Computational Studies on the Mechanisms of Nonenzymatic Intramolecular Cyclization of the Glutamine Residues Located at N-Termini Catalyzed by Inorganic Phosphate Species

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ABSTRACT: Glutamine (Gln) residues located at N-termini undergo spontaneous intramolecular cyclization, causing the formation of pyroglutamic acid (pGlu) residues. pGlu residues have been detected at the N-termini in various peptides and proteins. The formation of pGlu residues during the fermentation and purification processes of antibody drugs is one of the concerns in the design and formulation of these drugs and has been reported to proceed rapidly in a phosphate buffer. In this study, we have examined the phosphate-catalyzed mechanisms of the pGlu residue formation from N-terminal Gln residues via quantum chemical calculations using B3LYP density functional methods. Single-point energies were calculated using the second-order Møller−Plesset perturbation theory. We performed the calculations for the model compound in which an uncharged N-terminal Gln residue is capped with a methyl amino group on the C-terminal. The activation energy of the formation of pGlu residues was calculated as 83.8 kJ mol−1, which was lower than that of the typical nonenzymatic reaction of amino acid residues. In addition, the computational results indicate that the flexibility of the main and side chains in N-terminal Gln residues was necessary for the formation of pGlu residues to proceed. In the obtained pathway, inorganic phosphate species act as the catalyst by mediating the proton transfer.

INTRODUCTION

Pyroglutamic acid (pGlu) residues, which include γ-lactam rings, have been detected at the N-termini of peptides and proteins, including fibrinogen,1−3 collagen,4−6 and monoclonal antibodies.7−10 The formation of the pGlu residues occurs via intramolecular cyclization of glutamine (Gln) residues located at the N-termini; this post-translational modification is called “pyroglutamylation”. Specifically, pGlu residues are formed by the nucleophilic attack of the N-terminal amino nitrogen on the side-chain carbonyl carbon of N-terminal Gln residues, with the release of an ammonia molecule (Scheme 1).11 As yet, pGlu residues have been observed only at the N-termini of peptides and proteins. By the formation of pGlu residues, the N-terminal amino group is incorporated into the γ-lactam ring, and the basicity and nucleophilicity of the N-terminal amino group are reduced substantially. Thus, the formation of pGlu residues interferes with the determination of the primary structure of peptides and proteins by Edman degradation.12−14

pGlu residues play important roles in the unique biological functions of several regulatory peptides, such as gonadotropin-releasing hormone,15 thyrotropin-releasing hormone (TRH),16−18 luteinizing hormone-releasing hormone,19 and the human monocyte chemotactic protein.20,21 In particular, the replacement of pGlu residues with other chemical species at the N-termini of TRHs has been reported to decrease significantly both the hormonal potency and receptor-binding ability of TRH.18 Zhang and Henzel applied Edman analysis to a set of 270 secreted recombinant human proteins and demonstrated that Gln is located at the N-termini with a frequency of 10.7%.22 They suggested that the conversion from Gln to pGlu residues helped protect the secreted proteins from degradation by extracellular aminopeptidase. Although pGlu residues have been observed in a broad range of peptides and proteins, they are usually located only at the N-termini of peptides and proteins.
proteins with Gln residues at the N-termini, little is known about the biological roles of pGlu residues.

The glutamic acid (Glu) residues located at the N-termini can also form pGlu residues by intramolecular cyclization.23,24 Intramolecular cyclization of N-terminal Glu residues is considered to be involved in neurodegenerative diseases, including Alzheimer’s disease (AD).25–31 One of the pathological hallmarks of AD is the accumulation of amyloid plaques, with amyloid β (Aβ) peptides as the main components; the most abundant of these is Aβ with the pGlu residue at the N-terminal.27 In Aβ, the Glu that is pyroglutamylated most often is Glu3, which undergoes pyroglutamylation once Asp1 and Ala2 are truncated and exposed to the N-terminal.27,32 The formation of pGlu residues from N-terminal Gln residues tends to proceed faster than that from N-terminal Glu residues. Dammers et al. demonstrated that the formation of pGlu residues was significantly faster in the variant in which the Glu3 of Aβ was replaced with Gln than it was in the wild type.33 In addition, using QFR and EFRH peptides, Seifert et al. demonstrated that the rate constant for the pyroglutamylation of N-terminal Gln residues was approximately three orders of magnitude higher than that for N-terminal Glu residues.23

This pyroglutamylation is accelerated significantly in the presence of an enzyme glutaminyl cyclase (QC).34,35 Calvaresi et al. studied the mechanisms of catalysis of the enzyme for the N-terminal Gln residues by QC.36 In addition, it has been reported that the formation of pGlu residues can proceed not only enzymatically but also nonenzymatically.32,37–42 pGlu residues have also been detected in several monoclonal antibodies, and it has been reported that these residues can be formed during the fermentation and purification of monoclonal antibody drugs.37–41,43 Thus, the formation of pGlu residues has been one of the concerns in the formulation of such drugs. Although the formation of pGlu residues at the N-termini of antibodies has not been reported to have a significant impact on safety and efficacy, the details remain unclear due to the absence of comprehensive analysis.

Dick et al. demonstrated that the formation of pGlu residues from N-terminal Gln residues depended strongly on buffer composition and temperature and that it proceeds particularly rapidly in ammonium carbonate and sodium phosphate buffers.39 As the formation of pGlu residues is slow in water and Tris-HCl buffer, the chemical species contained in the buffer are considered to act as a catalyst for pyroglutamylation. Recently, Seifert et al. demonstrated that the rate of the pGlu residue formation from N-terminal Gln residues increases with increasing phosphate concentration, and they suggested that inorganic phosphate species can catalyze pyroglutamylation.42

However, the roles of inorganic phosphate species in the pyroglutamylation and the geometries of the transition states in the elementary reactions constituting the entire pyroglutamylation have not been fully elucidated at the atomic and molecular levels. Phosphate buffers have been used widely in studies using peptides and proteins, and it has been suggested that inorganic phosphate species play a role in nonenzymatic modifications of various amino acid residues.39,42,44–49 In the present study, we have used density functional theory (DFT) calculations to investigate the phosphate-catalyzed mechanisms of the formation of nonenzymatic pGlu residues from N-terminal Gln residues in the aqueous phase. Previous computational studies have indicated that inorganic phosphate species could catalyze nonenzymatic reactions of amino acid residues by acting as both proton donors and proton acceptors,50–57 and similar roles are anticipated in the formation of pGlu residues.

## RESULTS AND DISCUSSION

Human cells have an abundance of inorganic phosphate species, which have four types of charge states: H3PO4, H2PO4−, HPO42−, and PO43−, and the values of pK_a of H3PO4, H2PO4−, and HPO42− are 2.15, 6.82, and 12.38, respectively.58 Seifert et al. recently reported that pyroglutamylation is sufficiently catalyzed by inorganic phosphate species at pH 7.0, although the reaction rates are virtually pH-independent between pH 4.0 and pH 10.0 without inorganic phosphate species.59 Therefore, H2PO4− and/or HPO42− are considered to play important roles as catalysts for pyroglutamylation. As the nucleophilic attack by the N-terminal amino nitrogen on the side-chain carbonyl carbon is necessary for the pyroglutamylation of N-terminal Gln residues to proceed, the N-terminal amino group is preferably in a deprotonated form (NH2) rather than a protonated form (NH3+). Therefore, the N-terminal amino group was taken as deprotonated, and we adopted the optimized geometry of a complex comprising an uncharged N-terminal Gln residue with H2PO4− as the reactant. Figure 1 presents the model compound of the uncharged N-terminal Gln residue used in this study in which an uncharged N-terminal Glu residue is capped with an Nme group on the C-terminus. The N-terminal amino group is depicted as a deprotonated form. The dihedral angle ψ (N−Cα−C−N), which characterizes the conformation of the main chain, and the dihedral angles χ1 (N−Cα−Cβ−Cγ) and χ2 (Cβ−Cγ−Cδ−Cε), which characterize the conformation of the side chain, are indicated.

Figure 1. Model compound used in this study in which an uncharged N-terminal Gln residue is capped with an Nme group on the C-terminal. The N-terminal amino group is depicted as a deprotonated form. The dihedral angle ψ (N−Cα−C−N) characterizes the conformation of the main chain, and the dihedral angles χ1 (N−Cα−Cβ−Cγ) and χ2 (Cβ−Cγ−Cδ−Cε) characterize the conformation of the side chain.

In this study, we identified the pathways for the pyroglutamylation of N-terminal Gln residues; this pathway was divided roughly into two processes: cyclization and dehydration (Scheme 2). Figure 2 presents the energy profile for the overall process in forming pGlu residues catalyzed by H2PO4−. The energy minima and TS geometries were optimized using B3LYP exchange–correlation functional and the 6-31+G(d,p) basis set, and the single-point energy calculations were performed for all optimized geometries using the second-order Møller–Plesset perturbation theory (MP2) with the 6-311+G(d,p) basis set. All relative energies were compared using MP2 single-point energies corrected by zero-point energies (ZPEs) and Gibbs energies (given at 1.00 atm at 298.15 K). The B3LYP and MP2 total energies, zero-point energies (ZPEs), and Gibbs energies of all energy minima and TS geometries are presented in Table S1 in the Supporting Information. The formation of the product...
complex (PC) from the reactant complex (RC) was estimated to progress in four steps computationally. The four TSs are numbered consecutively as TS1, TS2, TS3, and TS4. IS1 and IS2 stand for the inner salt (zwitterionic) intermediates, and TH stands for the tetrahedral gem-hydroxylamine intermediate. The Cartesian coordinates of all energy minima and TS geometries are shown in Tables S2−S10 in the Supporting Information.

Cyclization Process. The cyclization from the RC to the TH involved two steps. The RC was converted to TH via a single energy minimum IS1 and two TSs (TS1 and TS2). The optimized geometry of RC is presented in Figure 3. RC consisted of a capped N-terminal Gln residue and an H₂PO₄⁻, and H₂PO₄⁻ was placed to connect the N-terminal amino and side-chain carbonyl groups by a hydrogen bond network. In the RC, the dihedral angles χ₁ and χ₂, which characterize the side chain, were 66° and −84°, respectively, and the distance between the N-terminal amino nitrogen and the side-chain carbonyl carbon (C₆−N distance) was 3.270 Å. Moreover, a main-chain conformation of the RC was extended (the dihedral angle ψ was −169°).

First, there was a nucleophilic attack by the N-terminal amino nitrogen on the side-chain carbonyl carbon, and the RC was converted to a zwitterionic (inner salt) intermediate IS1 via single TS TS1. During this step, a new covalent bond was formed between the N-terminal amino nitrogen and the side-chain carbonyl carbon of the N-terminal Gln residue, and a five-membered ring was formed. The optimized geometries of TS1 and IS1 are presented in Figures 4 and 5, respectively. In TS1, the C₆−N distance was shortened to 1.907 Å. In addition, the hydrogen bond between the N-terminal amino nitrogen and H₂PO₄⁻ in the RC (1.891 Å) was cleaved, and a new hydrogen bond was formed between the side-chain carbonyl nitrogen and H₂PO₄⁻ in TS1 (1.898 Å). Vibrational frequency calculations showed that TS1 had a single imaginary frequency of 179i cm⁻¹. In IS1, the N-terminal Gln residue was in the zwitterionic form, and the N-terminal amino nitrogen and the side-chain carbonyl carbon were close compared with the RC and TS1 (the C₆−N distance was 1.626 Å in IS1). Moreover, when IS1 was formed from TS1, all the hydrogen bonds connecting the model compound and H₂PO₄⁻ observed in TS1 were shortened. When IS1 was formed from the RC, the changes in the dihedral angles χ₁ and χ₂ were 56° and 48°, respectively, and the conformation of the side chain changed significantly. The relative energies of TS1 and IS1 with respect to the RC were 59.1 and 47.7 kJ mol⁻¹, respectively. As

Figure 2. Energy profile for the formation of pGlu residues from N-terminal Gln residues catalyzed by H₂PO₄⁻. Relative energies were calculated at the MP2/6-311+G(d,p) level of theory and were corrected for ZPEs and Gibbs energies. All relative energies are presented in kJ mol⁻¹.

Figure 3. Optimized geometries of the RC. The dihedral angles ψ, χ₁, and χ₂ were −169, 66, and −84°, respectively. Selected interatomic distances are presented in Å.

Figure 4. Optimized geometries of TS1. The dihedral angles ψ, χ₁, and χ₂ were 161, 19, and −74°, respectively. Selected interatomic distances are presented in Å.

Figure 5. Optimized geometries of IS1. The dihedral angles ψ, χ₁, and χ₂ were 160, 10, and −36°, respectively. Selected interatomic distances are presented in Å.

Scheme 2. Investigated Mechanism for Pyroglutamylation from N-terminal Gln to pGlu Residues via a gem-Hydroxylamine Intermediate

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described above, the progression of pyroglutamylation requires high nucleophilicity of the N-terminal amino groups. The nucleophilicity of the main-chain amide nitrogen is poorer than that of the N-terminal amino nitrogen due to the adjacent electron-withdrawing carbonyl group. This may explain why pyroglutamylation proceeds only at the N-termini.

Then, double proton transfer mediated by $\text{H}_2\text{PO}_4^-$ occurred, and IS1 was converted to TH via TS2. The optimized geometries of TS2 and TH are illustrated in Figures 6 and 7, respectively. Vibrational frequency calculations estimated that TS2 had a single imaginary frequency of 716$\text{cm}^{-1}$. In this process, the double proton transfer proceeded along the two hydrogen bonds: one between the side-chain carbonyl oxygen and $\text{H}_2\text{PO}_4^-$ and the other between the N-terminal amino group and $\text{H}_2\text{PO}_4^-$. The $\text{C}_\text{q}$-$\text{N}$ distances in TS2 and TH were 1.577 and 1.488 Å, respectively, and the $\text{C}_\text{q}$-$\text{N}$ distance decreased as the proton transfer progressed. When TH was formed from IS1, the changes in all dihedral angles defined for the main and side chains ($\psi$, $\chi_1$, and $\chi_2$) were less than 10$^\circ$, and there was no occurrence of large conformational changes of the main- and side-chain skeletons.

Based on the computational results, we considered that, in the process of conversion from RC to TH, the formation of covalent bonds between the N-terminal amino nitrogen and the side-chain carbonyl carbon of the N-terminal Gln residue was “stepwise” rather than “concerted” and that the new covalent bonds were formed before the double proton transfer mediated by $\text{H}_2\text{PO}_4^-$. In the process of cyclization (i.e., during conversion from the RC to TH), the changes of the dihedral angles $\psi$, $\chi_1$, and $\chi_2$ were 31, 60, and 58$^\circ$, respectively. This suggests that the conformational changes of the main- and side-chain skeletons took place in this step. Thus, it is considered that the main- and side-chain flexibilities were necessary for the formation of a gem-hydroxylamine intermediate from the N-terminal Gln residue.

**Deammoniation Process.** The gem-hydroxylamine intermediate TH, the product of the cyclization step, was the reactant in the deammoniation step in which TH was converted to PC in two steps via the single energy minimum IS2 and two TSs, TS3 and TS4.

First, IS2 was formed from TH via TS3 by double proton transfer mediated by $\text{H}_2\text{PO}_4^-$. In this process, the OH proton in the gem-hydroxylamine moiety was abstracted by $\text{H}_2\text{PO}_4^-$, and one of the protons in $\text{H}_2\text{PO}_4^-$ was transferred to NH$_2$ nitrogen in the gem-hydroxylamine moiety. In general, the amino group (NH$_3^+$) has very weak leaving ability, which is enhanced by the formation of primary ammonium cations (NH$_4^+$). That is, the enhancement of the leaving ability of the amino groups by the formation of IS2 is considered to contribute to the formation of pGlu residues. TS3 connected directly to the two energy minima TH and IS2; the optimized geometries of TS3 and IS2 are presented in Figures 8 and 9, respectively. According to the vibrational frequency calculations, the estimated single imaginary frequency of TS3 was 656$\text{cm}^{-1}$. According to IRC calculations, these $\text{H}_2\text{PO}_4^-$-mediated proton transfers proceeded along the hydrogen bond network. In addition, in the process of conversion from TH to IS2, the distances between carbon and nitrogen in the gem-hydroxylamine moieties ($\text{C}_\text{q}$-$\text{N}_\text{i}$ distance) increased gradually; the $\text{C}_\text{q}$-$\text{N}_\text{i}$ distances of TH, TS3, and IS3 were 1.471, 1.538, and 1.568 Å, respectively. Conversely, the distance between carbon and oxygen in the gem-hydroxylamine moieties ($\text{C}_\text{q}$-$\text{O}_\text{j}$ distance) decreased gradually; the $\text{C}_\text{q}$-$\text{O}_\text{j}$ distances of TH, TS3, and IS3 were 1.398, 1.344, and 1.321 Å, respectively. These changes in the lengths of the covalent bond can be explained by resonance effects in organic chemistry. On the
other hand, when IS2 was formed from TH, the changes in all the defined dihedral angles for the main and side chains were less than $5^\circ$, and there were no substantial conformational changes of the main chain and the five-membered ring. The relative energies of TS3 and IS2 with respect to the RC were 48.6 and 54.4 kJ mol$^{-1}$, respectively.

Then, PC was formed from IS2 via TS4. The optimized geometries of TS4 and PC are presented in Figures 10 and 11.

![Figure 10. Optimized geometries of TS4. The dihedral angles $\psi$, $\chi_1$, and $\chi_2$ were 154, 8, and $-28^\circ$, respectively. Selected interatomic distances are presented in Å.](image1)

![Figure 11. Optimized geometries of PC. The dihedral angles $\psi$, $\chi_1$, and $\chi_2$ were 143, 21, and $-20^\circ$, respectively. Selected interatomic distances are presented in Å.](image2)

respectively. In this process, the covalent bond connecting carbon and nitrogen in the gem-hydroxylamine moiety was cleaved, and an ammonia molecule was newly released. TS4 connected the two energy minima IS2 and PC. Vibrational frequency calculations estimated a single imaginary frequency of TS4 as being 2061 cm$^{-1}$. When PC was formed from TH, the dihedral angles $\psi$, $\chi_1$, and $\chi_2$ remained nearly unchanged; however, the dihedral angles of $\chi_{i}$, $\psi$, $\chi_1$, and $\chi_2$ were 12, 15, and $6^\circ$, respectively, and it is conceivable that there were no large conformational changes of the main and side chains. The relative energies of TS4 and PC with respect to the RC were 83.8 and $-41.4$ kJ mol$^{-1}$, respectively.

**Discussion.** In this study, the mechanism of the formation of pGlu residues was roughly divided into two processes—cyclization and deammoniation processes—both of which consisted of two steps. Although we identified four TSs in the pathway from the RC to PC, the energies of IS1 and IS2 were slightly higher than those of TS2 and TS3, respectively. The geometries of IS1 and IS2 were obtained by energy minimization, and it was confirmed that IS1 and IS2 did not have any significant frequencies by vibrational frequency calculations. Furthermore, using IRC calculations and subsequent geometry optimization, it was confirmed that IS1 and IS2 were directly connected to TS2 and TS3, respectively. Prior to the ZPE and Gibbs energy correction, the total energies (i.e., nuclear repulsion plus electronic energies) of IS1 calculated by B3LYP/6-31+G(d,p) and MP2/6-311+G(d,p) levels of theory were 3.41 and 2.02 kJ mol$^{-1}$ lower, respectively, than those of TS2. Moreover, those of IS2 were 2.38 and 2.36 kJ mol$^{-1}$ lower, respectively, than those of TS3. This indicates that IS1 and IS2 were located at very shallow stationary points on the potential energy surfaces and that, in essence, the conversions both from IS1 to TH and from IS2 to TH were barrierless. However, considering that the relative energies corrected by ZPEs and Gibbs energies for IS1 and IS2 were higher than those of TS2 and TS3, respectively, it is conceivable that IS1 and IS2 are artificial intermediates and that the IS1 and IS2 do not actually exist as energy minima.

The energy of TS4 was significantly higher than that of the other three, as is shown in Figure 2. Therefore, the rate-determining step was considered to be the deammoniation step, and the calculated activation energy in this pathway was 83.8 kJ mol$^{-1}$. To date, the activation energies of nonenzymatic reactions of amino acid residues has been experimentally reported to be typically approximately 90–120 kJ mol$^{-1}$, and it is possible that the formation of pGlu residues can proceed rapidly compared with the other nonenzymatic modification of amino acid residues. To date, some of the mechanisms for the nonenzymatic reactions of amino acid residues have been computationally explored, and these results suggest that inorganic phosphate species are superior catalysts to water molecules for several reactions, such as the Asp residue stereoinversion, Glu residue deamidation, and Ser residue stereoinversion. In the present study, we estimate that the activation energy of the phosphate-catalyzed pyroglutamylation is low enough to proceed under the physiological conditions. Conversely, there were great changes in the defined dihedral angles $\psi$, $\chi_1$, and $\chi_2$.
and $\chi_2$ when RC was converted to TH during cyclization, and there was conformational change of the five-membered ring skeleton during deammoniation. Thus, it is considered that the flexibilities of the main and side chains contribute significantly to the progress of pyroglutamylation. In the obtained pathway, an $\text{H}_3\text{PO}_4^-$ was always involved in intermolecular hydrogen bonds and functioned as a proton relay mediator in both cyclization and deammoniation.

Experimental data from Seifert et al. have shown that the addition of inorganic phosphate species accelerates the formation of pGlu residues from N-terminal Gln residues, and our study supports these experimental data. In addition, Seifert et al. recently reported that the isotope effects decreased with increasing concentration of phosphate. In this study, the release of the ammonia molecule and the substantial conformational change of the five-membered ring skeleton occurred in the determining step, and the proton transfer between the substrate and $\text{H}_2\text{PO}_4^-$ did not occur in this step. On the other hand, the experimentally observed large isotope effects indicate that proton transfers occur in the rate-determining step in the absence of phosphate. That is, the reaction mechanisms of pyroglutamylation in the presence of phosphate may be different from those in the absence of phosphate, and the difference in the reaction mechanisms is considered to be the cause of the decrease in the isotope effects with the increase in the concentration of phosphate. Conversely, Seifert et al. used very short peptides in their research, whereas the N-terminal regions of short peptides are likely to be exposed to solvents, and those of large proteins are not always exposed. Moreover, the N-termini of short peptides are flexible, whereas those of large proteins are sometimes not flexible. Given that the progress of the formation of pGlu residues from N-terminal Gln residues is slow in water, nonenzymatic pyroglutamylation is unlikely to be rapid if the N-terminal Gln residues in proteins and the inorganic phosphate species in the solution cannot form the appropriate reactant complexes.

## CONCLUSIONS

This study has explored the mechanism of nonenzymatic pyroglutamylation of the N-terminal Gln residue catalyzed by $\text{H}_3\text{PO}_4^-$ in aqueous conditions. In the present study, the amino group of the N-terminal Gln residue was taken as deprotonated. Dick et al. reported that the rate of pyroglutamylation at pH 6.2 was slightly slower than that at pH 7.2. The present study is consistent with these experimental results as computational results indicate that uncharged N-terminal amino groups are important for pyroglutamylation progression. The pyroglutamylation was roughly divided into two steps: cyclization and deammoniation. In this pathway, an $\text{H}_3\text{PO}_4^-$ always interacted with the N-terminal residue and acted as both a proton donor and a proton acceptor in double proton transfer. The rate-determining step was predicted to be a deammoniation step, and the obtained activation energy of the rate-determining step with respect to RC was 83.8 kJ mol$^{-1}$. As the typical activation energies for nonenzymatic reactions of amino acid residues have been reported to be 90–120 kJ mol$^{-1}$, it is predicted that $\text{H}_3\text{PO}_4^-$-catalyzed pyroglutamylation can proceed more rapidly than the other nonenzymatic modification of amino acid residues. Furthermore, pyroglutamylation required a large conformational change of the main and side chains in N-terminal Gln residues. Therefore, the main- and side-chain flexibilities contribute significantly to the formation of pGlu residues from N-terminal Gln residues.

There are four types of inorganic phosphate species—$\text{H}_2\text{PO}_4^-$, $\text{H}_3\text{PO}_4^-$, $\text{HPO}_4^{2-}$, and $\text{PO}_4^{3-}$—the abundance ratios of which depend on pH. That is, changing the pH alters the composition of the buffer. Although the $\text{H}_3\text{PO}_4^-$, the inorganic phosphate species abundant in the phosphate buffer at pH 7.0, was used as a catalyst in this study, it is necessary to clarify the catalytic abilities of other inorganic phosphate species for pyroglutamylation. We are planning to do this. Very recently, we investigated the pyroglutamylation of N-terminal Glu residues (not N-terminal Gln) in aqueous conditions and reported that these activation energies were calculated to be 108 and 107 kJ mol$^{-1}$ when two or three water molecules act as catalysts. Although the types of amino acids and catalytic species are different, the computational results of the present study support that the N-terminal Gln residues are more prone to intramolecular cyclization than N-terminal Glu residues and that inorganic phosphate species are superior to water molecules as catalysts for pyroglutamylation.

The formation of pGlu residues from N-terminal Gln residues is rapid even in an ammonium carbonate buffer. Previous computational study has revealed that bicarbonate ions can act as a preferred catalyst for the nonenzymatic deamidation of Gln residues. We also intend to examine the mechanisms of pyroglutamylation catalyzed by carbonate species. In addition, Seifert et al. experimentally reported spontaneous pyroglutamylation in water. Although deuterium oxide showed the large solvent isotope effect, the isotope effects were reduced in phosphate buffers. This is one of the most interesting experimental results, and the reaction mechanisms of pyroglutamylation in the presence of phosphate may be different from those in the absence of phosphate. We are planning to explore water-catalyzed pathways for pyroglutamylation of N-terminal Gln residues in future studies.

## COMPUTATIONAL METHODS

In this study, all calculations were performed using Gaussian 16. Optimized energy minima and transition state (TS) geometries were obtained without any constraints by DFT calculations using B3LYP/6-31+G(d,p) level of theory. For all the optimized geometries, vibrational frequency calculations were conducted to confirm them as energy minima (with no imaginary frequency) or TSs (with a single imaginary frequency). Moreover, intrinsic reaction coordinate (IRC) calculations were performed to confirm that each TS was connected to energy minima. For all calculations, the polarizable continuum model (PCM) was employed to reproduce the aqueous condition, and the dielectric constant of water for the PCM was set to 78.355 (default setting in Gaussian 16). Furthermore, to obtain more reliable energies, single-point calculations were performed using the MP2/6-311+G(d,p) level of theory for all the optimized geometries. The relative energies of all energy minima and TS geometries calculated at the MP2/6-311+G(d,p) level of theory were corrected by the ZPEs and the thermodynamic corrections (to give the Gibbs energies at 1.00 atm and 298.15 K) calculated at the B3LYP/6-31+G(d,p) level of theory. In the Results and Discussion, all relative energies have been reported for values calculated by MP2/6-311+G(d,p)//B3LYP/6-31+G(d,p).
B3LYP and MP2 total energies, zero-point energies (ZPEs), Gibbs energies, and thermal-corrected energies of optimized geometries and Cartesian coordinates of the optimized geometries (PDF)

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**Notes**

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