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ORIGINAL PAPER

Theoretical study on isomerization and peptide bond cleavage at aspartic residue

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Abstract Isomerization and peptide bond cleavage at aspartic residue (Asp) in peptide models have been reported. In this study, the mechanisms and energies concerning the isomerization and peptide bond cleavage at Asp residue were investigated by the density functional theory (DFT) at B3LYP/6-311++G(d,p). The integral equation formalism-polarizable continuum model (IEF-PCM) was utilized to calculate solvation effect by single-point calculation of the gas-phase B3LYP/6-311++G(d,p)-optimized structure. Mechanisms and energies of the dehydration in isomerization reaction of Asp residue were comparatively analyzed with the deamidation reaction of Asn residue. The results show that the succinimide intermediate was formed preferentially through the step-wise reaction via the tetrahedral intermediate. The cleavage at C-terminus is more preferential than those at N-terminus. In comparison to isomerization, peptide bond cleavage is ~20 kcal mol^{-1} and lower in activation barrier than the isomerization. So, in this case, the isomerization of Asp is inhibited by the peptide bond cleavage.

Keywords Aspartic residue · Density functional theory · Isomerization · Peptide bond cleavage

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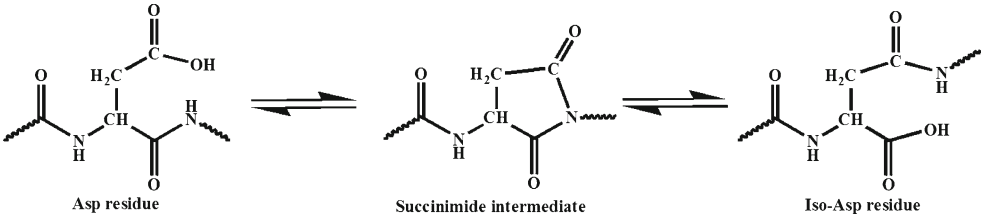
Introduction

Deamidation of asparagine (Asn) and glutamine (Gln) are well-known spontaneous non-enzymatic reactions under physiological condition [1–3]. The deamidation of relatively unrestrained Asn residues through a succinimide intermediate have been proposed by Capasso et al. [4, 5]. The intermediate is then hydrolyzed at either one of the two carbonyls giving Asp and iso-Asp residue as deamidation products. The ratio of Asp:iso-Asp was experimentally found to be 1:3 [6]. However, the ratio of Asp:iso-Asp greatly depends on pH, neighboring amino acid sequence and conformation of the protein/peptide. At low pH (< 2), the reaction pathway to form Asp:iso-Asp is direct hydrolysis which undergoes succinimide-mediated pathway at philological pH. The direct hydrolysis is most favorably indicated by the marked drop of the iso-Asp/Asp ratio [7]. Ordinary base catalysis also occurs rapidly at high pH but the mechanism of the imide intermediate is still obscure.

While Asn and Gln undergo deamidation to form the succinimide intermediate, acidic amino acid residues; Asp and Glu can also undergo dehydration forming succinimide intermediate. Therefore, an iso-Asp can arise from a spontaneous deamidation or dehydration following Asn or Asp residue. The chemical equilibrium of isomerization of Asp was shown in Scheme 1. Deamidation and iso-Asp formation in proteins has been reviewed [8]. Formation of iso-Asp residue results from the hydrolyzation of the succinimide intermediate (typically half-life 4 h at physiological condition) in a mixture of ~30 % Asp and ~70 iso-Asp residues [3, 9, 10]. The iso-Asp residue is a predominant product due to the asymmetry of the succinimide structure [11]. Isomerization related to deamidation of Asn typically occurs more rapidly than hydration of Asp residue within the same amino acid sequence [3, 12].

Scheme 1 Isomerization equilibria of Asp-isoAsp residues through the stable cyclic succinimide intermediate



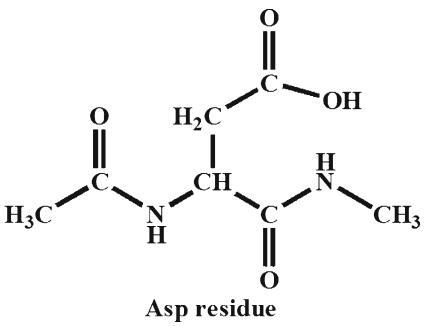
The rate of iso-Asp formation is affected by C-terminal amino acid sequence. A high rate of iso-Asp formation is found at small and hydrophilic neighboring amino acid sequence whilst there is less at bulky or hydrophobic ones. Comparison of the rate of succinimide formation of Asn/Asp peptides with various C-terminal amino acid sequences has been summarized [13]. However, not only the C-terminal amino acid sequence affects the rate of iso-Asp formation and also the flexibility of peptide/protein structures. Iso-Asp occurs preferentially within the flexible proteins/peptide whilst less formation was found within secondary structure such as α-helix and β-sheet [14]. Deamidation of Asn and hydrolysis of Asp caused changes in amino acid sequence, conformation and also biological function in peptide/proteins. For example, aggregation and neurodegenerative disorders affected from iso-Asp formation in β-amyloid have been found [15–17]. The presence of iso-Asp even in low abundance may also initiate autoimmune response [18, 19], likely due to altered antigen presentation [20]. However, all organisms possess the mechanism to reduce the level of iso-Asp. The mechanism may be proceeding either via an action of protein isoaspartic methyltransferase (PIMT or PCMT) or putative proteolytic pathway [8, 21–23].

Nevertheless, peptide bond cleavage is also occurring in peptides/proteins as non-enzymatic reaction. Peptide bond cleavage at Asn residue has been studied [24, 25]. For Asn-Pro sequence, the peptide bond cleavage occurs without deamidation at the elevated temperature in which a succinimide intermediate was not formed [3, 26–29]. Peptide bond cleavage was found in most peptide deamidation generally with a slower rate than that of the deamidation. Experimental half-life of Asn deamidation reaction was found between 1 to 400 days while peptide fragmentation ranged from 200 to >10000 days [30]. Peptide bond cleavage at Asn-Pro is the fastest among all other sequences due to a lack of amino proton of Pro leading to slower hydrolysis of the Asn-Pro deamidates [3, 27]. Peptide bond cleavage at Asp and Glu residues in peptides and proteins with a higher rate than Asn and Gln has also been observed [31–34]. Peptide bond cleavage at Asp residue was first reported by Partridge and Davis [35]. The experimental observation showed that the preferential hydrolysis of peptide bond at Asp residue generally occurred at C-terminal. The hydroxyl oxygen of carboxylic side-chain is acting as a proton donor which catalyzed the C-terminal cyclization at a pH value under pK_a of Asp residue [36]. Pyrolysis-induced peptide bond cleavage of Asp residue in peptides and proteins at C- and N-terminal were investigated by mass spectrometry [37]. The result demonstrated that pyrolysis at temperatures from 220 to 250 °C favor cleavage at C-terminal occurs via the five-membered ring intermediate. Peptide bond cleavage at Asp residue with 30.5 kcal mol^{-1} activation barrier calculated with DFT/B3LYP/6-31++G(d,p) method has been reported [38].

Cases of deamidation of Asn have been widely studied with computational method [39–41], the mechanisms and energies of dehydration associated to isomerization and peptide bond cleavage of Asp residue has been less studied. The aim of this work is to computationally explore mechanisms and energies of the isomerization reaction and peptide bond cleavage at different terminals of Asp residue in model peptide where the isomerization of Asp may be reasonably predicted for the likelihood of Asn. The calculated activation barrier found in the isomerization reaction will then be compared to the peptide bond cleavage reaction. The result is useful for prediction of the Asp residue ability to compete between isomerization and C- or N-terminal cleavage. Knowledge on mechanism and energies of isomerization when peptide bond cleavage of Asp may also be useful in case of isomerization and peptide bond cleavage is obstructed by protein structure.

Computational methods

Even though Asp residue is presented as anion in physiological conditions due to its pK_a, the carboxylic form is used in this work rather than the carboxylate form. It is noted that carboxylate group is a better candidate for nucleophilic attack than its acid form, so the calculation results are considered in



Scheme 2 Asp residue modeled peptide used in this work

the scope of the process based on carboxylic attack. The Asp residue in model peptide used in this calculation is shown in Scheme 2. The geometries of reactants, transition states, intermediates and products were fully optimized in the gas phase using density functional theory (DFT) [41–44] at B3LYP/6−311++G(d,p) [45–47] level. Zero-point energy corrections and thermodynamic properties of all relevant structures were obtained by vibrational frequencies calculation at 1 atm and 298.15 K, the frequency are not scaled. Local minima and first-order saddle points were identified by the number of imaginary vibrational frequency. Solvent effect was taken into account by utilizing the polarize continuum model (PCM) with integral equation formalism-polarizable continuum (IEF-PCM) model [48–52]. Solvation free energy (ΔG_{sol}) were obtained by single-point calculation of the gas phase-B3LYP/6−311++G(d,p) geometrical structure at the same level in H_2O medium (ε=78.37). The cavity model used for solvent effect calculation is united atom topological model for Khon-Sham (UAKS) [53, 54]. All calculations were performed with the Gaussian 03 program [55]. The molecular graphics of all related species were generated with the MOLEKEL 4.3 program [56].

Results and discussion

Isomerization at Asp residue

Based on the likelihood of deamidation, there are two pathways of succinimide intermediate formation due to dehydration reaction. The concerted (A) and stepwise (B) reactions of isomerization of Asp residue was shown in Scheme 3. Iso-Asp residue is formed from the hydrolysis of the intermediate as shown in reaction C. The mechanisms and energies for each reaction steps are discussed in this part.

Concerted reaction of succinimide formation (asp→suc)

For the concerted succinimide formation (Pathway A), the amino backbone attacks to side-chain carbonyl carbon and the amino proton transfers to the side-chain hydroxyl oxygen formed the succinimide intermediate (INT1) in one step. Dehydration occurred in this ring closure step. Figure 1a is the relative free energy profiles with optimized-structures for concerted and step-wise succinimide intermediate formation in water-less systems. The energy profile for water-assisted systems is shown in Fig. 1b. Based on the optimized-B3LYP/6-311++G(d,p) structures, the difference between transition states (TS1/TS1_w) of both systems is the forming of a carbonyl side-chain-amino backbone (C-N) bond, breaking C-OH bond and breaking N-H bond distances. The C-N bond distance found in TS1_w (1.695 Å) is shorter than TS1 (2.178 Å). The C-OH and N-H bond distances in TS1_w (1.722, 1.268 Å) is also shorter than found in TS1 (1.844, 1.471 Å). The closure of C-N bond provides the ring closure step conveniently affected by the present of water molecule. The activation free energies for water-less and water-assisted systems are 59.0(62.4) and 50.4(50.0) kcal mol^{-1}, respectively (The value in parenthesis is due to continuum solvation effect). As can be seen, it is ~9(12) kcal mol^{-1} lower in an activation barrier found

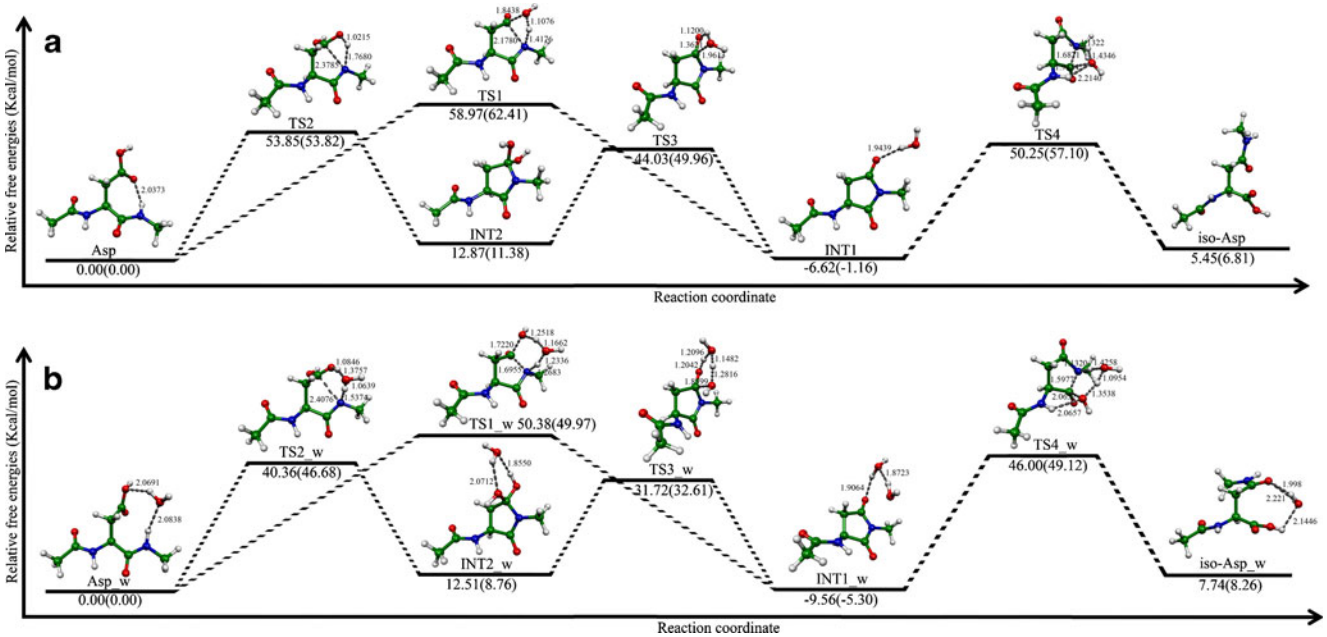
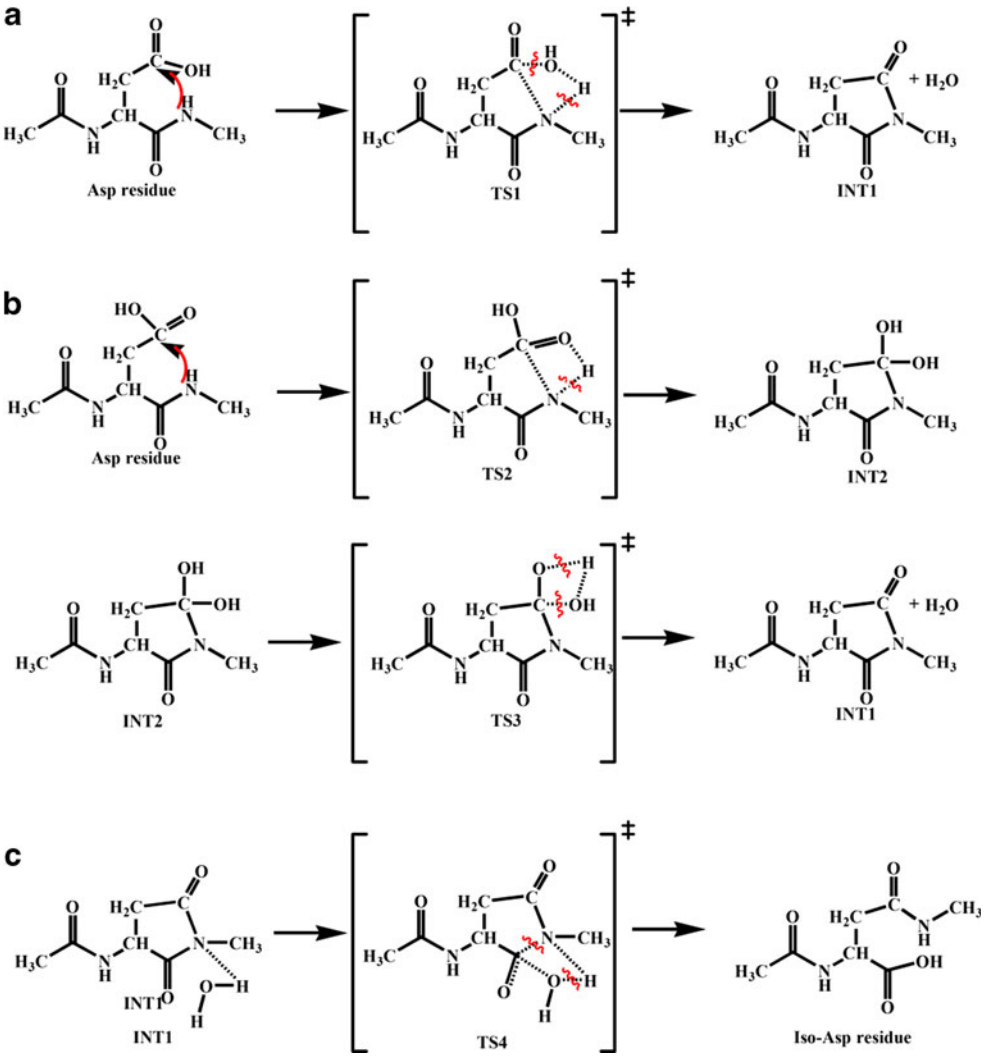


Fig. 1 The relative free energy profile with optimized-structures for water-less (**a**) and water-assisted (**b**) cyclization step in isomerization of Asp residue (relative free energies in continuum aqueous media are in parenthesis, bond distances are in Å)



Scheme 3 The reaction pathways of the isomerization of aspartic residue

for water-assisted compared to water-less system. It can be said that the presence of explicit solvent molecule participates in the reaction can lower the energy barrier. In addition, the polarization due to continuum environment also has an important effect.

Step-wise reaction of succinimide formation (asp→tet→suc)

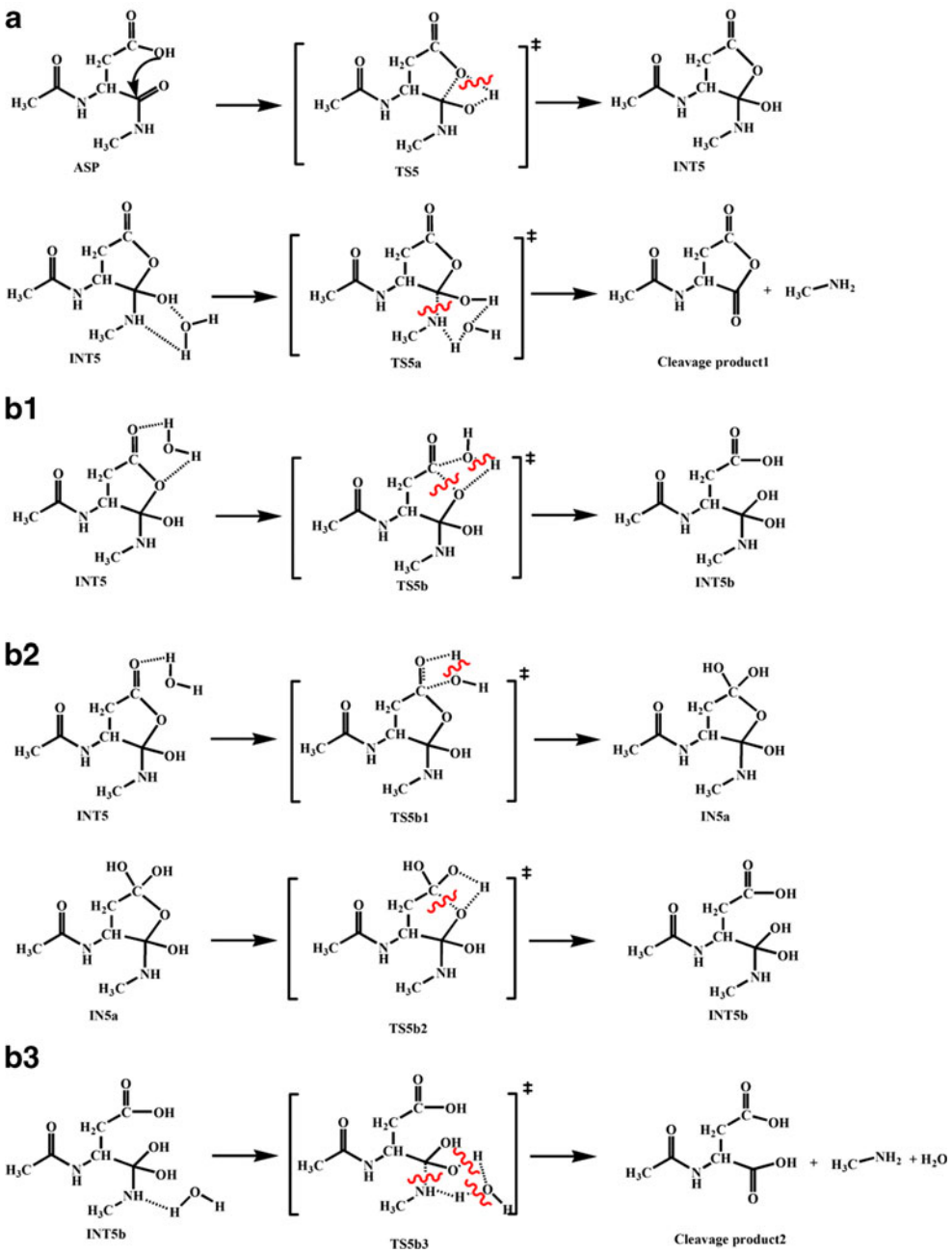
For the step-wise reaction, the tetrahedral intermediate (TS2/TS2_w) was formed in the first step, then succinimide intermediate will be formed after dehydration of the tetrahedral intermediate in the second step (pathway B). The formation of the tetrahedral intermediate occurring by the amino backbone attack on carbonyl side-chain and amino proton transfers to the carbonyl oxygen. The longer C-N bond and N-H bond distances of TS2 (2.378, 1.768 Å) and TS2_w (2.407, 1.537 Å) with respect to TS1 and TS1_w are found.

Activation barriers for TS2 and TS2_w are 53.8(53.8) and 40.8(36.4) kcal mol^{-1}, respectively for water-less and water-assisted systems. It is ~13.0(17.4) kcal mol^{-1} lower in barrier for water-assisted against to water-less. The tetrahedral intermediate (INT2) is then converted to INT1 through dehydration reaction with relative 44.0(49.9) (TS3) and 31.7(32.6) (TS3_w) kcal mol^{-1} activation barrier. As can be seen, the activation barrier for forming INT2 is approximately 5(9) and 10(13) kcal mol^{-1} in water-less and water-assisted systems being lower than those of the INT1 formation. This indicates that the step-wise forming of INT1 is more energetically preferential than the concerted reaction. Thus, the step-wise reaction of succinimide formation is the rate determining step for the isomerization reaction of Asp residue.

Hydrolysis of succinimide intermediate

Hydrolysis of succinimide intermediate on either side of carbonyl groups causes forming of iso-Asp or Asp residue. In some case, the succinimide residue is stable enough to be a major end product especially in mildly acidic condition [57, 58]. The major product of succinimide hydrolysis is an iso-Asp residue due to the asymmetric effect of the structure as mentioned in the Introduction. In case of Asp, hydrolysis of

Scheme 4 Reaction pathway for C-terminal peptide bond cleavage at Asp residue



succinimide intermediate is the equilibrium of Asp and iso-Asp (Scheme 1). So, the reverse hydrolysis of INT1 via TS1 as well as INT1_w via TS1_w is the forming of original Asp residue. In path C (Scheme 3), iso-Asp is formed via TS4 and TS1_w for water-less and water-assisted systems. For the water-assisted system, one additional water molecule is involved in the INT1_w hydrolysis reaction. It was found that relative barriers for formation of iso-Asp via TS4 and TS4_w are 50.2(57.1) and 46.0(49.1) kcal mol^{-1}, respectively. Thus, the formation of iso-Asp via water-assisted system is more energetically preferential than the waterless system. In comparison to the reverse reaction to form an original Asp via a concerted pathway, the reaction is inhibited because of higher barrier found in TS1 (~45 kcal mol^{-1}) and TS1_w (~39 kcal mol^{-1}) regarding INT1 and INT1_w. Thus, reformation of Asp is also due to the stepwise backward reaction via either INT1→TS3→INT2→TS2→Asp or INT1_w→TS3_w→INT2_w→TS2_w→Asp, respectively. In this case, it can be noted that the number of water molecules plays an important role in assessing the ratio of Asp:iso-Asp.

Peptide bond cleavage at Asp residue

For peptide bond cleavage, there are three major mechanisms which have been identified. Preferential hydrolysis of peptide bond at Asp residues under acidic condition, succinimide

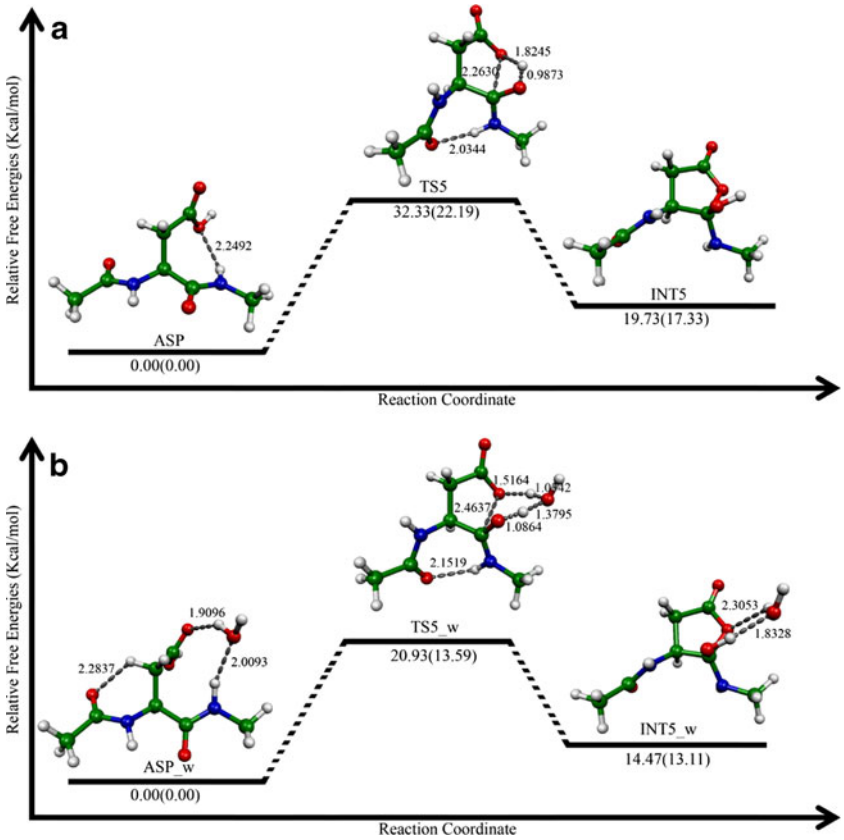


Fig. 2 The relative free energy profile with optimized-structures for water-less (**a**) and water-assisted (**b**) cyclization step forming INT5 in C-terminal cleavage of Asp residue (Relative free energies in continuum aqueous media are in parenthesis, Bond distances are in Å)

formation of Asn residue at more physiological pH and enzymatic proteolysis included autolysis [59]. The cleavage of peptide bond obviously disrupts the linear sequence of the amino acid residue in proteins, however, this may or may not affect the higher ordered structure in proteins. Based on the previous work [37] where the cleavage of peptide bond at Asp residue with two competitive pathways, C- and N-terminal has been investigated. The computational results of this study are discussed as the following.

C-terminal cleavage

Reaction pathway for C-terminal cleavage of Asp residue is shown in Scheme 4. The reaction mechanism of the cleavage through concerted formation and hydrolysis of a five-membered ring intermediate (INT5). The C-terminal cleavage undergoes the formation of INT5 by nucleophilic attack of hydroxyl oxygen of carboxylic side-chain on backbone carbonyl carbon. The C-O bond is formed where the hydroxyl proton is transferred to backbone carbonyl oxygen. The corresponding transition state for the initial ring closure step is TS5 and TS5_w for water-less and water-assisted systems. Reaction energy profile with optimized structures for water-less and water-assisted cyclization steps formed INT5 and INT5_w and is depicted in Fig. 2. Activation barrier for the water-less and water-assisted are 32.3(22.2) and 20.9(13.6) kcal mol^{-1}, respectively. Based on the transition state structures, the forming C-O bond in

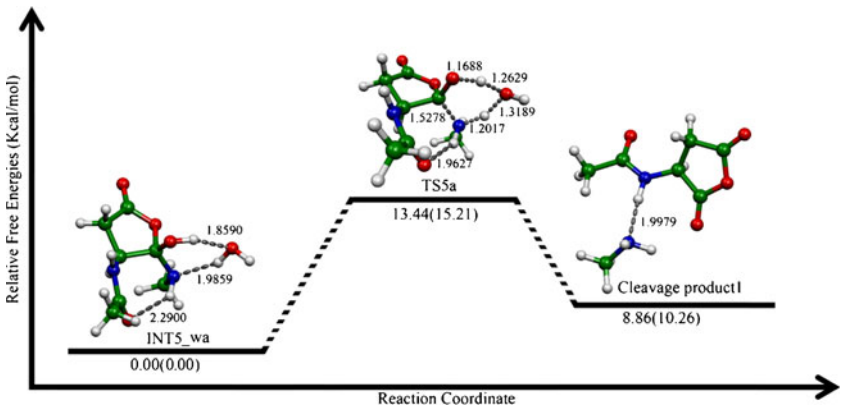


Fig. 3 The relative free energy profile with optimized-structures for concerted hydrolysis of INT5 in C-terminal cleavage of Asp residue (Relative free energies in continuum aqueous media are in parenthesis, Bond distances are in Å)

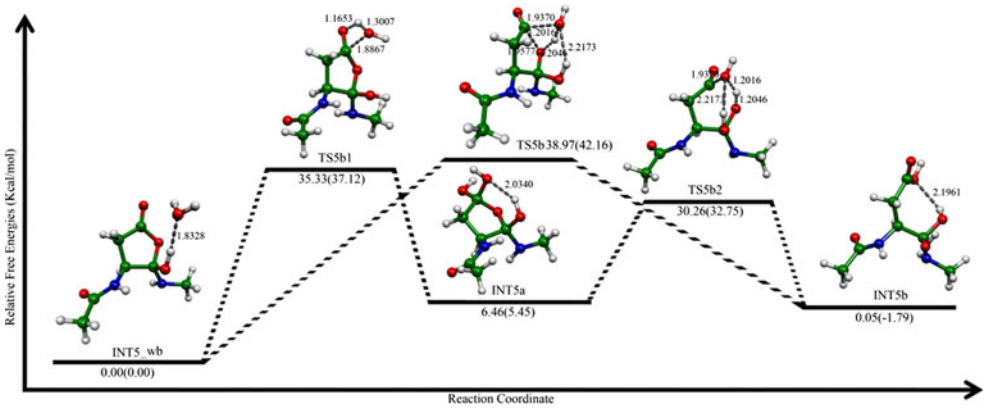


Fig. 4 The relative free energy profile with optimized-structures for concerted hydrolysis of INT5 forming INTb5 in C-terminal cleavage of Asp residue (relative free energies in continuum aqueous media are in parenthesis, bond distances are in Å)

TS5_w (2.463 Å) is longer than that found in TS5 (2.263 Å). Additionally, H-bond between backbone skeleton is found in both TS structures. For peptide bond cleavage, hydrolysis of INT5 is required. Two pathways for hydrolysis of INT5_w based on the conformation of INT5_w are proposed in this study. There are two conformations of INT5_w including INT5_wa and INT5_wb, respectively. Figure 3 shows the relative free energy profile of the concerted hydrolysis of INT5_wa via TS5a to form cleavage product 1. The concerted hydrolysis of INT5_wa via TS5a requires a 13.4(15.2) kcal mol^{-1} activation barrier.

Based on hydrolysis of INT5_wb, the concerted hydrolysis occurred via INT5_wb→TS5b→INT5b reaction while the stepwise one is due to the reaction INT5_wb→TS5b1→INT5a→TS5b2→INT5b, respectively, the cleavage is then due to hydrolysis of INT5b. The relative energy profile with optimized structures for concerted and step-wise hydrolysis of INT5_wb forming INT5b is depicted in Fig. 4. The concerted reaction involves the breaking of ester bond by water attack on carbonyl carbon and an asynchronous proton from water is transfered to the ester oxygen leading to the ring opening intermediary (INT5b). This step requires 45.3(37.1) kcal mol^{-1} (TS5b) activation barriers regards to INT5_wb. For the step-wise, nucleophilic attack by water molecule without breaking of the ester bond (TS5b1) is the first step. The outcome of this step is INT5a where carbonyl carbon in the five-membered ring is found as tetrahedral carbon with two hydroxyl groups. The ring opening on ester bond occurs via proton process from hydroxyl group on tetrahedral carbon to ester oxygen (TS5b2) forming INT5b. The relative barriers 35.3(37.1) and 30.2(32.7) kcal mol^{-1} are required for theses two steps. According to hydrolysis of INT5b_w, 14.9 kcal mol^{-1} activation barriers requires the fragmentation of the peptide bond (Fig. 5). Even though the peptide fragmentation due to INT5b_w hydrolysis is comparable to those of INT5_wa, the first path is less favorable due to higher energy requirements for INT5b formation. It can be concluded that the concerted hydrolysis of INT5_wa is more energetically preferential for the peptide bond cleavage at C-Terminus than those of both concerted and stepwise reactions of INT5_wb. This may be due to the flexibility of geometrical structure INT5_wa which is higher than those of INT5_wb, leading to the lowering in the activation barrier for forming TS5a.

N-terminal cleavage

The reaction mechanism of N-terminal cleavage is shown in Scheme 5. For cleavage at N-terminal, the N-terminal carbonyl

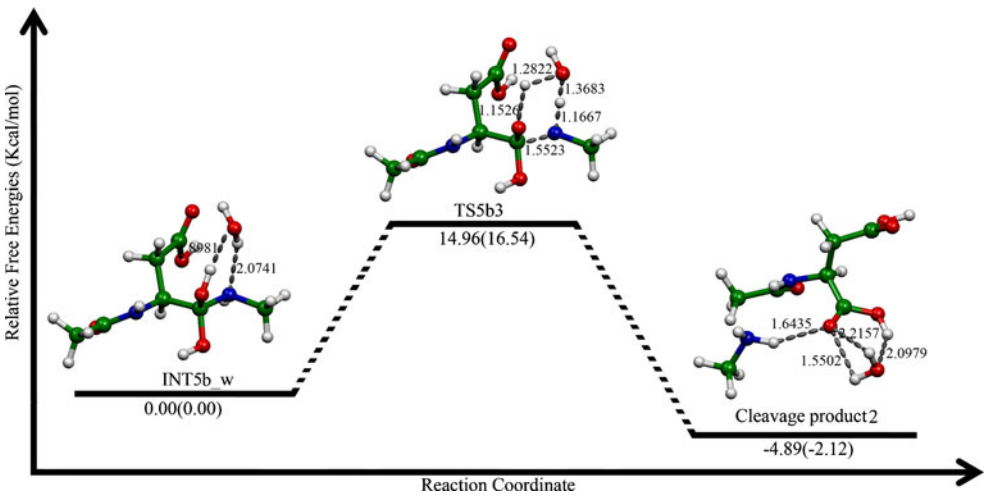
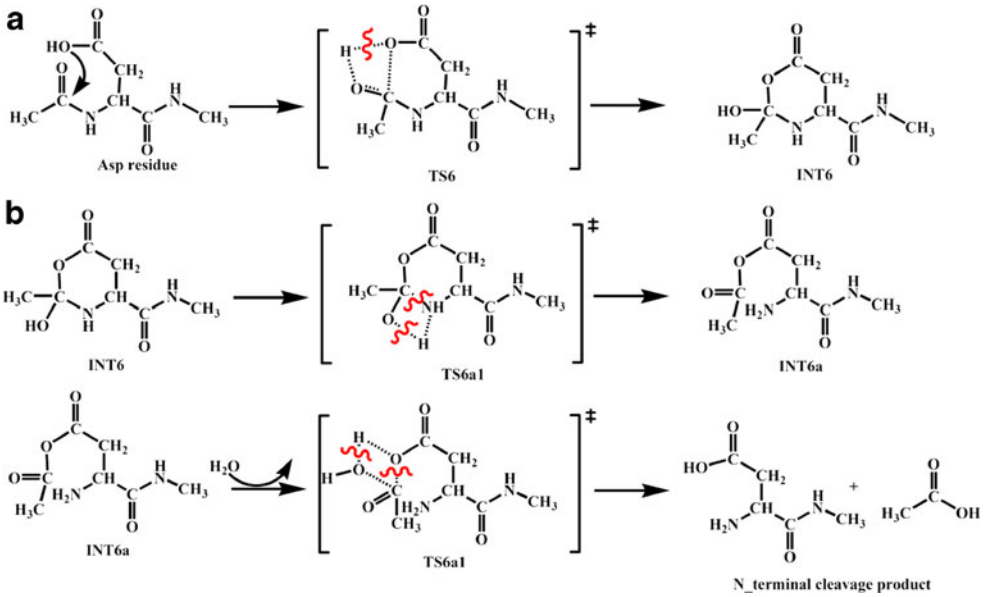


Fig. 5 The relative free energy profile with optimized-structures for concerted hydrolysis of INT5b in C-terminal cleavage of Asp residue (relative free energies in continuum aqueous media are in parenthesis, bond distances are in Å)

Scheme 5 Reaction pathway for N-terminal peptide bond cleavage at Asp residue



carbon is attacked by side-chain hydroxyl oxygen. The hydroxyl proton is transfered to carbonyl oxygen prior to ring closure forming six-membered ring intermediate (INT6) (pathway A). N-terminal cleavage is then followed by the hydrolysis of INT6 (pathway B.). The reaction energy profile of waterless and water-assisted systems of N-terminal cleavage of Asp residue is shown in Fig. 6. It is obvious that water molecule assists in the N-terminal ring closure step which can lower ~10 kcal mol^{-1} activation barriers in comparison to the water-less system. The forming C-N bond in TS6_w (2.530 Å) is longer than TS6 (2.299 Å) due to the insertion of water molecules as found in the isomerization and C-terminal cyclization. The relative energies of the INT6 and INT6_w are 24.7(22.5) and 22.2(23.6) kcal mol^{-1} higher than those of their corresponding ground state Asp residue. This may be due to high strain in the six-membered ring.

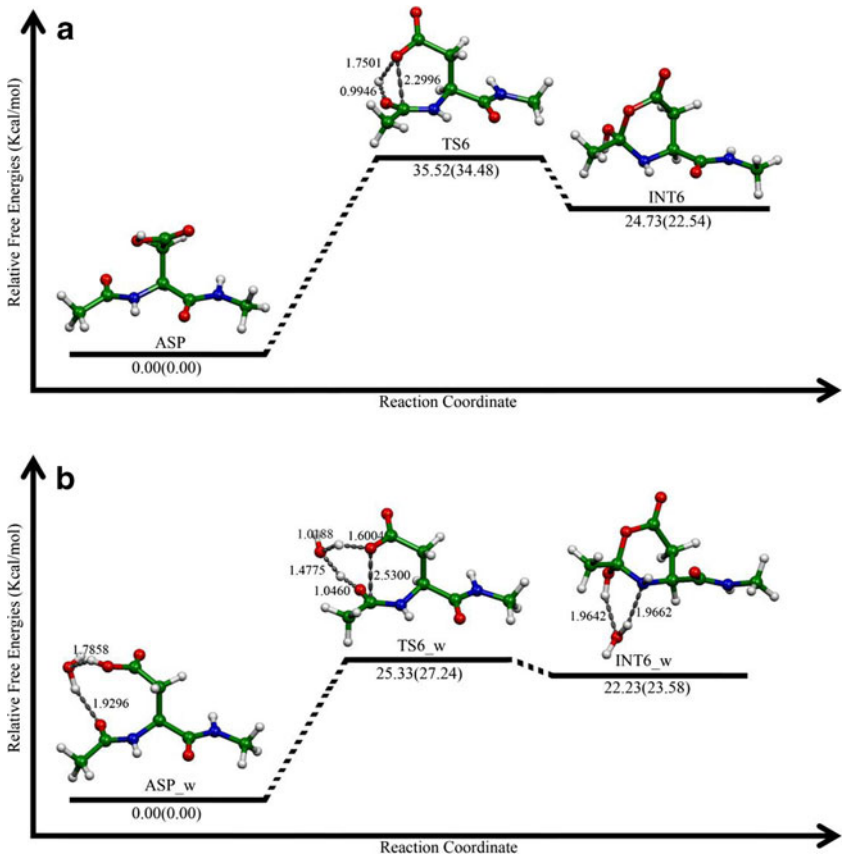


Fig. 6 The relative free energy profile with optimized-structures for water-less (**a**) and water-assisted (**b**) cyclization step forming INT6 in N-terminal cleavage of Asp residue (relative free energies in continuum aqueous media are in parenthesis, bond distances are in Å)

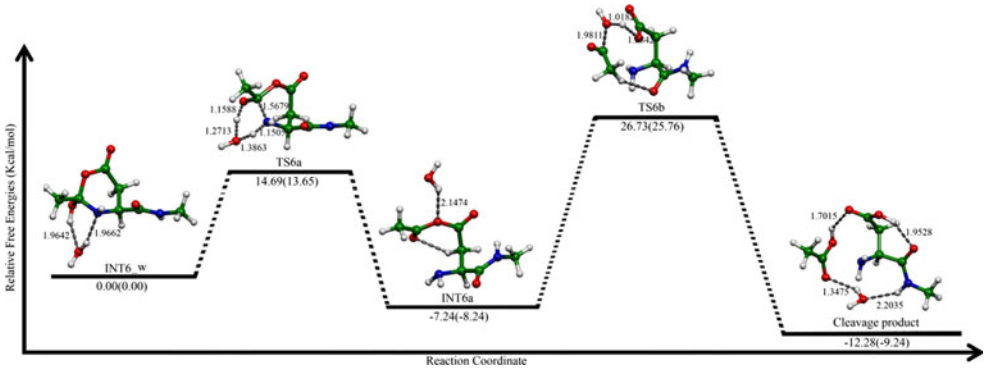


Fig. 7 The relative free energies profile for step-wise hydrolysis of INT6 in N-terminal cleavage of Asp residue (relative free energies in continuum aqueous media are in parenthesis, bond distances are in Å)

The reaction energy profile for hydrolysis of INT6 is depicted in Fig. 7. According to reaction, the first hydrolysis at N-terminal peptide bond of INT6 via TS6a forming INT6a was found, then the cleavage product is formed after hydrolysis of INT6a via TS6b. The relative barriers for TS6a and TS6b are respectively, 14.9(13.6) and 26.7(25.8) kcal mol^{-1}. Also, the relative barriers for TS6a and TS6b in comparison to reactant Asp are 41.4(39.4) and 48.9(49.3) kcal mol^{-1}, respectively. It is noted that the hydrolysis INT6a via TS6b is assigned to the rate-determining step for N-terminal cleavage.

However, although the six-membered ring (INT6) is expected to be more thermodynamically stable than those of the five-membered ring (INT5) [60], the experiment has not found the cleavage at N-terminus [37]. So, this calculation work supports the experimental observation that the peptide bond cleavage of Asp residue at N-terminus is thermodynamically favored but not energetically and kinetically favored and likely found in the C-terminal cleavage.

Conclusions

In this study, mechanisms related to non-enzymatic isomerization and peptide bond cleavage of Asp residue in peptide model were investigated by density functional theory at the B3LYP/6-311++G(d,p) level of the theory. The results can be concluded as the following:

Isomerization:

- The step-wise ring closure reaction step of succinimide intermediate formation is more preferential than the concerted ones by ~9(12) kcal mol^{-1} activation barrier.
- Iso-Asp formed by one water-assisted pathway INT1→TS4→iso-Asp (Fig. 1a) is suggested to be more stable than those of the two water-assisted pathways INT1_w→TS4_w→iso-Asp_w (Fig. 1b) due to its higher barrier for the backward reaction to form the intermediate.
- The number of water molecules assisted in the hydrolysis of INT1 and INT1_w suggested assessment at the ratio of Asp:iso-Asp.

Peptide bond cleavage:

- Peptide bond cleavage at C-terminus (with ~20.93(13.59) kcal mol^{-1} activation barrier) is more energetically preferential than those at N-terminus (with ~25.33(27.24) kcal mol^{-1} activation barrier) even though the six-membered ring intermediate in N-terminal cleavage is believed to be more stable than the five-membered ring intermediate found in C-terminal cleavage.
- The isomerization is obstructed by C-terminal cleavage of Asp by approximately ~20 kcal mol^{-1} barrier lowering. This may be due to more flexibility of the backbone conformation in peptide bond cleavage leading to lower energy barriers than those of isomerization.
- The catalytic effect of explicit water molecule as well as the dielectric effect of the continuum media plays an important role in the reaction energies.

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