The Effects of a Histidine Residue on the C-Terminal Side of an Asparaginyl Residue on the Rate of Deamidation Using Model Pentapeptides

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ABSTRACT: The effects of a histidine (His) residue located on the C-terminal side of an asparaginyl (Asn) residue on the rate of deamidation were studied using Gly-Gln-Asn-X-His pentapeptides. The rates of deamidation of the pentapeptides were determined at 37 °C ($I = 0.5$) as function of pH, buffer species, and buffer concentration. A capillary electrophoresis stability-indicating assay was developed to monitor simultaneously the disappearance of the starting peptides and the appearance of the degradation products. The rates of degradation of the peptides were pH dependent, increasing with pH, and followed apparent first-order kinetics. At pH values <6.5, Gly-Gln-Asn-His-His degraded faster than Gly-Gln-Asn-Gly-His, suggesting that the N+1 His residue is catalyzing the deamidation of the Asn residue. The His side chain at these pH values could function as a general acid catalyst, stabilizing the oxyanionic transition state of the cyclic imide intermediate formation. In contrast, at pH values >6.5, Gly-Gln-Asn-Gly-His deamidates more rapidly than Gly-Gln-Asn-His-His. The bulk of the side chain of the N+1 His residue versus the N+1 Gly residue apparently inhibits the flexibility of the peptide around the reaction site and, consequently, reduces the rate of the reaction. The significance of this steric hindrance effect of the N+1 His residue on the rate of deamidation was examined further. It was observed that at pH >6.0, Gly-Gln-Asn-His-His undergoes deamidation faster than Gly-Gln-Asn-Val-His. This observation indicated that, at the higher pH values, the N+1 His residue is also acting as a catalyst. Thus, at basic pH, the N+1 His residue influences the rate of deamidation via two opposing effects; that is, general base catalysis and steric interference. The pentapeptide Gly-Gln-Asn-His-His, in addition to undergoing the deamidation reaction, also undergoes bond cleavage at the Asn-His peptide bond. The enhanced rate of Asn-His peptide bond cleavage can be attributed to the general base behavior of the His residue, leading to increased nucleophilicity of the Asn side-chain amide group. Finally, we have shown that the His residue that is two amino acids removed from the Asn, the N+2 position, has little or no effect on the rate of deamidation. J Pharm Sci 89: 818–825, 2000

Keywords: deamidation; VEGF (vascular endothelial growth factor); histidine catalysis; protein degradation

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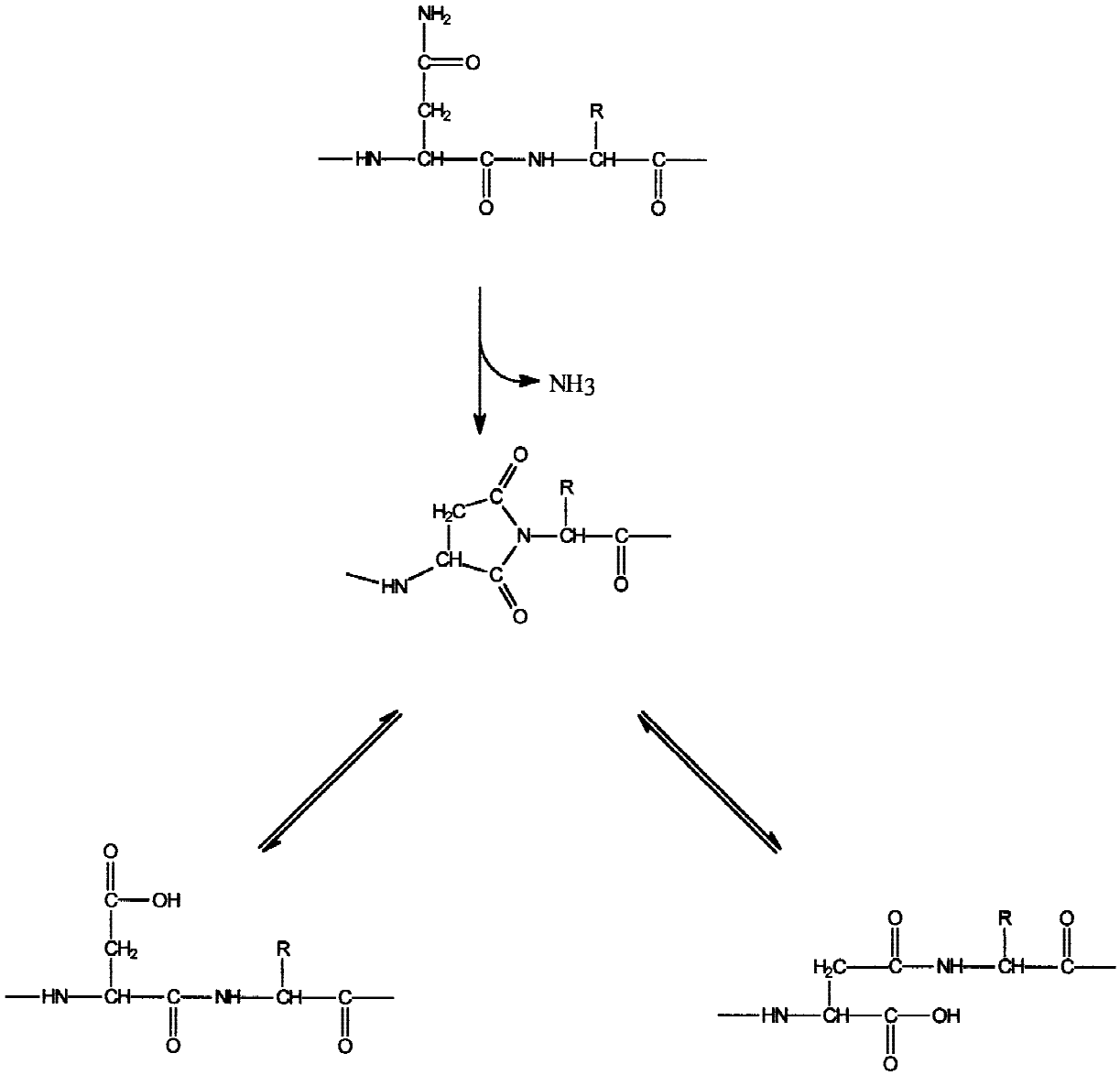
INTRODUCTION

The nonenzymatic deamidation of asparaginyl (Asn) residues is one of the major chemical deg-

radation pathways of peptides and proteins and has been an area of continuing interest.[1–5] Under slightly acidic to basic pH conditions (pH 5–10), mechanistic studies suggested that deamidation of an Asn residue proceeds through a 5-membered cyclic imide intermediate[6–8] (Scheme I). The intermediate is formed by intramolecular attack of the peptide-bond nitrogen of the succeeding amino acid on the side-chain carbonyl carbon of Asn in a reaction that releases ammonia.[9–12] It is generally assumed that the reaction pathway for the formation of the cyclic imide involves ionization of the peptide-bond nitrogen, followed by its nucleophilic attack on the Asn side-chain carbonyl carbon.[13,14] The transition state of this reaction is an oxyanionic tetrahedral intermediate; subsequent proton transfer to the leaving group from a general acid and the concerted breakdown of the tetrahedral intermediate generate the product.[14] These cyclic imides are unstable under slightly acidic to basic pH conditions (pH 5–10) and spontaneously hydrolyze to form a mixture of Asp and isoAsp peptides.[7–12]

Peptide bond cleavage between Asn and the succeeding residue has also been observed in peptides and proteins.[8,15,16] The reaction is believed to occur through a pathway similar to deamidation, via a cyclic imide intermediate. This cyclic



Scheme 1.

imide, however, is formed by attack of the side-chain amide of Asn on the peptide-bond carbonyl group.

It is well documented in the literature that the amino acid located on the C-terminal side of the Asn residue has a significant effect on its rate of deamidation. For example, peptides with amino acids with bulky side chains on the C-terminal side of the Asn residue have slower rates of deamidation.[8,11,12] Other physicochemical properties of the C-terminal residue can also alter the rate of deamidation. For example, His, Ser, and Thr residues can enhance the rate of the reaction by involvement of their respective side chains in the mechanism.[13,17] Using a series of hexapeptides, Brennan and Clarke[17] argued that at pH 7.4, the presence of a His residue succeeding an Asn residue (the N+1 position) did not affect the rate of succinimide formation. However, the N+1 His residue increases the rate of the competing side-chain amide nitrogen attack on the peptide backbone, leading to cleavage of the peptide bond.[17]

In this study, we are interested in looking at the effect of a His residue on the C-terminal side of an Asn over a wider pH range than that studied by Brennan and Clarke,[17] encompassing the His residue in its protonated and unprotonated states. The model peptide that we chose for our study, Gly-Gln-Asn-His-His, constitutes the sequence of residues 8–12 of vascular endothelial growth factor (VEGF). This sequence was chosen because the predominant degradation pathway observed for VEGF is the deamidation of the Asn-10 residue (Cleland JL, Keck R, Jones AJS, unpublished work). This labile Asn residue in VEGF is followed on the C-terminal side by a His residue, and its rate of deamidation appears to be greater than that predicted by simply considering the steric bulk provided by the His side chain. The kinetics of the deamidation of Gly-Gln-Asn-His-His and its derivatives, in which the N+1 and N+2 residues are varied, were investigated in aqueous buffer solutions as a function of pH, buffer species, and concentration.

MATERIALS AND METHODS

Materials

The pentapeptides L-Gly-L-Gln-L-Asn-L-His-L-His (GQNHH), L-Gly-L-Gln-L-Asp-L-His-L-His (GQDHH), L-Gly-L-Gln-L-Asn-L-Gly-L-His (GQNGH), L-Gly-L-Gln-L-Asn-L-Val-L-His (GQNVH), and L-Gly-L-Gln-L-Asn-L-Gly-L-Gly (GQNGG) were synthesized by California Peptide Research, Inc. (Napa, CA). Sodium phosphate monobasic, sodium phosphate dibasic, and sodium chloride were purchased from Sigma Chemical Company (St. Louis, MO). Deuterium oxide (99.9 atom % D) was obtained from Cambridge Isotope Laboratories (Woburn, MA). All other chemicals were of analytical grade and used as received. The water used in all studies was from a Millipore MILLI-Q™ water system (Bedford, MA).

Buffers

Buffer solutions were prepared with sodium phosphate monobasic and dibasic for pH values 5.0–8.0 and glycine for pH values 9.0–10.0. The buffer concentration range was 0.15–0.05 M. The buffer solutions were adjusted to a constant ionic strength of 0.5 M by adding the appropriate amount of sodium chloride. The buffers were prepared at the experimental temperature, and the pH value for a given solution remained unchanged throughout the investigation. An Orion (420A) pH meter (Beverly, MA) equipped with an Orion automatic temperature compensation (ATC) electrode was used to measure the pH (±0.05) of the buffer solutions.

Kinetic Measurements

All kinetic experiments were carried out in aqueous buffer solutions at 37 °C unless otherwise indicated. The pentapeptides were dissolved in the appropriate buffer solution to yield an initial concentration of ~1 mM. Aliquots of 200 μL were transferred to 250-μL vials, which were then sealed with a screw cap fitted with a butyl rubber stopper and stored in a 37 °C incubator. At time zero and predetermined time intervals, a vial was removed and cooled to room temperature and the contents were analyzed by capillary electrophoresis. All reactions were carried out for three or more half-lives.

Solvent Isotope Study

The kinetic studies in D_2O were carried out at pH 5.0 and 8.0 at 37 °C using 0.1 M phosphate buffer in deuterium oxide. The ionic strength was adjusted to 0.5 M by adding a calculated amount of NaCl. The pD was determined from the pH meter reading after applying the appropriate correc-

tions.[18] The kinetic solvent isotope effect (KSIE) is expressed as k_{H_2O}/k_{D_2O}, where k_{H_2O} and k_{D_2O} are the rate constants in H_2O and D_2O, respectively.

Sample Analysis

An electrophoretic method was developed for the separation of the pentapeptides and their degradation products. The capillary electrophoresis (CE) system used was an ISCO Model 3140 Electropherograph™ (Lincoln, NE). The temperature of the CE was maintained at 25 °C by a Fisher Scientific Isotemp Refrigerated Circulator Model 900 (Pittsburgh, PA). A 50 μm i.d. fused-silica capillary (Polymicro Technologies Inc., Phoenix, AZ) with a total length of 75 cm and an effective length of 40 cm was used. The separations were performed with a running buffer of 40 mM sodium phosphate monobasic (pH 3.5). A constant voltage of 25 kV was applied, yielding a current of 30 μA. The detection wavelength was 214 nm. The data were collected and analyzed by an ISCO ChemResearch Data Analysis System 150 program on a 486 SX computer.

RESULTS AND DISCUSSION

The kinetics of the degradation of the pentapeptides GQNXZ (X = H, G, or V, and Z = H, or G) were studied in aqueous buffer solutions at 37 °C over the pH range 5–10. The disappearance of the initial peptide and the appearance of degradation products were monitored by a stability-indicating capillary zone electrophoretic method (see Materials and Methods). A typical electropherogram showing the separation of GQNHH and its degradation products at pH 7.0 is shown in Figure 1. The degradation product that elutes immediately after the starting material (t = 9.6 min) was identified as the Asp-containing pentapeptide by comparing its retention time with that of an authentic sample. The second degradation peak at a retention time of 10.6 min was assigned as the isoAsp-containing peptide because its molecular weight as determined by mass spectrometry was identical to that observed for the Asp degradation product (data not shown). Over the pH range 5–10, the disappearance of the pentapeptides follows pseudo-first-order kinetics. The first-order rate constants, k_{obs}, were obtained from semilogarithmic plots of peak area versus time.

In the neutral and basic pH regions, investigators[7–10,19] have previously shown that deamida-

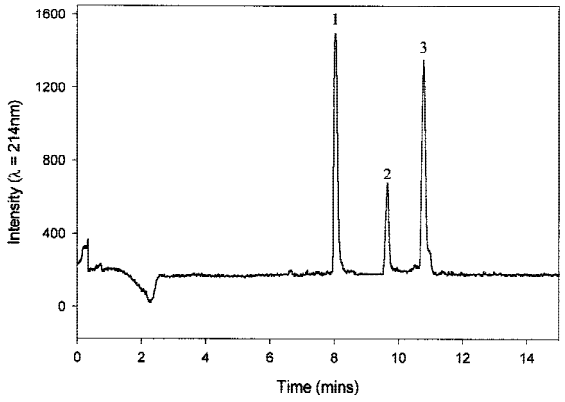


Figure 1. Electropherogram for the deamidation of GQNHH at pH 7.0. Peak 1 is the parent peptide (GQNHH), and peaks 2 and 3 are degradation products GQDHH and GQ(isoD)HH, respectively.

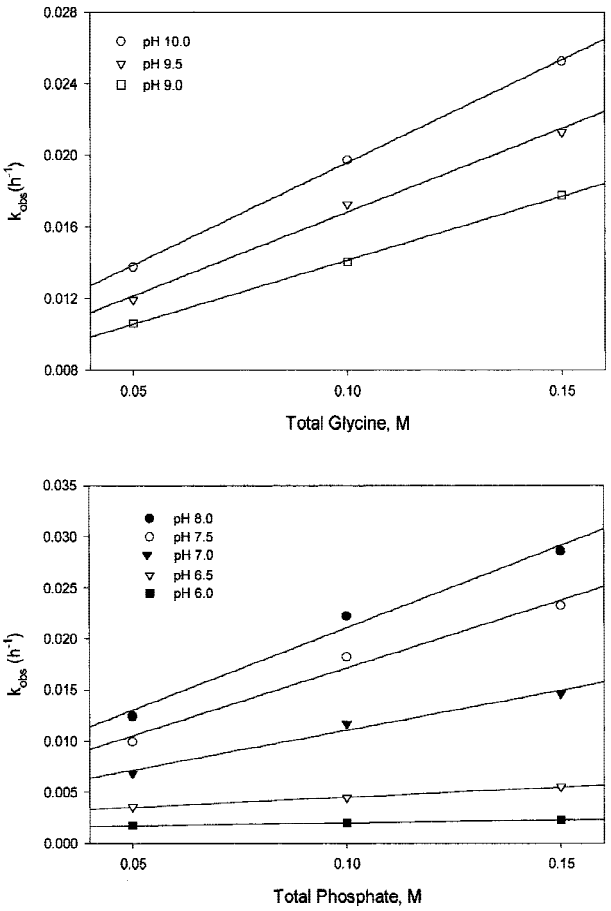


Figure 2. Dependence of rate constants of the deamidation of GQNHH at 37 °C, I = 0.5, on the buffer concentration and the pH of the solution.

tion of Asn residues proceeds exclusively through the formation of a cyclic imide intermediate followed by degradation to Asp and isoAsp products. In this study, incubation of the peptides resulted in the Asp and isoAsp degradation products in an approximately 1:3.5 ratio.

Degradation of GQNXH

The kinetics of degradation of the pentapeptides GQNHH and GQNGH were studied as a function of pH, buffer species, and concentration. The rates of deamidation of both peptides increase with increasing pH, which is consistent with literature reports.[19] In the pH range studied, the degradation of GQNGH generated the Asp- and isoAsp-containing peptides as degradation products. However, the degradation of GQNHH also generated detectable levels of Asn-His peptide bond cleavage products at pH values >7.0.

The catalytic effects of the buffers were determined by measuring the rate of deamidation at constant pH, ionic strength, and temperature while varying the buffer concentration. The observed pseudo-first-order rate constants, when plotted against total buffer concentrations, $[B_t]$, yielded linear plots (Figure 2). The slopes of these lines represent the second-order catalytic rate constants, k_{cat}, and the y-axis intercepts are the buffer-independent rate constants, k_o. The results indicated that both peptides are subject to buffer catalysis at pH values >7.0.

pH Dependence

The rate constants at zero buffer concentration, k_o, were used to generate the pH–rate profile. The

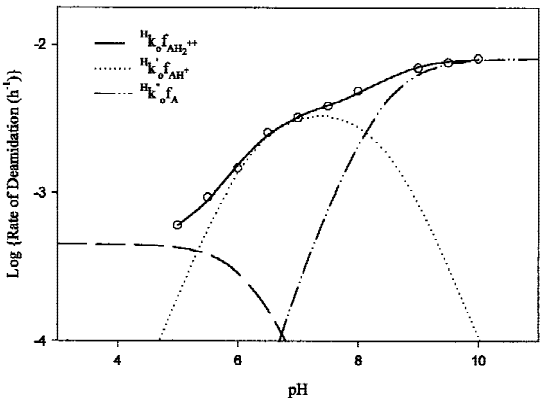


Figure 3. The pH–rate profile for the deamidation of GQNHH at 37 °C in aqueous solution. The contributions of the $^{H}k_o$, $^{H}k'_o$, and $^{H}k''_o$ steps in Scheme II to $^{H}k_{obs}$ are illustrated. The sum of these contributions gives the solid line.

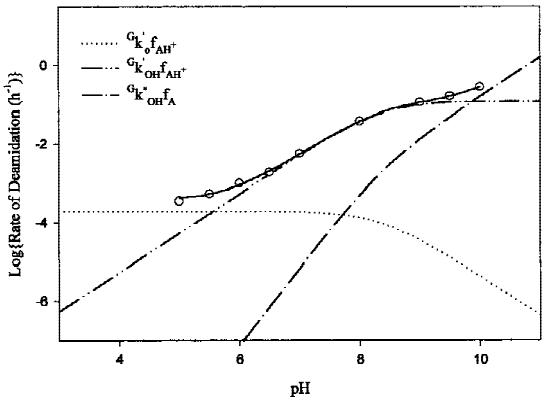


Figure 4. The pH–rate profile for the deamidation of GQNGH at 37 °C in aqueous solution. The contributions of the different steps in Scheme III to $^{G}k_{obs}$ are shown.

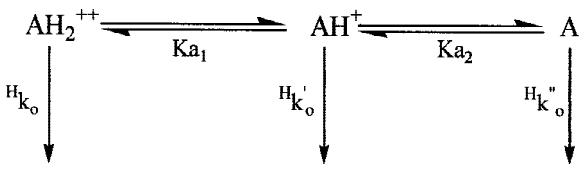
pH–rate profiles for the deamidation of GQNHH and GQNGH at 37 °C and an ionic strength of 0.5 M are shown in Figures 3 and 4, respectively.

The pentapeptide GQNHH contains four ionizable groups. However, in the pH range studied, only three undergo ionization; that is the α-amino group of the N-terminus and the imidizole rings on the two His residues. The approximate doubly sigmoidal shape of the profile (Figure 3) suggests that two of the ionizable groups significantly influence the kinetics of the reaction. An explanation of the profile is shown in Scheme 2, where AH_2^+, AH^+, and A represent the different ionic species, all of which may have a different propensities for deamidation.

The shape of the overall pH–rate profile suggests that the rate of deamidation of GQNHH may be expressed by eq 1:

$$^{H}k_{obs} = {}^{H}k_o f_{AH_2^{++}} + {}^{H}k'_o f_{AH^+} + {}^{H}k''_o f_A \qquad (1)$$

where $^{H}k_o$, $^{H}k'_o$ and $^{H}k''_o$ are the microscopic first-order rate constants for the water catalysis of the different ionic species present, and $f_{AH_2^+}$, f_{AH^+}, and f_A represent the fraction of the respective species. Substituting the fraction of each ionic species of



Scheme 2.

Table 1. Calculated Rate Constants[a] for the Deamidation of GQNHH at 37°C

Parameter	Value (±SD)
$^{H}k_{o}$	4.53 (±0.22) × 10^{-4} h^{-1}
$^{H}k'o$	3.86 (±0.12) × 10^{-3} h^{-1}
$^{H}k''o$	8.13 (±0.14) × 10^{-3} h^{-1}
Ka_1	5.49 (±0.35) × 10^{-7}
Ka_2	3.55 (±0.41) × 10^{-9}

[a] Rate constants are for the ionic species shown in Scheme II and eq 2.

GQNHH into eq 1 results in the following equation:

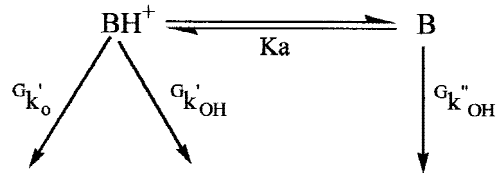
$$^{H}k_{o1bs} = \{^{H}k_{o}[H^+]^2 + {}^{H}k'_{o}Ka_1[H^+] + {}^{H}k''_{o}Ka_1Ka_2\}/ \{[H^+]^2 + Ka_1[H^+] + Ka_1Ka_2\} \quad (2)$$

The experimental data were fitted by a least-squares procedure to eq 2, generating the values shown in Table 1 for the parameters. The Ka_1 value was determined by NMR experiments to correspond to the ionization constant of the His imidizole side chain adjacent to the Asn residue. This assignment was made based on the shift that occurred in the NMR signal for the C-2 proton on the imidazole of histidine in the pH range 5–7 (data not shown). The Ka_2 value was then assigned to the α-amino group of the N-terminus.

The pH–rate profile for the deamidation of GQNGH (Figure 4) suggests the involvement of an ionizable group in the kinetics of the reaction. The kinetic model shown in Scheme III could be proposed to describe this profile. The overall velocity of the degradation of GQNGH at a given pH can be expressed as:

$$^{G}k_{obs} = {}^{G}k'_{o}f_{BH^+} + {}^{G}k'_{OH}[^{-}OH]f_{BH^+} + {}^{G}k''_{OH}[^{-}OH]f_{BH^+} \quad (3)$$

The term $^{G}k'_{o}f_{BH^+}$ is kinetically equivalent to the acid-catalyzed term $^{G}k''_{H}f_{B}$. Similarly,



Scheme 3.

Table 2. Calculated Rate Constants[a] According to Equation 4 for the Deamidation of GQNGH at 37°C

Parameter	Value (±SD)
$^{G}k'_{o}$	3.74 (±0.28) × 10^{-4} h^{-1}
$^{G}k'_{OH}$	6.05 (±0.13) × 10^{4} M^{-1} h^{-1}
$^{G}k''OH$	1.69 (±0.07) × 10^{3} M^{-1} h^{-1}
Ka	4.36 (±0.45) × 10^{-9}

[a] Rate constants are for the ionic species shown in Scheme III and eq 4.

$^{G}k'_{OH}[-OH]f_{BH}$ is equivalent to $^{G}k''_{o}f_{B}$. The terms used in eq 3 were chosen because of the tendency for deamidation to be a base-catalyzed reaction. Equation 3 can be rewritten as follows:

$$^{G}k_{obs} = \{^{G}k'_{o}[H^+] + {}^{G}k'_{OH}Kw + {}^{G}k''_{OH}[^{-}OH]Ka\}/ \{[H^+] + Ka\} \quad (4)$$

The best fit of the theoretical pH–rate profile (solid line in Figure 4) was obtained by using the values in Table 2 for the parameters of eq 4. The Ka value was assigned as the ionization constant of the N-terminal amino group.

Effect of N+1 His Residue

At neutral to basic pH, the amino acid located at the N+1 position has been shown to have a significant effect on the rate of deamidation of an Asn residue.[4,11,12] The results from these studies suggested that as the steric bulk of the side chain on the N+1 residue increases, the rate of deamidation decreases. To elucidate the effect of the His

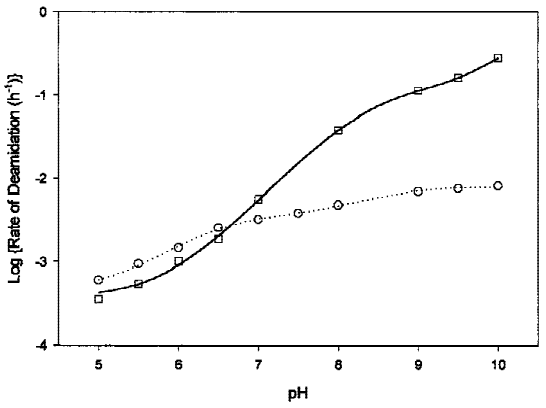


Figure 5. The pH–rate profiles for the deamidation of GQNHH (○) and GQNGH (□) in aqueous solution at 37 °C and I = 0.5.

Table 3. The Kinetic Solvent Isotope Effect for the Deamidation of GQNHH and GQNGH at 37°C

	k_{H_2O}/k_{D_2O}	
pH	GQNGH	GQNHH
5.0	1.21	1.64
8.0	1.12	1.34

residue at the N+1 position on the rate of deamidation of Asn residue, the pH–rate profiles of GQNHH and GQNGH were compared (Figure 5).

At pH values <6.5, GQNHH degraded slightly faster than GQNGH, suggesting that the N+1 His residue is acting as a catalyst in this pH region. The transition state for the formation of cyclic imide is assumed to be an oxyanionic tetrahedral intermediate whose stabilization will result in an enhancement of the rate of deamidation. Below pH 6.5, the His side chain can function as a general acid group, providing a proton to stabilize the transition state and, consequently, increasing the rate of the reaction. In an effort to distinguish whether the imidazole is facilitating the deprotonation of the peptide-bond nitrogen or facilitating cyclic imide formation by protonation of the leaving group, kinetic studies were conducted in H_2O and D_2O at pH 5.0 and 8.0, and the ratios of the rates k_{H_2O}/k_{D_2O} were calculated. The magnitude of the kinetic solvent isotope effect shown in Table 3 suggests that the proton on the imidizole is hydrogen bonded to the transition state, rather than being involved in a proton transfer step. A similar general acid catalytic behavior has been proposed for the side chains of Ser and Thr residues.[13,20,21]

Above pH 6.5, GQNGH was more sensitive to changes in the pH of the solution and degraded more rapidly than GQNHH (Figure 5). The differences in the rates at higher pH could result from the greater steric bulk of His side chain, inhibiting flexibility and, consequently, the rate of formation of the cyclic imide. To examine the effects of steric hindrance of the N+1 residues, we compared the rates of deamidation of GQNHH, GQNVH, and GQNGH. The His side chain occupies a larger volume than that of Val;[22] as such and based on previous observations, one would expect the Val analogue to undergo cyclization faster than the His analogue. However, at all pH values studied, GQNHH deamidated faster than GQNVH (Table 4), suggesting that the N+1 His residue is also acting as a catalyst in the higher pH region. Thus, at basic pH, the N+1 His residue influences the rate of deamidation via two opposing effects, general base catalysis and steric interference.

For the pentapeptide GQNHH, at pH >7.0, detectable levels of Asn-His peptide bond cleavage products (retention times: t = 6.8 and 7.2 min) were observed. This observation is consistent with that of Brennan and Clarke,[17] who also observed spontaneous cleavage of the Asn-His peptide bond at pH 7.4. The enhanced rate of Asn-His peptide bond cleavage can be attributed to the ability of the His side chain to hydrogen bond with the Asn side-chain amide, increasing its nucleophilicity and, thus, the rate of peptide bond cleavage. The effects of N+2 His residue on the rate of deamidation were also examined. As shown in Table 4, the rate of deamidation of GQNGH is comparable to that of GQNGG. This noninvolvement of the N+2 His residue in the kinetics of the reaction is in agreement with the pH–rate profile information.

In conclusion, these results suggest that Asn-His sites in peptides and proteins may be highly

Table 4. Summary of the Rate of Deamidation of the GQNXZ Peptide Derivatives in 100 mM Phosphate Buffer (I = 0.5) at 37°C

	Rate of Deamidation ($h^{-1} \times 10^3$)			
pH	GQNGG	GQNGH	GQNVH	GQNHH
5.0	0.385 (0.02)	0.358 (0.04)	—	—
6.0	2.55 (0.21)	1.77 (0.14)	0.159 (0.01)	1.96 (0.09)
7.0	18.58 (0.009)	12.41 (0.002)	1.46 (0.04)	11.69 (0.17)
8.0	51.76 (3.1)	52.97 (2.6)	4.56 (0.13)	22.21 (0.13)
9.0	143.4 (0.51)	121.15 (9.2)	—	—
10.0	268.5 (4.1)	293.30 (2.8)	6.72 (0.09)	19.75 (0.04)

susceptible to deamidation and peptide bond cleavage reactions. We found that the His residue on the C-terminal side of Asn catalyzes the rate of deamidation in the pH range studied (i.e., pH 5–10).

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