Computational studies on cyclic imide formation mechanism of glutamic acid residues catalyzed by two water molecules

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Abstract. Aspartic acid (Asp) residues in peptides and proteins are prone to nonenzymatic stereoinversion and/or isomerization to form three types of isomerized Asp residues (L-β-Asp, D-α-Asp, and D-β-Asp) via a five-membered ring succinimidyl intermediate. These isomerized Asp residues are detected more frequently in aged tissues. However, stereoinversion and/or isomerization of glutamic acid (Glu) residues having a chemical structure similar to Asp residues are hardly detected in proteins. In this study, we investigate computationally the formation mechanism of the cyclic imide, i.e., amino-glutarimidyl (Agl) from Glu residues, with water molecules as catalyst. We study the reaction mechanism by using quantum chemical B3LYP/6-31+G(d,p) density functional theory calculations. All calculations are performed by using model compounds in which a Glu residue is capped with acetyl and methylamino groups on the N- and C-termini, respectively. Agl formation consists of the three steps of iminolization, cyclization, and dehydration, and two water molecules acting as proton-relay mediators catalyze all three steps. The calculated activation energy for Agl formation from Glu residues is 30.3 kcal mol⁻¹, which is somewhat greater than found experimentally for Asp-residue stereoinversion. This calculation suggests that in vivo Glu-residue stereoinversion is unlikely to occur because of the high activation barrier.

1. Introduction
Aspartic acid (Asp) residues in peptides and proteins are prone to isomerization [1-7]. The three-dimensional structure of proteins is maintained by interactions between amino acid residues. Asp-residue stereoinversion and/or isomerization disturb these interactions, and these reactions may cause undesirable conformational changes in proteins [6,8,9]. Nonenzymatic Asp-residue stereoinversion and/or isomerization proceeds via the five-membered ring aminosuccinimidyl (Asu) intermediate and yield three types of isomerized Asp residues (L-β-Asp, D-α-Asp and D-β-Asp; see Scheme 1) [1-7]. Three types of isomerized Asp residues have been detected from various aging tissues, and the amounts detected increase with aging. Therefore, Asp-residue isomerization is
suspected as one cause of protein denaturation. Fujii et al. showed experimentally that the activation energy for Asp-residue stereoinversion in distilled water is 25.7–29.0 kcal mol\(^{-1}\) [10]. Recently, we reported that water molecules can catalyze Asp-residue stereoinversion [11-13]. The Asu intermediate is considered to form by nucleophilic attack of the side-chain carboxyl carbon by the amide nitrogen of the adjacent residue on the C-terminal side, i.e., the \((N + 1)\) residue. However, the nucleophilicity of the amide nitrogen is known not to be very high. Via a quantum chemical calculation, we investigated the cyclization pathway in which Asp residue directly forms Asu intermediates, and the calculated activation barrier is much higher than the experimental value [11]. We have proposed a novel Asu-intermediate formation mechanism via iminol tautomer. Specifically, the \((N + 1)\)-residue amide bond changes to iminol tautomer and iminol nitrogen attacks of the side-chain carboxyl carbon of the Asp residue. It is expected to proceed the Asu-intermediate formation easily because the electron density on the iminol nitrogen increases by the resonance effect. The calculated activation barrier of Asu-intermediate formation from Asp residue via iminol tautomer is consistent with the experimental values [11].

![Scheme 1. Nonenzymatical stereoinversion and isomerization pathway of Asp residue via Asu intermediate.](image)

Glutamic acid (Glu) is an acidic amino acid constituting proteins and has a chemical structure similar to that of Asp. In the isomerization mechanism of Glu presumed from the analogy with the Asp isomerization mechanism, the Glu residue is considered to form a cyclic imide intermediate, i.e., an aminoglutarimidyl (Agl) intermediate similar to the Asp residue and to yield three types of Glu isomers (\(L-\gamma\)-Glu, \(D-\alpha\)-Glu, \(D-\gamma\)-Glu; see Scheme 2). However, few reports claim to detect \(D\)-Glu residues in proteins [14]. It remains unclear why Glu-residue stereoinversion is less likely to occur compared with Asp-residue stereoinversion. Based on computations, we proposed the Agl-residue stereoinversion mechanism model catalyzed by a water molecule and estimated its activation energy [15]. We investigated the possibility that water molecules catalyze Agl-stereoinversion and reported that the activation barriers for the stereoinversions of Asu and Agl are nearly equal [15]. Therefore, we consider that the difficulty of Glu-residue stereoinversion may be due to the Agl-intermediate formation step and not to the Agl-stereoinversion step. In the present study, we investigate the reaction mechanisms of Agl-intermediate formation catalyzed by two water molecules by using quantum
chemical calculations, and the resulting activation barrier is compared with the experimentally determined activation energy of Asp stereoinversion.

Scheme 2. Presumed stereoinversion and isomerization pathway of Glu residue via Agl intermediate.

2. Computational methods
All calculations were performed using Gaussian 09 [16]. In this study, all calculations were performed using model compound shown in Figure 1, in which a Glu residue was capped with an acetyl (Ace) and a methylamino (Nme) groups on the N- and C-termi, respectively. The dihedral angles $\phi$ (C-N-C$_\alpha$) and $\psi$ (N-C$_\alpha$-C-N) characterize the main-chain conformation, and the $\chi_1$ (N-C$_\alpha$-C$_\beta$-C$_\gamma$) and $\chi_2$ (C$_\alpha$-C$_\beta$-C$_\gamma$-C$_\delta$) dihedral angles characterize the side-chain conformation. In the model compound, the Glu-residue side chain is protonated because the protonated form is more likely to undergo nucleophilic attack by the amide nitrogen of the (N+1) residue to form the Agl intermediate. Catak et al. reported that the calculated activation energies for Asu-intermediate formation are 53.3 and 46.1 kcal mol$^{-1}$ in the absence of catalyst and presence of one water molecule [17], however the deviation from the experimental value is large. We have reported that Asp-residue stereoinversion can proceeds catalyzed by two water molecules [11] and its activation barrier falls within experimental range [10]. Thus, we assumed that two water molecules also catalyze the Glu-residue stereoinversion and use as initial structure a reactant complex in which two water molecules are arranged at appropriate positions around the Glu residue.

Energy-minimum and transition-state (TS) geometries are optimized with no constraints by density functional theory (DFT) calculations using the B3LYP exchange-correlation functional [18] and the 6-31+G(d,p) basis set using Gaussian 09. The vibrational frequency calculations were performed for all optimized geometries to confirm them as energy-minimum geometries (with no imaginary frequency) or TS (with a single imaginary frequency) and to correct the relative energies for the zero-point energy. Intrinsic reaction coordinate (IRC) calculations were performed from the TSs, followed by full geometry optimizations, to confirm that each TS connects two energy-minimum geometries.
3. Results and Discussion

We presume that the Agl-intermediate formation consists of three steps: iminolization, cyclization, and dehydration (Scheme 3) and estimate the activation energy in each process by using DFT calculations.

3.1 Iminolization on C-terminal side amide group of Asp

Figure 2 shows the geometries calculated for the reaction system for the iminolization step. The reactant complex (1-AM) is composed of the Glu residue (amide form) and two water molecules (Figure 2a). In 1-AM, the hydrogen bridge forms among the C-terminal amide group, two water molecules, and the side-chain carboxyl group. One of the water molecules forms an additional hydrogen bond with the amide oxygen of the \((N-1)\) residue. In this process, the \((N+1)\)-residue amide NH proton is pulled out and transferred to the Glu-residue amide C=O oxygen. Iminolization proceeds by quadruple proton transfer mediated by the Glu-residue side-chain carboxyl group and two water molecules, and transition state 1-TS forms (Figure 2b). When 1-TS forms from 1-AM, the change in all dihedral angles defined for Glu residues is small, and no significant conformational change occurs (Table 1). The calculated activation barrier for iminolization is 21.0 kcal mol\(^{-1}\), and it is considered that 1-AM can undergo sufficient iminolization under physiological conditions. As a result of quadruple proton transfer mediated by the two water molecules and the Glu-residue side-chain carboxyl group, iminolization completes and the iminol tautomer 1-IM forms (Figure 2c). The 1-IM complex is composed of Glu residue (iminol tautomer) and two water molecules. When 1-IM forms from 1-TS, a small change occurs in all dihedral angles defined for the Glu residue (Table 1). The hydrogen-bond bridge observed in 1-AM remains throughout the iminolization. It is considered that the hydrogen-bond bridge serves to immobilize the two water molecules and the Glu residue. The immobilization of a reactant complex seems to lead to the catalytic actions of the water molecules and hinders a large conformational change during the iminolization. The relative energy of 1-IM with respect to 1-AM is calculated to be 14.0 kcal mol\(^{-1}\).
3.2. Cyclization of iminol tautomer

Figure 3 shows the calculated geometries of the reaction system. The iminol tautomer 2-IM, which differs from 1-IM, forms by Cα–C bond rotation of the Glu-residue main chain and water-molecule migration from 1-IM (Figure 3a). The 2-IM complex is composed of a Glu residue (iminol tautomer) and two water molecules. In 2-IM, two water molecules connect iminol OH and the Glu side-chain carboxyl C=O by a hydrogen-bond bridge. One of the water molecules forms an additional hydrogen bond with the amide oxygen of the (N – 1) residue. In this process, nucleophilic attack by the (N + 1) residue iminol nitrogen and proton transfer from the Glu-residue iminol C–OH proton to the side-chain carboxyl group occurs by using two water molecules as catalyst, and the transition state 2-TS forms (Figure 3b). When 2-TS forms from 2-IM, the change in dihedral angles $\phi$ and $\chi_1$ is small; however, $\psi$ and $\chi_2$ change by 82° and 63°, respectively (Table 1). The large conformational change in this process is attributed to the approach of the side-chain carboxyl group to the main chain, and the conformation of the main and side chains changes remarkably. In 2-TS, N–C bonds are being newly formed by nucleophilic attack of the (N + 1)-residue iminol nitrogen and the Glu-residue side chain (the interatomic N–C distance is 1.690 Å). The calculated activation energy for the cyclization process is 30.3 kcal mol$^{-1}$ when using 1-AM as energy reference. As the nucleophilic attack occurs by the iminol nitrogen and triple proton transfer mediated by two water molecules, cyclization completes and the tetrahedral gem-diol intermediate 2-TH forms (Figure 3c). When 2-TH forms from 2-TS, the change in all dihedral angles defined for the Glu residue is less than 10° (Table 1). In 2-TS, C–N bond forms by cyclization, and the interatomic distance shrinks to 1.480 Å. The calculated relative energy of 2-TH with respect to 1-AM is 9.65 kcal mol$^{-1}$.
3.3. Dehydration of tetrahedral intermediate

Figure 4 shows the calculated geometries of the reaction system. The tetrahedral gem-diol 3-TH forms from 2-TH by water migration (Figure 4a). The 3-TH complex is composed of a gem-diol intermediate and two water molecules. In 3-TH, two OH groups in gem-diol and two water molecules are connected by a hydrogen-bond bridge. In this process, triple proton transfer mediated by two water molecules occurs and one of the gem-diol C=O bonds is cleaved, and the transition state 3-TS forms (Figure 4b). When 3-TS forms from 3-TH, the change in all dihedral angles defined for the Glu residue is small (Table 1); however, the length of the C–O bond that was cleaved increases from 1.457 to 1.873 Å. The calculated activation energy for the dehydration process is 28.4 kcal mol⁻¹ with respect to 1-AM. As triple-proton transfer and cleaving of one of the gem-diol C–O bonds proceeds, the Agl intermediate 3-GI yields (Figure 4c). When 3-GI forms from 3-TS, the gem-diol OH oxygen receives a proton from a water molecule and one water molecule is newly eliminated, accompanying the gem-diol C–O bond cleavage. Changes are small for the dihedral angles defined for the main and side chains of the Glu residues for 3-TH formation from 3-TS (Table 1). However, since the C₆ carbon transforms from sp³ to sp² hybrization, the dihedral angle χ₂ changes by 32° over the dehydration process. The Agl-intermediate piperidinedione ring forms a boat-type conformation. The calculated relative energy of 3-GI with respect to 1-AM is −3.98 kcal mol⁻¹.
4. Conclusion
In this study, we investigated the Agl-intermediate formation pathway from Glu residues as a part of Glu-residue stereoinversion. The reaction pathway analyses indicate that our presumed pathway, which includes the three steps of iminolization, cyclization, and dehydration, is appropriate for Agl formation. We show that Agl-intermediate formation can be potentially catalyzed by two water molecules, which act as proton-transfer mediators. Although Agl-intermediate formation consists of three steps, significant conformational changes of the main and side chains occur only in the cyclization process. Therefore, it is considered that Glu-residue stereoinversion only occurs in an environment where such a conformational change is possible. Figure 5 shows the entire energy profile throughout the iminolization-cyclization-dehydration process. The calculated activation energy required for Glu-residue cyclization is 30.3 kcal mol⁻¹; however, this value slightly exceeds the experimentally observed activation energy of Asp-residue stereoinversion, which is reported to be 25.7–29.0 kcal mol⁻¹ [10]. The result of this calculation supports the fact that few reports have detected D-Glu residues.
Figure 5. Energy profile of Agl-intermediate formation mechanism. The relative energies with respect to 1-AM are shown in kcal mol\(^{-1}\).

Acknowledgments
This work was supported by grants-in-aid for scientific research [15H01064] and [17K08257] from the Japan Society for the Promotion of Science. The authors would like to thank Enago (www.enago.jp) for the English language review.

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