Computational Studies on the Nonenzymatic Deamidation Mechanisms of Glutamine Residues

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Supporting Information

ABSTRACT: The nonenzymatic deamidation reactions of asparagine (Asn) and glutamine (Gln) residues in proteins are associated with protein turnover and age-related diseases. The reactions are also believed to provide a molecular clock for biological processes. Although Gln deamidation is assumed to occur through the glutarimide intermediate, the mechanisms for this are unclear because under normal physiological conditions, Gln deamidation occurs relatively less frequently and at a lower rate than Asn deamidation. We investigate the mechanisms underlying glutarimide formation from Gln residues, which proceeds in two steps (cyclization and deammoniation) catalyzed by phosphate and carbonate. We also compare these reactions with noncatalytic mechanisms and water-catalyzed mechanisms. The calculations were performed on the model compound Ace–Gln–Nme (Ace = acetyl, Nme = methylamino) using the density functional theory with the B3LYP/6-31+G(d,p) level of theory. Our results suggest that all the catalysts used in our study can mediate the proton relays required for glutarimide formation. We further determined that the calculated activation barriers of the reactions catalyzed by phosphate ions (115 kJ mol⁻¹) and carbonate ions (112 kJ mol⁻¹) are sufficiently low for the reactions to occur under normal physiological conditions. We also show that nucleophilic enhancement of Nme nitrogen is essential for the cyclization of Gln residues.

INTRODUCTION

The deamidation reactions of asparagine (Asn) and glutamine (Gln) residues are nonenzymatic post-translational modifications of proteins that occur under normal physiological conditions. The introduction of negatively charged residues by deamidation results in structural changes and aggregation of proteins.¹⁻⁷ Some of these deamidated proteins are targeted for degradation by the ubiquitin-proteasome pathway.⁵ By regulating the protein turnover,⁶⁻⁸ Asn/Gln deamidation behaves like a molecular clock involved in the timing of biological processes. In addition, deamidation reactions are involved in aging processes. For example, the deamidation of eye lens crystallin results in denaturation and aggregation, wherein the formed insoluble crystallin causes cataracts.¹⁰⁻¹³ In addition, deamidation of residues in the complementarity-determining region of therapeutic antibodies alters the binding affinity and specificity of the antibodies for the target antigen, causing unexpected degradation¹⁴⁻¹⁶ of the antibody. Peptide drugs are assumed to be impaired by deamidation. Therefore, a better understanding of the deamidation mechanisms can be used to devise ways of (i) better controlling the quality of antibodies and the usefulness of peptide drugs, (ii) better understanding processes that promote physiological development, and (iii) clarifying pathogenic mechanisms that promote some diseases.

Asn deamidation has been observed to occur in several proteins such as in ribonuclease and phenylalanine hydroxylase.⁷⁻⁹,¹⁷,¹⁸ The deamidation of Gln appears to occur less frequently than that of Asn. Gln is also detected in very long-lived proteins such as crystallins in eye lenses.⁴,¹⁰,¹³ Experimental studies using model peptides indicate that the deamidation rates of Gln are much slower than those of Asn.¹⁹ For example, the average rate constant for Asn with a following (N + 1) glycine residue (Gly) peptide deamidation is 7.037 × 10¹² s⁻¹, whereas the rate constant for Gln–Gly peptide deamidation under the same conditions is 1.216 × 10¹⁰ s⁻¹ (i.e., the deamidation of Asn is about 580 times more rapid than that of Gln). The Asn deamidation rate in a peptide with (i.e., the deamidation of Gln is assumed to occur through the glutarimide intermediate, whereas the rate constant for Gln–Gly peptide deamidation under the same conditions is 1.216 × 10¹⁰ s⁻¹ (i.e., the deamidation of Asn is about 580 times more rapid than that of Gln). The Asn deamidation rate in a peptide with any following (N + 1) residues (except for proline residue) is also >20-fold faster than Gln deamidation.¹⁹ Asn deamidation is believed to proceed via the formation of a five-membered succinimide ring intermediate, generated by a nucleophilic attack of peptide-bond nitrogen of the following (N + 1) residue to the amide carbon of the side chain. Then, the succinimide intermediate is converted into an α-/β-aspartate residue (Asp) via hydrolysis.²⁰⁻²² It is presumed that Gln deamidation occurs via a mechanism similar to Asn.
deamidation, wherein the deamidation proceeds through the six-membered glutarimide ring intermediate, which then generates the α-/γ-glutamate residue (Glu) (Scheme 1).

![Scheme 1. Glutarimide-Mediated Deamidation Pathway of Glutamine Residues](image)

Although γ-Glu has been detected in experimental studies in vitro using model peptides and the results were consistent with the presumed deamidation mechanism. Gln deamidation studies are limited to peptide-level studies because of the infrequency of Gln deamidation in vivo. In addition, atomic or molecular-level studies for the Gln deamidation mechanism have not yet been conducted, and there have been no adequate studies of catalytic molecules involved in Gln deamidation.

In this paper, we focus on the formation of glutarimide from Gln residues to better understand the Gln deamidation pathway. Recently, mechanisms involving the formation of succinimide from Asn have been investigated using density functional theory (DFT) calculations of Asn-histidine model pathways. These calculations did not converge except as illustrated in these figures. In the optimized geometry for the reactant complex in the phosphate-catalyzed reaction (pathway A), two hydrogen bonds were observed between Gln and the H₂PO₄⁻ ion (at 1.71 and 1.77 Å, respectively). In

| R-A | TS-A1 | INT-A1 |
|-----|-------|--------|
| 1.56 | 1.30 | 1.05 |
| 1.57 | 1.21 | 1.45 |
| 1.77 | 1.71 | 2.25 |
| 1.64 | 1.54 | 1.64 |

2. In the noncatalytic and water-catalyzed reactions (pathways C and D, respectively), the optimized geometries are shown in Figures S1 and S2. Many calculations for complexes with different catalyst orientations were performed; however, the calculations did not converge except as illustrated in these figures. In the optimized geometry for the reactant complex in pathway A (R-A), two hydrogen bonds were observed between Gln and the H₂PO₄⁻ ion (at 1.71 and 1.77 Å, respectively). In
the cyclization step, a proton was transferred from the Nme nitrogen atom to the phosphate ion at an early stage to enhance the nucleophilicity of the Nme nitrogen, and the transfer completed in the transition state (TS) of cyclization (TS-A1) (Figure 1). Following this proton transfer, another proton then shifted from the phosphate ion to the amide oxygen. The distance between the Nme nitrogen and the amide carbon of the side chain decreased from 3.30 to 2.07 Å, whereas the C—N distance in the cyclic intermediate (INT-A1) was 1.54 Å after the double proton transfers have been completed. Therefore, the phosphate-catalyzed cyclization step is initiated by the enhancement of Nme nitrogen nucleophilicity via proton transfer, wherein the Nme nitrogen nucleophilically attacks the amide carbon of the side chain. In the reaction step from R-A to TS-A1, the largest dihedral angle change was \( \psi \) (40.9°) in the conversion from R-A to TS-A1 (Table S1). Therefore, a large conformational change of the main chain is required for the cyclization reaction. In pathways B, C, and D, the cyclization step progressed by almost the same mechanisms as in pathway A. Thus, the enhancement of Nme nitrogen nucleophilicity occurs at an early stage and results in the progress of the cyclization steps in all pathways. A large conformational change was observed in pathways A, B, and D (Tables S1, S2, and S4); however, the change of the dihedral angle \( \psi \) in pathway B (36.9°) was slightly smaller than that in pathways A (40.9°) and D (42.5°). Therefore, the proceeding of pathways A, B, and D may be affected by the structural flexibility of the main chain. Although a large conformational change of the main chain was not required for pathway C and that of the side chain was accompanied by a noncatalytic reaction (Table S3).

**Deammoniation Step.** In the second step of the deammoniation reaction, the reaction proceeds from the intermediate INT-2 of each pathway (Figures 3, 4, S3, and S4). In INT-A2, the conformation of the gem-hydroxylamine moiety and the location of the \( \text{H}_2\text{PO}_4^- \) ion differ from those in the INT-A1 intermediate. Because phosphate ions are abundantly present in a physiological environment, this rearrangement of the \( \text{H}_2\text{PO}_4^- \) ion is expected to occur without a high energy barrier. INT-A2 is the predicted reactant complex of the deammoniation step. The gem-hydroxylamine nitrogen and OH hydrogen form hydrogen bonds with the \( \text{H}_2\text{PO}_4^- \) ion (1.77 and 1.63 Å, respectively). From the INT-A2, the deammoniation step was initiated by two proton transfers between the hydrogen-bonded atoms. In the TS of the deammoniation step (TS-A2) (Figure 3), each proton transfer did not complete, but the distance between the gem-hydroxylamine nitrogen and proton (1.11 Å) was shorter than that between oxygen and proton (1.21 Å). This result and our IRC calculation suggested that the proton transfer between the NH\(_2\) nitrogen and the \( \text{H}_2\text{PO}_4^- \) ion occurred first, and the transfer between the OH oxygen and the \( \text{H}_2\text{PO}_4^- \) ion occurred a little later. In the TS-A2, the C—N distance in the gem-hydroxylamine moiety stretched slightly (by 0.09 Å) from the C—N distance in INT-A2. The product complex (P-A) was formed when the proton transfer was completed and ammonia was released (with a C—N distance of 4.91 Å). The sequence of proton transfer by HCO\(_3^-\) differed from the phosphate-catalyzed reaction. The abstraction of the hydrogen atom from the OH of gem-hydroxylamine by the HCO\(_3^-\) ion occurs first, and then another proton shifts from HCO\(_3^-\) to the nitrogen of gem-hydroxylamine. This is mostly due to the difference in the tendency of ions to form. That is, the HCO\(_3^-\) ion reluctantly transfers its hydrogen atom to nitrogen (due to the instability of CO\(_3^{2-}\) at neutral pH), whereas \( \text{H}_2\text{PO}_4^- \) easily converts to HPO\(_4^{2-}\). The dihedral angles \( \phi, \psi, \chi_1, \chi_2 \) remain almost unchanged through the deammoniation step of pathway A (Table S5); hence, a large conformational change is unnecessary for this step to occur in the phosphate-catalyzed reaction. As with this pathway, the deammoniation step of carbonate-catalyzed, noncatalytic, and water-catalyzed reactions can occur without a large conformational change because...
each dihedral angle change was <10° in this step (Tables S6–S8).

**Energy Profiles.** The energy profile for phosphate-catalyzed deamidation differs from those for noncatalytic and water-catalyzed reactions (Figures 5 and S5). The barrier height of the cyclization step in the phosphate-catalyzed reaction (115 kJ mol⁻¹) was significantly higher than that of the deammoniation step (89 kJ mol⁻¹). Therefore, the activation barrier of the cyclization step is considered to be the rate-determining step. According to experimental data in the literature, Gln deamidation is slower than Asn deamidation ranging from 80 to 100 kJ mol⁻¹. Therefore, the activation barrier calculated in this study is considered to be reasonable. Although the phosphate-catalyzed deamidation of Gln is very slow, it can proceed at normal physiological temperatures. In pathway B, the rate-determining step is same as in pathway A, that is, the cyclization step, and the activation barrier was 112 kJ mol⁻¹ (Figure 5). Therefore, carbonate ions may play an important role in Gln deamidation, similar to phosphate ions. On the other hand, in pathway C, the barrier height for deammoniation (204 kJ mol⁻¹) was higher than that for cyclization (179 kJ mol⁻¹). The deammoniation step is considered to be the rate-determining step in the noncatalytic reactions. In pathway D, the barrier heights of deammoniation (131 kJ mol⁻¹) and cyclization (134 kJ mol⁻¹) were almost the same. Owing to the large amount of water available in vivo, the water-catalyzed deamidation of Gln can proceed under normal physiological conditions, even though the activation energy is high.

**CONCLUSIONS**

In this study, we investigated the mechanisms underlying the formation of glutarimide from glutamine residues, which proceed through cyclization and deammoniation. For our calculations, three types of small catalyst molecules were included in the reactant complexes. The gem-hydroxylamine intermediates were generated by the cyclization reaction. Then, proton transferred on the gem-hydroxylamine led to deammoniation. All the catalysts used in this study mediated the proton transfers. Although these catalysts abstract the Nme nitrogen, especially in the water-catalyzed cyclization, where the difference in ψ angles before and after the reaction was 42.5°. In the noncatalytic reaction, the alteration of χ₁, which is one of the dihedral angles of the side chain, was the largest changed (63.5°). In contrast to Gln deamidation, larger dihedral angle changes are needed for Glu stereoinversion (ψ: 65°; χ₁: 113°) and Asp stereoinversion (ψ: 167°; χ₁: 114°). Therefore, a large conformational change may not be necessary for Gln deamidation to occur, and the deamidation can occur on a more rigid structure of protein than with stereoinversion. In fact, deamidated Gln has not only been detected in residues located in loop structures but has also been detected in residues forming secondary structures. Although Gln120 of γS-crystallin forms an α-helix, approximately 60% of this residue in age-related cataracts is deamidated. The frequency at which deamidation of Gln120 occurs is higher than the frequency of Gln63 and Gln92, which are also located in loop structures. Therefore, we assume that the accessibility of the catalyst is more important than its structural flexibility.

The calculated activation barriers for deamidation were sufficiently low for reactions to occur under normal physiological conditions. However, the barriers are higher in Gln than in Asp stereoisomerizations catalyzed by phosphate ions (100 kJ mol⁻¹). In addition, the activation barrier for Asn deamidation is 108 kJ mol⁻¹ when catalyzed by glycolic acid, 84 kJ mol⁻¹ when catalyzed by N + 1 His residue, and 91 kJ mol⁻¹ when catalyzed by phosphate. Therefore, the activation barrier for Gln deamidation is relatively high, and this helps explain that the deamidation is detected from mainly very long-lived proteins. In a previous study, the water molecule-mediated mechanisms of cytosine and guanine deamination, in which the reactions proceed via gem-hydroxylamine intermediate, were computationally analyzed. Although multiple pathways were investigated in these studies, the same pathways as in the present study were included (NH₃ is released after the proton transfers of the NH₂ nitrogen—catalyst and OH oxygen—catalyst). The possibilities of NH₃ formation after it is released as NH₄⁺ were also reported. In addition, the deamidation rate of Gln is affected by the bulkiness of adjacent residues. For example, deamidation is accelerated when the N + 1 residues are glycine or cysteine residues. Furthermore, the ionization state of catalysts may affect the mechanisms of Gln deamidation. For example, OH⁻ ions are thought to easily abstract Nme hydrogen to enhance the nucleophilicity of the Nme nitrogen. In addition, the direct hydrolysis of Gln is catalyzed by OH⁻ / H₂O. Therefore, additional studies are needed to help clarify...
the influence of ionization states of catalysts and adjacent residues.

In a previous study, phosphate intake was reported to be involved in an acceleration of aging, but carbonate was not implicated. Our study suggests that the carbonate ion is related to the nonenzymatic, post-translational modification of proteins. Therefore, regulation of the carbonate-ion concentration in cells may also be important in affecting age-related diseases. The concentration of carbonate ions in cells is adjusted to maintain pH homeostasis and by carbonic anhydrase, which is involved in the control of intraocular pressure and urinary volume. In addition, changes in the partial pressure of carbon dioxide by respiration and acidosis/alkalosis may control carbonate concentrations. Therefore, the computational results of this study are expected to be useful for investigating the relationships between age-related diseases and inorganic ions.

**COMPUTATIONAL METHODS**

The model compound used in this study was an L-Gln residue capped with acetyl (Ac) and methylamino (Nme) groups on the N- and C-termini, respectively (i.e., Ace-Gln-Nme) (Figure 6). This figure shows the dihedral angles $\psi$ (C-N-Ca-C) and $\psi$ (N-Ca-C-N), which characterize the main-chain conformation, and $\chi_1$ (N-Ca-C$\beta$-C$\gamma$) and $\chi_2$ (Ca-C$\beta$-C$\gamma$-C$\delta$), which characterize the side-chain conformation. All calculations were performed using Gaussian 16 software. The $\psi$ (C-N-Ca-C-N) and $\psi$ (N-Ca-C-N) dihedral angles characterize the main-chain conformation of the Gln residue, whereas the $\chi_1$ (N-Ca-C$\beta$-C$\gamma$) and $\chi_2$ (Ca-C$\beta$-C$\gamma$-C$\delta$) dihedral angles comprise the side chain of the Gln residue. The energy-minimum and TS geometries were optimized without any constraints by DFT calculations using the B3LYP/6-31+G(d,p) level of theory, this is considered to be reasonable for calculations of Gln deamidation. In addition, for simple comparison with the results of previous studies, the same level of theory as those studies was used. We performed vibrational frequency calculations for all the optimized geometries to confirm them as energy-minimum geometries (with no imaginary frequency) or TS geometries (with a single imaginary frequency) and to obtain the relative energies for the zero-point energy. We performed intrinsic reaction coordinate (IRC) calculations to confirm the energy minima connected with each TS.

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