Kinetics and Mechanism of the Reversible Isomerization of Aspartic Acid Residues in Tetrapeptides

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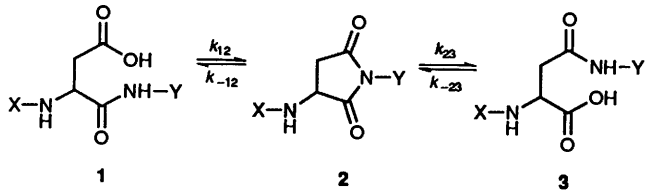
The influence of pH and buffer concentration on the rate of the isomerization of Asp residues has been analysed using model aspartic acid-containing tetrapeptides, in the pH range 1.5–10 at 37 °C and μ = 1 mol dm^{-3}. The reaction involves the reversible formation of an amino-succinimide intermediate.

Kinetic evidence indicates that of the various forms of the Asp-peptide in acid–base equilibrium, only one, having the carboxylic Asp side chain in the neutral state and the N–H peptide group next to the Asp residue in the deprotonated state, reacts at an appreciable rate. The reaction involves nucleophilic attack by the nitrogen atom of the peptide bond on the carbonyl carbon of the Asp side chain, giving a cyclic tetrahedral intermediate. At pH values lower than about 3 the cyclization step is rate-determining, but at higher pH the rate-determining step is the general acid-catalysed departure of the leaving group. This change of rate-determining step, together with the ionization of the carboxylic side chain of the Asp residue, produces a bell-shaped curve in the pH–rate profile for the cyclization reactions.

The deamidation of asparaginyl residues and the isomerization of aspartyl residues in peptides and proteins are topics of continuing interest.[1–4] It is well established that the major pathway of these post-translational modifications is *via* an aminosuccinimidyl residue (Asu) resulting from intramolecular nucleophilic attack on the β-carbonyl group of Asn or Asp residues by the amido NH group of the next residue, with the elimination of ammonia or water, respectively. In aqueous solution the succinimide is unstable and its hydrolysis may occur on either side of the imide nitrogen, giving a mixture of α- and β-linked peptides.[5–7] Scheme 1 shows the isomerization reaction *via* the succinimide derivative for the aspartyl peptide Ac-Gly-Asp-Gly-Gly-NHMe.†



1 2 3

Scheme 1 X = AcNHCH$_2$CO–, Y = MeNHCOCH$_2$NHCOCH$_2$

Kinetic studies[8–11] on the deamidation reaction have shown that above pH 4–5 the rates of cyclization to succinimide and the subsequent hydrolysis to aspartyl peptides increase with increasing pH, and that the cyclization step is rate-limiting for the overall process. Therefore, in this pH region, the succinimide derivative is always a small fraction of the reacting mixture. In more acidic media (pH < 2) the deamidation pathway involves mainly direct hydrolysis of the Asn side chain to produce only α-linked peptides.[10,12] Recently, a mechanism involving an ionic tetrahedral intermediate has been proposed for the cyclization of Asn-peptides to succinimide derivatives.[13]

The reversible isomerization in aqueous solution of Asp residues, either already present in the native form of the peptide chain, or produced by the deamidation of Asn, has been less extensively studied. Previous results,[7,14–16] show that this isomerization reaction also occurs *via* a succinimide derivative, but that it is much slower than deamidation at neutral and basic pH. Moreover, slow formation of Asu residues is observed in Asp-peptides stored in acidic media.[17] In this paper we report the results of a kinetic study on the reversible isomerization of aspartyl-containing peptides. Preliminary results have been reported.[18]

† Tetrapeptides (rather than tripeptides) are used in this work because it forms part of a much larger study, of the effects of neighbouring residues on the deamidation and isomerization reactions of the Asn-Gly and Asp-Gly sequence.

Experimental

Synthesis.—The peptide Ac-Gly-Asp-Gly-Gly-NHMe (**1**, Scheme 1), Ac-Gly-Asu-Gly-Gly-NHMe (**2**) and Ac-Gly-β-Asp-Gly-Gly-NHMe (**3**) used in this study were synthesized by a solution-phase procedure and purified by preparative high performance liquid chromatography (HPLC). The compounds were checked for homogeneity by thin-layer chromatography and analytical HPLC, and characterized by ^{1}H NMR spectroscopy, amino-acid composition of the acidic hydrolysate and optical rotation.

Preparative HPLC was carried out with a Waters Delta Prep 3000 (preparative chromatography system) equipped with a Delta-Pak C18 3000 Å (30 mm × 30 cm, 15 μm, spherical) column. The compounds were eluted over 30 min with a 0–10% linear gradient of 0.1% (w/v) trifluoroacetic acid, in water (solvent A) and in acetonitrile (solvent B). The flow rate was 30 cm^3 min^{-1}. ^{1}H NMR spectra were recorded on a Bruker (200 MHz FT) instrument. Amino acid analyses were conducted in a Carlo Erba automatic amino-acid analyser, model 3A29, using ninhydrin as the amino-acid derivatization reagent. TLC analyses were performed on precoated plates of silica gel F254 (Merck) using the following solvent systems: (A) butan-1-ol–AcOH–H$_2$O (6:1:5); (B) CH$_2$Cl$_2$–MeOH–benzene (85:10:5). Ninhydrin 1%, fluorescamine and/or chlorine were used as spray reagents. HPLC analyses were performed on a Beckman Model System Gold using a C18 column eluted with 0.1% (w/v) trifluoroacetic acid in water. Optical rotations were determined with a Perkin-Elmer 241 polarimeter with a 10 cm water-jacketed cell.

Boc-Gly-Gly-NH-CH$_3$ (**4**).—At room temperature Boc-Gly-Gly-OSu[19] (10 mmol: OSu = *N*-oxysuccinimido) was added as a solid in small portions to a solution containing 40% aqueous methylamine (1.6 cm^3) and dioxane (8.4 cm^3). After 4 h the solvent was removed, and the residue was purified by silica gel column chromatography using CH_2Cl_2–MeOH (20:1) as the eluent: yield 81%; R_f (A) 0.87, R_f (B) 0.64.

Boc-Asp(OBzl)-Gly-Gly-NH-CH$_3$ (**5**).—The peptide **4** (2 mmol) was deprotected by treatment with 5 mol dm^{-3} HCl in dioxane (10 cm^3) at 0 °C for 1 h. The solvent was removed *in vacuo* and the residue triturated with dry ether. The solid product was dissolved in dimethylformamide (DMF) (20 cm^3) containing *N*-methylmorpholine (NMM, 2 mmol). The solution was cooled at 0 °C and Boc-Asp(OBzl)-OH (2 mmol), dicyclohexylcarbodiimide (DCC) (2.2 mmol) and hydroxybenzotriazole (HOBt, 2.2 mmol) were added. The reaction mixture was stirred for 2 h at 0 °C and overnight at room temperature. *N,N'*-Dicyclohexylurea was filtered off, and the residue crystallized from AcOEt–Et_2O: yield 85%; R_f (B) 0.53; $[\alpha]_D^{20}$ −13.8 (*c* 1.0, MeOH).

Ac-Gly-Asp(OBzl)-Gly-Gly-NH-CH$_3$ (**6**).—0.5 mmol of **5** was treated with 5 mol dm^{-3} HCl in dioxane (5 cm^3) at 0 °C for 1 h. Then the mixture was allowed to stand at room temperature for 30 min, concentrated *in vacuo* and the residue triturated with ether. The solid product was dissolved in DMF (10 cm^3) containing Ac-Gly-ONp (0.5 mmol, ONp = 4-nitrophenoxy). The solution was cooled in an ice–water bath, and NMM (0.5 mmol) added. The solution was left to stand overnight at room temperature and then concentrated. The precipitate crystallized from AcOEt, yield 79%; R_f (A) 0.39; $[\alpha]_D^{20}$ −18.6 (*c* 1.0 MeOH).

Asp-peptide **1**.—Compound **6** (0.4 mmol) was dissolved in MeOH (10 cm^3) containing 1% (v/v) of acetic acid and hydrogenated at 1 atm using 5% Pd–C. The catalyst was removed by filtration, the solution concentrated *in vacuo* and the residue triturated with ether. The crude product was purified by preparative HPLC: yield 92%. R_f (A) 0.15, $[\alpha]_D^{20}$ −8.6 (*c* 1.0 MeOH). Amino-acid analysis: Asp 0.98, Gly 3.01; δ_H(DMSO) 1.65 (s, 3 H, Ac), 2.55 (d, 3 H, NHC*H*$_3$), 2.67 (dd, 1 H, Asp β-CH$_2$), 2.81 (dd, 1 H, Asp β-CH$_2$), 3.66 (m, 6 H), 4.55 (m, 1 H, Asp α-CH), 7.54 (m, 1 H), 7.90 (m, 1 H) 8.16 (m, 2 H) and 8.61 (m, 1 H).

Asu-peptide **2**.—Triethylamine (TEA, 1 mmol) was added to a solution of **6** (1 mmol) in dry DMF (10 cm^3) and the solution stirred for 2.5 days at room temperature. The crude product was purified by preparative HPLC: yield 65%. R_f (A) 0.28. Amino-acid analysis: Asp 1.01, Gly 3.00. δ_H(DMSO) 1.68 (s, 3 H, Ac), 2.48 (d, 3 H, NHC*H*$_3$), 2.69 (dd, 1 H, *J* 5.1 and 18.2 Hz, Asu β-CH$_2$), 3.42 (dd, 1 H, *J* 4.49 and 18.01 Hz, Asu β-CH$_2$), 3.64–3.70 (m, 4 H), 4.07 (m, 2 H), 4.61 (q, 1 H, *J* 7.7 and 12.7 Hz, Asu-CH), 7.8 (m, 1 H), 8.2 (t, 1 H) and 8.4 (t, 1 H, 8.54 (d, 1 H).

Boc-β-Asp(OBzl)-Gly-Gly-NHMe (**7**).—H-Gly-Gly-NHMe·HCl (5 mmol) was dissolved in DMF (10 cm^3) containing NMM (5 mmol) at 0 °C and Boc-Asp-OBz (5.5 mmol), DCC (5.5 mmol) and HOBt (5.5 mol) added to the solution. Work-up and purification as reported for compound **5** gave a pure product: yield 68%. R_f (B) 0.49, $[\alpha]_D^{20}$ 18.2 (*c* 1.0, MeOH).

Ac-β-Asp(OBzl)-Gly-Gly-NHMe (**8**).—Compound **7** (1.5 mmol) was treated with 5 mol dm^{-3} HCl (5 cm^3) in dioxane as reported for **6**. The residue, after trituration with ether, was dissolved in DMF at 0 °C (10 cm^3) containing NMM (1.5 mmol). To the solution was added Ac-Gly-ONp (1.4 mmol). Work-up and purification as reported for compound **4** gave a pure product: yield 68%. R_f (B) 0.55, $[\alpha]_D^{20}$ −18.2 (*c* 1.0, MeOH).

β-Asp-peptide **3**.—Compound **8** (1 mmol) was dissolved in MeOH (15 cm^3) and hydrogenated and purified as for **1**: yield 85%. R_f (A) 0.17, $[\alpha]_D^{20}$ 11.4 (*c* 1.0, MeOH). Amino-acid analysis: Asp, 0.98, Gly 3.03; δ_H(DMSO) 1.65 (s, 3 H, Ac), 2.61 (d, 3 H, NHC*H*$_3$), 2.63–2.71 (m, 2 H, Asp β-CH$_2$), 3.60–3.79 (m, 6 H), 4.57 (m, 1 H, Asp α-CH), 7.65 (m, 1 H), 7.99 (m, 1 H) 8.16 (m, 2 H) and 8.35 (m, 1 H).

pK_a *Measurements*.—The pK_a values for the dissociation of the carboxy group of the Asp side chain of **1** and **3** were determined under the conditions of the kinetic experiments by titration with 1 × 10^{-2} mol dm^{-3} KOH. The values obtained from the half-neutralization points were 4.1 ± 0.1 and 3.4 ± 0.1 for **1** and **3**, respectively.

Kinetic and Equilibrium Concentration Measurements.—Aqueous solutions of purified peptides (3.0 × 10^{-4}–1.2 × 10^{-3} 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 4.0 × 10^{-2}–0.21 mol dm^{-3}: HCO_3^-/CO_3^{2-}, pH 9.78; TrisH$^+$/Tris, pH 8.48–7.52; $H_2PO_4^-$/HPO_4^{2-}, pH 7.08–5.88; CH_3COOH/CH_3COO^-, 5.34–3.96; HCOOH/$HCOO^-$, 4.18–2.98; $CH_2ClCOOH$/CH_2ClCOO^-, 3.29–2.09. Dilute HCl was used in the pH range 1.5–2.0. The pH values were measured by means of a glass electrode at the same temperature and ionic strength as the rate measurements.

At preselected times the reacting mixtures were analysed by HPLC performed as previously described.[13] The rate constants k_{12} and k_{-23} (Scheme 1) were calculated by least-squares fits of the experimental data for the disappearance of the starting peptide, usually **1**, to the rate equation for two consecutive reversible first-order reactions given by Connors.[20] To reduce the number of the rate constants to be determined from four to two, the values of the equilibrium constants relating the concentrations of the peptides **1**, **2** and **3** were measured. These equilibrium constants were obtained from HPLC analyses of mixtures after the changes in peptide concentrations under the reaction conditions were complete. k_{-12} and k_{23} were obtained from the values of k_{12} and k_{-23} and the values of the equilibrium constants. The values of rate constants obtained from different runs carried out under the same experimental conditions were reproducible to within 5%. In several cases both the concentrations at equilibrium and the rate constants were also determined in samples obtained starting from **2** and **3**. The results obtained in these experiments were in good agreement with those obtained starting from **1**.

Results and Discussion

The isomerization reactions of aspartyl peptides were studied by incubating aqueous solutions of the Asp-peptides **1** and **3**, and of the Asu-peptide **2** (Scheme 1) at 37 °C and ionic strength 1 mol dm^{-3}, and monitoring the course of the reaction by HPLC. Example progress curves obtained from a sample of **1** followed at pH 4.2 are shown in Fig. 1. The progress curves show that the isomerization of Asp-peptides is a reversible reaction, and occurs *via* the succinimide derivative over the whole pH range explored, 1.5–10. Moreover, the rates of the two consecutive reversible reactions of the isomerization process show very similar dependences on pH and buffer concentration.

pH Dependence of the Reaction Rate.—Plots of the pseudo-first-order rate constants k_{12} and k_{-23} (defined in Scheme 1) of the cyclization reactions, and of the logarithms of the pseudo-first-order rate constants k_{-12} and k_{23} of the reverse, ring-opening reactions, *versus* the pH, are shown in Figs. 2 and 3, respectively.

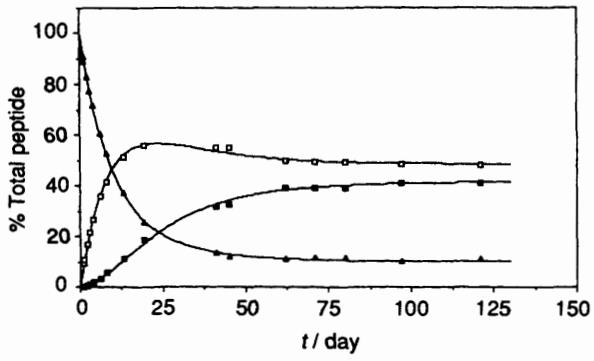


Fig. 1 Typical time profiles for the isomerization of Ac-Gly-Asp-Gly-Gly-NHMe: T = 37 °C, pH 4.2, 0.1 mol dm^{-3} formate buffer, μ = 1 mol dm^{-3} with KCl: Ac-Gly-Asp-Gly-Gly-NHMe (△); Ac-Gly-Asu-Gly-Gly-NHMe (□); Ac-Gly-β-Asp-Gly-Gly-NHMe (■)

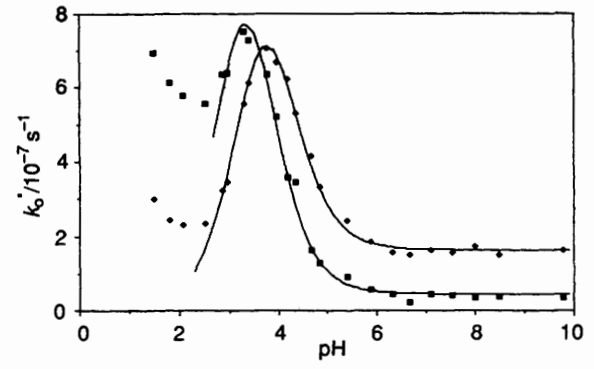


Fig. 2 pH–rate profiles for the cyclization of Ac-Gly-Asp-Gly-Gly-NHMe (◆) and Ac-Gly-β-Asp-Gly-Gly-NHMe (■) to Ac-Gly-Asu-Gly-Gly-NHMe at zero buffer concentration, T = 37 °C, μ = 1 mol dm^{-3}

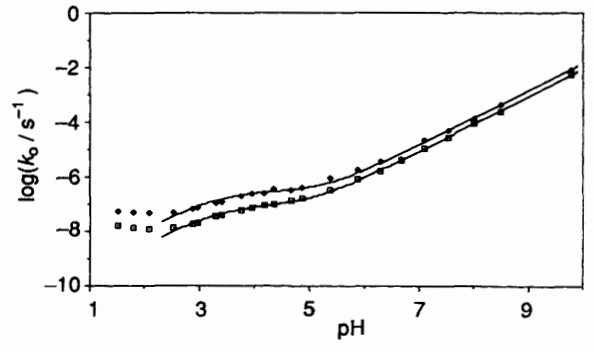


Fig. 3 pH–rate profile for the hydrolysis of Ac-Gly-Asu-Gly-Gly-NHMe to Ac-Gly-Asp-Gly-Gly-NHMe (□) and Ac-Gly-β-Asp-Gly-Gly-NHMe (◆) at zero buffer concentration, T = 37 °C, μ = 1 mol dm^{-3}

In the pH range 3–7 the reaction rate depends significantly on buffer concentration, and the values in the figures have been obtained by extrapolation to zero buffer concentration. The plots of k_{12} and k_{-23} show regions above pH 6–7 where both rate constants are independent of pH, and bell-shaped pH–rate profiles between pH 2–6. Below pH 2.5 the curves show a negative slope, suggesting efficient catalysis by hydronium-ion at low pH.

For the hydrolysis reactions at pH > 2.5, the rate constants k_{-12} and k_{23} increase with pH (Fig. 3), as already reported for succinimide derivatives.[9,14] At pH > 6 the plots of log (k_{-12}) and log (k_{23}) are linear with unit slopes (1.00 + 0.01 and 0.99 + 0.03, respectively), indicating reactions that are first order with respect to hydroxide-ion concentration. At pH < 6 the slopes are markedly lower.

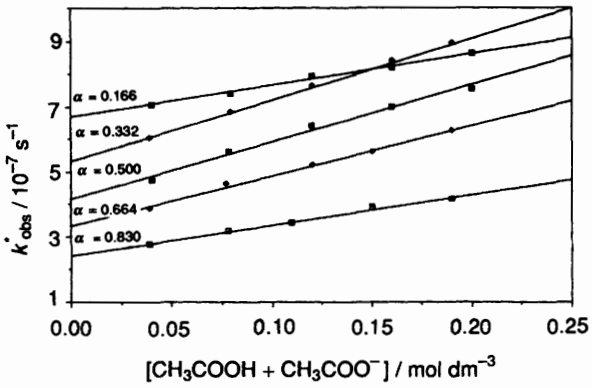


Fig. 4 Dependence of the pseudo-first-order rate constants for the cyclization of Ac-Gly-Asp-Gly-Gly-NHMe to Ac-Gly-Asu-Gly-Gly-NHMe on the total concentration of acetate buffer at the fraction (α) of free base indicated

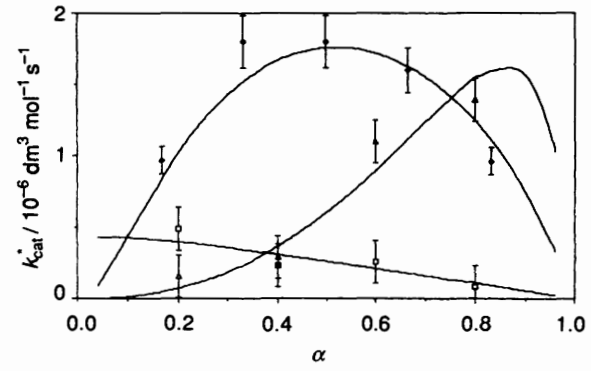


Fig. 5 Plot of the catalytic constants k_{cat} for the cyclization of Ac-Gly-Asp-Gly-Gly-NHMe to Ac-Gly-Asu-Gly-Gly-NHMe against the fraction (α) of free base in the buffers HCOOH/CHCOO$^-$ (△); CH_3COOH/CH_3COO^- (◆); $H_2PO_4^-/HPO_4^{2-}$ (□)

Buffer Dependence of the Reaction Rate.—In the pH range 3–7 the pseudo-first-order rate constant, k_{obs}, determined at constant pH by varying the total concentration of the buffer (up to 0.21 mol dm^{-3}), increases linearly with buffer concentration. However, a significant deviation from linearity was found in some experiments using acetate buffers at higher concentrations (up to 0.5 mol dm^{-3}). No significant buffer effect was detected at pH values below 3 or above 7. Fig. 4 depicts the influence of acetate buffer on the cyclization reaction of **1** in the pH range 4.0–5.4. The slopes of the lines, which measure the catalytic efficiency of the buffer (k_{cat}), show an unusual dependence on the fraction (α) of the buffer in the free base form: the plots of k_{cat} *vs.* α for the cyclization of **1** are shown in Fig. 5. In order to rationalize this dependence, the values of k_{cat} were divided at each pH by the fraction (f) of the peptide with the Asp side chain in the acid, COOH form. On the reasonable hypothesis that the buffers act only on the acidic form, this ratio, k_{cat}/f, should represent the effective catalytic rate constant for the buffer. Plots of k_{cat}/f *vs.* α are shown in Fig. 6. All the curves increase with α and pass through the origin. Thus when the reaction is expressed in terms of the acidic form of the Asp residue, the buffer catalysis involves only the basic form of the buffer; *i.e.*, the reaction proceeds by apparent general base catalysis. The non-linear relationships between k_{cat}/f and α for the buffers HCOOH/HCOO$^-$ and CH_3COOH/CH_3COO^-, together with the absence of buffer catalysis at low pH, suggest a change of the rate-determining step with pH, from a catalysed step at high pH to an uncatalysed one at low pH (below about 3).

Concentrations at Equilibrium.—The equilibrium concentrations of **1**, **2** and **3** change significantly with pH. At neutral

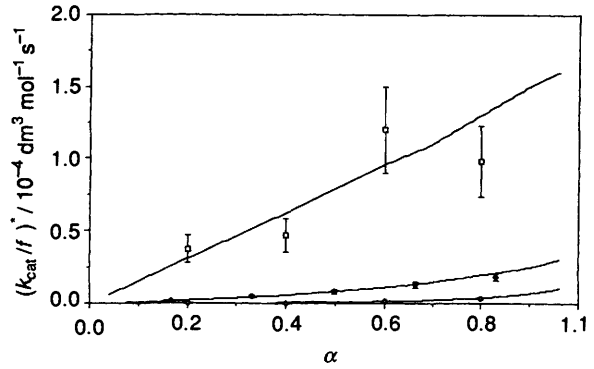


Fig. 6 Plot of k_{cat}/f for the cyclization of Ac-Gly-Asp-Gly-Gly-NHMe to Ac-Gly-Asu-Gly-Gly-NHMe against the fraction (α) of free base in the buffers $HCOOH/HCOO^-$ (△); CH_3COOH/CH_3COO^- (◆); $H_2PO_4^-/HPO_4^{2-}$ (□); k_{cat} and f are the catalytic constant and the fraction of the Asp-peptide in the acidic form, respectively

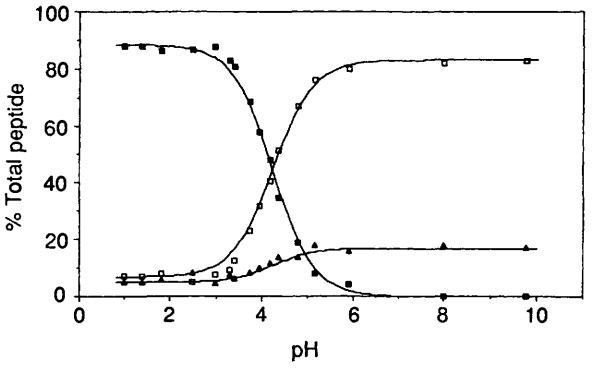
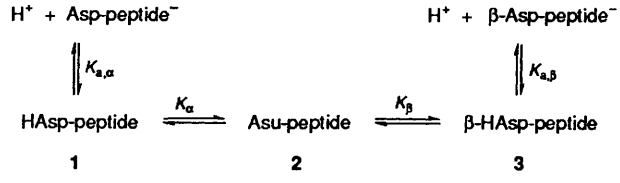


Fig. 7 Distribution diagram for the isomerization of aspartyl peptides as a function of pH: Ac-Gly-Asp-Gly-Gly-NHMe (▲); Ac-Gly-Asu-Gly-Gly-NHMe (■); Ac-Gly-β-Asp-Gly-Gly-NHMe (□)

and basic pH the concentration of the Asu-peptide **2** is very low, and the β-Asp-peptide is predominant. On the other hand, at acidic pH the Asu-peptide is the most abundant compound (Fig. 7). For the two aspartyl peptides the values reported in Fig. 7 refer to total concentrations (protonated plus deprotonated form), as determined by HPLC analysis. Least-squares fit of the experimental data to the equations describing the pH-dependence of the concentrations in the multi-equilibria reported in Scheme 2 [eqns. (1) and (2)], gave values of 18 ± 1



Scheme 2 HAsp-peptide and β-HAsp-peptide indicate the uncharged forms of the two aspartyl peptides, Asp-peptide$^-$ and β-Asp-peptide$^-$ are the anions

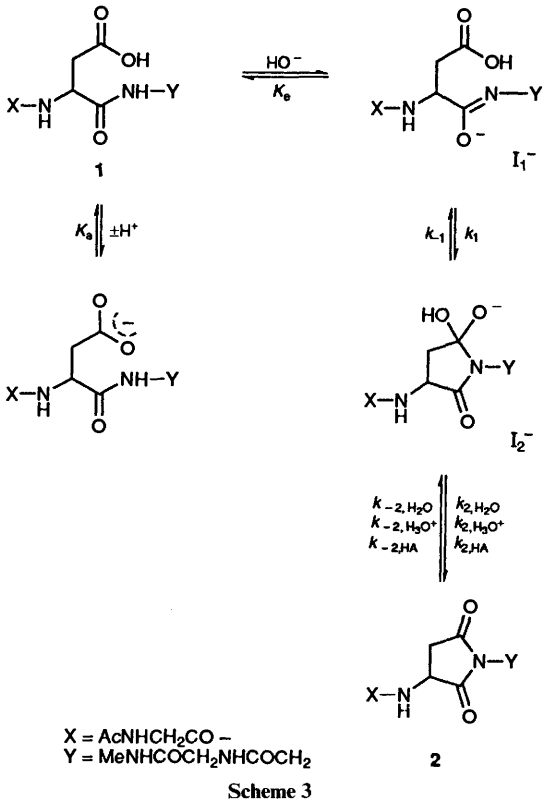
$$\% \text{ of Asp-peptide} = \frac{\{K_{a,\alpha}/K_\alpha[H^+] + 1/K_\alpha\}}{\{K_{a,\alpha}/K_\alpha[H^+] + 1/K_\alpha + 1 + K_{a,\beta}/K_\beta[H^+] + 1/K_\beta\}} \quad (1)$$

$$\% \text{ of } \beta\text{-Asp-peptide} = \frac{\{K_{a,\beta}/K_\beta[H^+] + 1/K_\beta\}}{\{K_{a,\beta}/K_\beta[H^+] + 1/K_\beta + 1 + K_{a,\alpha}/K_\alpha[H^+] + 1/K_\alpha\}} \quad (2)$$

and 13 ± 1 for the equilibrium constants K_α = [Asu-peptide]/[HAsp-peptide] and K_β = [Asu-peptide]/[β-HAsp-peptide], respectively, where [HAsp-peptide] and [β-HAsp-peptide] indicate specifically the concentrations of the neutral forms of the two aspartyl peptides. In the calculation of equilibrium apparent dissociation constants of $10^{-4.1}$ mol dm^{-3} and $10^{-3.4}$ mol dm^{-3} have been used for the carboxy groups of Asp-peptide ($K_{a,\alpha}$) and β-Asp-peptide ($K_{a,\alpha}$), respectively. These were evaluated from the half-neutralization point of the titration curves. The curves in Fig. 7 have been drawn using the constants obtained from the fit.

Mechanism of Succinimide Ring Formation.—The two reversible cyclization reactions (Scheme 1) probably proceed through identical mechanisms: they involve the same functional groups and their reaction rates show similar pH and buffer dependences. The unit slopes at pH > 6 of the plots *vs.* pH of $\log k_{-12}$, and $\log k_{23}$, (Fig. 3) show that in this pH region the two hydrolysis reactions are first order with respect to hydroxide ion concentration. The aspartyl peptides **1** and **3** have a single proton titrating in the pH range explored. The bell-shaped pH–rate profiles characterizing the two cyclization reactions (Fig. 2) therefore reflect multi-step processes, involving either intermediates with ionizable groups, or a change of the rate-determining step with pH (or a combination of both).[21] The data on the buffer dependence of reaction rate suggest a change of the rate-determining step with pH.

A pathway for the formation of **2** from **1**, which fits the experimental data over the whole pH range, is shown in Scheme 3. Of the forms of the aspartyl peptide in acid–base equilibrium,



Scheme 3

only the monoanion $\mathbf{I}_1^-$, derived from the neutral form by deprotonation of the NH peptide bond next to the Asp residue, reacts at an appreciable rate. Intramolecular nucleophilic attack by the deprotonated nitrogen atom on the carbon atom of the carboxy group of the Asp side chain gives the tetrahedral intermediate $\mathbf{I}_2^-$. Subsequent proton transfer to the leaving

Table 1 Apparent dissociation constants (K_a) of the Asp side chains of Ac-Gly-Asp-Gly-Gly-NHME (**1**) and Ac-Gly-β-Asp-Gly-Gly-NHMe (**3**), and microscopic rate constants for the reversible cyclization to the succinimide derivative: T = 37 °C, μ = 1.0 mol dm^{-3}; the rate constants are defined in Scheme 3

	Asn-peptide **1**	β-Asp-peptide **3**
K_a/dm^3 mol^{-1}	7.9 × 10^{-5}	4.0 × 10^{-4}
k_1/dm^3 mol^{-1} s^{-1}	(5.8 ± 0.2) × 10^6	(1.40 ± 0.06) × 10^7
$k_{2,H_2O}/k_{-1}$	0.029 ± 0.002	0.016 ± 0.003
$(k_{2,H_3O^+}/k_{-1})$/dm^3 mol^{-1}	(2.8 ± 0.2) × 10^3	(2.4 ± 0.2) × 10^3
k_{-2,H_2O}/s^{-1}	(9.0 ± 0.3) × 10^1	(1.5 ± 0.1) × 10^2
k_{-2,H_3O^+}/dm^3 mol^{-1} s^{-1}	(9.3 ± 0.3) × 10^6	(3.0 ± 0.2) × 10^7
k_2 (Formic acid)/dm^3 mol^{-1} s^{-1}	(8.1 ± 0.6) × 10^{-5}	(5.9 ± 0.5) × 10^{-5}
k_2 (Acetic acid)/dm^3 mol^{-1} s^{-1}	(2.0 ± 0.2) × 10^{-5}	(1.4 ± 0.2) × 10^{-5}
k_2 (Phosphoric acid)/dm^3 mol^{-1} s^{-1}	(1.4 ± 0.3) × 10^{-6}	(9.3 ± 0.7) × 10^{-7}

group from a general acid leads to the product. At pH above about 3 the conversion of I_2^- to the succinimide ring **2** (elimination of OH^-) is rate-determining, so when the rate is expressed in terms of the acid form of the Asp side chain, the reaction is specific base–general acid catalysed. This is kinetically equivalent[22] to the general base catalysis observed. At basic and neutral pH in the absence of buffer the reaction is mainly water-catalysed, because of the low concentration of hydrogen ion. Consequently, as in this pH region the concentration of I_1^- does not depend on pH, k_{obs} for the cyclization reaction is pH-independent at zero buffer concentration (and k_{obs} for the hydrolysis is first-order in hydroxide). At pH > 7 buffer catalysis is not measurable because of the low strength of the buffer acids, Tris·H^+ and HCO_3^-, used in this pH region.

As the pH decreases, the increasing concentration of the hydronium-ion makes the cyclization to I_2^- rate determining. As a consequence of this change, we find (*a*) a break in the plot of the pH dependence of the rate constant for the hydrolytic reaction; (*b*) a bell-shaped profile in the pH dependence of the cyclization, and (*c*) no detectable buffer catalysis below 3. Additional support for this mechanism is the saturation behaviour of the plots of k_{cat} *versus* the total concentration of buffer found in some experiments carried out using acetate buffer at higher concentration ($\leqslant$0.5 mol dm^{-3}, data not shown). Such a non-linear dependence is consistent with the catalysed step being rate controlling only at low buffer concentration.[23] Assuming the validity of the steady-state approximation for the concentration of I_2^-, the proposed mechanism leads to eqns. (3) and (4) for the observed first-order

$$k_{obs}(\text{cyclization}) = \frac{K_e}{([H^+] + K_a + K_e)} \times \frac{k_1 - k_1k_{-1}}{k_{-1} + k_{2,H_2O} + k_{2,H_3O^+}[H^+] + k_{2,HA}[HA]} \quad (3)$$

$$k_{obs}(\text{hydrolysis}) = \frac{k_{-1}(k_{-2,H_2O} + k_{-2,H_3O^+}[H^+] + k_{-2,HA}[HA])[OH^-]}{(k_{-1} + k_{2,H_2O} + k_{2,H_3O^+}[H^+] + k_{2,HA}[HA])} \quad (4)$$

rate constant for the cyclization and hydrolysis reactions, respectively.

The acid–base equilibrium constants and microscopic kinetic constants are defined in Scheme 2; [HA] is the concentration of the acidic form of the buffer. The curves in Figs. 2 and 3 were drawn using the values of the dissociation constants K_a (Table 1) determined by titration, a value of 10^{-16} mol dm^{-3} for the equilibrium constant K_e,[24] and values of kinetic constants obtained by least-squares fits of the experimental points to eqns. (3) and (4). The difference between the experimental and the calculated points at low pH indicates the appearance of acid catalysis at low pH, not considered in the kinetic equations. The curves in Figs. 5 and 6 show the dependence of k_{obs} on the buffer concentration calculated at different values of α using eqn. (3) and the data of Table 1. In these plots, as well as those of Figs. 2 and 3, the agreement between experimental and computed points is satisfactory. The large errors for phosphate buffer are due to the relatively low values of k_{cat}, and the consequently relatively large errors for this buffer.

Finally, we underline the substantial similarity between the mechanism of succinimide derivative formation from Asp-peptides proposed here and the mechanisms previously proposed for the related cyclizations to succinimide derivatives of Asn-peptides[13] and Asp-peptide esters.[25] It was suggested in these latter cases that the reactions also involve initial deprotonation of the NH group next to the Asn or Asp ester residue, followed by nucleophilic attack of the nitrogen atom on the carbonyl carbon of the side chain. In the reactions of Asn-peptides a change of rate determining step with pH produces a break in the pH rate profile. In the cyclization of the Asp-peptide the additional break due to the ionization of the side chain of the Asp residue causes the bell-shaped pH–rate profile.

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