inhibitor were not known yet. Free energy simulations were performed in order to estimate the ratio of the binding constants of the *S* and *R* isomers of JG365 by two independent groups.[11,12] Both groups predicted that the *S* isomer formed a decisively more stable complex with HIV-1 PR. While the calculations were in progress, the experimental binding affinities of the *S* and *R* isomers of JG365 became available.[8] In both theoretical studies,[11,12] the calculated values of the relative binding free energies of the *S* vs *R* isomers agreed fairly well with the experimental results. The free energy perturbation method has been also successfully applied to estimate relative binding constants of other HIV-1 PR inhbitors,[13] as well as to several other ligand–receptor systems.[14,15] Although in most reported cases the researchers attempted to reproduce available experimental data, this method was also used recently to predict the relative binding free energy of a

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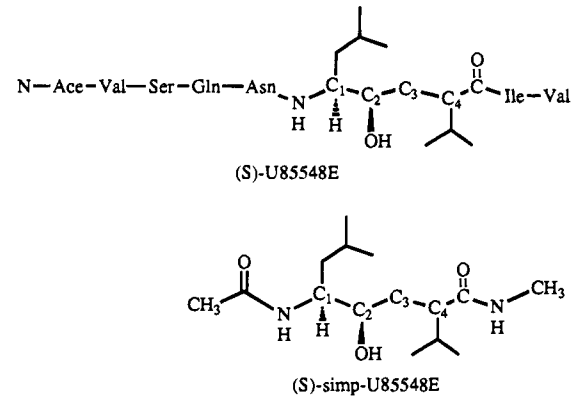


(S)-U85548E

(S)-simp-U85548E

**Figure 1.** The structure of the HIV-1 protease inhibitor U85548E (Ace-Val-Ser-Gln-Asn-LES-Ile-Val) and its simplified form simp-U85548E (Ace-LES-NMe) in the *S* configuration. The *R* isomer of LES (not shown) is called LER.

novel rigid HIV-1 PR inhibitor that has not been synthesized yet.[16]

Despite progress in molecular simulations and experimental studies of HIV-1 PR and HIV-1 PR–inhibitor complexes, the question of protonation state of the catalytic aspartic acid residues in the complex of the protease with its inhibitors remains controversial. On the basis of the commonly accepted mechanism of the protease action, it is believed that upon ligand binding one of the active site aspartates is protonated and neutral while the other one is negatively charged.[4,17] Recent detailed analysis of the geometry of the protease active site in its complex with U85548E inhibitor led to a similar conclusion.[3] Nevertheless, both aspartic acid residues were treated as neutral species in the first reported unconstrained molecular dynamic simulations of the free enzyme in aqueous solution[18] and in recent simulations of protease–inhibitor complexes.[19] Only one aspartic acid residue was considered neutral in several simulations of protease–inhibitor complexes.[11,12] Finally, in recent extensive molecular dynamic simulation studies of the protease in aqueous solution with counterions, both aspartic residues were treated as negatively charged.[20]

In this paper, we have applied free energy simulation approach in order to estimate quantitatively the effects of both the chirality of inhibitors and the protonation state of the catalytic aspartic acid residues on the relative binding free energies of *S* vs *R* isomers of U85548E inhibitor. U85548E is an eight residue peptide containing a hydroxyethylene moiety that mimics the transition state of substrate hydrolysis (Figure 1). This inhibitor has one of the highest absolute binding constant to the protease,[21] but we are not aware of any experimental studies that provide information about the difference in binding affinities of the *S* and *R* isomers of U85548E. The complex of this inhibitor with the protease was characterized by X-ray crystallography, where the inhibitor appears to have the *S* configuration.[3] Different mono- and diprotonated states of the catalytic aspartic residues were considered in the course of our simulations. We find that the location of proton(s) substantially influences the calculated difference in binding constants of the two stereoisomers. However, we show that the *S* isomer forms a decisively more stable complex with the protease regardless of the protonation state of the aspartic acid residues. On the basis of these calculations and the comparisons between binding of U85548E and JG365 with the protease, we also suggest that the protease is monoprotonated in its complexes with the two inhibitors.

## Computational Methods

**Molecular Models and Force Field Parameters.** The coordinates for the X-ray structure of the HIV protease–U85548E complex were kindly provided by Dr. A. Wlodawer (presently available as the 8HVP entry from the Brookhaven Crystallographic Database). All calculations in this paper were performed using the Cedar molecular mechanics and dynamics package (developed by Professor J. Hermans at University of North Carolina.) with an all-atom force field. Cedar force field uses the same nonbonded parameters as the GROMOS force field[22] and the bonded parameters that were developed independently.[23] In order to facilitate calculations, U85548E was modeled in a simplified form (simp-U85548E) in the simulations of unbound inhibitor (Figure 1). The force field parameters for the nonstandard residues LES and LER (which are isomers of each other) were based on those of leucine and valine since structurally LES may be viewed as a combination of leucine and valine connected by hydroxyl ethylene bridge instead of an amide bond. The atomic charges of the hydroxyl were the same as for the hydroxyl in serine.

As mentioned above, the common belief is that only one of the catalytic aspartic residues in the protease active site is protonated,[4,17] although the diprotonation state of the aspartic acid residues was also considered.[19] Thus, *a priori*, in treating a monoprotonated system, due to nonsymmetrical structure of the protease/inhibitor complex, the proton can be placed onto any of the four oxygen atoms of the two carboxyl groups of the catalytic aspartates. In placing the proton, we followed the methodology, adopted in our earlier studies,[11] that relies on the accuracy of the crystallographic determination of the HIV-1 PR–U85548E complex. Thus, starting from the X-ray structure of the protease–inhibitor complex, we placed a proton successively at all four oxygen atoms and applied molecular mechanics minimization in order to optimize the location of both this proton and the proton of the inhibitor hydroxyl group with respect to the rest of the structure. We found (data not shown) that the lowest energy was achieved when the proton was placed at the OD1 atom of Asp125 (the names of the atoms correspond to those of Jaskolski et al.[3]). Consequently, this protonated form of Asp125 was used in all simulations. Similar considerations in treating the diprotonated HIV-1 PR–U85548E complex resulted in placing protons on OD1 of Asp25 and OD2 of Asp125 for the diprotonation model 1 and on OD2 of Asp25 and OD1 of Asp125 for the diprotonation model 2.

**Molecular Dynamic Simulations.** Molecular dynamic simulations were performed using the Cedar program. The SHAKE algorithm[24] was used to keep bond lengths constant, with the time step of 2 fs. The inhibitor and inhibitor–protease complex were solvated with SPC water,[25] and an 6 Å nonbonded interaction cutoff and periodic boundary conditions were used. (In our earlier studies[11] we found that this relatively low cutoff was sufficient to reproduce the experimentally observed relative binding free energies of the *S* vs *R* isomers of the JG365 inhibitor.) Average temperature of 300 K and pressure of 1 atm were kept constant by adjustment of the kinetic energy and the dimensions of the periodic box at each dynamic step.[25] The water molecules were initially equilibrated with immobile solutes for 10K steps of dynamic simulation. The geometry of the whole system was then optimized, followed by gradual heating of the system via equilibration for 10K steps at 100 K, 10K steps at 200 K, and 20K steps at 300 K. The unbound inhibitor simp-U85548E was solvated in a cubic box (25 × 25 × 25 Å) containing 488 SPC water molecules. The initial atomic coordinates of the simp-U85548E were based on those of equivalent atoms in the crystal structure of the inhibitor–protease complex. The crystal structure of the inhibitor (U85548E)–protease complex[3] contained 80 crystal water molecules; it was solvated in a rectangular box (64 × 42 × 46 Å) with additional 2233 SPC water molecules. During molecular simulations of the pro-

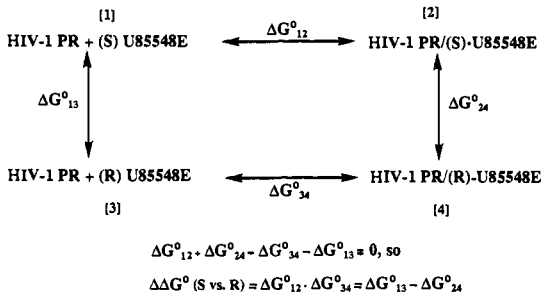


**Figure 2.** The scheme of the thermodynamic cycle used in this study. $\Delta G°_{12}$ and $\Delta G°_{34}$ represent binding free energies and can be obtained experimentally whereas $\Delta G°_{14}$ and $\Delta G°_{23}$ are computed by free energy simulations.

tease–inhibitor complex, only water molecules and neutral groups of the protease and inhibitors with at least one atom within an 8 Å sphere around all C-α carbon atoms of U85548E were allowed to move.

**Free Energy Simulations.** The free energy simulations/thermodynamic cycle approach was used to estimate the difference in binding free energy between the *S* and *R* isomers of the U85548E inhibitor. Figure 2 illustrates the scheme of the thermodynamic cycle. According to this scheme and the thermodynamic cycle concept,[26,27] the relative free energy of binding of the *S* vs *R* isomers with the protease was calculated as the difference of the free energies in two calculations in which LES residue was changed into LER residue, first in solution ($\Delta G_{13}$, Figure 2) and then in the protease-bound form ($\Delta G_{24}$, Figure 2). Following the strategy employed in our previous study,[11] we assumed that the use of the truncated form of the U85548E inhibitor (simp-U85548E on Figure 1) for the solution free energy calculations will not influence the calculated free energy change value. The individual free energy changes were calculated using the slow-growth method.[28] In this method, both interchanging residues (i.e., LES and LER) are represented. All equivalent atoms of the two residues (i.e., all atoms except those of the hydroxyl groups which are unique) are maintained at the identical positions during the whole simulation. The potential energy of the system is calculated with varying contribution from both residues by making it dependent on the special parameter, lambda ($\lambda$). All nonbonded molecular mechanics terms are multiplied by $\lambda$ for one residue and by $1 - \lambda$ for another, and $\lambda$ varies continuously in the molecular dynamic simulation from 1 to 0 (forward replacement) or from 0 to 1 (reverse replacement). Thus, at the end points (i.e., when $\lambda$ is equal to 0 or 1) the structure of only one residue is represented. The free energy difference between the two states is calculated from the following equation:

$$\Delta G = \sum_{i=1}^{N} \langle \partial E(\lambda)/\partial\lambda \rangle_i \Delta\lambda_i \qquad (1)$$

where *N* represents the number of steps and *E* is the potential energy of the system. Further details of the implementation of this method in Cedar are described by Yun and Hermans.[29]

In order to prevent undesired conformational changes while the system undergoes the transition from one inhibitor to another through physically unrealistic states, torsional constraints were applied to the backbone of simp-U85548E. The implementation of this routine in Cedar is described by Yun and Hermans.[29] The ranges for these constraints were obtained from unconstrained dynamic simulations carried out for both the *S* and *R* isomers of simp-U85548E for 20K steps with the corresponding torsional angles being monitored. The overlapping ranges of torsion angles of the *S* and *R* inhibitors characterize stable conformations for both inhibitors. During all simulations no torsional constraints were applied to the enzyme–inhibitor complex. The typical simulation cycle involved 20 ps of equilibration of the whole system, 200 ps of the "forward" molecular replacement (i.e., from *S* to *R* inhibitor), 20 ps of equilibration, and 200 ps of the "reverse" replacement (i.e., from *R* to *S*). This cycle was repeated two times to obtain an estimate of the reproducibility of simulation.

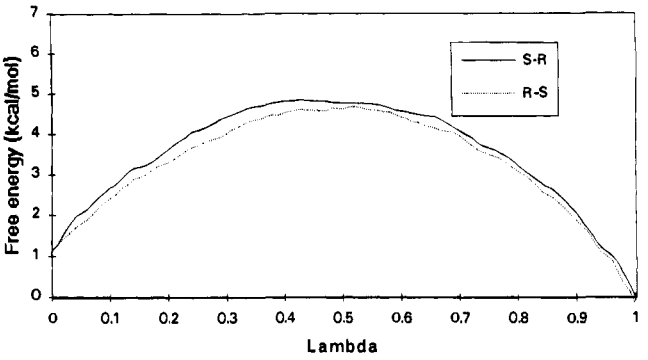


**Figure 3.** The progress of the free energy in transformation of the *S* isomer of simp-U85548E (at $\lambda = 1$) to the *R* isomer of simp-U85548E (at $\lambda = 0$) (solid line) and back (dotted line) in solution.

## Results

**Free Energy Changes between the Two Configurations of Simp-U85548E in Solution.** The unconstrained molecular dynamic simulations of the *S* and *R* isomers of simp-U85548E in solution resulted in the following overlapping ranges of the main chain dihedral angles (see Figure 1 for the atom nomenclature):

ACE(C)–LES(N)–LES(C1)–LES(C2)
from −175° to −65°

LES(N)–LES(C1)–LES(C2)–LES(C3)
from 15° to 105°

LES(C1)–LES(C2)–LES(C3)–LES(C4)
from −155° to 155° (i.e., 180° ± 25°)

LES(C2)–LES(C3)–LES(C4)–LES(C5)
from −105° to −25°

LES(C3)–LES(C4)–LES(C5)–NMe(N)
from 75° to 170°

These ranges were used to constrain the backbone conformation during the course of molecular replacement simulations. Two independent estimates of the free energy difference between *S* and *R* inhibitor in solution ($\Delta G_{13}$, Figure 2) were obtained; the typical progress of free energy in the course of these simulations is shown on Figure 3. The hysteresis (i.e., the difference in free energy of the forward and reverse simulations which ideally should be equal to zero) was small in both simulations (Table 1). This result indicates a good reversibility of the simulations. The results of the two independent evaluations of $\Delta G_{13}$ also agreed reasonably well (Table 1). From these simulations, the *S* isomer of simp-U85548E is ca. 1.3 kcal/mol more stable than the *R* isomer of simp-U85548E in water.

**Free Energy Difference between the *S* and *R* Configurations of Bound U85548E Inhibitor and Relative Free Energy of Binding of the Two Inhibitors.** The perturbations of the bound *S* into *R* U85548E inhibitor and back were performed for both mono- and diprotonated states of the protease. In each case, the calculations were repeated two times to estimate the reproducibility of the simulated free ener-

**Table 1.** Free Energy of Molecular Replacement and Relative Free Energy of Binding of *S* vs *R* Isomers of U85548E[a]

| molecular system | $\Delta G°$ of replacement (kcal/mol) $S \rightarrow R$ | $R \rightarrow S$ | average ± SEM ($S \rightarrow R$) | $\Delta\Delta G°$ of binding (*S* vs *R*) (kcal/mol) |
|---|---|---|---|---|
| simp-U85548E in solution | 1.13 | −1.32 | 1.3 ± 0.1 | |
| | 1.60 | −1.26 | | |
| HIV-1 PR−U85548E: monoprotonation model | 4.44 | −4.35 | 4.1 ± 0.2 | −2.8 |
| | 3.58 | −4.12 | | |
| HIV-1 PR−U85548E: diprotonation model 1 | 6.95 | −6.54 | 6.5 ± 0.2 | −5.2 |
| | 6.67 | −5.95 | | |
| HIV-1 PR−U85548E: diprotonation model 2 | 7.54 | −7.04 | 7.6 ± 0.2 | −6.3 |
| | 8.13 | −7.85 | | |

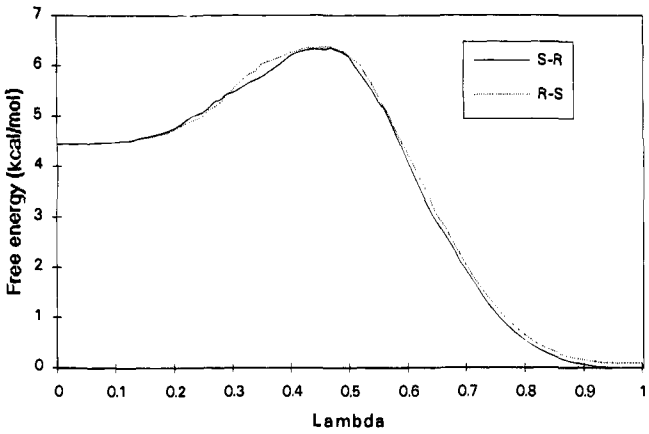


**Figure 4.** The progress of the free energy in transformation of HIV protease bound *S* isomer of U85548E (at $\lambda = 1$) to the *R* isomer of U85548E (at $\lambda = 0$) (solid line) and back (dotted line) for the monoprotonation model.

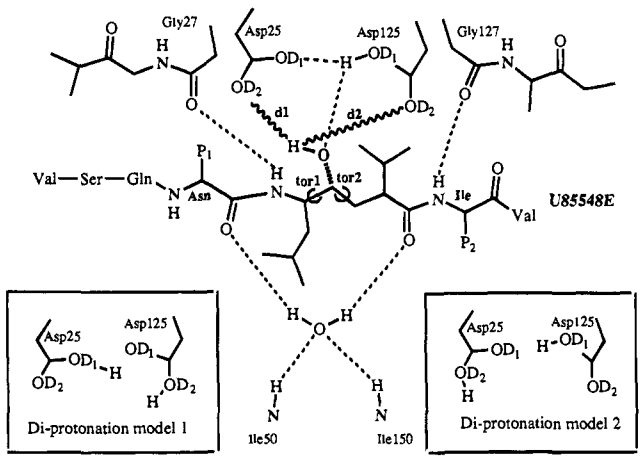


**Figure 5.** Schematic representation of the active site of HIV protease (monoprotonation model) with bound U8554E inhibitor. The distances d1 and d2 and torsion angles tor1 and tor2 are plotted vs simulation time in Figures 6 and 7. The diprotonation models 1 and 2 are shown on the lower left and the lower right insets, respectively.

gies. Figure 4 shows the typical progress of the free energy change for the monoprotonation model, and the results of the simulations for both mono- and diprotonation models are given in Table 1. These results indicate that the protonation state of the protease drastically influences the value of calculated free energy difference ($\Delta G_{24}$, Figure 2). These data were used then to calculate the relative binding free energy of *S* vs *R* isomers of U85548E inhibitor, according to the thermodynamic cycle of Figure 2. The calculations suggest (cf. Table 1) that the *S* isomer of U85548E binds more tightly to the protease than the *R* isomer of U85548E, regardless of the protonation state of the catalytic aspartic residues. However, quantitative estimates of the relative binding free energy of the *S* vs *R* inhibitor differ considerably (Table 1).

**Structural Comparison between *S* and *R* Isomers of U85548E Inhibitors Bound to the Protease.** Perturbation of the *S* isomer of U85548E inhibitor into the *R* inhibitor in the course of the "forward" molecular replacement calculations generates the (dynamically equilibrated) structure of the *R* inhibitor bound to the protease. We have performed two 100 ps unconstrained dynamics simulations of both complexes of *S* and *R* isomers of U85548E inhibitors with the protease, and the comparison of the results of these simulations offers some interesting suggestions as to why the former binds stronger than the latter. During these simulations the hydrogen bonds formed between the hydroxyl group of the inhibitors and the protease were monitored, as well as the two torsional angles of the backbone of the inhibitors, defined by rotation around two single bonds adjacent to the chiral hydroxyl (Figure 5). The hydrogen bond distance between the chiral hydroxyl and OD1 of Asp25 fluctuates around 1.5 Å for the *S* inhibitor−enzyme complex, but it swings back and forth between 1.5 and 4 Å three times during the simulation of the *R* inhibitor−enzyme complex (cf. Figures 6 and 7). Every time when a hydrogen bond is formed between the chiral hydroxyl and OD1 of Asp25 for the *R* inhibitor, the backbone torsional angles defined by single bonds adjacent to the chiral hydroxyl move away from their stable conformations (Figure 7). On the other hand, for the *S* inhibitor the backbone torsional angles remain near their initial values during the whole simulation (Figure 6). For the *R* inhibitor it is obvious that there is a conflict between the trend to form a hydrogen bond between the hydroxyl group of the inhibitor and the active site aspartic acid side chain and the trend to keep the backbone of the inhibitor in the stable conformation. These factors are probably responsible for the stronger interaction between the *S* inhibitor and the protease relative to the *R* inhibitor. From our simulations, this interaction is stronger by about 4 kcal/mol for the monoprotonation model and even more so for the diprotonation models (Table 1).

## Discussion

During the last decade, HIV protease has been a subject of extensive theoretical and experimental research. Many HIV-1 PR−inhibitor complexes were investigated by X-ray crystallography. These studies revealed that although binding of the inhibitors is accompanied by significant motion of the flaps of the protease, the structure of the protease in its complexes with inhibitors remains almost unchanged.[9] This feature of the HIV protease was successfully used recently

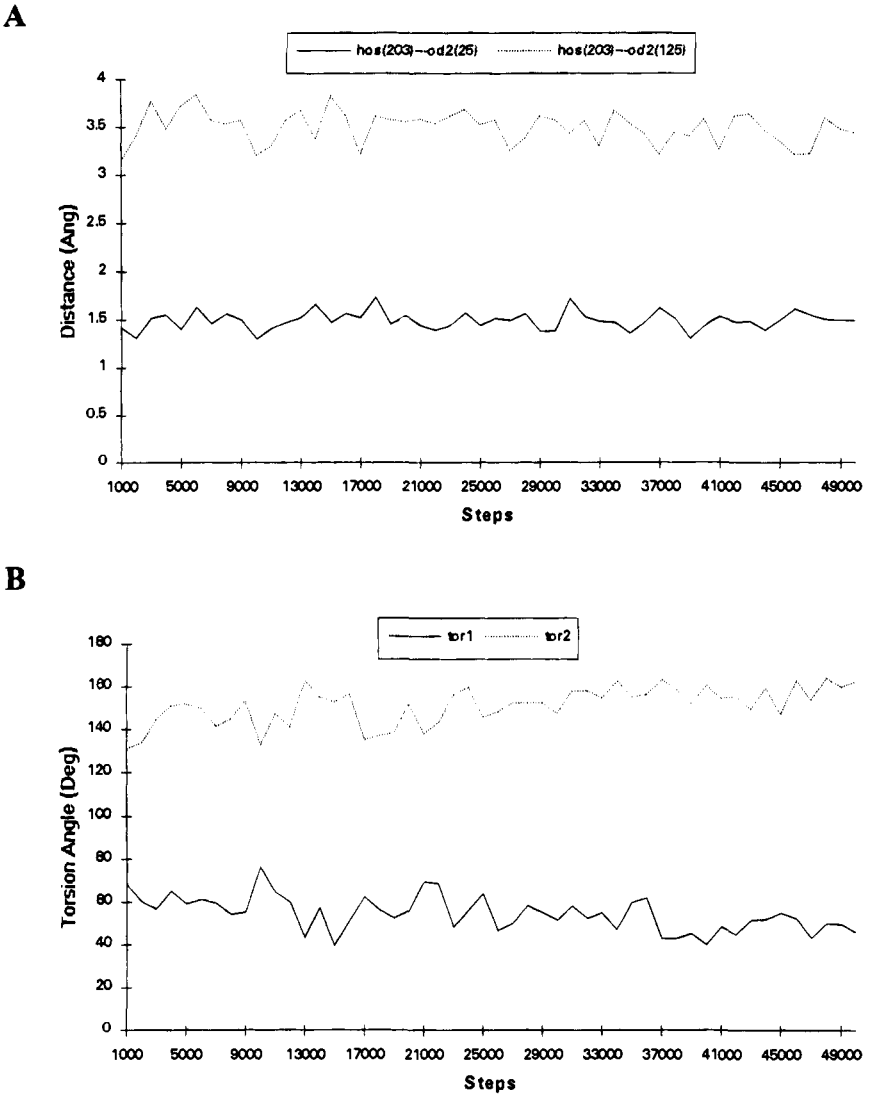


**Figure 6.** Conformational behavior of the *S* isomer of U8554E in the protease active site during 100 ps of unconstrained molecular dynamics simulation. (A) Distance changes between the hydroxyl hydrogen of the *S* isomer and OD2 atoms of Aps25 and Asp125 (cf. d1 and d2 on Figure 5, respectively). (B) Torsion angle changes (cf. tor1 and tor2 on Figure 5) of the *S* isomer.

in order to predict bound conformations of several protease inhibitors and use this prediction to generate a viable model of the protease inhibitors within the framework of comparative molecular field analysis (CoMFA).[30]

This structural feature of the protease justifies our use of the "active site" molecular dynamic simulations where only atoms located in the active site and in the vicinity of the active site are allowed to move during the course of MD simulations. So, we based our simulations on the notion that the protease active site is (relatively) inflexible, i.e., the geometry of the active site is about the same upon binding of both the *S* and *R* inhibitors. With this model, we further addressed the important question of the protonation state of the protease active site in its complexes with the inhibitors. We found that, regardless of the protonation state of the catalytic aspartic residue and distribution of proton(s) between side chain oxygen atoms, the *S* inhibitor (i.e., the one identified in the crystallographic structure[3]) binds tighter than the *R* inhibitor. However, quantitatively the difference in calculated binding free energy ranges from 2.8 to 6.3 kcal/mol (Table 1).

Free energy simulations have been considered traditionally as a computational technique that affords the quantitative estimates of the free energy difference between two molecular systems. However, from the structural viewpoint, this approach can be also considered as a tool for generating a new chemical structure in a very delicate way, while keeping the whole system equilibrated. Therefore, we suggest that our calculations predict the bound conformation of the *R* inhibitor from the structure of the *S* inhibitor and provide some explanation for the difference in binding constants. We show that unlike *S* inhibitor (Figures 6), the *R* inhibitor cannot form stable hydrogen bonds with the protease active site and oscillates between two conformations (Figure 7); neither one appears particularly favorable.

As we discussed earlier, most of the experimental studies and present knowledge about the mechanism of the protease action indicate a monoprotonated state of the protease in its complex with transition-state-analog inhibitors. Earlier, we were able to predict accurately the relative binding free energy of the *S* vs *R* isomers of JG365 inhibitor.[11] In that study, we assumed that the protease was monoprotonated, and we placed proton on that oxygen where the energy of

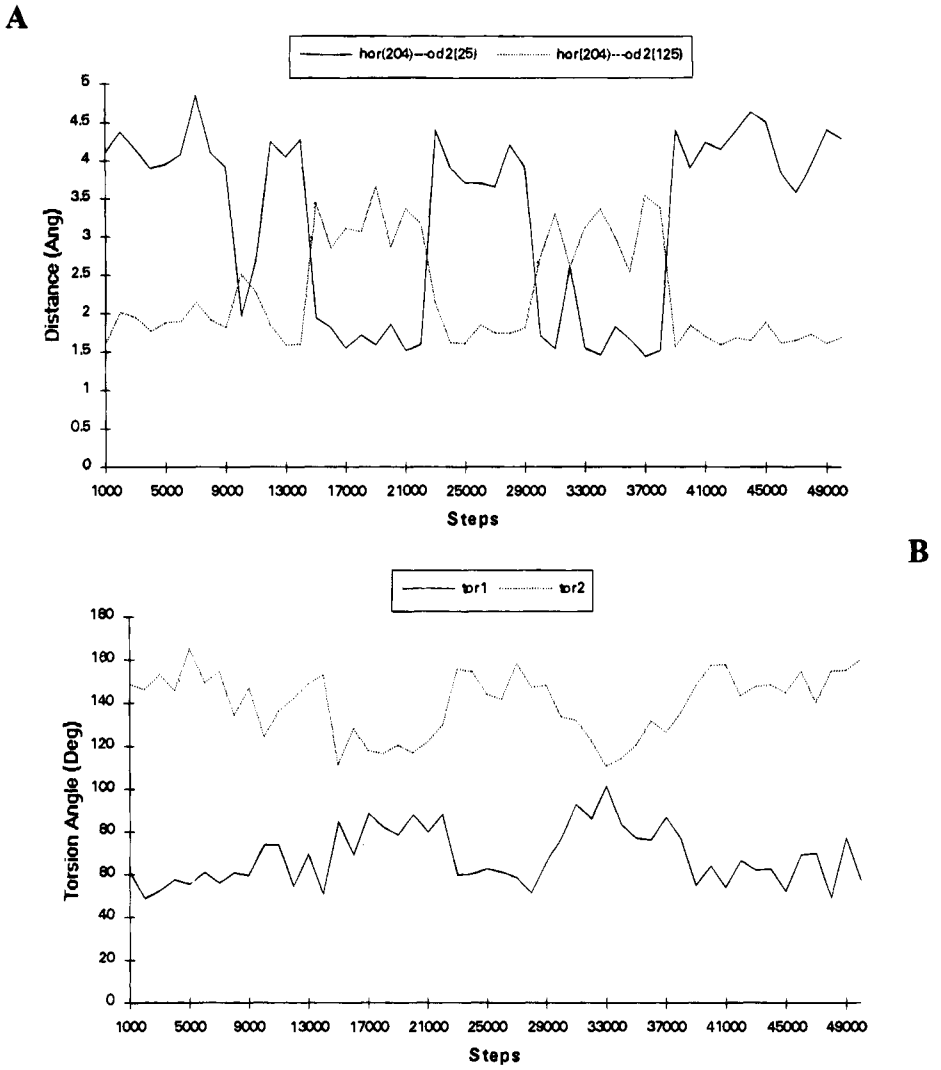


**Figure 7.** Conformational behavior of the *R* isomer of U8554E in the protease active site during 100 ps of unconstrained molecular dynamics simulation. (A) Distance changes between the hydroxyl hydrogen of the *R* isomer and OD2 atoms of Aps25 and Asp125 (cf. d1 and d2 on Figure 5, respectively). (B) Torsion angle changes (cf tor1 and tor2 on Figure 5) of the *R* isomer.

the protease–inhibitor complex was the lowest. Ferguson et al.[12] calculated the relative binding free energy of the *S* vs *R* isomers of JG365 for two locations of a single proton on the two catalytic aspartic acid residues as well as for dianionic system. They found that the location of the proton significantly influenced the calculated binding free energy difference, which ranged from 1.5 to 6.8 kcal/mol. However, only one location of a proton (the same that we used in our simulations[11]) generated the free energy difference that agreed with the experimental value.

The placement of a proton on OD1 of Asp125 in our monoprotonated model (Figure 5) is different from the one suggested by Jaskolski et al.[3] on the basis on their analysis of the active site geometry in the HIV PR/U85548E complex. Jaskolski et al.[3] reasoned that the proton should be located on the OD2 atom of one of the catalytic aspartic acid residues because placing it on the OD1 atom would require the other OD1 to accept three hydrogen bonds (from another protonated OD1, from Gly27, and from C–OH of the inhibitor). However, the hydrogen bond network in our model after the dynamic equilibration of the active site is different (Figure 5) in that the C–OH of the inhibitor forms hydrogen bond with the OD2 atom of Asp25, not with OD1 as suggested by Jaskolski et al.[3] This C–OH···OD2(Asp25) hydrogen bond is stable during the whole unconstrained MD simulation of the *S* isomer bound to the protease (Figure 6A). No oxygen atom in our model of the active site accepts more than two hydrogen bonds. Thus, from our simulations, the suggested arrangement of the hydrogen bond network (Figure 5) seems energetically favorable.

Recently, Harte and Beveridge[19] reported the results of unconstrained molecular dynamic simulation of the HIV-1 PR–U85548E complex where the two catalytic aspartic residues were treated in three different ways: both negatively charged, one charged and another neutral, and both neutral. They found that when both aspartates were neutral, the complex displayed the lowest rms deviation from the initial X-ray structure. They interpreted these findings as an indication that in HIV-1 PR–U85548E complex, both aspartic acid residues are found in their neutral form. Harte and Beveridge[19] used the GROMOS force field which is similar to the Cedar forcefiled (except that they used a united atom approach vs an all-atom approach in our simulations), which makes a comparison of their results with the results obtained in this paper sensible. Our results show (Table 1) that if both aspartates are treated

as neutral residues, the difference in computed binding free energy ($\Delta G_{12} - \Delta G_{34}$, Figure 2) changes from −2.8 kcal/mol (monoprotonation model) to −5.2 kcal/mol (diprotonation model 1) or −6.3 kcal/mol (diprotonation model 2), respectively. The value obtained for monoprotonation model of HIV-1 PR/U85548E complex (−2.8 kcal/mol) is similar to that obtained earlier in monoprotonation model simulations of HIV-1 PR−JG365 complex (−2.7 and −2.8 kcal/mol, as reported in ref 11 and ref 12, respectively) and in the experiment[8] (−2.6 kcal/mol for *S* vs *R* isomers of JG365 binding). This result is not surprising given the structural similarity between JG365 and U85548E inhibitors. On the basis of this structural similarity, we find it unlikely that the binding free energy difference of *S* vs *R* isomers of U85548E is as high as predicted by the diprotonation models (−5.2 kcal/mol for model 1 and −6.3 kcal/mol for model 2, respectively; see Table 1). Thus, we predict that U85548E binds to a monoprotonated HIV protease and that the *S* inhibitor binds stronger than the *R* inhibitor by ca. 2.8 kcal/mol. These predictions can be verified by stereo specific synthesis of the *S* and *R* inhibitors followed by binding studies and by a neutron diffraction study. Obviously, the latter experiment will directly address the question of protonation state of the protease in its complexes with inhibitors. However, we suggest that due to apparently strong effect of the protonation state on calculated binding free energy differences of *S* vs *R* inhibitors, the results of the former experiments should be sufficient to answer this question.

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