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Thermochimica Acta 286 (1996) 41–50

thermochimica acta

Thermodynamic parameters of the reversible isomerization of aspartic residues via a succinimide derivative

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Received 2 October 1995; accepted 28 February 1996

Abstract

Values of the thermodynamic quantities ΔH° and ΔS° for the reactions "Asp-peptide ⇆ Asu-peptide ⇆ β-Asp-peptide" in aqueous solution have been obtained for the model peptides Ac–Gly–X–Gly–Gly–NHMe and Ac–X–Gly–NHMe (X = Asp, β-Asp, Asu; Asu = aminosuccinyl residue) from the temperature-dependence of equilibrium constants. The ΔH° and ΔS° values for the cyclization reactions of the carboxylic-acid form of the Asp and β-Asp side chains of the dipeptides and tetrapeptides are positive and coincident within experimental error. Medium values are 34 kJ mol^{-1} and 127 J K^{-1} mol^{-1} for ΔH° and ΔS°, respectively.

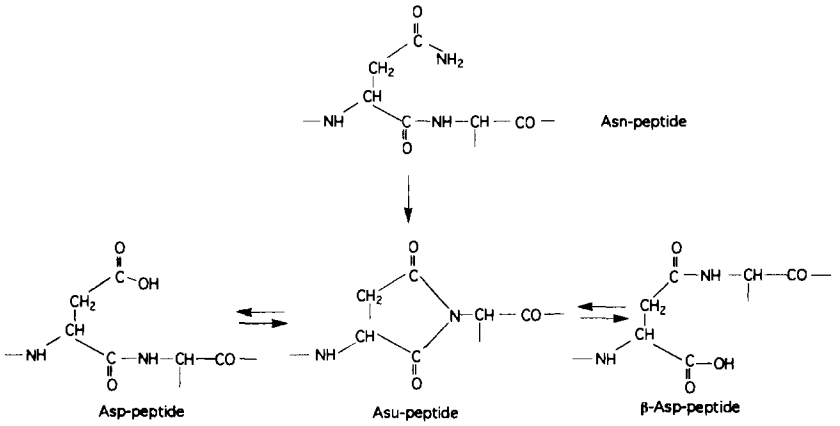
The molar enthalpies and molar entropies of the Asp-dipeptide and β-Asp-dipeptide, and of the Asp-tetrapeptide and β-Asp-tetrapeptide, did not exhibit significant differences.

Keywords: Aspartic residue; β-Aspartic residue; Isomerization; Succinimide ring

1. Introduction

The reversible isomerization of aspartic residues and the deamidation of asparagine to aspartic residues have proven to be among the most common chemical modifications resulting in covalent damage to polypeptide chains [1–3]. It is well known that these reactions occur via an aminosuccinyl residue (Asu) arising from intramolecular nucleophilic attack on the β-carbonyl group of Asp or Asn residues by the amido NH group of the next residue, with the elimination of water or ammonia, respectively [4–7]. In aqueous solution the succinimide ring is labile and its hydrolysis may occur on either side of the imide nitrogen, yielding a mixture of α- and β-linked aspartyl peptides (also referred to as isoAsp-peptides) [4, 5, 8, 9]. These reactions are enhanced by the absence

PII: S0040-6031(96)02937-1



Scheme 1. Deamidation of side chain of Asn residues and isomerization of Asp residues via succinimide derivative.

of bulky side chain or the residue next to Asn or Asp [10, 11]. Scheme 1 shows the deamidation and isomerization via the succinimide derivative. It has been shown that the observed first-order rate constant for the formation of the β-Asp-peptides from the Asu-peptides usually predominates at a ratio of about 3:1 [4, 5, 12]. Moreover at equilibrium at neutral and basic pH, the concentration of the Asu-peptide is always very low, whereas at moderately acidic pH it is by far the most abundant product [13]. Recently, it has been shown that the cyclization reactions from Asp or Asn residues proceed through deprotonation of the amido bond next to the Asn or Asp residue by fast acid–base equilibrium, followed by nucleophilic attack of the nitrogen atom on the carbonyl carbon of the Asn or Asp side-chain, giving a cyclic tetrahedral intermediate [14, 15]. Of course sequences like Asn–Sar and Asn–Pro, which do not have a hydrogen atom on the peptide bond, cannot react according to the above pathway. For peptides containing these sequences an alternative degradation pathway has been proposed, which also proceeds via a succinimide derivative [4, 11, 16]. This involves deprotonation of the amido group of the Asn side-chain followed by nucleophilic attack of the nitrogen atom on the α-carbonyl carbon atom of the Asn residue, resulting in the cleavage of the peptide chain and formation of an *N*-terminal succinimide fragment. Deamidation of the Asn side chain by direct interaction with water molecules, which does not involve intramolecular cyclization and subsequent hydrolysis, has been evidenced only in experiments carried out at low pH [6, 11, 12].

The cyclization reaction to a succinimide moiety is accompanied by the release of a mole of water or ammonia, thereby involving a substantial gain in translational entropy [17, 18]. Moreover, because of the atoms immobilized in the five-membered ring, the process probably also involves a loss of internal rotational entropy, and an increase of strain energy. For the succinimide ring, a strain energy of 35.6 kJ mol^{-1} is reported in the literature [19].

As a part of a current research program on the chemical stability of the Asn and Asp side-chain in peptides and proteins, this paper reports a thermodynamic study of the

reversible reactions between the Asp-, β-Asp- and the Asu-peptides in aqueous solution. Since the succinimide ring is very prone to racemization due to its electronic structure [20], this study was carried out on the *N*- and *C*-terminal-blocked peptides Ac–Gly–X–Gly–Gly–NHMe and Ac–X–Gly–NHMe (X = Asp, β-Asp or Asp or Asu; Asu = aminosuccinyl residue). These peptides have only one chiral centre and therefore their racemization does not produce diastereoisomers.

2. Experimental

2.1. Synthesis

The peptides were synthesized by conventional solution phase procedures [21], purified by semi-preparative high performance liquid chromatography (HPLC), checked for homogeneity by analitical HPLC and identified by ^{1}H NMR spectrometry, FAB (fast atom bombardment) mass spectrometry and, in the case of Asu-peptide, by second-derivative ultraviolet spectrometry [22, 23].

Preparative and analytical HPLC were carried out on a Beckman Model System Gold using a C_{18} column (10 × 250 mm, particle size 10 μm) and a C_8 column (4.6 × 250 mm, particle size 5 μm), respectively. The peptides were eluted with 0.1% (*w*/*v*) trifluoroacetic acid in water at a flow rate of 0.7 (analytical HPLC) and 3.5 (preparative HPLC) $cm^3\ min^{-1}$ and detected by absorption at 214 nm. ^{1}H NMR spectra were recorded on a Bruker WH-270 (270 MHz) spectrometer operating in the FT mode. Chemical shifts were related to tetramethylsilane. FAB mass spectra were recorded on a VG ZAB 2 SE double-focusing mass spectrometer equipped with a caesium gun operating at 1 mA (20 kV). The samples were dissolved in 5% acetic acid and loaded onto a glycerol–thioglycerol-coated probe tip. The second-derivative ultraviolet spectra were recorded on a Perkin–Elmer model 320 spectrophotometer in CH_3CN at a concentration of 0.01 mol dm^{-3}: band width 1 nm, $\Delta\lambda = 2$ nm.

2.2. Ac–Asp–Gly–NHMe (1)

Glycine-*N*-methylamide was coupled with *N*-Boc-L-aspartic acid-β-benzyl ester by the dicyclohexylcarbodiimide–1-hydroxybenzothiazole method in dimethylformamide. After 2 h at room temperature and 3 h at 4°C the precipitate was removed by filtration. The filtrate was evaporated in vacuo and the residue, dissolved in chloroform, was washed with water, aqueous HCl, 1 mol dm^{-3}, aqueous $NaHCO_3$, 1 mol dm^{-3}, and water, and then dried over $MgSO_4$. Pure Boc–Asp(OBzl)–Gly–NHMe was obtained by precipitation with petroleum ether. After Boc deprotonation by treatment with trifluoroacetic acid–CH_2Cl_2 for 1 h, the solution was evaporated in vacuo. The free *N*-terminal amino group of the dipeptide was acetylated with acetic anhydride (ninhydrin test negative). The solution was evaporated in vacuo and the residue purified by semi-preparative HPLC. $MH^+ = 246$; ^{1}H NMR (DMSO): d 1.70 (s, 3H, CH_3, Ac); 2.45 (d, 3H, CH_3, NHMe), 2.48 (m, 2H, $C^\beta H_2$Asp); 3.50 (m, 2H, $C^\alpha H_2$Gly); 4.35 (m, 1H, C^αHAsp); 7.41 (q, 1H, NH, NHMe); 8.02 (t, 1H, NH Gly), 8.11 (d, 1H, NH Asp).

2.3. *Ac-β-Asp-Gly-NHMe* (2)

The peptide was prepared as described for **1** except that *N*-Boc-L-aspartic acid-α-benzyl ester was used instead of *N*-Boc-L-aspartic acid-β-benzyl ester. $MH^+ = 246$; ^1H NMR (DMSO): d 1.66 (s, 3H, CH_3, Ac); 2.47 (d, 3H, CH_3, NHMe); 2.43 (m, 2H, $C^\beta H_2$, Asp); 3.50 (m, 2H, $C^\alpha H_2$, Gly); 4.36 (m, 1H, $C^\alpha H$, Asp); 7.50 (q, 1H, NH, NHMe); 7.98 (d, 1H, NH, Asp); 8.02 (t, 1H, NH, Gly).

2.4. *Ac-Asu-Gly-NHMe* (3)

An equimolar amount of triethylamine was added to Boc–Asp(OBzl)–Gly–NHMe (0.1 mol dm^{-3}) in dry dimethylformamide and the solution kept at room temperature for 2 days. The solution was evaporated in vacuo and the residue purified by semi-preparative HPLC: $MH^+ = 228$; ^1H NMR (DMSO): d 1.68 (s, 3H, CH_3, Ac); 2.48 (d, 3H, CH_3, NHMe); 2.68 (m, 1H, $C^\beta H_2$, Asu); 3.41 (m, 1H, $C^\beta H_2$, Asu); 3.49 (m, 2H, $C^\alpha H_2$, Gly); 4.56 (m, 1H, $C^\alpha H$, Asu); 7.40 (q, 1H, NH, NHMe), 7.80 (d, 1H, NH, Asu); second-derivative UV spectra: two minima at 246 and 254 nm, and two shoulders at 258 and 269 nm [23].

The peptides Ac–Gly–Asp–Gly–Gly–NHMe (**4**), Ac–Gly–β–Asp–Gly–Gly–NH Me (**5**), and Ac–Gly–Asu–Gly–Gly–NHMe (**6**) were synthesized and characterized as previously reported [15].

2.5. *Equilibrium concentration measurements*

Aqueous solutions of the Asp-peptides **1** and **4** (5×10^{-3} mol dm^{-3}) at the desired pH were filtered through a 0.45 μm membrane filter and then stored in thermostatted baths at 20.0, 28.0, 37.0, 48.0 and 60.0°C. A constant ionic strength of 1 mol dm^{-3} was maintained in each sample by adding an appropriate amount of a concentrated solution of KCl. The following buffers were used at a concentration of 0.1 mol dm^{-3}, $HCOOH–HCOO^-$, pH 3.00 and 4.00; $CH_3COOH–CH_3COO^-$, pH 3.96–5.34; $H_2PO_4^-–HPO_4^{2-}$, pH 6.00 and 7.00; Tris·H–Tris pH 8.00. The pH values were measured with a glass electrode at the same temperature and ionic strength as the rate measurements. The equilibrium concentration of the succinimide derivative and of the two aspartyl peptides were determined by analysing the reacting mixtures by HPLC until the concentrations became constant. The equilibrium concentrations obtained from different samples having the same pH values were reproducible to within 5%. In several cases the concentration at equilibrium were also determined in samples obtained starting from the β-Asp-peptides **2** and **5**, and from the Asu-peptides **3** and **6**. The results obtained in these experiments were in good agreement with those obtained starting from the Asp-peptides **1** and **4**.

2.6. *pK_a measurements*

The pK_a values of the dissociation of the carboxylic acid group of the Asp side-chain of **1**, **2**, **4** and **5** were determined by titration with 0.01 mol dm^{-3} KOH under conditions

Table 1
Negative logarithm (pK_a) of the apparent dissociation constants for the aspartyl peptides at several temperatures, ionic strength 1 mol dm^{-3}

Peptide	Temperatures/°C				
	20	28	37	48	60
Ac–Asp–Gly–NHMe	4.02	4.01	3.98	3.99	3.97
Ac–Asp–β-Gly–NHMe	3.37	3.33	3.33	3.31	3.28
Ac–Gly–Asp–Gly–Gly–NHMe	4.06	4.04	4.05	4.03	4.00
Ac–Gly–β-Asp–Gly–Gly–NHMe	3.36	3.36	3.33	3.31	3.30

of equilibrium measurements ($T = 20.0$, 28.0, 37.0, 48.0 and 60.0°C, μ(ionic strength) = 1 mol dm^{-3}). The method of the half-neutralization point gave the values reported in Table 1.

3. Results and discussion

The reversible isomerization reactions of aspartyl peptides were studied by incubating aqueous solutions of the model peptides Ac–Gly–X–Gly–Gly–NHMe or Ac–X–Gly–NHMe (X = Asp, β-Asp, Asu; Asu = aminosuccinyl residue) at several pH values and temperatures, and determining the equilibrium concentrations of the Asp-, Asu- and β-Asp-peptide in each sample by HPLC. As an example, the chromatogram obtained from a sample of Ac–Asp–Gly–NHMe stored at 37°C and pH 5.0 is shown in Fig. 1.

3.1. Temperature- and pH-dependence of the equilibrium concentrations

The equilibrium concentrations of both the dipeptides and tetrapeptides changed markedly with pH and temperature. The behavior of the dipeptides and tetrapeptides was substantially similar. In the range of temperature explored, at neutral and basic pH the concentration of the Asu-peptides was very low, and the β-Asp-peptides were predominant. Conversely, at acidic pH the Asu-peptides were the most abundant compounds. Moreover, an increase of the temperature caused the reactions to shift toward the formation of the Asu-peptides. Fig. 2 shows the distribution curves for the dipeptides at two temperatures. For the two aspartyl peptides the values reported in Fig. 2 refer to total concentrations (carboxylic-acid and carboxylate anion form), as determined by HPLC analysis. The equilibrium constants K_α and K_β between the Asu-peptide and the carboxylic acid forms of the two peptides, K_α = [Asu-peptide]/[H-Asp-peptide], K_β = [Asu-peptide]/[H-β-Asp-peptide], were obtained by least-squares fitting of the experimental data to the equation describing the pH-dependence of the concentrations in the multi-

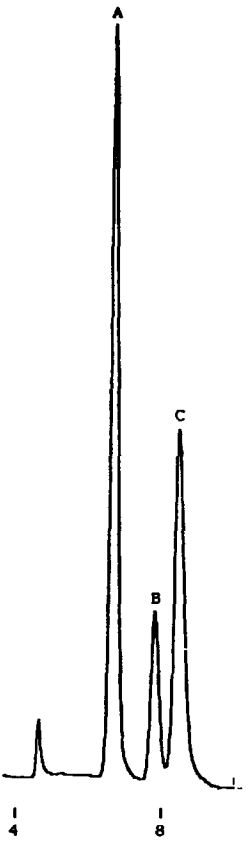


Fig. 1. Analytical HPLC traces of a sample at the equilibrium obtained by incubating Ac–Asp–Gly–NHMe at 20.0°C and pH 5.0 Ac–β-Asp–Gly–NHMe (A), Ac–Asp–Gly–NHMe (B) and Ac–Asu–Gly–NHMe (C); time scale: min.

equilibria reported in Scheme 2 [Eqs. (1) and (2)].

$$\%\ \text{of Asp-peptide} = \frac{100 \times \{K_{a,\alpha}/K_\alpha[H^+] + 1/K_\alpha\}}{\{K_{a,\alpha}/K_\alpha[H^+] + 1/K_\alpha + 1 + K_{a,\beta}/K_\beta[H^+] + 1/K_\beta\}} \quad (1)$$

$$\%\ \text{of } \beta\text{-Asp-peptide} = \frac{100 \times \{K_{a,\beta}/K_\beta[H^+] + 1/K_\beta\}}{\{K_{a,\beta}/K_\beta[H^+] + 1/K_\beta\} + 1/K_\beta + K_{a,\alpha}/K_\beta[H^+] + 1/K_\alpha\} \quad (2)$$

In the calculation of these equilibrium constants the apparent dissociation constants of the carboxylic group of the aspartyl peptides reported in Table 1 have been used. These values were evaluated from the half-neutralization point of the titration curves recorded under the conditions of equilibrium measurements. At each temperature the corresponding pK_a values of the dipeptides and tetrapeptides are substantially similar.

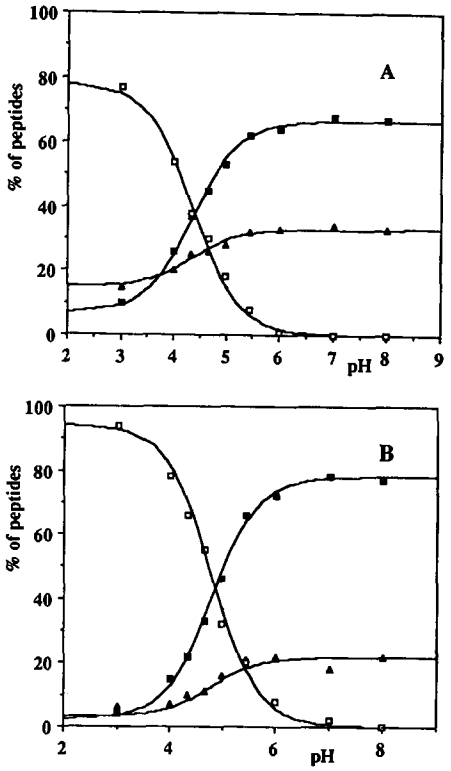
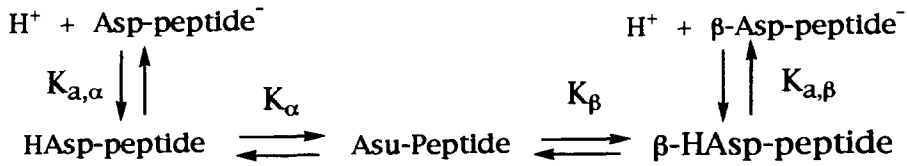


Fig. 2. Distribution diagram for the isomerization reaction of aspartyle peptides as a function of pH at 20°C (A) and 60°C (B). Ac–Asp–Gly–NHMe (Δ), Ac–Asu–Gly–NHMe (□), Ac–β-Asp–Gly–NHMe (■).



Scheme 2. HAsp-peptide and β-HAsp-peptide indicate the carboxylic-acid forms of the aspartyl peptides, Asp-peptide$^-$ and β-Asp-peptide$^-$ are the carboxylate anions.

However, as expected, there is a significant, though very small increase of the acidity constants with the temperature. The equilibrium constants between the carboxylic acid form of the peptides and the Asu-peptides (K_α and K_β) increased with the temperature and, within the temperature range explored, the plots ln K versus $1/T$ are linear with negative slopes (Fig. 3). The ΔH° and ΔS° values determined from the slopes and the intercepts of these straight lines are reported in Table 2.

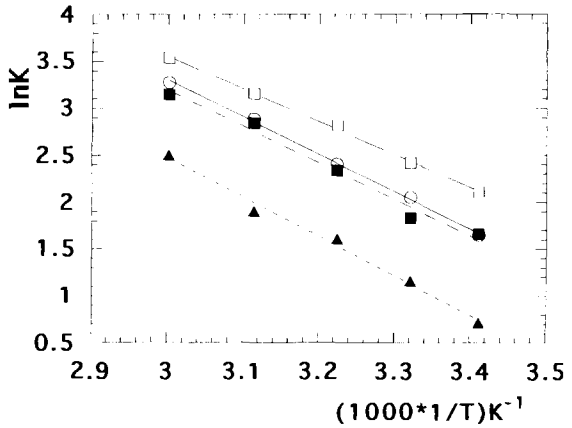


Fig. 3. Equilibrium constants for the formation of succinimide ring in water from Ac–Asp–Gly–NHMe (○), Ac–β-Asp–Gly–NHMe (□), Ac–Gly–Asp–Gly–Gly–NHMe (■) and Ac–Gly–β-Asp–Gly–Gly–NHMe (▲) plotted against $1/T$, $\mu = 1$ mol dm^{-3}.

Table 2
Thermodynamic parameters for the succinimide ring formation in water, $\mu = 1$ mol dm^{-3}. Standard states are: 1 mol dm^{-3}, for the peptides; pure component, for water

Starting peptide	$\Delta H°$/kJ mol^{-1}	$\Delta S°$/J K^{-1} mol^{-1}
Ac–Asp–Gly–NHMe	34.3	131
Ac–Asp–β-Gly–NHMe	30.1	121
Ac–Gly–Asp–Gly–Gly–NHMe	33.5	128
Ac–Gly–Asp–β-Gly–Gly–NHMe	36.2	130

Estimated error less than 10%.

Interestingly, for all the cyclization reactions examined, both the enthalpic and entropic changes are positive and coincident within experimental error.

4. Conclusion

The experimental results show that the cyclization reaction of Asp side chains to succinimide derivatives occurs with an increase in enthalpy and entropy. Thus, the succinimide ring formation is an entropy-driven reaction. The mean values are approximately 34 kJ mol^{-1} and 127 J K^{-1} mol^{-1} for $\Delta H°$ and $\Delta S°$, respectively. The gain of translational and rotational entropy, resulting from the yield of a new molecule of water and probably also from the release of water molecules incorporated into the

inner hydration shell of the reactant [24], exceeds the loss of internal rotational entropy accompanying the formation of the five-membered ring.

With reference to the relative stabilities of the Asp and β-Asp residues, the data show that the reaction "HAsp-peptide⇆β-HAsp-peptide" involves no significant change in enthalpy nor in entropy for both the dipeptides and tetrapeptides studied, indicating that the Asp and β-Asp residues have similar energy and flexibility. The higher concentration at basic and neutral pH of the β-Asp-peptides is due principally to the higher acidity of the side chain of β-Asp residue.

Although conclusions from studies of small peptides cannot be directly extrapolated to polypeptide chains, the notion of a possible intrinsic equal stability of the two isomeric aspartic residues should be taken into account when comparing homologous proteins, differing for having the normal Asp residue or the β-Asp residue [25].

Acknowledgements

I am grateful to Professor L. Mazzarella for helpful comments, and Professor P. Pucci and the staff of "Servizio di Spettrometria di Massa, Università-CNR, Napoli" for FAB mass spectra. This work was supported in part by the Italian MURST.

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