Spontaneous Cyclization of the Aspartic Acid Side Chain to the Succinimide Derivative

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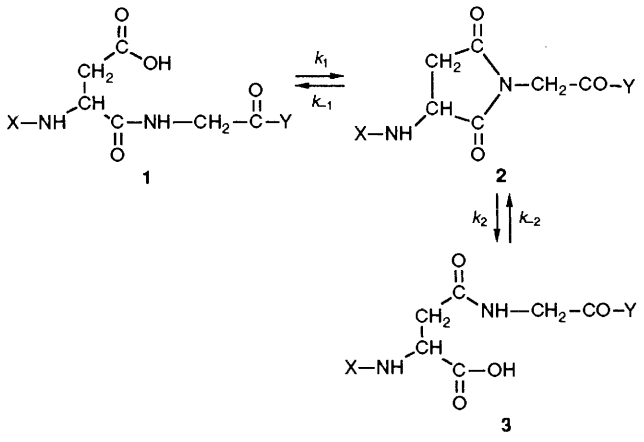
At equilibrium in acidic solution the succinimide derivative, formed by cyclization reaction of the Asp side chain, predominates; at a pH close to the apparent pK_a of the Asp residue this reaction is relatively fast with a $t_{1/2}$ of a few days.

It is well established[1,2] that an aminosuccinyl residue (Asu) can occur as an intermediate in the deamidation of Asn residues and in the reversible isomerization of Asp residues. The β-carbonyl group of Asp or Asn can acylate the amino group of the next residue producing the succinimide derivative. In aqueous solution the cyclic moiety is unstable and its hydrolysis may occur on either side of the imide nitrogen giving a mixture of α- and β-Asp-peptides. The hydrolysis is catalysed by hydrogen and hydroxide ions, and the stability of the Asu residue reaches a maximum in acidic media.[3,4]

Previous kinetic studies[4] on the pH dependence of the rate of deamidation of Asn-peptides have shown that the formation of the imide ring is always much slower than its hydrolysis; thus the concentration of the Asu-peptide during the reaction is always very low. Moreover, at basic and neutral pH, formation of the succinimide ring from an Asp residue is much slower than its formation from Asn. However, as the carboxylic acid hydroxy group is a good leaving group, it would be expected that at acidic pH the protonated Asp residue would give the Asu residue at a higher rate. Recently slow formation of Asu residues in Asp-peptides stored at acidic pH has been reported.[5]

In this study, we have investigated the pH dependence of the spontaneous reversible isomerization, *via* the succinimide derivative, of an α- to a β-Asp-peptide (Scheme 1). The results indicate that at pH close to the apparent pK_a of the Asp residue the cyclization rate is the fastest, the half-life being only a few days. Moreover, at equilibrium in acidic solution



Scheme 1 X = $AcNHCH_2CO$–
Y = $MeNHCOCH_2NH$–

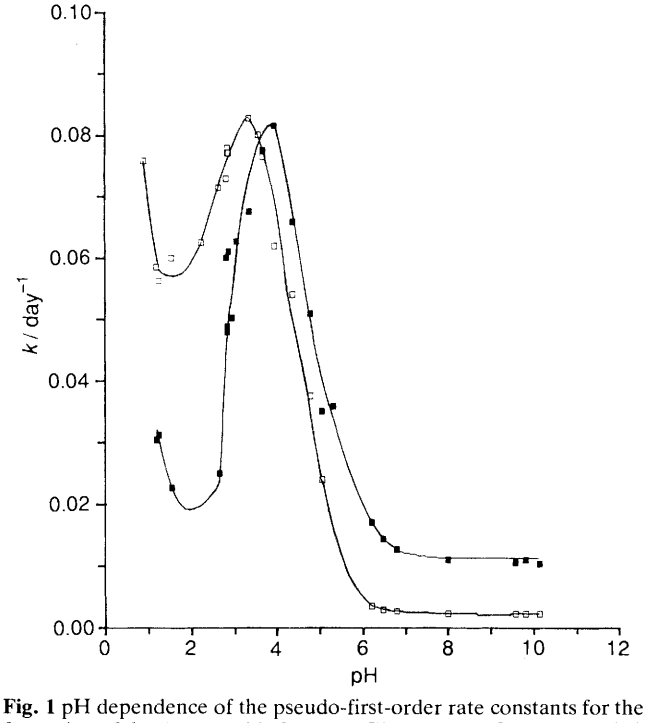


Fig. 1 pH dependence of the pseudo-first-order rate constants for the formation of the Asu-peptide from Ac-Gly-Asp-Gly-Gly-NH-Me (■) and Ac-Gly-β-Asp-Gly-Gly-NH-Me (□) at 37 °C and μ = 1 mol dm^{-3}

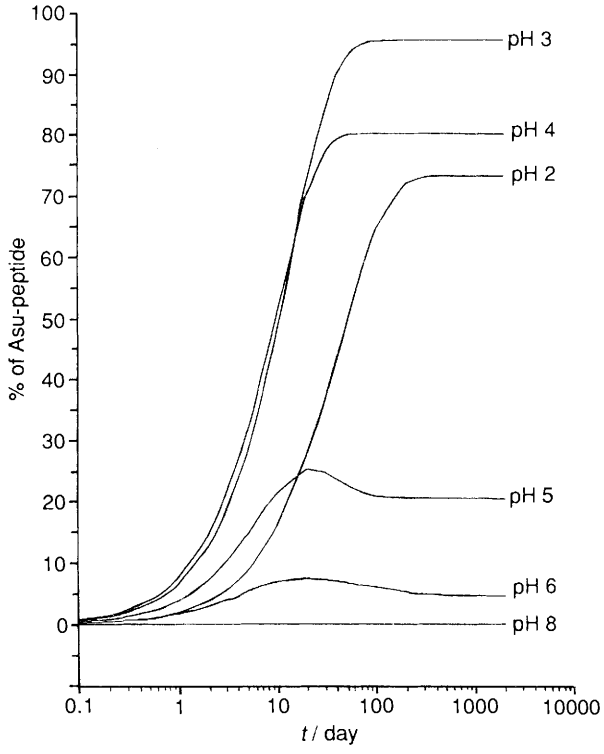


Fig. 2 Effect of pH on the time–concentration curves of the Asu-peptide in the cyclization reaction of Ac-Gly-Asp-Gly-Gly-NH-Me at 37 °C and μ = 1 mol dm^{-3}

the Asu-peptide is the most abundant compound, as a consequence of the different pH–rate profile of the formation and hydrolysis reactions.

The peptides were synthesized by classical solution phase method.[6] N^{α}-Boc-glycylglycine-*N*-hydroxysuccinimide ester[7] 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 (THF) for 1 h at 0 °C, glycylglycine-*N*-methylamide was coupled with N^{α}-Boc-L-aspartic acid-β-benzyl ester and N^{α}-Boc-L-aspartic acid-α-benzyl ester by the 1,3-dicyclohexylcarbodiimide–hydroxybenzotriazole method. Deblocking with hydrochloric acid, as reported above, gave deprotected peptides that were coupled with *N*-acetyl-glycine-4-nitrophenyl ester. Benzyl esters of peptides **1** and **3** were obtained in good yield, and after catalytic hydrogenation on 5% palladized charcoal, compounds **1** and **3** were finally obtained. The Asu derivative **2** was obtained by cyclization of the corresponding β-benzyl ester with a stoichiometric amount of triethylamine in dimethylformamide.[8] All the compounds were purified by HPLC and gave expected ^1H NMR and amino acid analysis: m.p.s 214, 240 and 175 °C for **1**, **2** and **3** respectively.

The apparent pK_a values of **1** and **3** were found to be 4.1 and 3.4 respectively from the half-neutralization point of titration curves.

Kinetic studies were carried out on the α-Asp peptide **1**, the β-Asp peptide **3** and the Asu-peptide **2** (0.2–0.8 mmol dm^{-3}), at ionic strength 1.0 mol dm^{-3} (with KCl), in the pH range 0.5–10.1. The following buffers were used at a concentration of 0.07 mol dm^{-3}: HCO_3^-/CO_3^{2-}, pH 9.5–10.1; Tris·H^+/Tris, pH 8.0; $H_2PO_4^-/HPO_4^{2-}$, pH 6.2–6.8; AcOH/AcO^-, 4.0–5.1; and HCO_2H/HCO_2^-, pH 2.2–4.0; HCl was used in the pH range pH 2.0–0.5.

The solutions of each peptide were filtered through a 0.45 μm membrane filter and then stored at 37 °C in the dark. At preselected times the reacting mixtures were analysed by HPLC on a C_{18} reversed-phase column (3.9 × 300 mm, 5 μm resin), eluted with 0.2% trifluoroacetic acid.

The reaction rates were obtained by the slope at zero concentration of the time–concentration plots. Several experiments, carried out at different concentrations of the peptides, showed that all the reactions involved were of first order with respect to the starting peptide. The kinetic constants were reproducible within ±5%.

In Fig. 1 the kinetic constants k_1 and k_{-2} (Scheme 1) of the cyclization reaction of **1** and **3** are plotted as function of pH. The increases at low pH (<2) are due to the onset of hydrogen ion catalysis; above pH 2 the rate constants for both peptides rapidly increase with pH, as a result of hydroxide ion catalysis, and show a sharp maximum at a pH close to the apparent pK_a of the corresponding carboxy groups. At this pH, the $t_{1/2}$ of the cyclization reaction of both peptides is about 8 days. Besides the progressive deprotonation of the aspartyl residues, the rapid decrease of the rate constants at higher pH is probably

caused by a change in the rate-limiting step of the reaction. Further increment of pH does not influence the reaction rates.

In order to determine accurately the pH dependence of the reversible reactions reported in Scheme 1, the Asu-peptide **2** was hydrolysed under the same conditions used for the cyclization of **1** and **3**. The shape of the pH–rate profiles of the kinetic constants k_{-1} and k_2 is in agreement with previous results on the hydrolysis of the succinimide ring.[3,4] The cyclic imide shows its maximum stability in the pH range 2.0–2.8.

The values of k_1, k_{-1}, k_2 and k_{-2} were used to compute the time–concentration curves of the Asu-peptide **2**, using a kinetic equation which describes two consecutive reversible reactions.[9] The agreement between the computed and experimental data is satisfactory. Some significant curves obtained starting from **1** are in Fig. 2. At neutral and basic pH, the concentration of the Asu peptide is always very low, whereas at moderate acidic pH it is by far the most abundant product at equilibrium.

In conclusion, we have shown that at pH values near the pK_a of the Asp residue the spontaneous cyclization of the aspartyl side chain in the model compound **1** is an efficient and relatively fast reaction. Although the cyclization rate is probably dependent on the specific amino acid sequence and molecular complexity of the peptide, our data suggest that Asp-peptides, which have been stored, or merely purified, at acidic pH may contain a considerable amount of the Asu-peptide. The importance of these results should be considered in connection with the conformational propensities of the Asu derivative to fold in a type II′ β bend[10] and with the relevant role of this conformation in the biological activity of some peptides.[11]

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