Kinetics and Mechanism of Succinimide Ring Formation in the Deamidation Process of Asparagine Residues

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The cyclization of Ac–Gly–Asn–Gly–Gly–NHMe to the succinimide derivative has been studied in the pH range 5.5–10.4 at 37 °C and μ = 1 mol dm^{-3}. Kinetic evidence indicates that the reaction is a multistep process with a change in the rate-determining step at pH 6.5–7.0.

The suggested mechanism involves the pre-equilibrium deprotonation of the NH group next to the Asn residue, followed by nucleophilic attack of the nitrogen atom on the carbonyl carbon of the Asn side chain giving a cyclic tetrahedral intermediate. At acidic pH the cyclization step is rate determining, whereas, the removal of the leaving group by apparent general-base catalysis is the rate-determining step at neutral and basic pH. The literature data on the deamidation rate are discussed in light of the proposed mechanism.

A succinimide derivative is known to occur as an intermediate in the deamidation reaction of peptides and proteins.[1–4] The β-carbonyl group of the Asn residue acylates the amino group of the next residue, producing an aminosuccinyl residue (Asu). This cyclic imide is unstable in aqueous solution and its rapid hydrolysis can occur on either side of the imide nitrogen, thus generating two compounds, α-Asp- and β-Asp-peptides in approximately a 1 : 3 ratio.[1,4,5] The β-Asp residues in peptides and proteins can be stoichiometrically methylated by eukaryotic protein-carboxyl methyltransferases.[6,7]

The formation of the succinimide ring may occur not only *in vivo* but also during purification, storage and manipulation of peptides and proteins.[8] For instance, the reduction in biological activity of growth hormone-releasing factors, stored in solution for an extended period of time, has been ascribed to a deamidation process *via* succinimide.[9]

The relevant biological role of the deamidation reaction has stimulated a large number of reports on the influence of the sequence and external factors on the deamidation *via* succinimide intermediate. Peptides and proteins bearing, next to Asn, residues with no bulky side chain, or containing a hydroxy group, are more prone to give the succinimide derivative.[4,10,11] Kinetic studies on peptides containing the Asn–Gly sequence have shown that the rate of the cyclization reaction strongly increases at basic pH,[4,12] and decreases by adding organic solvents to the aqueous solution of the Asn-peptides.[13] Experimental data have also indicated a substantial catalytic contribution of the buffer,[12,13] in apparent contrast with previous data[14] obtained on different peptides.

In order to get a deeper understanding of the kinetic behaviour of this reaction we have studied the formation of the succinimide derivative from the peptide Ac–Gly–Asn–Gly–Gly–NHMe and we propose a kinetic mechanism, which consistently explains previously reported data.

Experimental

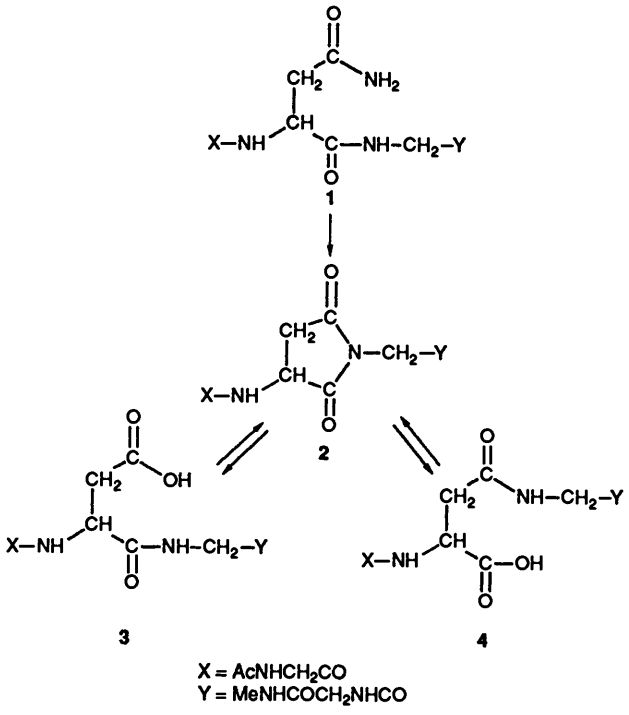
Materials.—Ac–Gly–Asu–Gly–Gly–NHMe (**2**) (Scheme 1), Ac–Gly–Asp–Gly–Gly–NHMe (**3**) and Ac–Gly–isoAsp–Gly–Gly–NHMe (**4**) were synthesized and characterized as previously reported.[15]

Synthesis of Ac–Gly–Asn–Gly–Gly–NHMe (**1**). N^{α}-Boc-glycylglycine-*N*-hydroxysuccinimide ester[16] was treated with 40% aqueous methylamine to give the intermediate N^{α}-Boc-glycylglycine-*N*-methylamide. After Boc deprotection with 4 mol dm^{-3} HCl in tetrahydrofuran for 1 h at 0 °C, glycylglycine-*N*-methylamide was coupled with N^{α}-Boc-L-asparagine by the 1,3-dicyclohexylcarbodiimide–hydroxybenzotriazole method. Deprotection with hydrochloric acid, as reported above, followed by treatment with *N*-acetylglycine 4-nitrophenyl ester gave the peptide **1**. Purification was carried out by preparative HPLC on a C18 column eluted with 0.1% trifluoroacetic acid in water. δ_H([$^2H_6$] DMSO, 200 MHz) 1.65 (s, 3 H, CH_3CO), 2.51 (d, 3 H, NH-CH_3), 2.82 (m, 2H, Asn β-CH_2), 3.6–3.75 (m, 6 H, Gly α-CH_2), 4.67 (m, 1 H, Asn α-CH), 6.95 (s, 1 H, $CONH_2$), 7.40 (s, 1 H, $CONH_2$), 7.5 (m, 2 H, CONH), 8.05 (t, 1 H, $CONHCH_3$), 8.4 (m, 2 H, CONH); amino acid analysis after hydrolysis in 6 mol dm^{-3} HCl for 8 h at 110 °C: Gly 3.0, Asp 0.97; m.p. 211–213 °C; $[\alpha]_D^{22}$ − 26.2 (*C* 1.0 water); TLC: R_f 0.23, Merck precoated 0.25 mm analytical silica gel plate 60F_{254}, butanol–acetic acid–water (3 : 1 : 1) as solvent system.

Kinetic Measurements.—Aqueous solutions of purified peptides **1**, **2**, **3** and **4**, (1 × 10^{-4}–5 × 10^{-4} mol dm^{-3}) at the desired pH and buffer concentration (ionic strength 1 mol dm^{-3}, KCl), were filtered through a 0.45 μm membrane filter and then stored in a thermostatted bath (37 ± 0.1 °C) in the dark. The following buffers were used in the concentration range 0.018–0.11 mol dm^{-3}; HCO_3^-/CO_3^{2-}, pH 10.4–9.2; morpholine·H^+/morpholine, pH 9.2–8.0; Tris·H^+/Tris, pH 8.6–7.4; $H_2PO_4^-/HPO_4^{2-}$, pH 7.1–5.9; AcOH/AcO^- 6.0–5.5. The pH values were measured by glass electrode at the same temperature and ionic strength as the rate measurements.

At preselected times the reacting mixtures were analysed by HPLC carried out on a Beckman Model System Gold, using a C18 reverse-phase column (3.9 mm × 300 mm, 4 μm resin), eluted with an aqueous solution of H_2SO_4 (0.01 mol dm^{-3}) and KOH, pH 3.6. Peptides were detected using a Beckman Model 166 variable wavelength monitor at 220 nm with a Shimadzu C-R6A integrating recorder. The rate constants of the cyclization reaction were calculated by least-squares analysis, assuming the rate to be first order in the Asn–peptide concentration. For all the samples, the fitting of the experimental data was satisfactory. The values of rate constants were reproducible within 5%. The results of a few tests carried out in the presence of sodium azide (1.0 mmol dm^{-3}), as a preservative, did not change significantly.

The intermediate and the final products of the reaction were identified by comparison with HPLC traces of authentic samples.



Scheme 1

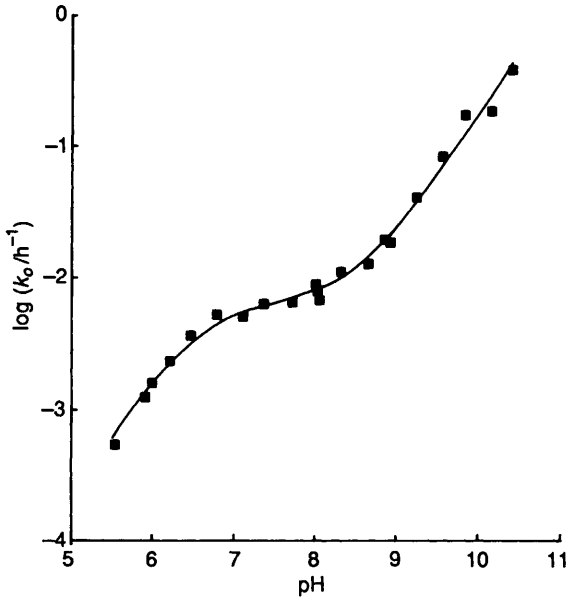


Fig. 1 Plot of log k_0 *vs.* pH for the cyclization of Ac-Gly-Asn-Gly-Gly-NHMe **1** to Ac-Gly-Asu-Gly-Gly-NHMe **2**; T = 37 °C and μ = 1 mol dm^{-3}

Results

The Asn-peptide **1**, Scheme 1, was incubated at 37.0 °C, μ = 1 mol dm^{-3} in the pH range 5.5–10.4, using several buffer concentrations.

As expected on the basis of literature data, the HPLC analyses of the reacting mixtures showed that the spontaneous deamidation of **1** proceeds prevalently *via* the succinimide intermediate **2** in the entire range of pH and buffer concentration used. The final product was a mixture of the α-Asp-**3** and the β-Asp-peptide **4**, with a relative yield that did not change detectably during the reaction. In all the experiments the intermediate **2** never exceeded 3% of the total peptide concentration and the ratio **3** to **4** was about 1:3. The cyclization reaction and the subsequent hydrolytic step are reported in Scheme 1.

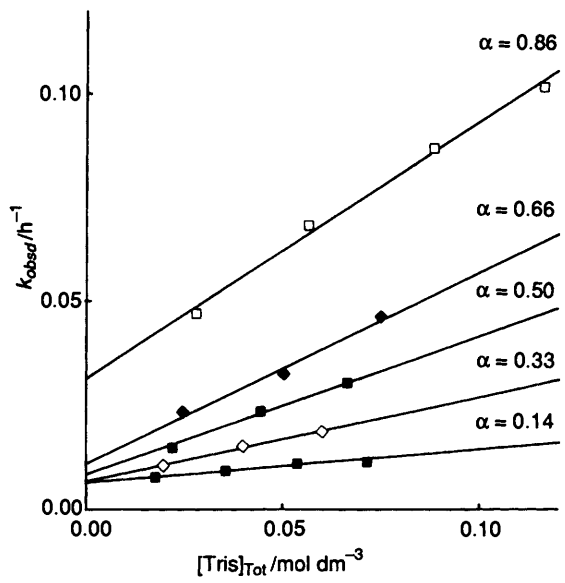


Fig. 2 Dependence of the pseudo-first-order rate constant on the total concentration of Tris buffer at the fraction (α) of free base indicated

In order to check the amount of **1** which deamidates *via* direct solvent hydrolysis, the succinimide peptide **2** and each of the two aspartyl peptides, **3** and **4**, were incubated separately under the same conditions as **1**.[4,17] In the experimental conditions, the aspartyl peptides were stable, and their relative amount obtained by hydrolysis of **2** was coincident, within experimental error, with the value obtained for the deamidation of **1**. These results show that direct solvent hydrolysis of **1**, which should give only the α-Asp-peptide **3**, does not occur to an appreciable extent. On this basis, the rate of the conversion of **1** to **2** was measured by monitoring the concentration of **1**. As reported elsewhere,[15] at acidic pH (< 5.5), the rate of the interconversion of the final products is not negligible in comparison with the deamidation rate. In this case, direct solvent hydrolysis of **1** to **3** cannot be excluded on the basis of the relative yield of **3** and **4**. Therefore the kinetic studies were limited to the pH values above 5.5.

pH Dependence.—In Fig. 1 is shown a plot of log k_0 *vs.* pH for the cyclization reaction of **1** at 37 °C and μ = 1 mol dm^{-3}, where k_0 is the observed first-order rate constant extrapolated to zero buffer concentration. At pH > 9 the plot is approximately linear with a slope of 1.0, suggesting hydroxide ion catalysis. At the intermediate pH (8.3–6.7), k_0 becomes substantially pH independent, which is indicative of an uncatalysed or water-catalysed reaction. Below pH 6.5, k_0 begins again to decline, almost linearly, with a slope of *ca.* 1.0.

Buffer Catalysis.—The influence of the buffer on the reaction rate was measured in both acidic and basic conditions. In the first case, pH < 6.5 (buffers $H_2PO_4^-/HPO_4^{2-}$ and AcOH/ AcO^-), no significant effects were detected. On the other hand, a marked influence on the reaction rate was observed at neutral and basic pH for each of the buffers used, HCO_3^-/CO_3^{2-}, morpholine·H^+/ morpholine, Tris·H^+/ Tris and $H_2PO_4^-/HPO_4^{2-}$. At constant pH, the observed pseudo-first-order rate constant, k_{obsd}, increases linearly with total concentration of the buffer. The coefficient of the linear correlation, which measures the catalytic efficiency k_{cat} of the buffer, also increases with the fraction α of the free base. The experimental data for Tris·H^+/Tris are presented in Fig. 2. Fig. 3 shows the plot of k_{cat} *vs.* α for all the buffers used in the neutral and alkaline range. The plots are linear and pass through the origin at α = 0, suggesting that the acidic form of the buffers is inactive; for the buffer $H_2PO_2^-$/

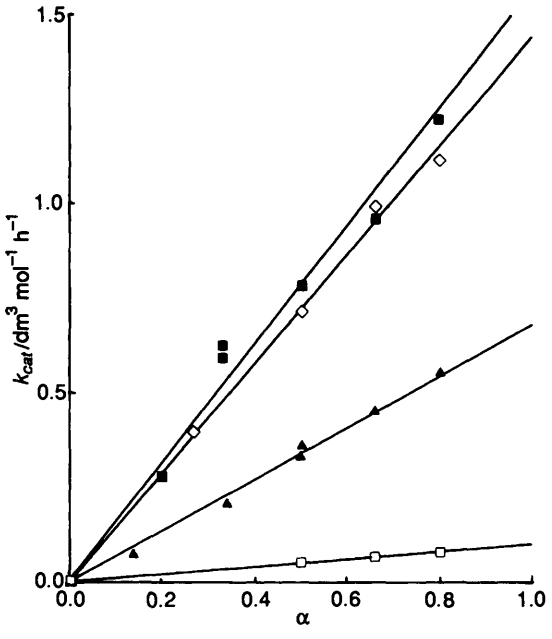
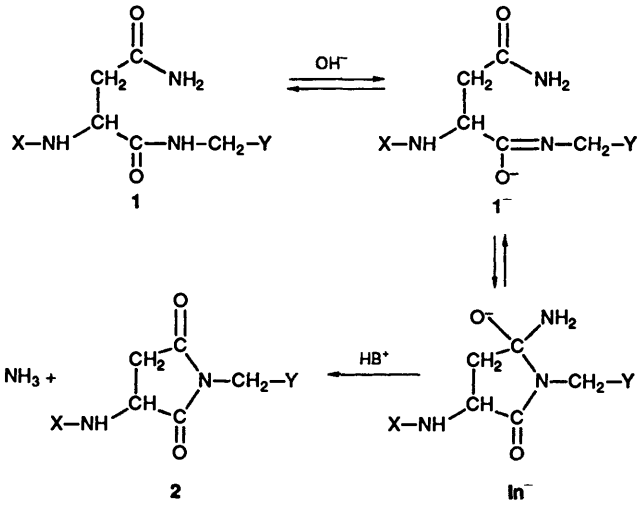


Fig. 3 Catalytic constant k_{cat} *vs.* the fraction, α, of free base in the buffers HCO_3^-/CO_3^{2-} ■; Tris·H^+/Tris ▲; morpholine·H^+/morpholine ◇; and $H_2PO_4^-/HPO_4^{2-}$ □. For presentation purposes k_{cat} values of carbonate have been scaled down by a factor of 9.



Scheme 2

Table 1 Rate constants[a] for the apparent general-base catalysis of the cyclization of **1** to **2**, pH > 6.5, T = 37 °C and μ = 1 mol dm^{-3} with KCl

k_{OH^-}/dm^3 mol^{-1} h^{-1}	4.5 × 10^2
$k_{CO_3^{2-}}$/dm^3 mol^{-1} h^{-1}	15
$k_{morpholine}$/dm^3 mol^{-1} h^{-1}	1.4
k_{Tris}/dm^3 mol^{-1} h^{-1}	0.68
$k_{HPO_4^{2-}}$/dm^3 mol^{-1} h^{-1}	0.10
k_{H_2O}/h^{-1}	5.9 × 10^{-3}

[a] Estimated error ≤ 5%.

HPO_4^{2-} the values of k_{cat} reported in Fig. 3 have been obtained at pH > 6.5.

These results, together with those described in the previous section, indicate that at neutral and basic pH the cyclization reaction of **1** is an apparent general-base-catalysed reaction. Accordingly, in this pH region the observed rate constant includes contributions from all the bases in solution [eqn. (1)].

$$k_{obsd} = k_{H_2O} + k_{OH^-}[OH^-] + \Sigma(k_{B_i}[B_i]) \quad (1)$$

The catalytic constants k_{B_i} were obtained from the values of k_{cat} reported in Fig. 3 at α = 1, whereas k_{H_2O} and k_{OH^-} were obtained by the pH dependence of k_0 in the neutral and basic range (Table 1). The Brønsted plot, log k_B *vs.* pK_a of the conjugate acid of the apparent general-base catalyst, is linear with a slope of 0.39 ± 0.02 and a correlation coefficient of 0.99.

Discussion

The profile of log k_0 *vs.* pH shown in Fig. 1 can be caused either by an acid–base equilibrium of the substrate, with different rate coefficients for reaction of the acidic and basic forms, or by a change of the rate-determining step, suggesting the formation of an intermediate in the pathway of the reaction.[18] In the intramolecular cyclization of **1** the chemical nature of the substrate excludes an acid–base equilibrium in the pH range explored.

The experimental results show that, at neutral and basic pH, the reaction of the formation of the succinimide derivative is affected by a general-base catalysis, or by a kinetically equivalent catalysis. Furthermore the low value of the Brønsted plot slope (0.39) suggests that this catalysis probably involves a proton transfer, concerted with bond making or breaking.[19] At acidic pH a change in the rate-determining step occurs: the reaction rate is not affected by buffers, and increases linearly with the hydroxide ion concentration, indicating that the reaction is specific-base catalysed.

Cyclization by base-catalysed intermolecular nucleophilic attack on the carbonyl carbon is a class of reactions which has been extensively studied. The most widely accepted mechanisms involve either ionization of the nucleophile followed by the attack on a carbonyl carbon,[20,21] or, alternatively, initial attack of a neutral group on the carbonyl carbon to form a neutral tetrahedral intermediate, which then ionizes before the expulsion of the leaving group.[22] In the cyclization reaction of the Asn residue to its succinimide derivative, the well known acidity of the hydrogen atom of peptide groups,[23] makes the first mechanism more probable. On this basis, our experimental results can be rationalized in terms of the reaction pathway depicted in Scheme 2. The cyclization of the Asn side chain involves pre-equilibrium formation of the anionic intermediate **1**$^-$, followed by intramolecular cyclization to **In**$^-$. Subsequent proton transfer to the leaving group from a general acid and the concerted breakdown of the tetrahedral intermediate **In**$^-$ finally gives the product.

At acidic pH the cyclization to **In**$^-$ is rate determining, and therefore the reaction is specific-base catalysed. Whereas, at higher pH the conversion of **In**$^-$ to **2** becomes rate-limiting and the reaction is general-acid–specific-base catalysed. This catalysis is kinetically equivalent to the general-base catalysis as shown in eqn. (2) where k_B is the second-order rate constant for

$$k_B[B] = k_B K_a (K_w)^{-1}[BH^+][OH^-] \quad (2)$$

the apparent general-base catalysis, K_a and K_w are the dissociation constants for the conjugate acid BH^+ and water, respectively.

According to this mechanism the function of the buffer in the catalysis is, therefore, to protonate the nitrogen atom of the leaving group thus facilitating its removal.

Conclusions

The proposed mechanism gives a sound interpretation of the

kinetic data previously published on the deamidation of peptides and proteins.

According to this mechanism an important role in the reaction rate is played by the acidity of the hydrogen atom of the NH group next to the asparagine residue. Therefore all the factors, which decrease the acidity of this group, such as polarity and/or negative residual charges of neighbouring residues, as well as the polarity of the solvent of the reaction mixture, should also decrease the reaction rate. In this light, our previous data, which show a marked decrease of the deamidation rate in water–organic solvent mixtures,[15] can be explained by the decreased stability of the anionic intermediates in organic solvents. On the other hand, the activating effect of Ser next to Asn [24] may result from the electronic inductive effect of the Ser hydroxy group, which stabilizes the intermediates.

For the peptide used in this work, the buffer catalysis is efficient only at pH > 6.5. However, the interval of pH in which the effect of the buffer is appreciable, may well depend on the specific sequence of the peptide. Thus, in some studies the buffer catalysis may not have been noted,[14] simply because the tests were not carried out in a sufficiently large pH range.

Finally, we want to underline two interesting aspects of the catalysis: first, in the presence of a high concentration of buffer the deamidation reaction may be relatively fast even at moderately basic pH, thus producing a significant amount of deamidated forms under conditions which are often used in the purification and crystallization of peptides and proteins;[25] secondly, in proteins and peptides with a stable three-dimensional structure, intramolecular catalysis, by a moderately weak acidic residue close in space to the deamidation centre, may make on its own an important contribution to the reaction rate.

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