First Evidence of Spontaneous Deamidation of Glutamine Residue *via* Cyclic Imide to α- and γ-Glutamic Residue under Physiological Conditions

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At 37 °C and pH 7.4 the spontaneous deamidation of Gln-peptide proceeds prevalently *via* a glutarimide intermediate and gives a mixture of α-Glu- and γ-Glu-peptide.

The side chains of peptides and proteins are subject to a variety of spontaneous chemical reactions under physiological conditions.[1] Among the most prevalent is the non-enzymatic deamidation of asparagine and glutamine residues.[2–4]

It is now well established that the deamidation of asparagine residues proceeds, in the absence of a bulky side chain on the next residue, *via* a succinimide intermediate:[4–8] the asparaginyl side chain acylates the NH group of the next residue producing an aminosuccinyl residue. The spontaneous hydrolysis of the succinimide ring can occur on either side of the imide nitrogen, thus generating two compounds, β-Asp- and α-Asp-peptides in approximately a 3:1 ratio.[4,6,7] The L-isoaspartyl sites in peptides and proteins can be selectively and stoichiometrically methylated by eukaryotic protein-carboxyl methyltransferases.[9,10]

On the other hand, the deamidation of glutamine residues has been studied much less extensively; the only general result that emerges from the studies reported is a lower deamidation rate,[2,11] but no indication of the reaction pathway is reported.

In this communication we present experimental results showing that, besides a direct hydrolytic pathway, the deamidation of glutamine residues at physiological temperature and pH can also proceed through a cyclic imide intermediate, a glutarimide derivative. The pathway of the reactions is shown in Scheme 1. The products were identified by comparison with authentic samples synthesized by classical solution phase methods.[12] Glycine-*N*-methylamide was coupled with *N*-acetyl-L-glutamine, *N*-Boc-L-glutamic acid-α-benzyl ester and *N*-Boc-L-glutamic acid-γ-benzyl ester by the 1,3-dicyclohexylcarbodiimide–hydroxybenzothiazole (DCC–HOBt) method, giving peptide **1** and the benzyl esters of peptides **3** and **4**, respectively. Treatment of the last two compounds with trifluoroacetic acid and acetic anhydride, followed by catalytic hydrogenation gave peptides **3** and **4**. The glutarimide derivative **2** was obtained by cyclization of the γ-benzyl ester with triethylamine in dimethylformamide.[13] All compounds were purified by HPLC and gave the expected ^{1}H NMR and FAB (fast atom bombardment) mass spectra and amino acid analysis; m.p. 137, 160, 143 and 127 °C for **1**, **2**, **3** and **4**, respectively.

The deamidation study was carried out incubating peptide **1** (0.2 mmol dm^{-3}) in phosphate buffer (10 mmol dm^{-3}), pH 7.4, ionic strength 0.5 mol dm^{-3} with KCl. The mixture was filtered through a 0.45 μm membrane filter and then stored in a set of glass microvials in the dark.

At preselected times, a vial was opened and analysed by HPLC on a C8 column eluted with 0.2% acetonitrile and 0.2% trifluoroacetic acid. The time–concentration curves (Fig. 1)

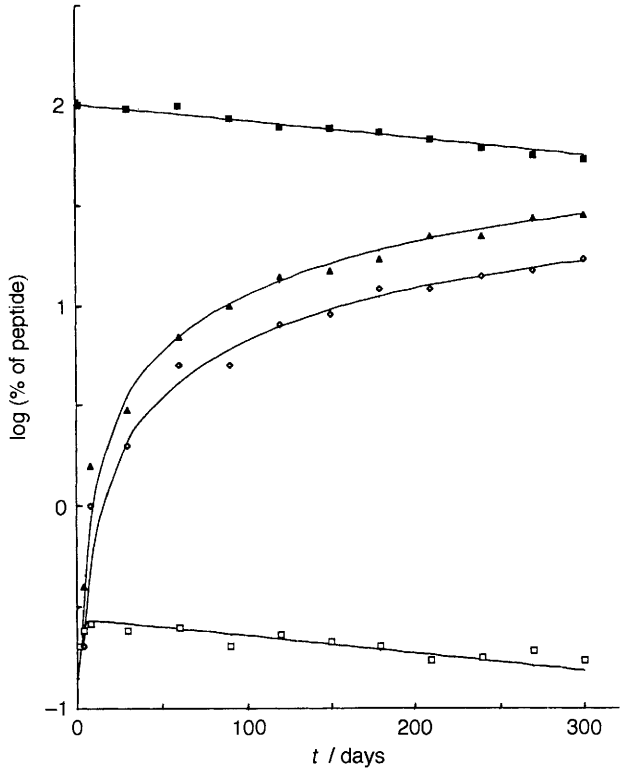
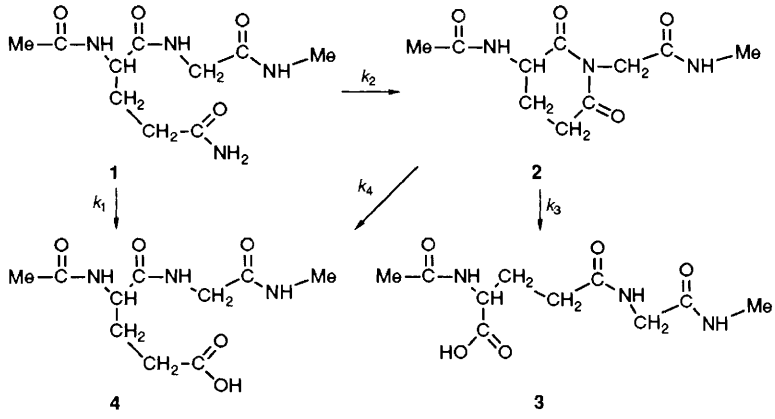


Fig. 1 Progress curves for the deamidation of Ac-Gln-Gly-NHMe at 37.0 °C and pH 7.4, phosphate buffer 10 mmol dm^{-3}; (■) Gln-peptide, (□) cyclic imide intermediate, (▲) γ-Glu-peptide, (◇) α-Glu-peptide



Scheme 1 Proposed pathway for the non-enzymatic deamidation of glutamine residues

showed a first-order loss of the glutaminyl peptide, and the formation of the final products, γ-Glu-peptide **3** and α-Glu-peptide **4**, in a 1.7:1 ratio. The fast accumulation of the glutarimide intermediate **2** at the beginning of the reaction and its slow decay are also shown. The results of several tests carried out in the presence of sodium azide (1.0 mmol dm^{-3}), as a preservative, did not change significantly.

In order to test the amount of peptide **1** which deamidates *via* direct solvent hydrolysis, giving only the α-Glu-peptide **4**, the glutarimide peptide **2** was incubated under the same conditions as peptide **1**. The hydrolysis of the glutarimide ring also proceeds with a first-order loss of the starting peptide ($t_{1/2}$ = 0.96 day), and gives peptides **3** and **4**, in a 3.3 : 1 ratio. From the relative yields obtained in the two reactions the percentage of the glutaminyl peptide which deamidates *via* glutarimide was estimated to be about 82%. On this basis the following values of the kinetic constants (Scheme 1) were obtained: $k_1 = 3.8 \times 10^{-4}$, $k_2 = 1.6 \times 10^{-3}$, $k_3 = 5.5 \times 10^{-1}$, $k_4 = 1.7 \times 10^{-1}$ day^{-1}. The associated errors are less than 10%. It is worth noting that the rate of the cyclization to glutarimide is much lower than the corresponding cyclization to succinimide[4,6] from asparagine residue. The difficulty in obtaining a six-membered imide ring is probably one of the reasons for the lower rate of deamidation reaction of glutamine residues. The presence in peptides, and presumably in proteins, of γ-Glu residues, as a result of the deamidation process, should be carefully considered, particularly in those stored for extended periods of time or having a long half-life, as the proteins obtained from tissues with limited capacity of new proteins synthesis.

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