Hydrolysis Mechanism of the Phenylalanine−Proline Peptide Bond Specific to HIV-1 Protease: Investigation by the ab Initio Molecular Orbital Method

Noriaki Okimoto,*,† Toshiyuki Tsukui,‡ Masayuki Hata,‡ Tyuji Hoshino,‡ and Minoru Tsuda‡

Contribution from the Laboratory of Physical Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan

Received November 30, 1998. Revised Manuscript Received May 18, 1999

Abstract: The protein hydrolysis mechanism by the HIV-1 protease (HIV-1 PR) was studied using ab initio molecular orbital calculations with a model compound. The initial model compound was constructed based on the result of a 100 ps molecular dynamics (MD) simulation of the enzyme−substrate (ES) complex under physiologic conditions, and consists of an acetate/acetic acid pair for the two catalytic-site Asp residues, a water molecule acting on the hydrolysis, and a dimethylacetamide for the substrate. This study suggests that the hydrolysis mechanism of the Phe-Pro peptide bond specific to the HIV-1 PR consists of three elementary reactions: first, the reaction of the formation of the amide hydrate intermediate; second, the reaction of the protonation of the proline nitrogen of the substrate; and third, the reaction of the C−N bond cleavage of the substrate. The rate-determining step is the protonation of the substrate proline nitrogen, and its activation energy is 23.95 kcal/mol. This result strongly suggests that protein hydrolysis by the HIV-1 PR occurs in vivo.

Introduction

In the process of the replication of HIV type 1 (HIV-1), two precursor polyproteins, $Pr55^{gag}$ and $Pr160^{gag-pol}$, are transformed into structural proteins and replication enzymes by HIV-1 PR. HIV-1 PR belongs to the family of aspartic proteases, which have two aspartic acid residues at the catalytic site. The active form of the HIV-1 PR is a dimer of two identical polypeptide chains, each of which consists of 99 amino acid residues. Previous analysis of the proteolytic processing of the HIV-1 PR[1−5] revealed that three of the cleavage sites contained Phe-Pro or Tyr-Pro at P1−P1′ residues (notation of Schecter and Berger[6]). Because it is unusual for mammalian endopeptidases to cleave the N-terminal of proline,[5,7] elucidation of the hydrolysis mechanism of these peptide bonds by the HIV-1 PR is of great interest. This elucidation is also important for the development of effective HIV-1 PR inhibitors. Despite several theoretical studies of the protein hydrolysis mechanism by the HIV-1 PR,[8−12] no paper has ever elucidated the entire hydrolysis mechanism of the peptide bond containing proline. In the present study, on the basis of the fact that the HIV-1 PR cleaves the peptide bond containing proline, we clarify the protein hydrolysis mechanism by HIV-1 PR through ab initio molecular orbital calculations.

Methods

Quantum Chemical Calculation. The Schrödinger equation of the model compound was solved with the Hartree−Fock method using the 6-31G** basis set. The model structures at the minimum points and transition states on the potential energy hypersurface were fully optimized using the energy gradient method. The frequency analysis on the structure of the transition state indicated the presence of an imaginary frequency. The lowest energy reaction path was determined by calculating the steepest descent paths from each transition state in both the forward and reverse directions following the normal vibrational mode of the imaginary frequency. The structures of a reactant and a product connected to the transition state resulted from this calculation. The intrinsic reaction coordinate in Figure 2 represents the lowest energy reaction path expressed in the mass-weighted coordinate. The computational program used was Gaussian 94.[13]

In all optimized structures, some constraints were applied to prevent the corruption of the basic conformation of the ES complex.[14]

MD Simulation. Energy minimization and MD simulation were performed with the program package AMBER, Version 4.1.[15] Calculations were performed using an all-atom force field.[16] The solvent was the TIP3P water model[17] and 3935 water molecules were generated to

† Current address: Computational Science Laboratory, Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako-shi, Saitama, 351-0198, Japan.

‡ Chiba University.

(1) Gowda, S. D.; Stein, B. S.; Engleman, E. G. *J. Biol. Chem.* **1989**, *264*, 8459−8462.

(2) Debouck, C.; Gorniak, J. G.; Strickler, J. E.; Meek, T. D.; Metcalf, B. W.; Rosenberg, M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 8903−8906.

(3) Kräusslich, H.-G.; Ingraham, R. H.; Skoog, M. T.; Wimmer, E.; Pallai, P, V.; Carter, C. A. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 807−811.

(4) Veronese, F. D.; DeVico, A. L.; Copeland, T. D.; Oroszlan, S.; Gallo, R. C.; Sarngadharan, M. G. *Science* **1985**, *229*, 1402−1404.

(5) Darke, P. L.; Nutt, R. F.; Brady, S. F.; Garsky, V. M.; Ciccarone, T. M.; Leu, C.-H.; Lumma, P. K.; Freidinger, R. M.; Veber, D. F.; Sigal, I. S. *Biochem. Biophys. Res. Commun.* **1988**, *156*, 297−303.

(6) Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157−162.

(7) Pearl, L. H.; Taylor, W. R. *Nature* **1987**, *328*, 482.

(8) Lee, H.; Darden, T. A.; Pedersen, L. D. *J. Am. Chem. Soc.* **1996**, *118*, 3946−3950.

(9) Liu, H.; Müller-plathe, F.; van Gunsteren, W. F. *J. Mol. Biol.* **1996**, *261*, 454−469.

(10) Chatfield, D. C.; Eurenius, K. P.; Brooks, B. R. *J. Mol. Struct. (THEOCHEM)* **1998**, *423*, 79−92.

(11) van Gunsteren, W. F.; Liu, H.; Müller-plathe, F. *J. Mol. Struct. (THEOCHEM)* **1998**, *432*, 9−14.

(12) Venturini, A.; López-Ortiz, F.; Alvarez, J. M.; González, J. *J. Am. Chem. Soc.* **1998**, *120*, 1110−1111.

10.1021/ja9841106 CCC: $18.00
Published on Web 07/30/1999

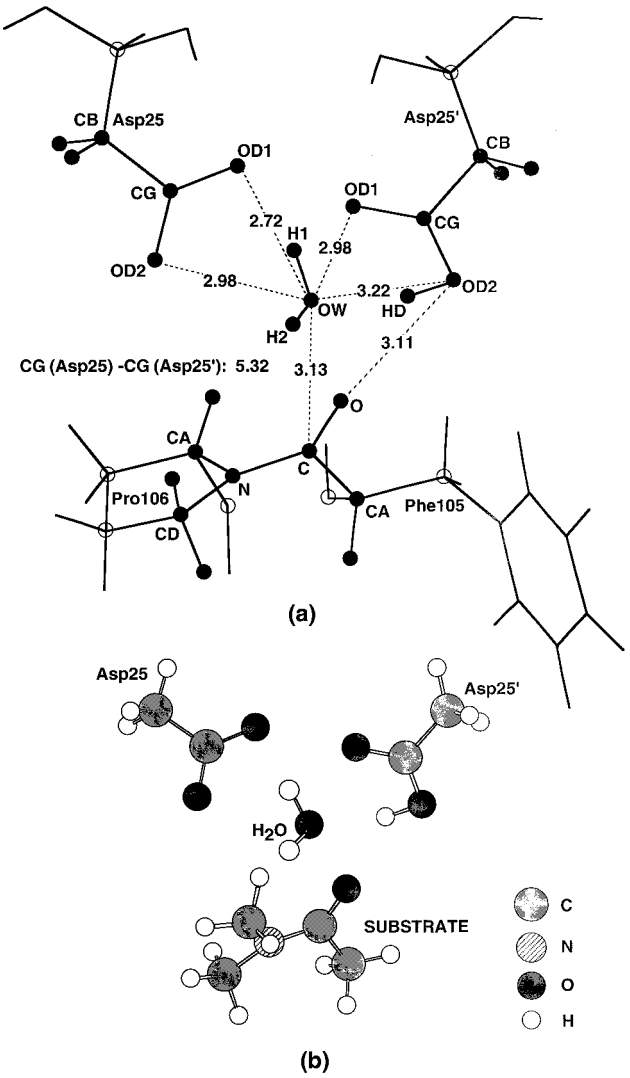

Figure 1. (a) Catalytic site in the average structure of a 100 ps MD simulation for the ES complex at physiologic temperature. Numerals are the interatomic distances (in Å). (b) Initial model compound. The initial model compound consists of an acetate/acetic acid pair for two catalytic-site Asp residues, a water molecule acting on the hydrolysis, and a dimethylacetamide for the substrate.

surround the solute. To simplify this calculation, the SHAKE procedure[18] was used. The calculations of the nonbonded term were accelerated with a hardware accelerator, called MD-Engine.[19] The MD simulation was performed starting from the energy-minimized structure. The temperature 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 trajectory at that temperature (300 K for 100 ps) was considered to be the most probable structure under physiologic conditions and the average structure was obtained.

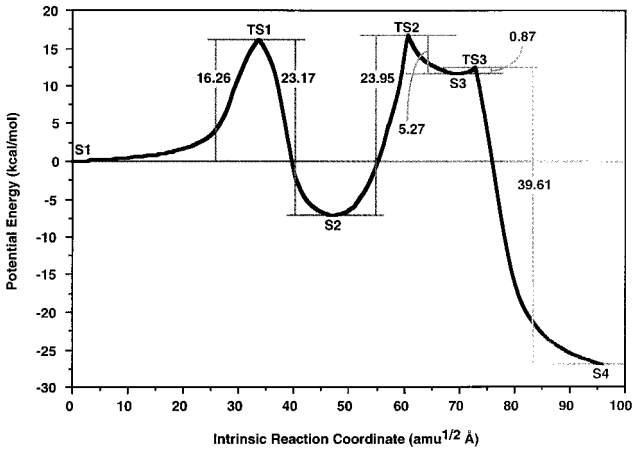

Figure 2. Total potential energy curve for the hydrolysis of the Phe−Pro peptide bond by the HIV-1 PR. The ordinate is the potential energy differences (kcal/mol) and the abscissa is the intrinsic reaction coordinate (amu$^{1/2}$ Å).

Construction of the Initial Model Compound. To obtain the initial model compound for quantum chemical calculations, the MD simulation was performed on the ES complex that was constructed based on the X-ray crystallographic structure of the HIV-1 PR-inhibitor (JG-365) complex (code in Protein Data Bank (PDB):[20−22] 7HVP[23]). The initial structure of the ES complex used for the MD simulation was as follows. The substrate molecule, Ac-Thr-Leu-Asn-Phe-Pro-Ile-Ser-NMe, represents the PR−RT cleavage site in the pol region of the HIV-1 PR. Kinetic studies of the HIV-1 PR[24−27] indicate that one of the two catalytic-site Asp residues is protonated and the other is not. Hence, the initial structure of the HIV-1 PR was constructed to satisfy this situation.[28] A water molecule acting on this hydrolysis was set in the X-ray crystallographic oxygen position of the pepsin which belongs to the family of aspartic proteases.[29] In addition, all the crystal water molecules, which were observed in the X-ray crystallographic structure (code in PDB:[20−22] 7HVP[23]), were set in the constructed ES complex.

The average structure of a 100 ps MD simulation under physiologic conditions (Figure 1a) revealed the following: The two catalytic-site Asp residues held the water molecule by forming hydrogen bonds

(13) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Gill, P. M. W.; Johnson, B. G.; Robb, M. A.; Cheeseman, J. R.; Keith, T. A.; Petersson, G. A.; Montgomery, J. A.; Raghavachari, K.; Al-Lahman, M. A.; Zakrzewski, V. G.; Ortiz, J. V.; Foresman, J. B.; Cioslowski, J.; Stefanov, B. B.; Nanayakkara, A.; Challacombe, M.; Peng, C. Y.; Ayala, P. Y.; Chen, W.; Wong, M. W.; Andres, J. L.; Replogle, E. S.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Binkley, J. S.; Defrees, D. J.; Baker, J.; Stewart, J. P.; Head-Gordon, M.; Gonzalez, C.; Pople, J. A. *Gaussian 94*, Revision E.2; Gaussian Inc.: Pittsburgh, PA, 1995.

(14) To prevent the corruption of the basic conformation of the ES complex, four constraints were applied to model compound (Z-matrix form): distance, CB(Asp25)-CB(Asp25′); dihedral angles, CB(Asp25′)−CB(Asp25)−C(Phe105)−N(Pro106), CA(Phe105)−C(Phe105)−N(Pro106)−CD(Pro106), CA(Pro106)−N(Pro106)−C(Phe105)−CD(Pro106). These atom labels are given in Figure 1a.

(15) Pearlman, D. A.; Case, D. A.; Caldwell, J. W.; Ross, W. S.; Cheatham, T. E., III; Ferguson, D. M.; Seibel, G. L.; Singh, U. C.; Weiner, P. K.; Kollman, P. A. *AMBER 4.1*; University of California: San Francisco, 1995.

(16) Weiner, S. J.; Kollman, P. A.; Nguyen, D. T.; Case, D. A. *J. Comput. Chem.* **1986**, *7*, 230−252.

(17) Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D. *J. Chem. Phys.* **1983**, *79*, 926−935.

(18) Ryckaert, J. P.; Ciccotti, G.; Berendsen, H. J. C. *J. Comput. Phys.* **1977**, *23*, 327−341.

(19) Toyoda, S.; Hashimoto, E.; Ikeda, H.; Miyakawa, N. *FUJI XEROX Tech. Rep.***1995**, *10*, 61−71.

(20) Abola, E. E.; Bernstein, F. C.; Bryant, S. H.; Koetzle, T. F.; Weng, J. In *Crystallographic Databases-Information Content, Software Systems, Scientific Applications*; Allen, F. H., Bergerhoff, G., Sievers, R., Eds.; Data Commission of the International Union of Crystallography: Bonn/Cambridge/Chester, 1987; pp 107−132.

(21) Abola, E. E.; Manning, N. O.; Prilusky, J.; Stampf, D. R.; Sussman, J. L. *J. Res. Natl. Inst. Stand. Technol.* **1996**, *101*, 231−241.

(22) Bernstein, F. C.; Koetzle, T. F.; Williams, G. J. B.; Meyer, E. F., Jr.; Brice, M. D.; Rodgers, J. R.; Kennard, O.; Shimanouchi, T.; Tasumi, M. *J. Mol. Biol.* **1977**, *112*, 535−542.

(23) Swain, A. L.; Miller, M. M.; Green, J.; Rich, D. H.; Schneider, J.; Kent, S. B. H.; Wlodawer, A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 8805−8809.

(24) Hyland, L. J.; Tomaszek, T. A., Jr.; Roberts, G. D.; Carr, S. A.; Magaard, V. W.; Bryan, H. L.; Fakhoury, S. A.; Moore, M. L.; Minnich, M. D.; Culp, J. S.; DesJarlais, R. L.; Meek, T. D. *Biochemistry* **1991**, *30*, 8441−8453.

(25) Hyland, L. J.; Tomaszek, T. A., Jr.; Meek, T. D. *Biochemistry* **1991**, *30*, 8454−8463.

(26) Rodriguez, E. J.; Angeles, T. S.; Meek, T. D. *Biochemistry* **1993**, *32*, 12380−12385.

(27) Meek, T. D.; Rodriguez, E. J.; Angeles, T. S. *Methods IN ENZYMOLOGY* **1994**, *241*, 127−156.

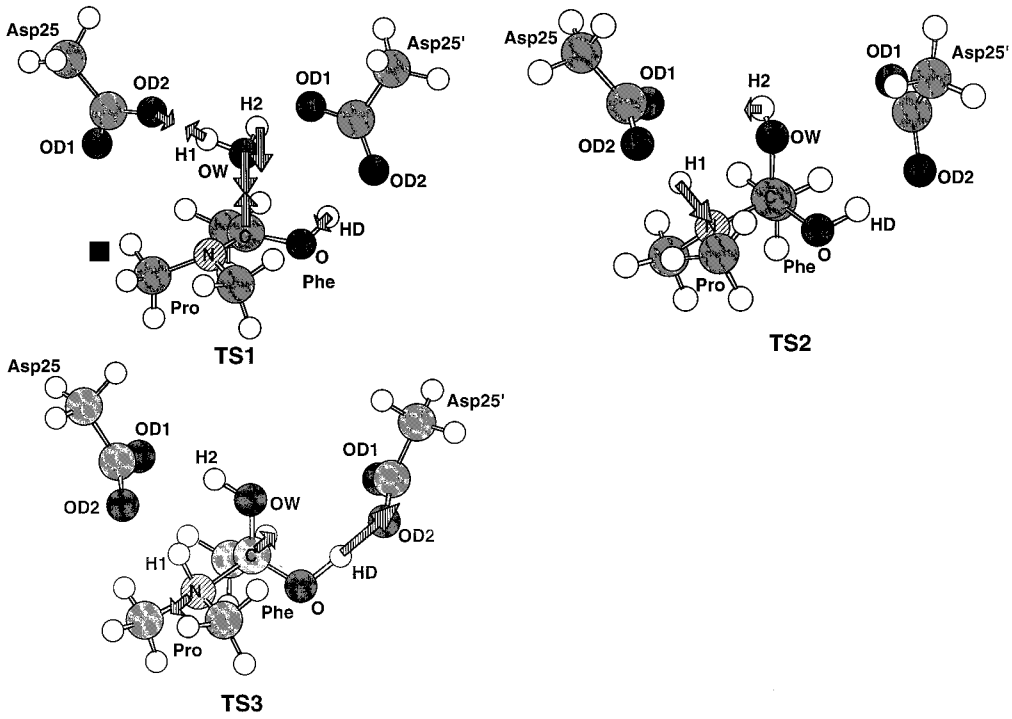

Figure 3. The normal vibrational mode of the imaginary frequency of the structure of each transition state (TS1, TS2, TS3) that appears in the hydrolysis. These arrows indicate the forward direction of the hydrolysis.

(OD1(Asp25)−OW, 2.72 Å; OD2(Asp25)−OW, 2.98 Å; OD1(Asp25′)−OW, 2.98 Å; and OD2(Asp25′)−OW, 3.22 Å) and the hydrogen bond between the OD2 atom of protonated Asp25′ and the carbonyl oxygen O of the substrate was formed (OD2(Asp25′)−O(Phe105), 3.11 Å). The oxygen atom OW of the water molecule strongly interacted with the carbonyl carbon C of the substrate (OW−C(Phe105), 3.13 Å). In addition, the CG(Asp25)−CG(Asp25′) distance was 5.32 Å.

The initial model compound for the quantum chemical calculations was constructed by extracting closed and open circle atoms from the structure shown in Figure 1a and replacing open circle atoms with hydrogen atoms. The positions of the replaced hydrogen atoms were adjusted to match the C−H distance of the methyl group. This constructed initial model compound is shown in Figure 1b. The initial model compound consists of an acetate/acetic acid pair for two catalytic-site Asp residues, a water molecule acting on the hydrolysis, and a dimethylacetamide for the substrate.

Results

The hydrolysis mechanism in this study consists of three elementary reactions that are characterized by each transition state (TS1, TS2, TS3), as seen in the potential energy curve (Figure 2). Figure 3 shows the normal vibrational mode of the imaginary frequency of the structure of each transition state (TS1, TS2, TS3). The structures of the stable states (S1, S2, S3, S4) and the transition states (TS1, TS2, TS3) that appear in the hydrolysis are shown in Figures 4−7. These elementary reactions and these structures are described below.

The First Elementary Reaction. The first step of the hydrolysis mechanism is the formation of the amide hydrate intermediate. The structure of the transition state TS1 (Figure 4) was determined from the initial model compound constructed based on the result of the MD simulation of the ES complex under physiologic conditions. This structure is obtained theoretically through the geometry optimization to search a saddle point on the potential energy hypersurface by solving the Schrödinger equation. The normal vibrational mode of the imaginary frequency indicates the attack of the water molecule on the carbonyl carbon of the substrate (see Figure 3). A steep energy drop in the direction of the separation of the OW atom of the water molecule and the C atom (carbonyl carbon) of the substrate (this direction is opposite to the arrows in Figure 3) transforms the structure of TS1 to the structure of the initial state of the hydrolysis S1, that is, the ES complex (Figure 4). Since the structure of the ES complex (S1, Figure 4) obtained theoretically from the structure of TS1 is independent of the initial model compound, it is meaningful to compare the result of the quantum chemical calculation with that of the 100 ps MD simulation of the ES complex under physiologic conditions. In contrast, a steep energy drop in the direction of the attack of the OW atom of the water molecule on the C atom of the substrate (this direction is the same as the arrows in Figure 3) transforms the structure of TS1 to the structure of the intermediate S2, that is, the enzyme-bound amide hydrate intermediate (Figure 5). The activation energy of this reaction is 16.26 kcal/mol (Figure 2).

The Second Elementary Reaction. The second step of the hydrolysis mechanism is the reaction of the protonation of the proline nitrogen of the substrate. Regarding the transition state TS2 (Figure 5), the normal vibrational mode of the imaginary frequency indicates the transfer of the H1 atom to the N atom of the substrate and the H2 atom approaching the OD1 atom of Asp25 (see Figure 3). A steep energy drop in the direction of

(28) The present study is performed based on the assumption that Asp25′ is protonated in the ES complex. The protonation of Asp25′ was already suggested to explain well the experimental data by Meek and coworkers.[24−27] In our reaction mechanism, the proton initially attached to Asp25′ is transferred to the substrate in the first step, which successfully enhances the dissociation of a water molecule for the hydrolysis. However, it is not possible from the experimental data on pK_a values[25] to determine which of two catalytic-site Asp residues is protonated. Hence, there still remains a possibility of the protonated state of Asp25 instead of Asp25′ in the ES complex. The reaction pathway initiated from the protonation of Asp25 should also be revealed for the complete understanding of the present protein hydrolysis, and this point will be an interesting subject in our future study.

(29) Cooper, J. B.; Khan, G.; Taylor, G.; Tickle, I. J.; Blundell, T. L. *J. Mol. Biol.* **1990**, *214*, 199−222.

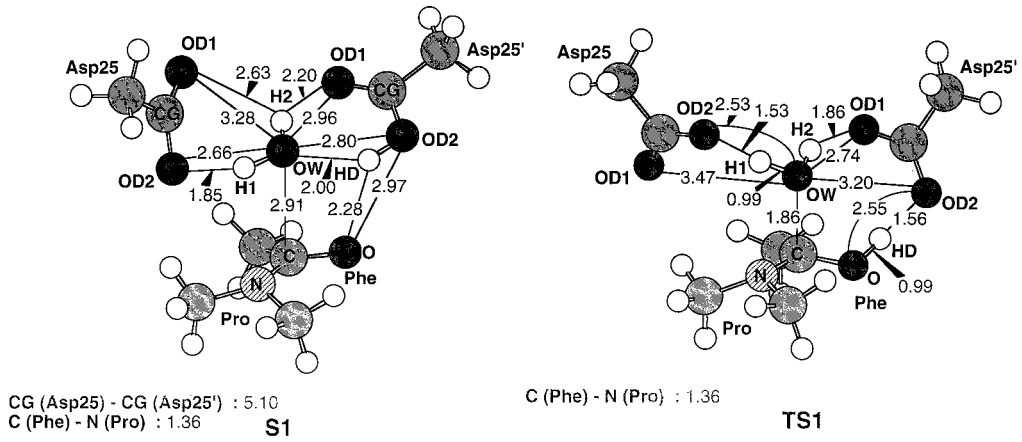

Figure 4. The structures of the stable state and the transition state which appear in the hydrolysis. S1 is the structure of the initial state of the hydrolysis and TS1 is the structure of the transition state of the first elementary reaction. Numerals are the interatomic distances (in Å).

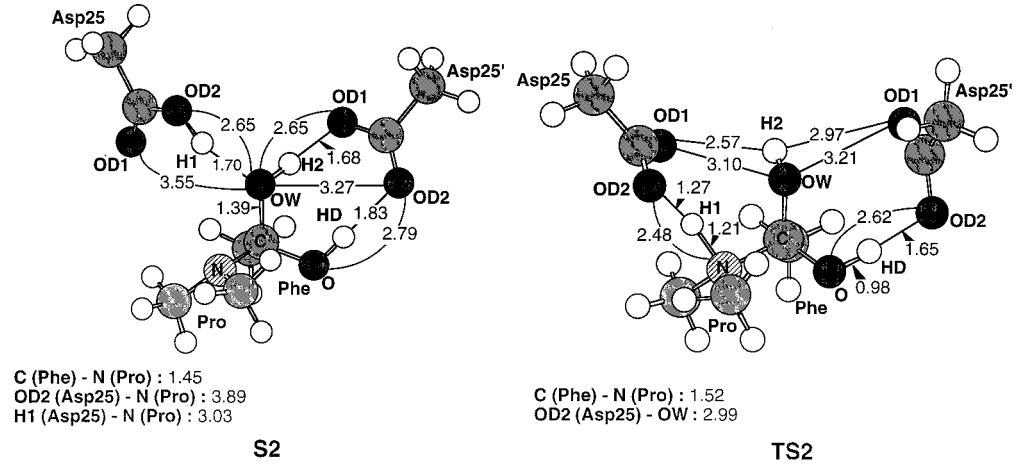

Figure 5. The structures of the stable state and the transition state which appear in the hydrolysis. S2 is the structure of the product of the first elementary reaction and TS2 is the structure of the transition state of the second elementary reaction. Numerals are the interatomic distances (in Å).

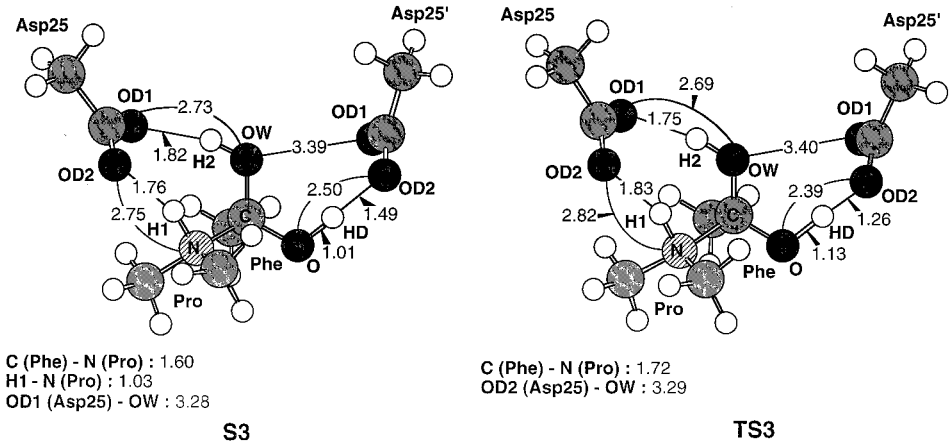

Figure 6. The structures of the stable state and the transition state which appear in the hydrolysis. S3 is the structure of the product of the second elementary reaction and TS3 is the structure of the transition state of the third elementary reaction. Numerals are the interatomic distances (in Å).

the separation of the H1 atom and the N atom of the substrate (this direction is opposite to the arrows in Figure 3) transforms the structure of TS2 to the structure of the intermediate S2, the product of the first elementary reaction (Figure 5). This result reveals that TS2 is connected to the first elementary reaction. In contrast, a steep energy drop in the direction of the transfer of the H1 atom to the N atom of the substrate (this direction is the same as the arrows in Figure 3) transforms the structure of TS2 to the structure of the intermediate S3 (Figure 6), in which the N atom of the substrate is protonated. The activation energy of this reaction is 23.95 kcal/mol (Figure 2). This reaction is the rate-determining step for the hydrolysis mechanism.

The Third Elementary Reaction. The third step of the hydrolysis mechanism is the reaction of the C−N bond cleavage of the substrate. In the structure of the transition state TS3 (Figure 6), the normal vibrational mode of the imaginary frequency indicates the C−N bond cleavage and the transfer of the HD atom to the OD2 atom of Asp25′ (see Figure 3). A steep energy drop in the direction of the shrinkage of the C−N distance (this direction is opposite to the arrows in Figure 3)

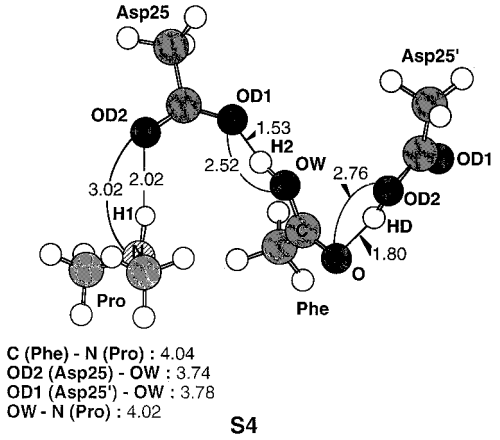

Figure 7. The structure of the stable state which appears in the hydrolysis. S4 is the structure of the final state of the hydrolysis. Numerals are the interatomic distances (in Å).

transforms the structure of TS3 to the structure of the intermediate S3, the product of the second elementary reaction (Figure 6). The result reveals that the transition state TS3 is connected to the second elementary reaction. In contrast, a steep energy drop in the direction of the C−N bond cleavage (this direction is the same as the arrows in Figure 3) transforms the structure of TS3 to the structure of the final state of the hydrolysis S4 (Figure 7). The activation energy of this reaction is 0.87 kcal/mol (Figure 2).

1. Initial State of the Hydrolysis S1. The initial state of the hydrolysis S1 (Figure 4) obtained in the above theoretical approach is the ES complex. In the structure of S1, Asp25 and Asp25′ at the catalytic site hold the water molecule by forming hydrogen bonds (OD1(Asp25)−OW, 3.28 Å; OD2(Asp25)−OW, 2.66 Å; OD1(Asp25′)−OW, 2.96 Å; OD2(Asp25′)−OW, 2.80 Å) and there is another hydrogen bond between the OD2 atom of protonated Asp25′ and the carbonyl oxygen O of the substrate (OD2(Asp25′)−O(Phe), 2.97 Å). The distance between the OW atom of the water molecule and the C atom of the substrate is 2.91 Å. Since this distance, 2.91 Å, is shorter than the sum of the van der Waals radius of carbon and oxygen by approximately 0.3 Å, there is an effective interaction to initiate the hydrolysis reaction. In addition, the CG(Asp25)−CG(Asp25′) distance is 5.10 Å. These geometric values suggest that the ES complex obtained by the quantum chemical calculations is consistent with the ES complex of the MD simulation under physiologic conditions (Figure 1a).

2. Transition State TS1. The transition state TS1 appears when the OW atom of the water molecule approaches the C atom of the substrate from the S1 structure (Figure 4). The structure of TS1 (Figure 4) indicates that the distance between the OW atom of the water molecule and the C atom of the substrate is reduced to 1.86 Å. This distance is shorter than that of the structure of S1 by 1.05 Å. The OW atom approaching the substrate causes the structural change of the carboxyl groups of the two catalytic-site Asp residues, so that the HD atom attached to the OD2 atom of Asp25′ in the structure of S1 is transferred to the O atom of the substrate and the hydrogen bond between the OW atom of the water molecule and the OD2 atom of Asp25 becomes stronger.

3. Intermediate S2. The product of the first elementary reaction is the intermediate S2, that is, the enzyme-bound amide hydrate intermediate (Figure 5). In the structure of S2, the OW atom from the water molecule is linked with the C atom of the substrate and the H1 atom initially attached to the water molecule is transferred to the OD2 atom of Asp25. Thus, the amide hydrate intermediate (S2, Figure 5) is formed by the linkage of the OW atom and the C atom, accompanied by two concerted proton transfers. In the formation of the amide hydrate intermediate by two concerted proton transfers, the protonation states of the catalytic-site Asp residues are opposite to those of the structure of S1 (Figure 4). These protonation states enable the second elementary reaction, that is, the reaction of the protonation of the proline nitrogen of the substrate. In addition, there are three hydrogen bonds among the two catalytic-site Asp residues and the substrate (OD2(Asp25)−OW, 2.65 Å; OD1(Asp25′)−OW, 2.65 Å; and OD2(Asp25′)−O(Phe), 2.79 Å). This indicates that the two catalytic-site Asp residues hold the substrate.

4. Transition State TS2. Following the intermediate S2, the structure of the transition state TS2 (Figure 5) appears, in which the H1 atom is just transferring to the N atom of the substrate. It is notable that the OD1(Asp25′)−OW distance is longer than that of the intermediate S2 by 0.56 Å, whereas the OD1-(Asp25)−OW distance is shorter than that of the intermediate S2 by 0.45 Å.

5. Intermediate S3. TS2 leads to formation of the structure of the intermediate S3 (Figure 6), in which the proton H1 is completely transferred to the N atom of the substrate. It is obvious from the comparison between the structures of the intermediates S2 and S3 that the rotation of the hydroxy group (OW−H2) occurs. This rotation induces the disappearance of the original hydrogen bond between the OD1(Asp25′) and OW atoms and the formation of the new hydrogen bond between the OD1(Asp25) and OW atoms. In addition, a remarkable structural change is observed at the point that the hydrogen bond between the OD2(Asp25′) and O(Phe) atoms becomes stronger (the HD atom effectively contributes to the formation of this low-barrier hydrogen bond between the OD2(Asp25′) and O(Phe) atoms) and the C−N distance becomes 0.15 Å longer in intermediate S3 compared to the intermediate S2. This expansion of the C−N bond indicates that the reaction proceeds to the direction of the collapse of the substrate.

6. Transition State TS3. The further expansion of the C−N bond of the structure of S3 leads to the structure of the transition state TS3 (Figure 6), in which the C−N distance is 1.72 Å. The distance between the OD2 atom of Asp25′ and the O atom of the substrate is reduced to 2.39 Å.

7. Final State of the Hydrolysis S4. The C−N bond cleavage occurs via the transition state TS3. The substrate is decomposed into acetic acid (phenylalanine) and dimethylamine (proline) by the C−N bond cleavage. In the structure of the final state of the hydrolysis S4 (Figure 7), Phe of the C-terminal product is bonded to Asp25 and Asp25′ by forming two hydrogen bonds. Pro of the N-terminal product is also bonded to Asp25 by forming a hydrogen bond. After this protein hydrolysis reaction, these products are released from the HIV-1 PR. Note that the proton HD is transferred from the O atom of the substrate to the OD2 atom of Asp25′ in the collapse of the substrate. Accordingly, the protonation states of the two catalytic-site Asp residues return to those of the structure of the initial state of the hydrolysis S1 so that the HIV-1 PR is able to hydrolyze another substrate.

Discussion

1. Reaction of the Formation of the Amide Hydrate Intermediate. The formation of the amide hydrate intermediate is supported by the isotope kinetic experiments by Meek and co-workers[24,25,27] and the theoretical study at the MP2/6-31+G* level by Venturini and co-workers.[12] However, Meek et al.

proposed that an additional intermediate (O-protonated amide) was present in the process of the formation of the amide hydrate intermediate. We were not able to detect this additional intermediate, although it was very similar to the structure of the transition state TS1. It is unclear whether the structure of the ES complex determined by Venturini and co-workers is consistent with our 100 ps MD simulation results under physiologic conditions (see Figure 1a) because the structure was not described in detail. In contrast to the proposal of the amide hydrate intermediate, Wlodawer et al.[30] and Lee et al.[8] described that the hydrolysis of the peptide bond was a single elementary reaction. The structure of the ES complex determined theoretically at the HF/6-31G* level by Lee et al. is quite different from that of this study. In the structure of Lee et al., neither of the two catalytic-site Asp residues were protonated and the nitrogen atom of the substrate was protonated. This structure, however, was not found in the isotope kinetic experiments.[24−27]

2. Reaction of the Protonation of the Proline Nitrogen of the Substrate. The hydrolysis mechanism proposed in this study involves the reaction of the proton transfer from the aspartic acid to the proline nitrogen prior to the C−N bond cleavage. This mechanism is supported by isotope kinetic experiments.[26,27] On the other hand, Venturini and co-workers proposed that the C−N bond cleavage was accompanied by the proton transfer to the nitrogen atom of the substrate.[12] This proposal suggests that the second elementary reaction in our mechanism does not occur. This is likely due to the inadequacy of the model compound as pointed out by Venturini and co-workers.[12] Because the substrate molecule (dimethylacetamide) of our model compound works as the substrate of the specific cleavage site containing proline, the hydrolysis mechanism of the Phe−Pro peptide bond by the HIV-1 PR would involve the second elementary reaction, that is, the reaction of the protonation of the proline nitrogen of the substrate. One reason that this reaction is the rate-determining step might be the presence of the proline in the amide hydrate intermediate, that is, the proline would cause the steric hindrance for the protonation reaction.

3. Reaction of the C−N Bond Cleavage of the Substrate. In the reaction of the C−N bond cleavage of the substrate, the final state of the hydrolysis S4 is more stable than the transition state TS3 by 39.61 kcal/mol so that this reaction is not readily reversed. The irreversibility of the reaction of the C−N bond cleavage is supported by the isotope kinetic experiments.[24]

Conclusion

We conclude the following from this study.

1. The hydrolysis mechanism of the Phe−Pro peptide bond specific to the HIV-1 PR consists of three elementary reactions.

2. The rate-determining step of the protein hydrolysis mechanism by the HIV-1 PR is the process of the protonation of the proline nitrogen of the peptide bond. This activation energy is 23.95 kcal/mol, which suggests that this hydrolysis occurs in vivo.

Acknowledgment. This work was partly supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan. The authors thank the Computer Center of the Institute for Molecular Science, Okazaki. The computations were also carried out by the DRIA System at the Faculty of Pharmaceutical Sciences, Chiba University.

JA9841106

(30) Jaskólski, M.; Tomasseli, A. G.; Sawyer, T. K.; Staples, D. G.; Heinrikson, R. L.; Schneider, J.; Kent, S. B. H.; Wlodawer, A. *Biochemistry* **1991**, *30*, 1600−1609.