Metal(II)-Ion Promoted Hydrolysis of Glycylglycine

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The hydrolysis of glycylglycine was studied in the presence of Cu(II) and Zn(II) at various pH values between 3.4 and 6.3. Glycine produced by the hydrolysis and remaining glycylglycine were trimethylsilylated and their relative quantities were determined by gas-liquid chromatography. The reaction is promoted by both metal ions. In the presence of Cu(II) the pH-rate profile has a maximum at pH *ca.* 4.4, whereas in the presence of Zn(II) the rate increases as pH becomes higher. These kinetic results are discussed in terms of the structures of metal(II)-glycylglycine complexes in aqueous solution.

Some metal ions are known to have catalytic activities in the hydrolysis of peptide bond.[1,2] Grant and Hay[2] have studied the hydrolysis of glycylglycine in the presence of Cu(II) and have found that the reaction rate is clearly dependent upon pH, showing a maximum at pH 4.2. The purpose of the present study is to examine more quantitatively the pH dependence of catalytic activities of Cu(II) and Zn(II) in the hydrolysis of glycylglycine and to discuss the relationship between the reactivity and structure of the metal(II)-glycylglycine complex. The structures of Cu(II) and Zn(II) complexes of glycylglycine in aqueous solution have been reported in a previous paper.[3]

Experimental

Materials. Glycylglycinatocopper(II) chloride monohydrate [Cu(HGG)Cl·H_2O][4] and glycylglycinatozinc(II) chloride hemihydrate [Zn(HGG)Cl·0.5H_2O] were prepared from glycylglycine and corresponding metal(II) chloride according to the method of Bair and Larsen.[5]

Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was synthesized by adding trimethylsilyl chloride to a mixture of trifluoroacetamide and trimethylamine. The product was purified by distillation.[6] Bp 52.5 °C (30 mmHg).

Reaction. The hydrolysis of glycylglycine was studied for 0.01 and 0.1 M solutions of Cu(HGG)Cl·H_2O and Zn(HGG)Cl·0.5H_2O. The samples were dissolved in sodium acetate–acetic acid buffer solution. The total concentrations of acetate ion plus acetic acid were 0.2 and 1 M, respectively, for the sample concentrations of 0.01 and 0.1 M. Sealed reaction tubes containing 5 ml solutions were kept in an oil bath at 85 °C for 100 hr. After the reaction each solution was diluted with several ml of water, treated with hydrogen sulfide, and then freed of hydrogen sulfide with a stream of nitrogen. The precipitate (CuS or ZnS) was removed after centrifugation (2000 g, 10 min).

Thin-layer Chromatography. The solution thus obtained was analysed qualitatively by thin-layer chromatography, using phenol–water–ammonium hydroxide (3:1:trace) mixture as solvent and a 20×20 cm TLC plate covered with microcrystalline cellulose. Glycine produced by the hydrolysis and glycylglycine were both identified by the coloration with ninhydrin, R_f values being 0.37 and 0.57, respectively.

Trimethylsilylation. Trimethylsilylation of glycine and glycylglycine was carried out in order to obtain volatile derivatives which could be analysed by gas–liquid chromatography. By adjusting the pH value of the solution (from which metal sulfide was already separated) to about 2.5 with 1 M hydrochloric acid, sodium acetate was converted to acetic acid, which was then removed by freeze-drying. After dissolving the remaining solid substance in a small amount of water (1 ml), an aliquot (100—200 μl) was put into a vial for trimethylsilylation (purchased from Pierce Co., Ltd. in Rockford, Illinois, U.S.A.). The solution in the vial was frozen and evaporated completely to dryness in a vacuum desiccator over phosphorus pentoxide. To this dry sample were added 150 μl of dry acetonitrile and 150 μl of BSTFA. The vial was sealed with a silicon-lined cap and heated for 15 min at 125—130 °C. Under such a condition both glycine and glycylglycine were trimethylsilylated to a satisfactory degree. However, two kinds of trimethylsilylated derivatives were obtained as described later.

Gas-liquid Chromatography. Gas-liquid chromatographic analysis was carried out with a Shimadzu gas chromatograph GC-4BM equipped with a flame ionization detector. Glass columns of 2.0 m×4 mm i.d. containing 5.0% w/w SE-30 coated on 80/100 mesh AW-DMCS Chromosorb W were used. The flow rate of carrier gas (nitrogen) was 60 ml/min and the temperature was elevated from 100 °C to 220 °C at a programmed rate of 5 °C/min.

Mass Spectrometry. The characterization of trimethylsilylated derivatives was carried out by mass spectrometry. A Hitachi RM 50 (combined GLC-MS instrument) was used with the temperature of ion source and manifold 220 °C, accelerating voltage 3 kV, and ionizing energy 70 eV.

Results

Analysis of Trimethylsilylated Glycine and Glycylglycine by Gas-liquid Chromatography. By the reaction with BSTFA both glycine and glycylglycine gave two kinds of trimethylsilylated (TMS) derivatives as shown below.

(1) $H_2NCH_2COOH + CF_3CON[Si(CH_3)_3]_2 \longrightarrow$ TMS–$NHCH_2COO$–TMS (Di-TMS-glycine, TG-1) + $(TMS)_2NCH_2COO$–TMS (Tri-TMS-glycine, TG-2)

(2) $H_2NCH_2CONHCH_2COOH + CF_3CON[Si(CH_3)_3]_2 \longrightarrow$ TMS–$NHCH_2CONHCH_2COO$–TMS (Di-TMS-glycylglycine, TGG-1) + $(TMS)_2NCH_2CONHCH_2$–COO–TMS (Tri-TMS-glycylglycine, TGG-2)

This was proved by GLC-MS analysis. The results of our GLC-MS analysis for trimethylsilylated glycine were in agreement with those reported by Bergström *et al.*[7] Both di-TMS- and tri-TMS-glycylglycine have fragmentation patterns very similar to di-TMS- and tri-TMS-glycine, respectively. Tri-TMS-glycine(M=348) gave the following fragment ions: *m/e* 333, $[(M-CH_3)^+]$; *m/e* 174, $[(TMS)_2N^+{=}CH_2]$; *m/e* 147, $[TMS{-}O^+{=}Si(CH_3)_2]$; *m/e* 102, $[TMS{-}N^+H{=}CH_2]$; *m/e* 86, $[(CH_3)_2Si{=}N^+{=}CH_2]$; *m/e* 73, $[TMS^+]$; *m/e* 59,

$[HSi^+(CH_3)_2]$; *m/e* 45, $[H_2Si^+CH_3]$.

It was found that trimethylsilylation of glycine at 100 °C for 15 min gave TG-1 alone, whereas the addition of trimethylsilyl chloride besides BSTFA yielded only TG-2 under the same reaction condition. Using fluorene as internal standard, the relative molar sensitivity of TG-1 and TG-2 for the flame ionization detector used in this experiment was determined to be 1.00: 1.39. In a similar manner the relative molar sensitivity of TGG-1 and TGG-2 was determined to be 1.00: 1.40. Various mixtures of glycine and glycylglycine were trimethylsilylated at 125 °C for 15 min, and the ratios of peak areas of TG-1 and TGG-1 were plotted against the molar ratio of glycine and glycylglycine. A linear relationship was obtained and from the slope the relative molar sensitivity of TG-1 and TGG-1 was determined to be 1.87: 1.00. Consequently, the relative molar sensitivity of TG-1, TG-2, TGG-1, and TGG-2 was 1.87: 2.60: 1.00: 1.40.

Hydrolysis of Glycylglycine. The solutions (0.01 and 0.1 M) of glycylglycine, $Cu(HGG)Cl\cdot H_2O$, and $Zn(HGG)Cl\cdot 0.5H_2O$ having various pH values were kept at 85 °C for 100 hr. The percentage of hydrolysis (ρ) was calculated by the following equations and the results are given in Tables 1 and 2 and Fig. 1.

$$\rho(\%) = \left\{1-\frac{[\text{Glygly}]_{100}}{[\text{Glygly}]_0}\right\}\times 100 = \left\{1-\frac{[\text{Glygly}]_{100}}{[\text{Glygly}]_{100}+(1/2)[\text{Gly}]_{100}}\right\}\times 100 = \left\{\frac{1}{2([\text{Glygly}]_{100}/[\text{Gly}]_{100})+1}\right\}\times 100 \quad (1)$$

where [Glygly] and [Gly] denote the concentrations of glycylglycine and glycine, respectively, and suffix indicates the reaction time (hr). The ratio $[\text{Glygly}]_{100}/[\text{Gly}]_{100}$ can be obtained from the gas-liquid chromatographic analysis as follows.

$$\frac{[\text{Glygly}]_{100}}{[\text{Gly}]_{100}} = \frac{A_{\text{TGG-1}}+(1/1.40)A_{\text{TGG-2}}}{(1/1.87)A_{\text{TG-1}}+(1/2.60)A_{\text{TG-2}}} \quad (2)$$

where $A_{\text{TG-1}}$, $A_{\text{TG-2}}$, $A_{\text{TGG-1}}$, and $A_{\text{TGG-2}}$ denote the peak areas of TG-1, TG-2, TGG-1, and TGG-2, respectively.

Table 1. Hydrolysis of glycylglycine in aqueous solutions (0.01 M) of glycylglycine, glycylglycinatocopper(II) chloride monohydrate, and glycylglycinatozinc (II) chloride hemihydrate.[a]

pH	Percent hydrolysis (ρ)[b]		
	Glygly	Cu(HGG)Cl·H_2O	Zn(HGG)Cl·0.5 H_2O
3.52	1.6	—	1.9
3.83	—	28.7	—
4.00	0.4	30.9	1.6
4.27	—	40.1	—
4.48	0.6	42.0	4.3
5.00	0.0	17.3	7.8
5.51	0.7	8.3	20.0
6.01	0.6	5.0	57.7

a) In 0.2 M CH_3COONa–CH_3COOH buffer solutions, heated at 85 °C for 100 hr. b) Defined in Eq. (1).

Table 2. Hydrolysis of glycylglycine in aqueous solutions (0.1 M) of glycylglycinatocopper (II) chloride monohydrate and glycylglycinatozinc(II) chloride hemihydrate[a]

pH	Percent hydrolysis (ρ)[b]	
	Cu(HGG)Cl·H_2O	Zn(HGG)Cl·0.5 H_2O
3.41	72.7	5.3
3.74	77.3	—
3.96	78.3	5.9
4.25	87.3	—
4.47	85.9	9.3
5.00	69.5	15.3
5.51	44.1	40.0
6.01	29.0	59.5
6.32	—	70.0

a) In 1M CH_3COONa–CH_3COOH buffer solutions, heated at 85 °C for 100 hr. b) Defined in Eq. (1).

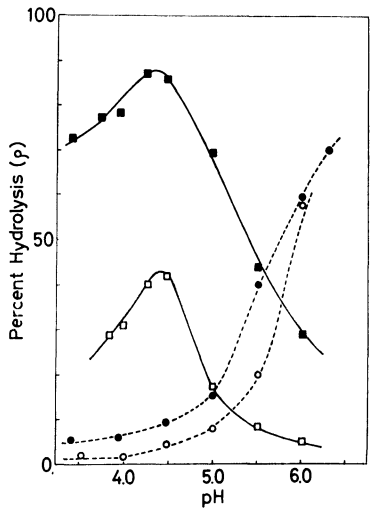

Fig. 1. The pH-rate profiles for the hydrolysis of glycylglycine in the presence of Cu(II) and Zn(II). (□), $Cu(HGG)Cl\cdot H_2O$ (0.01 M); (■), $Cu(HGG)Cl\cdot H_2O$ (0.1 M); (○), $Zn(HGG)Cl\cdot 0.5H_2O$ (0.01 M); (●), $Zn(HGG)Cl\cdot 0.5H_2O$ (0.1 M).

Discussion

The kinetic results obtained above may be summarized as follows.

(1) In the absence of the metal ion, glycylglycine is hardly hydrolysed under the present experimental conditions (Table 1).

(2) Both the Cu(II) and Zn(II) ions promote the hydrolysis of glycylglycine (Tables 1 and 2).

(3) In the presence of Cu(II) the pH-rate profile is a "bell-shape" having maximum at pH *ca.* 4.4. In contrast to this, the rate increases as pH becomes higher in the presence of Zn(II) (Fig. 1). The pH-rate profile in this case may be called "half bell-shape." (The pH range beyond 6.3 was not examined due to the lack of suitable buffer solution.)

(4) The rate of hydrolysis is greater for the solution having higher initial concentration (0.1 M *vs.* 0.01 M).

The bell-shaped pH-rate profile for the Cu(II)-glycylglycine system is in good agreement with the results reported previously by Grant and Hay[2] using an analytical method (ninhydrin technique) quite different from ours.

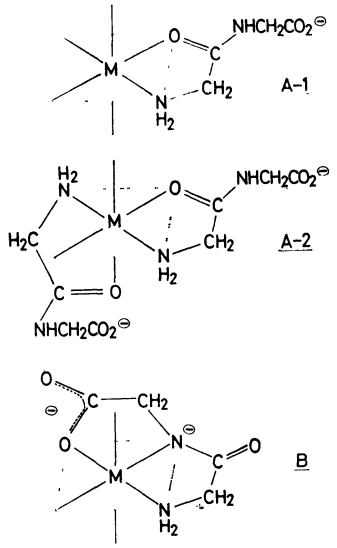

Fig. 2. Structures of metal(II)-glycylglycine complex.

An interesting aspect of the results obtained above is the correlation between the rate of hydrolysis and the structure of metal(II)-glycylglycine complex in aqueous solution. Previously we have studied the infrared spectra of D_2O solutions of the Cu(II) and Zn(II) complexes of glycylglycine and have derived the following conclusions on their structures.[3] (a) In the Cu(II) complex formed in the pD range between 3 and 5 the metal-ligand bonding occurs through the amino nitrogen and peptide oxygen (Structure A-1 of Fig. 2). (b) As pD is increased further, the structure of the Cu(II) complex changes into a form in which the metal-ligand bonding occurs through the amino nitrogen, peptide nitrogen (deprotonated), and carboxyl oxygen (Structure B of Fig. 2). (c) In contrast to the Cu(II) complex, the Zn(II) complex is formed for pD>5. Its structure is similar to the Cu(II) complex formed for 3<pD<5, the metal-ligand bonding ocurring through the amino nitrogen and peptide oxygen (Structure A-1 and/or A-2 of Fig. 2). A correlation between the rate and the complex structure is then obvious; *i.e.*, the hydrolysis occurs through the complex in which the peptide oxygen is coordinated to the metal ion (Structure A-1 and/or A-2). This suggests that the mechanism of hydrolysis might be described as follows. Due to the coordination of peptide oxygen to the metal ion, the double bond character of peptide C=O decreases. Although the bond order of peptide C–N increases somewhat at the same time,[3] the net effect of coordination results in the decrease of electron density around the peptide carbon atom. The electron-deficient carbon atom is susceptible to nucleophilic attack of the water molecule which will induce the scission of peptide bond. On the contrary, the electron density around the peptide carbon atom of Structure B would not be reduced (in comparison with free glycylglycine) due to the presence of negative charge on the nitrogen atom. Accordingly, the complex with Structure B is not as susceptible to nucleophilic attack of the water molecule.

It is interesting to compare our results with the peptidase activity of carboxypeptidase A (CPA).[8] Native CPA which has Zn(II) at its active site shows a bell-shaped pH-rate profile with maximum at pH 7.5. It is established that the peptide oxygen of the substrate molecule is coordinated to Zn(II) in the enzyme-substrate complex. If Zn(II) is replaced by Cu(II) (CuCPA), the enzymatic activities are lost. Our present results seem to suggest that a complex analogous to Structure B is formed between CuCPA and the substrate peptide. It is not surprising that this type of complex is inactive to hydrolysis. Although much more studies are needed, the investigation of the properties of metal-substrate complexes may supply useful information in delineating fundamental aspects of metalloenzyme activities.

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