Molecular Dynamics Study of HIV-1 Protease−Substrate Complex: Roles of the Water Molecules at the Loop Structures of the Active Site

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Abstract: Several molecular dynamics (MD) simulations of HIV-1 protease (HIV-1 PR)−substrate complex were performed. The initial structure of the enzyme−substrate (ES) complex was constructed based on the X-ray crystallographic structure of the HIV-1 PR−inhibitor (JG-365) complex. First, we investigate which of Asp25 and Asp25′ at the catalytic site (two catalytic Asp residues) is protonated in the ES complex. These MD simulations have revealed that the protein hydrolysis mechanism is initiated from the ES complex in which Asp25′ is protonated. This protein hydrolysis mechanism was already studied using quantum chemical calculations, which suggested that the specific conformation of the ES complex was essential for enzymatic activity. Next, we investigate the mechanism for the maintenance of specific conformation of the ES complex. The MD simulations suggest that two water molecules at the loop structures of the active site have a substantial role in maintaining the specific conformation for initiation of the enzyme reaction. This indicates that the enzymatic activity of HIV-1 PR cannot be induced by only the protease encoded by the RNA gene of HIV-1, but this also requires the incorporation of water molecules into the active site.

Introduction

HIV-1 PR has an important role in the replication of HIV-1 by processing two precursor polyproteins, $Pr55^{gag}$ and $Pr160^{gag-pol}$, into structural proteins and replication enzymes. This function is essential for the replication of the virus. This enzyme is a type of aspartic protease, which has a characteristic active site triad Asp-Thr(Ser)-Gly, and is a C_2 symmetric homodimer. Each monomer consists of 99 amino acid residues and contributes a loop structure containing the active site triad Asp25(25′)-Thr26-(26′)-Gly27(27′). A cavity for the insertion of the substrate is formed by the loop structures containing the active site triads and flap regions (flaps) which are presumably related with the entry and affinity of the substrate to the enzyme. Two catalytic Asp residues hold a lytic water molecule[1] that induces protein hydrolysis. The catalytic mechanisms of aspartic proteases have been studied,[2,3] and recent experimental[4−9] and theoretical data[10−12] proposed the most probable mechanism was a general acid/general base-catalyzed protein hydrolysis shown in Scheme 1 or Scheme 2.[13] In Scheme 1, Asp25′ acting as an acid provides a proton to the carbonyl oxygen of the scissile bond (substrate) and Asp25 acting as a base pulls up a proton from the lytic water molecule. Consequently, the nucleophilic attack of the water molecule results in an amide hydrate intermediate, and then the scissile bond collapses. This mechanism is supported by recent experimental evidence.[4−7] In Scheme 2, Asp25 acting as an acid provides a proton to the scissile nitrogen of the substrate, and Asp25′ acting as a base pulls up a proton from the lytic water molecule. Subsequently, the cleavage of the scissile bond is completed via the zwitterion intermediate. From the MD simulations of the ES complex structures of Scheme 1 and/or Scheme 2 (the results are described in the text), we determined that the protein hydrolysis of the HIV-1 PR started from the ES complex (Asp25′ protonated), that is, the mechanism of Scheme 1 occurred. The mechanism of Scheme 1 has

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(13) The early studies on the mechanism of aspartic proteases proposed that the unprotonated Asp residue attacked on the scissile bond of the substrate, so that a covalently bound intermediate complex between the enzyme and the substrate was formed. However, the intermediate complex has not been supported by the experimental evidence.

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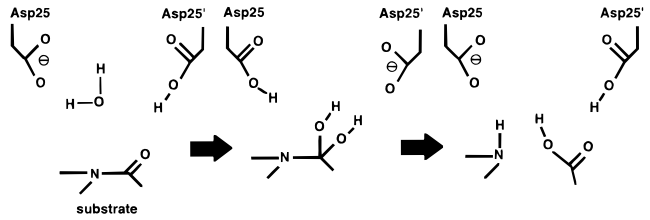
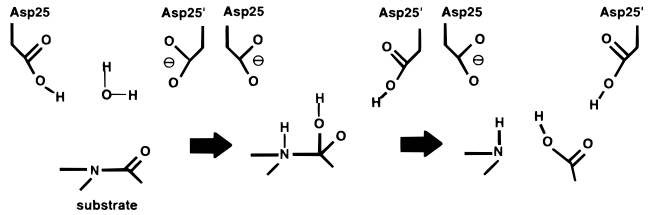
Scheme 1



Scheme 2



been elucidated through quantum chemical calculations[14,15] (ab initio molecular orbital method) using a model compound for the peripheral region of the catalytic site. The model compound consisted of an acetate/acetic acid pair for two catalytic Asp residues, a lytic water molecule, and a dimethylacetamide for the substrate. The outline of this hydrolysis mechanism obtained by the ab initio molecular orbital method is shown in Figure 1. The mechanism consists of three elementary reactions. The first step was the the formation of the enzyme-bound amide hydrate intermediate from the ES complex. This reaction is consistent with the experimental results obtained by Meek and coworkers[4,5] and the theoretical results by Venturini and coworkers.[11] The second and third steps were the proline N protonation and the scissile bond cleavage of the substrate. These steps are in agreement with the isotope kinetic experiments.[4,6] This study[14] revealed that it was necessary for the catalytic site of the ES complex (Figure 2) to maintain the specific conformation to be capable of leading the protein hydrolysis reaction. In this structure (Figure 2), two catalytic Asp residues held the lytic water molecule (WAT05) by forming hydrogen bonds. The distance between the oxygen of WAT05 attacking the scissile carbon of the substrate and the scissile carbon of Phe105 (Phe105 C) was 2.91 Å, which supported the interaction for initiation of the protein hydrolysis reaction. In addition, there was another hydrogen bond between protonated Asp25′ OD2 and the carbonyl oxygen of Phe105 (Phe105 O). This hydrogen bond is necessary for the proton transfer that triggers the formation of the enzyme-bound amide hydrate intermediate. This specific conformation of the ES complex is transformed into the enzyme-bound amide hydrate intermediate on the lowest energy reaction path of the protein hydrolysis. Hence, the ES complex should maintain the specific conformation (Figure 2) to present the enzymatic activity. We call this specific conformation the "active conformation". This paper reports the results of MD simulations that investigate (i) which of two catalytic Asp residues is protonated in the ES complex and (ii) the key mechanism for the ES complex (Asp25′ protonated) maintaining the active conformation. Some studies of MD simulations on HIV-1 PR−substrate complex were reported.[12,16] Those previous MD simulations were performed under the conditions of the

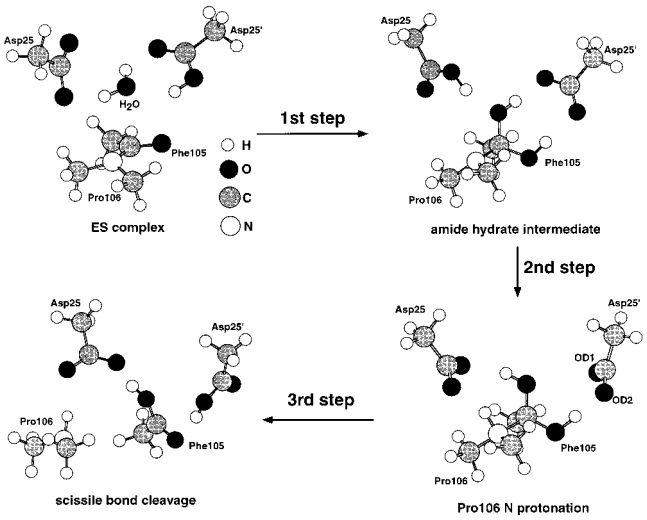


Figure 1. Outline of the protein hydrolysis mechanism elucidated with the ab initio molecular orbital method. This mechanism was studied at the Hartree−Fock level with use of the 6-31G** basis set.

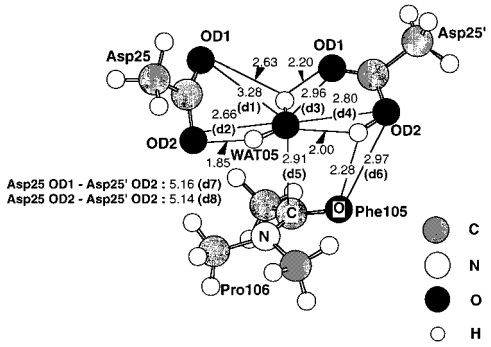


Figure 2. ES complex resulting from ab initio molecular orbital calculations. Numerals are the interatomic distances (in Å). Distances d1 through d8 were measured to judge whether the enzyme (Asp25′ protonated) is active.

modified charge distribution of the unprotonated Asp residue at catalytic site and/or several restraints to maintain the conformation of the catalytic site. Those techniques were required to keep the subtle balance of the catalytic site folding a water molecule adequately. To solve this problem, we perform the MD simulations, applying no cutoff technique for the calculations of the electrostatic forces. No papers have ever reported on the MD simulations of the HIV-1 PR−substrate complex with use of the cutoff technique and restraints.

Methods

Construction of Initial Structure. The MD simulations of seven different structures are performed in this study. Table 1 summarizes these seven structures. The initial structure of the ES complex was constructed based on the X-ray crystallographic structure of the HIV-1 PR−inhibitor (JG-365) complex (Protein Data Bank (PDB):[17−19] 7HVP[20]). The conversion from the inhibitor (JG-365) to the substrate was as follows. The structure of the inhibitor (JG-365) is Ac-Ser-Leu-Asn-Phe-Ψ[CH(OH)CH_2N]-Pro-Ile-Val-OMe. The molecule was modi-

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Table 1. Seven Structures for MD Simulations (We Generate Solvent Water Molecules around Each Structure)

structure	protonated Asp	crystal water molecules
1[a]	Asp25′	all crystal water molecules
2[a]	Asp25	all crystal water molecules
3[b]	Asp25′	no crystal water molecules
4[c]	Asp25′	absence of WAT00
5[d]	Asp25′	absence of WAT01-04
6[e]	Asp25′	absence of WAT01-02
7[e]	Asp25′	absence of WAT03-04

[a] ES complex with 95 crystal water molecules plus WAT05. [b] ES complex with no crystal water molecules plus WAT05. [c] ES complex with 94 crystal water molecules plus WAT05. [d] ES complex with 91 crystal water molecules plus WAT05. [e] ES complex with 93 crystal water molecules plus WAT05.

fied by replacing Ser with Thr, Val with Ser, OMe with NMe, and the non-hydrolyzable bond [CH(OH)CH_2N] of the inhibitor with the peptide bond. The selected substrate, Ac-Thr-Leu-Asn-Phe-Pro-Ile-Ser-NMe, represents the PR−RT cleavage site in the pol region of HIV-1 PR.

According to the kinetic studies of HIV-1 PR,[5] either of two catalytic Asp residues was protonated and the other was not protonated. The initial structure of HIV-1 PR was produced to satisfy this situation. Two types of HIV-1 PRs (Asp25′ protonated or Asp25 protonated) were built for the respecive MD simulations of the ES complex of Schemes 1 and 2. The lytic water molecule was set based on the data from the X-ray crystallographic structures of the HIV-1PR[1] and pepsin[21] that belongs to the family of the aspartic protease. This water molecule was placed in a position surrounded by two catalytic Asp residues. In addition, all the crystal water molecules, which were observed in the X-ray crystallographic structure (PDB:[17−19] 7HVP[20]), were set in the constructed ES complex (Table 1 (structures 1 and 2)). In this paper, protein atoms are designated by the amino acid abbreviation, residue number, and atom type (Brookhaven notation).[17,19] The amino acid residues of the respective monomers (A and B) of the HIV-1 PR were numbered 1 through 99 and 1′ through 99′. The residues of the substrate were numbered 101 through 109. To discuss the roles of the water molecules (crystal water molecules) in the ES complex where Asp25′ is protonated (i.e., ES complex of Scheme 1), the water molecule existing between the substrate and the flaps of the enzyme, the four water molecules existing at the loop structures of the active site, and the lytic water molecule were termed WAT00, WAT01 through WAT04, and WAT05, respectively (see Table 1 (structures 3−7)).

Calculation. The molecular mechanics (MM) potential energy minimizations and MD simulations were carried out with the program package AMBER, Version 4.1.[22,23] Calculations were performed using the all-atom force field.[24] The solvent was the TIP3P water model[25] and approximately 6170 water molecules were generated isotropically with respect to WAT05. The generation of a solvent water molecule is prohibited when the oxygen atom was within 2.5 Å from the enzyme, substrate, or crystal water molecules and likewise the hydrogen atom was within 1.8 Å. To simplify this calculation, the SHAKE procedure[26] was used. An integration time step of the MD simulations was 1 fs. The calculations of the nonbonded term were accelerated by the use of a hardware accelerator called MD-Engine,[27,28] which has a special processor chip, MODEL, to calculate the nonbonded term. A cutoff distance (11.5 Å) was applied for the computation of the van der Waals forces. The electrostatic term was calculated with no cutoff, taking full advantage of the MD-Engine because simplification in the computation of this term causes noticeable error.

The computational procedure of this study was as follows. First, the potential energy minimizations were performed on the initial systems. In the potential energy minimization, the steepest descent method was used for the early cycles and then the conjugate gradient method was used later. Next, MD simulations were performed on the energy-minimized systems, taking careful consideration of the temperature setting. After 10-ps MD simulations at 300 K only for the solvent water molecules with the enzyme, substrate, and the crystal water molecules fixed, the temperature of the whole system was gradually increased by heating to 300 K for the first 60 ps, and then it was kept at 300 K for the next 100 ps. The temperature was kept constant according to the Berendsen algorithm[29] (the separate scaling factor for the solute and the solvent was used) with a coupling time of 0.2 ps. The trajectories at the temperature (300 K for 100 ps) were considered to be the most probable structure under physiologic conditions and were analyzed in detail.

Definition of the Active Conformation. The result of the quantum chemical calculations shown in Figure 2 revealed that the catalytic site of the ES complex (Asp25′ protonated) of Scheme 1 maintained the specific conformation (i.e., active conformation) to produce enzymatic activity. The active conformation should satisfy the following four conditions.

Condition 1: Two catalytic Asp residues hold WAT05 by forming hydrogen bonds (Figure 2: d1, d2, d3, and d4).

Condition 2: The interactive distance (within 3.3 Å) between the oxygen of WAT05 and Phe105 C of the substrate is maintained (Figure 2: d5).

Condition 3: Asp25′ OD2 forms the hydrogen bond with Phe105 O (Figure 2: d6).

Condition 4: The relative position of two catalytic Asp residues is properly maintained (Figure 2: d7 and d8).

To judge if these four conditions were satisfied, the distances d1 through d8, shown in Figure 2, were measured. In the following discussion, the hydrogen bond was regarded to be effective when the time average of the distance between heavy atom (proton donor) and heavy atom (proton acceptor) was less than 3.5 Å. The value 3.3 Å in Condition 2 is decided on the basis of the van der Waals radius of carbon and oxygen.

Results

Protonation States of Two Catalytic Asp Residues. To investigate which of two mechanisms (Scheme 1 or 2) occurs, we carried out the MD simulations of the ES complex (Asp25′ protonated) of Scheme 1 and the ES complex (Asp25 protonated) of Scheme 2. First, the MD simulation of the ES complex (Asp25′ protonated) of Scheme 1 is described (Table 1 (structure 1)). The distances d1 through d8 during the 100 ps simulation are shown in Figure 3. The distances d1 through d4 were maintained almost within 3.5 Å for 100 ps, so that two catalytic Asp residues formed the hydrogen bonds with WAT05. As seen from the catalytic site in the average structure for the 100 ps simulation (Figure 4), two catalytic Asp residues held WAT05 by forming hydrogen bonds, that is, Condition 1 was satisfied. The average value of distance d5, which is closely related to Condition 2, was 3.16 Å, so that the interaction between WAT05 and Phe105 C of the substrate was effective. This value, 3.16 Å, was close to that of the quantum chemical calculations

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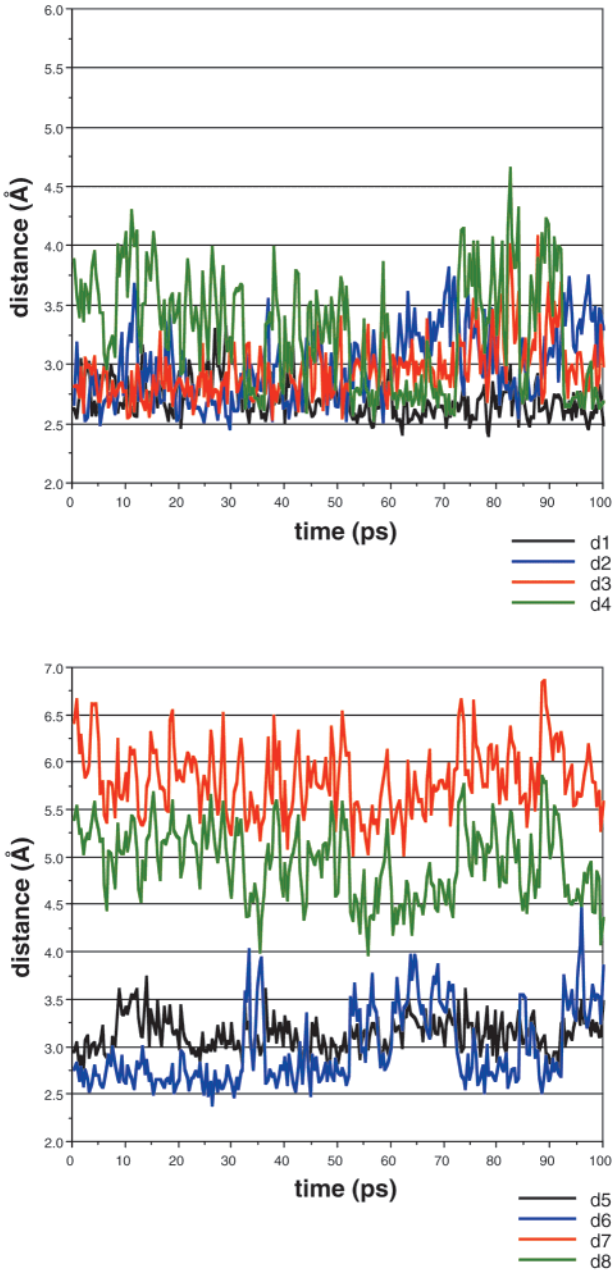

Figure 3. Change of distances d1 through d8 during 100 ps MD simulation for the ES complex where Asp25′ is protonated (Table 1 (structure 1)). The ordinate is the distance (Å) and the abscissa is the time (ps). The time average value of each distance is as follows: d1, 2.72 Å; d2, 2.97 Å; d3, 2.99 Å; d4, 3.22 Å; d5, 3.16 Å; d6, 3.04 Å; d7, 5.71 Å; and d8, 4.85 Å).

shown in Figure 2. The average value of distance d6, which is closely related to Condition 3, was 3.04 Å. Asp25′ OD2 formed a hydrogen bond with Phe105 O of the substrate. In addition, the average values of distances d7 and d8 were 5.71 and 4.85 Å, respectively. Each of these two values agreed approximately with that of the quantum chemical calculations (Figure 2). Hence, the relative position of two catalytic Asp residues was properly maintained for initiation of the hydrolysis reaction. Consequently, this MD simulation revealed that the ES complex (Asp25′ protonated) satisfied the four conditions required to maintain the active conformation.

Next, the MD simulation of the ES complex (Asp25 protonated) of Scheme 2 (Table 1 (structure 2)) is described. Figure 5 shows the catalytic site of the average structure for 100 ps

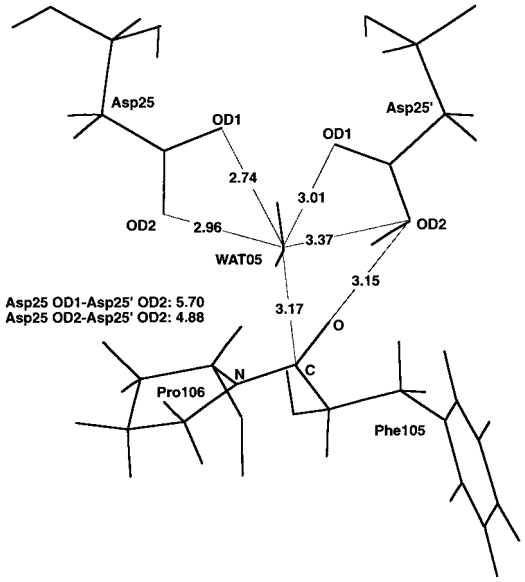

Figure 4. Catalytic site in the average structure for 100 ps MD simulation for the ES complex where Asp25′ is protonated (Table 1 (structure 1)). Numerals are the interatomic distances of the average structure (in Å), rather than the time average value of each distance.

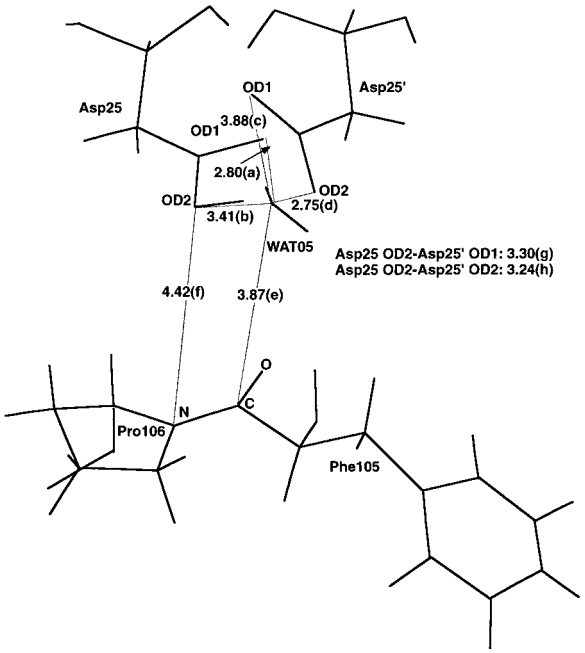

Figure 5. Catalytic site in the average structure for the 100 ps MD simulation for the ES complex where Asp25 is protonated (Table 1 (structure 2)). Numerals are the interatomic distances of the average structure (in Å), rather than the time average value of each distance. The time average value of each distance is as follows: a, 2.72 Å; b, 3.36 Å; c, 3.82 Å; d, 2.71 Å; e, 3.89 Å; f, 4.40 Å; g, 3.35 Å; and h, 3.20 Å).

simulation. As seen in this figure, WAT05 was detached from the scissile carbon of the substrate and two catalytic Asp residues formed hydrogen bonds with each other, so that the hydrogen bond between Asp25 OD2 and Pro106 N, which is necessary for the proton transfer that triggers the formation of zwitterion intermediate, is completely lost. It is difficult to induce the mechanism of Scheme 2 from this conformation. This MD simulation suggests that the ES complex (Asp25 protonated) cannot proceed in the mechanism of Scheme 2. The MD simulations of these two ES complex structures, Table 1

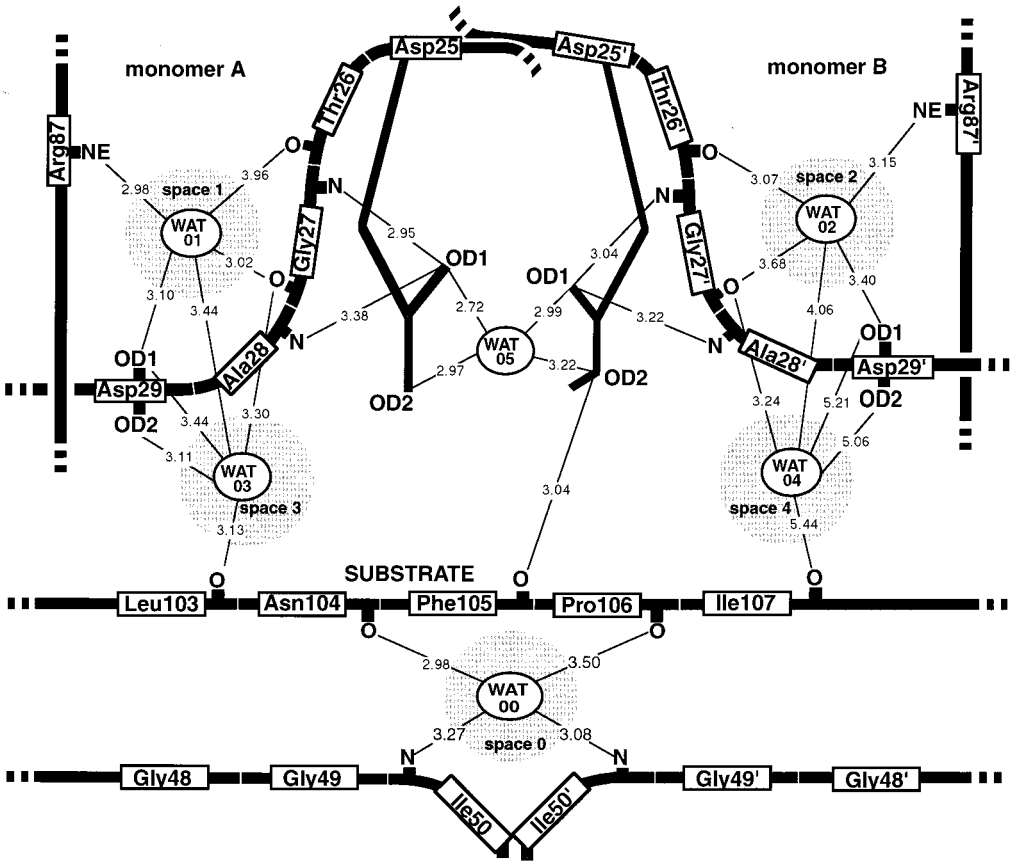

Figure 6. Sketch of the structures and the hydrogen bonds of the two loops containing active site triads for 100 ps MD simulation for the ES complex where Asp25′ is protonated (Table 1 (structure 1)). Numerals are the time average interatomic distances (in Å).

(structures 1 and 2), support the protein hydrolysis reaction starting from the ES complex (Asp25′ protonated), that is, the mechanism of Scheme 1 occurs. Accordingly, we consider that Asp25′ is protonated in the ES complex.

Factors Required To Maintain the Active Conformation. In the X-ray crystallographic structure of the HIV-1 PR, a number of structures with no crystal water molecules have been reported.[30,31] Therefore, it is important to investigate whether crystal water molecules contained in this enzyme crystal were necessary for the enzymatic activity of the HIV-1 PR. The MD simulation of the ES complex with no crystal water molecules (Table 1 (structure 3)) was performed and demonstrated that the ES complex with no crystal water molecules could not maintain the active conformation. In this case, WAT05 acting on the hydrolysis was detached from the catalytic site as the temperature rose to 300 K. This indicates that not only the protease encoded by the RNA gene of HIV-1 but also the crystal water molecules contained in the HIV-1 PR are necessary for maintaining the active conformation.

What mechanism allows for maintaining the active conformation of the ES complex with all the crystal water molecules (Table 1 (structure 1))? Also, what is the reason that the ES complex with no crystal water molecules (Table 1 (structure 3)) cannot maintain the active conformation?

To address these questions, two loops containing active site triads Asp25(25′)-Thr26(26′)-Gly27(27′) of the ES complex with all the crystal water molecules were investigated in detail. Figure 6 shows the structures and the hydrogen bonds of the two loops containing active site triads Asp25(25′)-Thr26(26′)-Gly27(27′) for the 100 ps simulation. Two structural features, which seem to be responsible for the retention of the active conformation, were determined from this figure. The first feature is that the hydrogen bonds of Asp25(25′) OD1-Gly27(27′) N and Asp25-(25′) OD1-Ala28(28′) N were maintained during the 100 ps MD simulation. These hydrogen bonds are present in the crystal structure and might help to maintain the conformation of Asp25-(25′) side chains. In particular, the hydrogen bonds of Asp25-(25′) OD1-Gly27(27′) N are short enough to have a large contribution to the retention of the active conformation. The second feature is that five water molecules (WAT00 through WAT04), which seem to be related to retention of the active conformation, exist at the two loops containing the active site triads Asp25(25′)-Thr26(26′)-Gly27(27′). WAT00 occupied space 0 forming four hydrogen bonds with Asn104 O and Pro106 O of the substrate and with Ile50 N and Ile50′ N of the flaps during 100 ps MD simulation.[32] These four hydrogen bonds seem to stabilize the scissile bond of the substrate, which is related in maintaining the active conformation. The role of this water molecule (WAT00) has attracted the interest of many researchers, but has not been completely determined. WAT01 formed hydrogen bonds with Gly27 O of the active site triad,

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(32) In the current study, the four hydrogen bonds involving WAT00 were formed in MD simulations. But the hydrogen bond of Pro106 N−WAT00 was weaker than the other hydrogen bonds. It is considered that this is due to the length of substrate which consists of seven (P4−P3′) residues, since P1′−P3′ residues are more mobile than P1−P4 residues. Accordingly, the MD simulation on large substrate will be a future subject.

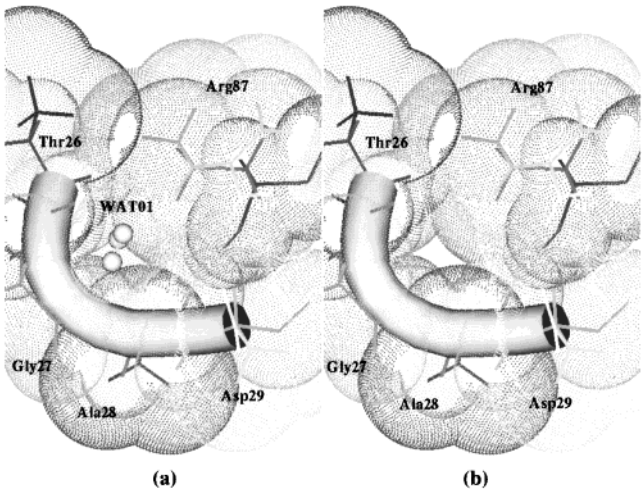

Figure 7. View of WAT01 for the 100 ps MD simulation for the ES complex with all the crystal water molecules (Table 1 (structure 1)). Part a is the rear view of the structure depicted in Figure 6. Part b indicates space 1 for the water molecules to occupy, depicted by eliminating WAT01 from part a. Dots represent the van der Waals radius.

Asp29 OD1, Arg87 NE, and WAT03. A number of hydrogen bonds involving WAT01 stabilize the loop containing the active site triad Asp25-Thr26-Gly27. In the same manner as WAT01, WAT02 formed hydrogen bonds with Thr26′ O of the active site triad, Asp29′ OD1, and Arg87′ NE. These two respective water molecules (WAT01 and WAT02) occupied space 1 and space 2 during the 100 ps MD simulation. Each space was between the loop containing the active site triad Asp25(25′)-Thr26(26′)-Gly27(27′) and Arg87(87′) in each monomer. Figure 7a shows the situation where WAT01 fit in space 1. Figure 7b, in the absence of WAT01, clearly indicates the space. A similar situation was observed for WAT02. On the other hand, WAT03 and WAT04 occupied spaces 3 and 4, respectively. Each space was between the loop containing the active site triad Asp25-(25′)-Thr26(26′)-Gly27(27′) in each monomer and the substrate. WAT03 formed hydrogen bonds with Gly27 O of the active site triad, Asp29 OD2(OD1), Leu103 O of the substrate, and WAT01, whereas WAT04 formed a hydrogen bond with only Gly27′ O of the active site triad. These findings indicate that these five water molecules did not directly participate in hydrolysis, but had an important role in maintaining the active conformation of the ES complex by stabilizing the scissile bond of the substrate and the two loops containing Gly27(27′) (the third residue in the active site triad), which were closely involved in the conformation of Asp25(25′) at the catalytic site. Absence of these five water molecules (WAT00 through WAT04) induced the collapse of the active conformation of the ES complex with no crystal water molecules (Table 1 (structure 3)) in the early stage of the MD simulation.[33] In other words, we thought that all the crystal water molecules were not necessary for maintaining the active conformation, but these five water molecules were substantially required. We consider WAT00 contributes much to the stabilization of the scissile bond (substrate), and investigate the role of WAT00 in maintaining the active conformation.

Effect of WAT00 for Active Conformation. We carried out the MD simulation of the ES complex without only WAT00

(33) Throughout MM minimizations and MD simulations on the structures in Table 1 (structures 3−7), the respective spaces of the deleted crystal water molecules (spaces 0−4) have been confirmed to be unoccupied by other water molecules supplied in the solvent generation.

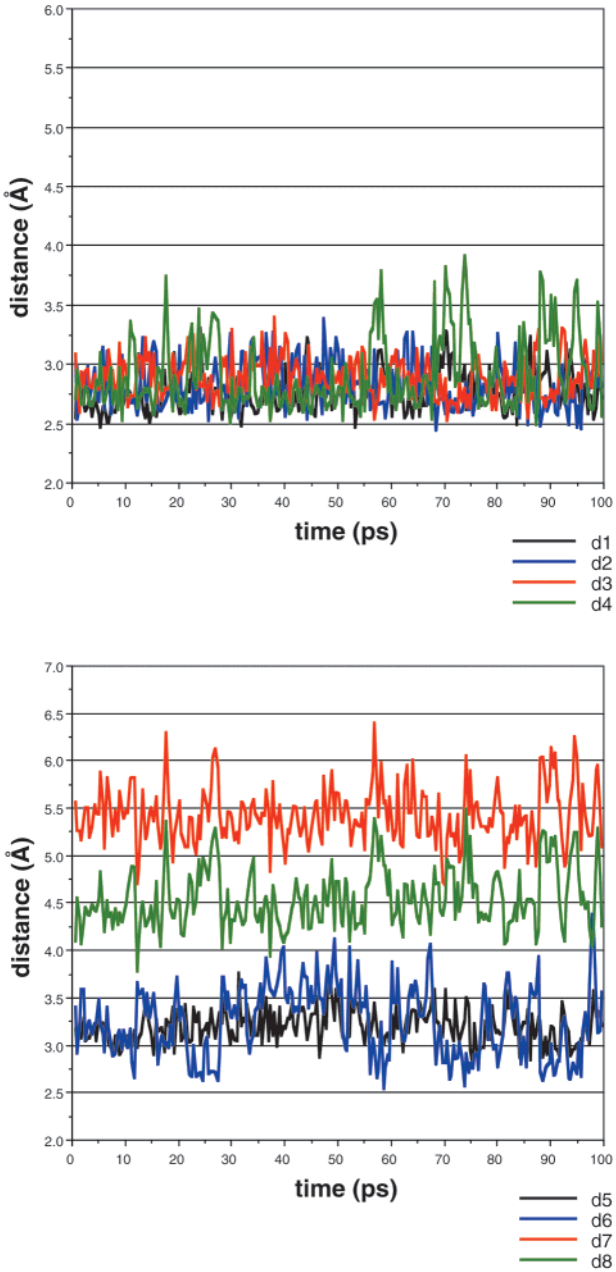

Figure 8. Change of distances d1 through d8 during 100 ps MD simulation for the ES complex in the absence of WAT00 (Table 1 (structure 4)). The ordinate is the distance (Å) and the abscissa is the time (ps). The time average value of each distance is as follows: d1, 2.97 Å; d2, 2.92 Å; d3, 2.84 Å; d4, 2.76 Å; d5, 3.22 Å; d6, 3.28 Å; d7, 5.50 Å; and d8, 4.82 Å).

(Table 1 (structure 4)). The distances d1 through d8 during the 100 ps simulation are shown in Figure 8. The distances d1 through d4 were maintained almost within 3.5 Å for 100 ps, so that two catalytic Asp residues formed the hydrogen bonds with WAT05. Since the average value of distance d5 was 3.22 Å, the interaction between WAT05 and Phe105 C of the substrate was effective. The average value of distance d6 was 3.28 Å, so Asp25′ OD2 formed a hydrogen bond with Phe105 O of the substrate. In addition, the average values of distances d7 and d8 were 5.50 and 4.82 Å, respectively. Hence, the relative position of two catalytic Asp residues was properly maintained for initiation of the hydrolysis reaction. This MD simulation suggests that this ES complex maintained the active conformation. For this 100 ps simulation, space 0 was not occupied by another water molecule, that is, WAT00 is not necessary for

maintaining the active conformation. However, we believe the water molecule (WAT00) plays a role in the opening and closing of the flaps and in the increase of the affinity between the enzyme and the substrate. In the MD simulation, four water molecules (WAT01 through WAT04) occupied stably spaces 1−4, respectively. This fact intimates the possibility that WAT01 through WAT04 are concerned in maintaining the active conformation. Therefore, to investigate whether these four water molecules are necessary for maintaining the active conformation of the ES complex, the MD simulation of the ES complex without only these water molecules (WAT01 through WAT04) was performed.

Necessity of the Four Water Molecules (WAT01 through WAT04) in Maintaining the Active Conformation. The MD simulation of the ES complex without only these four water molecules (WAT01 through WAT04), Table 1 (structure 5), is described as follows. The distances d1 through d8 during the 100 ps simulation are shown in Figure 9. The structure of the catalytic site changed drastically within the first 5 ps. The average values of distances d1 and d4 after 5 ps were 3.90 and 4.21 Å, respectively; thus, WAT05 was detached from the catalytic site. In this structural change, distance d6 became longer, while distances d7 and d8 became shorter. The hydrogen bond between Asp25′ OD2 and Phe105 O of the substrate was lost and two catalytic Asp residues formed hydrogen bonds with each other. In addition, the average value of distance d5 after this structural change was 4.05 Å. Because this value (4.05 Å) was longer than that of the quantum chemical calculations by 1.14 Å, the interaction between WAT05 and Phe105 C of the substrate did not take place. From the catalytic site in the average structure shown in Figure 10, it can be seen that the carboxyl rotations of Asp25 and Asp25′ expelling WAT05 were responsible for the formation of the hydrogen bonds between Asp25-(25′) side chains and the disappearance of the hydrogen bond between Asp25′ OD2 and Phe105 O of the substrate. Accordingly, the ES complex without these four water molecules (WAT01 through WAT04) did not satisfy the four conditions above during the MD simulation, that is, the ES complex became inactive. For this 100 ps simulation, despite the existence of a great number of water molecules around the HIV-1 PR, four spaces (spaces 1−4) were not occupied by the other water molecules, so each of the four water molecules (WAT01 through WAT04) held a stable position and was not replaced by another molecule.[34] These findings indicate clearly that WAT01 through WAT04 are involved in maintaining the active conformation.

Why is it that the ES complex without the four water molecules (WAT01 through WAT04), Table 1 (structure 5), could not maintain the active conformation? To examine this, two loops containing the active site triads Asp25(25′)-Thr26-(26′)-Gly27(27′) of this ES complex after the 100 ps MD simulation were inspected in detail. The inspection indicated that the structure of two loops of the ES complex without the four water molecules (WAT01 through WAT04) was apparently different from that of the ES complex with these four water molecules (Table 1 (structure 1)). The structures of the two loops without these four water molecules started to sway widely at 5 ps during the MD simulation. The hydrogen bond between Gly27(27′) O of the active site triads and Arg87(87′) NE in each monomer was formed by this sway, so that spaces 1 and 2 vanished. Figure 11 shows the situation where space 1 vanished. This situation also occurred in space 2. This structural change separated the two loops of the active site triads (see Table 2) to change the relative position of two catalytic Asp residues. It is clear from Table 2 that values of the ES complex without these four water molecules (Table 1 (structure 5)) became longer than those of the ES complex with the four water molecules (Table 1 (structure 1)), especially for the Gly27(27′), which formed the hydrogen bonds with Asp25(25′) at the catalytic site. Accordingly, the sway of the two loops containing the active site triads is involved in the carboxyl rotations of two catalytic Asp residues, and then the active conformation collapsed. Thus, we hypothesize that WAT01 and WAT02, which occupied spaces 1 and 2, respectively, had a major role in maintaining the active conformation, whereas WAT03 and WAT04 were not involved in maintaining the active conformation. To investigate this hypothesis, we performed MD simula-

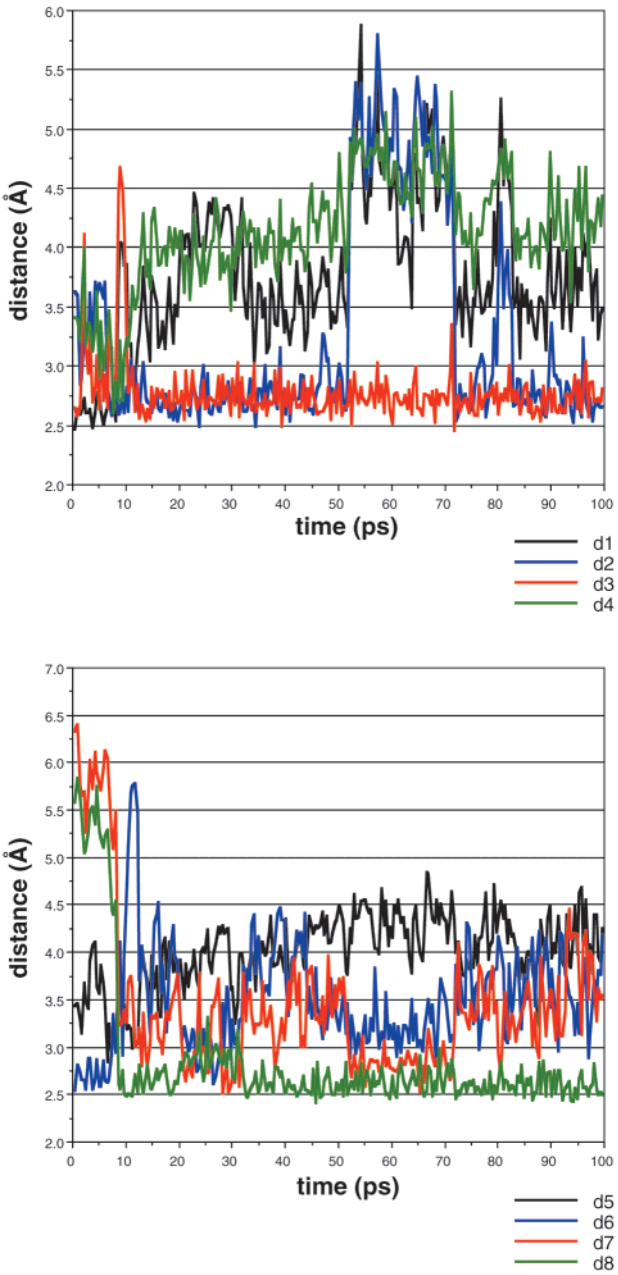

Figure 9. Change of distances d1 through d8 during the 100 ps MD simulation for the ES complex in the absence of the four water molecules (WAT01 through WAT04), Table 1 (structure 5). The ordinate is the distance (in Å) and the abscissa is the time (in ps). The time average value from 5 to 100 ps is as follows for each distance: d1, 3.90 Å; d2, 3.26 Å; d3, 2.80 Å; d4, 4.21 Å; d5, 4.05 Å; d6, 3.56 Å; d7, 3.27 Å; and d8, 2.66 Å.

(34) WAT00 also occupied space 0 during the 100-ps MD simulations.

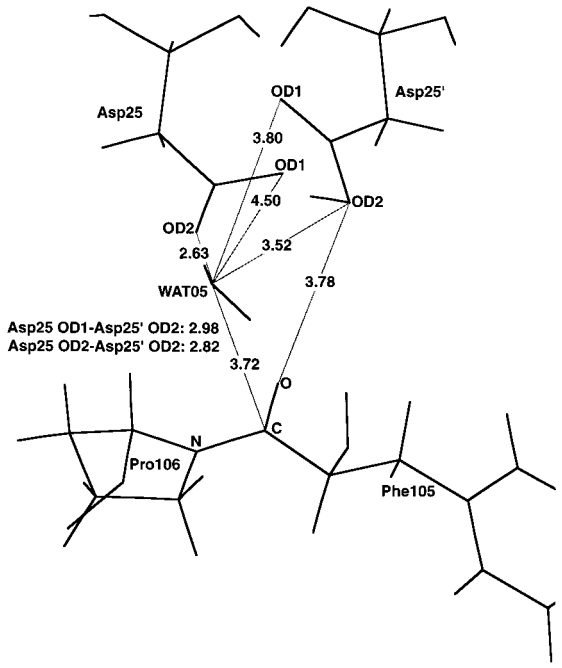

Figure 10. Catalytic site in the average structure for the 100 ps MD simulation for the ES complex in the absence of the four water molecules (WAT01 through WAT04), Table 1 (structure 5). Numerals are the interatomic distances of the average structure (in Å), rather than the time average value of each distance.

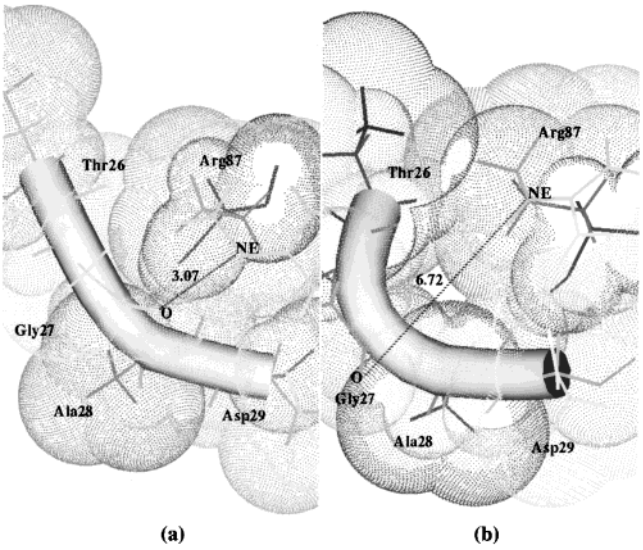

Figure 11. View of space 1 for the 100 ps MD simulation for the ES complex in the absence of the four water molecules (WAT01 through WAT04), Table 1 (structure 5). Part a is depicted in the same angle as Figure 7. Part b is in the same situation as Figure 7b. These figures indicate that space 1 vanished. Dots represent the van der Waals radius. Numerals are the interatomic distances between Gly27 O and Arg87 NE (in Å).

tions of the ES complex without only WAT01 and WAT02 and the ES complex without only WAT03 and WAT04.

Role of the Four Water Molecules (WAT01 through WAT04). We first describe the result of a 100 ps MD simulation of the ES complex without WAT01 and WAT02 (Table 1 (structure 6)). The distances d1 through d8 during the 100 ps MD simulation of this ES complex are shown in Figure 12. The structure of this catalytic site changed drastically within the first 5 ps in the same way as during the 100 ps MD simulation of the ES complex without all four water molecules

Table 2. Time Average Distances between the Main Chain Atoms of Two Loops Which Are within the Active Site of a Pair of Monomers of HIV-1 PR for 100 ps MD Simulations[e]

atom types of each monomer	no water absence[a]	absence of WAT01-04[b]	absence of WAT01-02[c]	absence of WAT03-04[d]
Thr26 C−Thr26′ C	6.64	7.15	7.30	6.70
Gly27 N−Gly27′ N	*5.33*	*6.21*	*6.45*	*5.46*
Gly27 CA−Gly27′ CA	*6.57*	*7.84*	*8.13*	*6.77*
Gly27 C−Gly27′ C	*7.88*	*9.10*	*9.34*	*7.84*
Ala28 N−Ala28′ N	8.18	8.64	8.68	7.81
Ala28 CA−Ala28′ CA	10.35	10.42	10.35	9.82
Ala28 C−Ala28′ C	13.00	13.24	13.21	12.58

[a] Table 1, structure 1. [b] Table 1, structure 5. [c] Table 1, structure 6. [d] Table 1, structure 7. [e] Numerals are the interatomic distances (in Å).

(WAT01 through WAT04), Table 1 (structure 5). The average values of distances d1, d3, and d4 after 5 ps were 4.45, 3.84, and 3.56 Å, respectively, so WAT05 was detached from the catalytic site. The average values of distances d6, d7, and d8 after 5 ps were 3.74, 2.97, and 2.87 Å, respectively. These values indicated that the hydrogen bond between Asp25′ OD2 and Phe105 O of the substrate was lost and two catalytic Asp residues formed hydrogen bonds with each other. The cause of this structural change was the carboxyl rotation of two catalytic Asp residues in the same way as the ES complex without the four water molecules (WAT01 through WAT04). The average value of distance d5 after 5 ps was 3.76 Å. This value (3.76 Å) was longer than that of the quantum chemical calculations by 0.85 Å. Accordingly, the interaction between WAT05 and Phe105 C of the substrate decreased. The above results indicate that the four conditions for maintaining the active conformation were not satisfied, that is, the ES complex without WAT01 and WAT02 became inactive. In addition, the structures of the two loops containing the active site triads Asp25(25′)-Thr26(26′)-Gly27(27′) of this ES complex also changed in the same way as the ES complex without the four water molecules (WAT01 through WAT04) (see Table 2). The two loops containing the active site triads without WAT01 and WAT02 swayed widely within the first 5 ps. The hydrogen bonds of Gly27(27′) O−Arg87(87′) NE were formed by this sway, and spaces 1 and 2 vanished. On the other hand, the two water molecules, WAT03 and WAT04, occupied spaces 3 and 4, which were between the two loops containing the active site triads and the substrate.[34]

Next, we describe the result of the 100 ps MD simulation of the ES complex without WAT03 and WAT04 (Table 1 (structure 7)). This result was similar to the case of the ES complex with all four water molecules (Table 1 (structure 1)). Distances d1 through d8 during this 100 ps simulation are shown in Figure 13. Distances d1 through d4 were maintained almost within 3.5 Å for 100 ps. The average value of distance d5 was 3.11 Å, which was close to that of the quantum chemical calculations. The average value of distance d6 was 2.98 Å, that is, Asp25′ OD2 formed a hydrogen bond with Phe105 O of the substrate. In addition, the average values of distances d7 and d8 were 5.57 and 4.66 Å, respectively. Each value agreed approximately with that of the quantum chemical calculations. Accordingly, the ES complex without WAT03 and WAT04 satisfied the four conditions necessary to maintain the active conformation. In addition, the structures of the two loops containing the active site triads are compatible with that of the ES complex with the four water molecules (Table 1 (structure 1)). WAT01 and WAT02 fit in spaces 1 and 2, respectively, to stabilize the two loops containing the active site triads, that is, the sway of two loops did not occur.[34] This fact was supported by the similarity of the values between the ES complex without

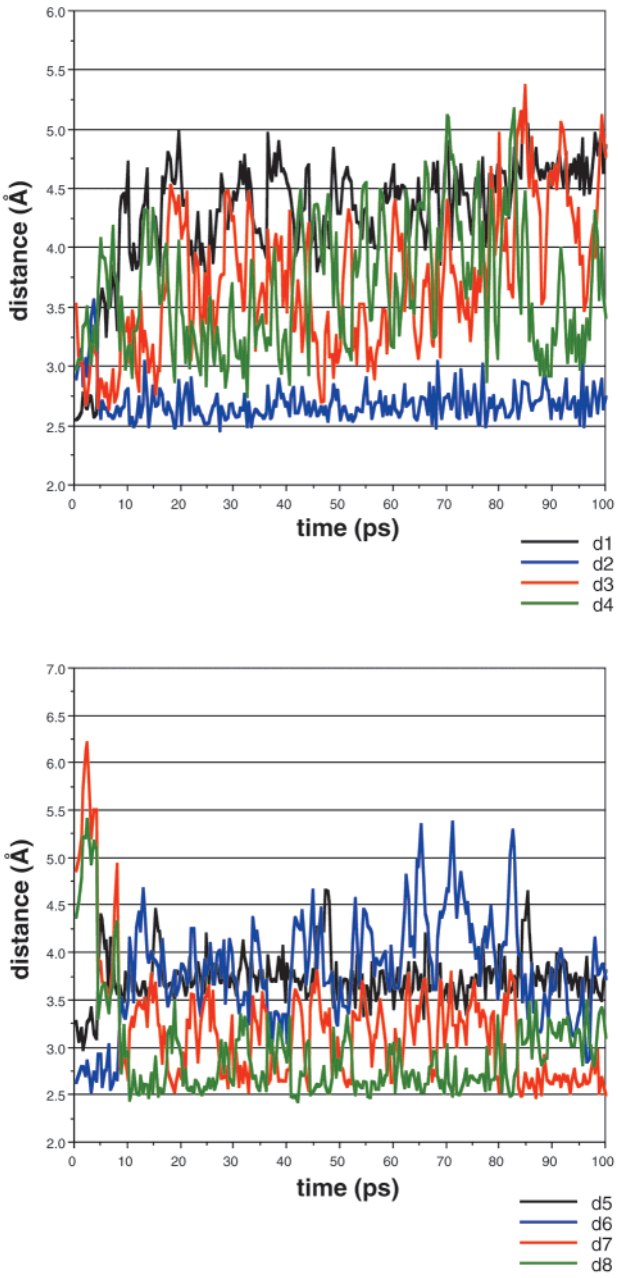

Figure 12. Change of distances d1 through d8 during the 100 ps MD simulation for the ES complex in the absence of WAT01 and WAT02 (Table 1 (structure 6)). The ordinate is the distance (in Å) and the abscissa is the time (in ps). The time average value from 5 to 100 ps is as follows for each distance: d1, 4.45 Å; d2, 2.68 Å; d3, 3.84 Å; d4, 3.56 Å; d5, 3.76 Å; d6, 3.74 Å; d7, 2.97 Å; and d8, 2.87 Å.

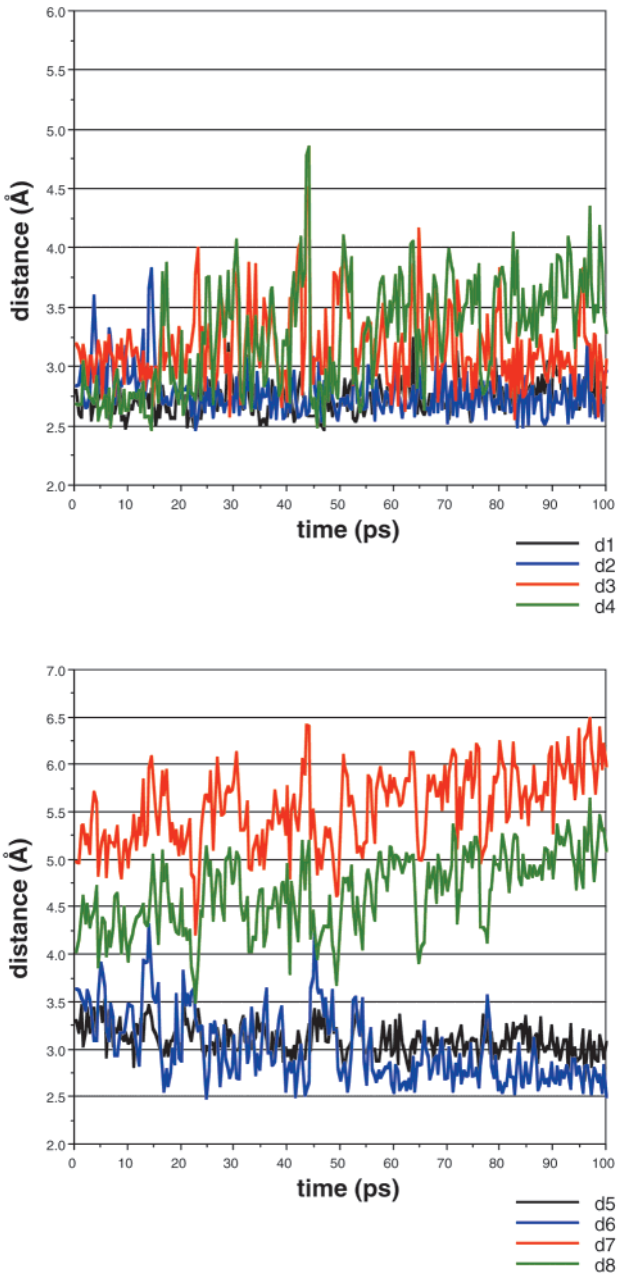

Figure 13. Change of distances d1 through d8 during the 100 ps MD simulation for the ES complex in the absence of WAT03 and WAT04 (Table 1 (structure 7)). The ordinate is the distance (in Å) and the abscissa is the time (in ps). The time average value of each distance is as follows: d1, 2.74 Å; d2, 2.78 Å; d3, 3.18 Å; d4, 3.27 Å; d5, 3.11 Å; d6, 2.98 Å; d7, 5.57 Å; and d8, 4.66 Å.

WAT03 and WAT04 (Table 1 (structure 7)) and the ES complex with all four water molecules (Table 1 (structure 1)) in Table 2.

Discussion

The MD simulations of the ES complex (Asp25′ protonated) of Scheme 1 and the ES complex (Asp25 protonated) of Scheme 2 suggested that the protein hydrolysis mechanism was initiated from the ES complex (Asp25′ protonated) of Scheme 1. This proposal is supported by the isotope kinetic[4−7] and theoretical studies.[12,16] However, the MD simulation studies by Liu et al.[12] and Chatfield et al.[16] report the catalytic site of the ES complex (Asp25′ protonated) of Scheme 1 is adequately maintained only in the condition of applying the modified charge distribution of the unprotonated Asp residue at the catalytic site and/or several restraints. In this work, other additional MD simulations were performed with the particle mesh Ewald (PME) method.[35] On the initial structure of the ES complex, the MA-CA region beside the PR−RT region was used for the substrate. The substrate (MA-CA) is Ac−Ser-Gln-Asn-Tyr-Pro-Ile-Val-Nme.

(35) The system was solvated in a rectangular box (whose size is approximately 90 Å × 71 Å × 62 Å) and 11080 TIP3P water molecules were generated in the box. In the PME system, a charge grid of 89 × 70 × 60 gridpoints was used with a spacing of approximately 1 Å for the gridpoints. The periodic boundary condition was applied and the pressure was kept constant in the system. The temperature was kept constant according to the Berendsen algorithm with a coupling time of 0.2 ps. The cutoff distance of the nonbonded term was 14 Å. An integration time step of the MD simulations was 0.5 fs. See the Methods section in the text for other details on the calculation.

Table 3. MD Simulations of the PME Method

structure[a]	substrate	H-bonds among WAT05 and two catalytic Asp residues	d5[b]	d6[b]	d7[b]	d8[b]
1	PR−RT	4	3.01	2.84	5.52	4.82
1	MA−CA	4	3.04	2.91	5.42	5.10
2	PR−RT	2	3.93	4.33[c]	3.31	2.90
2	MA−CA	2	4.33	4.44[c]	3.40	3.10
6	PR−RT	1	4.12	3.89	3.12	3.42
6	MA−CA	2	4.15	3.88	3.28	3.32
7	PR−RT	4	3.11	3.00	5.26	5.12
7	MA−CA	4	3.18	2.97	5.12	4.98

[a] This indicates the structure of Table 1. [b] Time average distances (in Å). [c] Asp25 OD2−Pro106 N distance.

The heating of the system was done up to 300 K for 60 ps, and then the MD simulation was continued at 300 K for 250 ps. These MD simulations (300 K, 250 ps) with the PME method (Table 3) also indicate that Asp25′ is protonated in the ES complex, and two water molecules at the active site are essential for the enzyme activity.[36] Therefore, our suggestions are supported by two different MD simulations. The investigation on the roles of the water molecules in the ES complex indicates that WAT01 and WAT02 are essential for the ES complex to maintain the active conformation. In other words, the conformation for the enzymatic activity is maintained through the stabilization of the two loops containing the active site triads Asp25(25′)-Thr26(26′)-Gly27(27′) by formation of a number of hydrogen bonds involving two water molecules (WAT01 and WAT02). On the other hand, WAT00 is not essential for maintaining the active conformation, but we believe this water molecule plays another role to control the opening and closing motion of the flaps and the affinity between the enzyme and the substrate. The role of this water molecule is not revealed from the current MD simulation. This point will be an interesting subject in our future study. WAT03 and WAT04 have only a minor role in maintaining the active conformation, in spite the fact that these two water molecules form hydrogen bonds with the two loops of the active site triads. This conclusion will be supported by the fact that the two water molecules exist at the same positions even in the X-ray crystallographic structures of HIV-1 PR,[37−39] pepsin,[40] and rhizopuspepsin,[9] all of which have similar loop structures in the active site.

(36) In addition, we carried out the MD simulations with use of the cutoff technique (18 Å). The initial structure of the ES complex was placed in a box filled with approximately 6430 TIP3P water molecules. A box the size of approximately 80 Å × 60 Å × 51 Å was chosen. The periodic boundary condition was applied. The pressure was kept constant in the system and the temperature was kept constant according to the Berendsen algorithm with a coupling time of 0.2 ps. An integration time step of the MD simulations was 0.5 fs. The MD simulations of seven structures shown in Table 1 indicated that the ES complex could not maintain the active conformation. It is not considered that this cutoff distance (18 Å) is long enough for representation of the maintenance of the active conformation. See the Methods section in the text for other details of the calculation.

Conclusion

We conclude the following from this study.

(1) The catalytic mechanism of HIV-1 PR starts from the ES complex (Asp25′ protonated).

(2) The enzymatic activity of HIV-1 PR depends not only on the protease encoded by the RNA gene of HIV-1 but also on the two water molecules, WAT01 and WAT02, that exist at the loop structures of the active site and are essential for the enzymatic activity of HIV-1 PR.

(3) Gly27(27′) of the active site triads forms hydrogen bonds with Asp25(25′) side chains to maintain the specific conformation of the catalytic site, which is required for the enzymatic activity of HIV-1 PR.

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