Spectroscopy of Hydrothermal Reactions. 27. Simultaneous Determination of Hydrolysis Rate Constants of Glycylglycine to Glycine and Glycylglycine−Diketopiperazine Equilibrium Constants at 310−330 °C and 275 bar

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Hydrolysis rate constants of glycylglycine to glycine and equilibrium constants between glycylglycine and diketopiperazine were determined simultaneously in situ using an FT-IR spectroscopy flow reactor at 310−330 °C and 275 bar. The hydrolysis of the peptide bond in neutral hydrothermal solution follows the first-order (or pseudo-first-order) rate law. The cyclization of dipeptides is very prominent at high temperatures. Pressure affects the hydrolysis rate and the enthalpy of cyclization of the dipeptide. Specifically, the activation energy for hydrolysis of the dipeptide is reduced by about 50 kJ/mol and the standard enthalpy change of cyclization of the dipeptide is increased by about 50 kJ/mol when pressure is increased from a steam pressure of 1−16 bar to a solution pressure of 275 bar. The data analysis method used in this work is general and could be applied to other dipeptides provided the decomposition rate of the free amino acid product of hydrolysis is monitored.

Introduction

The mechanisms of hydrolysis and the reverse formation of a single peptide bond have been studied experimentally[1,2] and theoretically.[3−7] The hydrolysis rate of peptide bonds is very slow at ambient conditions in neutral solution. It has been shown however that the presence of acids, bases,[8−10] and metal complexes[11−14] promotes the hydrolysis rate and that base catalysis is more efficient than acid catalysis. Less attention has been paid to the preferential hydrolysis of peptide bonds in polypeptides and proteins. With minimal assumptions, Martin[15] proposed that nucleation was required in the initial condensation of two amino acids to yield the dipeptide, which was slower than subsequent condensation to grow the chain. Experimentally, the formation of the dipeptide or its cyclic dipeptide via the condensation of amino acids and the hydrolysis of the polypeptide was found.[16−26] Cyclization of glycylglycine to diketopiperazine was more rapid than its hydrolysis.[19,20,25,26] In a neutral hydrothermal solution, the hydrolysis of glycylglycine via the formation of diketopiperazine[18−20] and the formation of the polypeptide mediated by diketopiperazine[22,23,27] indicate that the diketopiperazine might play an important role in prebiotic polypeptide formation.

Accurate nonenzymatic hydrolysis rates of peptide bonds are difficult to determine in neutral solution at room temperature because they are very slow. A method[28−31] was therefore developed to bind reactants and products with a polymeric fluorogenic substrate. The hydrolysis process was then monitored by determining the concentrations of complexes using HPLC. However, this method inevitably disturbs the state distribution of species in aqueous solution, accelerates or decelerates the hydrolysis of some peptide bonds because of the substrate, and may lead to inaccurate rate constants, especially for polypeptides. Raising the temperature can increase the hydrolysis rate of the peptide bond, but at the same time accelerate the formation of dipeptides from both amino acid condensation[16−21,25,29] and internal aminolysis of polypeptides[18−20] and reduce them by decomposition of the product amino acids.[32−36] The cyclization of the dipeptide was more prominent at high temperature. Thermodynamically, the formation of dipeptides from condensation of the amino acid is favored at high temperature.[37] Therefore, accurate determination of the rate constant of hydrolysis of the peptide bond requires simultaneous consideration of amino acid condensation, internal aminolysis (for a polypeptide), cyclization of the dipeptide, and decomposition of product amino acids. In the course of studies on hydrothermolysis of amino acids, it was found that the rate constant of hydrolysis of simple dipeptides, such as glycylglycine, to free amino acids and the equilibrium constant between the dipeptide and its cyclization product diketopiperazine could be determined simultaneously in situ by using a FT-IR spectroscopy flow reactor. In this paper, the hydrolysis rate constants of the simplest dipeptide, glycylglycine, and equilibrium constants between glycylglycine and diketopiperazine are described. In theory, this method could be applied to dipeptides of other amino acids if the decomposition of the product amino acid can be monitored.

Experimental Section

Glycylglycine (glycine anhydride) and alanylalanine (alanine anhydride) were purchased from Sigma-Aldrich Co. and used without further purification. Milli-Q deionized water was used. Solution pH values of the aqueous solutions were measured on an Orion model 330 pH meter. The concentrations of aqueous solutions were 0.1 *m*.

The flow reactor FT-IR spectroscopy cell constructed from titanium with sapphire windows and gold foil seals has been described in detail elsewhere.[38,39] The temperature and pressure were controlled within ±1 °C and ±1 bar, respectively. The flow rate in the 0.1−1.0 mL/min range at ambient temperatures and 275 bar was controlled with an accuracy of 1% by the use of an Isco syringe pump. Correction of the flow rate in the spectroscopy cell was made to account for the density change with temperature. Transmission IR spectra were recorded at 4

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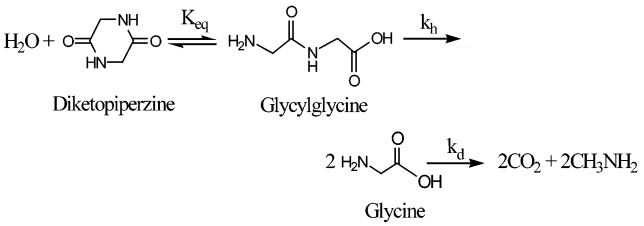
SCHEME 1



cm^{-1} resolution with a Nicolet 560 Magna FTIR spectrometer and an MCT-A detector. Background spectra recorded for pure water at the same conditions were subtracted. Thirty-two spectra were summed at each condition.

During the hydrothermolysis of the dipeptide, only the asymmetric stretch of the aqueous CO_2 product centered at 2343 cm^{-1} was observed in the band-pass of sapphire. The band area of CO_2 was converted into concentration by using the Beer−Lambert law and the previously determined molar absorptivity of aqueous CO_2.[40] The CO_2 concentrations were corrected for hydrolysis[41] as expressed in eqs 1−4. The total CO_2 concentration was replaced by the observed CO_2 concentration in acidic and neutral media at temperatures of 310−330 °C because buffering by the reactants, products, and water prevents a significant change in the pH and hydrolysis of CO_2.[32]

$$CO_2 + H_2O \overset{K_{a1}}{\leftrightarrow} HCO_3^- + H^+ \quad (1)$$

$$HCO_3^- \overset{K_{a2}}{\leftrightarrow} CO_3^{2-} + H^+ \quad (2)$$

The total CO_2 concentration $[CO_2]_T$ is

$$[CO_2]_T = [CO_2]_{obs} + [HCO_3^-] + [CO_3^{2-}] \quad (3)$$

$$= [CO_2]_{obs}\left(1 + \frac{K_{a1}}{[H^+]} + \frac{K_{a1}K_{a2}}{[H^+]^2}\right) \quad (4)$$

The calculation of pH at high temperature was performed on the basis of charge balance and the ionization constants of the species. The ionization constants were obtained by extrapolation using the iso-Coulombic method[42] given the ionization constant at room temperature[43] and specific volume of water.[43]

Results and Discussion

A rapid equilibrium exists between glycylglycine (Gly-Gly) and diketopiperazine (DKP) compared to the hydrolysis rate of the Gly-Gly. The rate of the forward reaction (i.e., Gly-Gly → DKP) is faster than the backward reaction. It is therefore reasonable to assume that equilibrium always exists between Gly-Gly and DKP during the hydrolysis of the peptide bond. Scheme 1 applies to the hydrolysis of Gly-Gly at hydrothermal conditions. At the early stage of decarboxylation of glycine, the analytic kinetic expressions eqs 5−7 apply:

$$[\text{Gly-Gly}] = \frac{C_0}{K_{eq}} e^{-k_h t} \quad (5)$$

$$[\text{Gly}] = \frac{C_0 k_h}{K_{eq}(k_d - k_h)} e^{-k_d t}(e^{(k_d - k_h)t} - 1) \quad (6)$$

$$[CO_2] = \frac{C_0}{K_{eq}}\left(1 - \frac{k_d e^{-k_h t} - k_h e^{-k_d t}}{k_d - k_h}\right) \quad (7)$$

C_0 is the initial concentration of DKP, k_h is the hydrolysis rate of Gly-Gly, k_d is the decarboxylation rate constant, and K_{eq} is the equilibrium constant. k_h and k_d are first-order rate constants. Fitting of the nonlinear eq 7 with the previously determined decarboxylation rate constants of glycine at the same conditions[32] provides the hydrolysis rate constant of Gly-Gly to glycine and equilibrium constant between Gly-Gly and DKP simultaneously. These constants are given in Table 1. The variation of the hydrolysis rate constant with temperature followed the Arrhenius equation (Figure 1), and the variation of the equilibrium constant with temperature followed the van't Hoff equation (Figure 2). Also plotted are results from previous work. The data at water stream pressure from Qian et al.[25] (pressure change is in the range of 1−16 bar when temperature changed from 100 to 220 °C) and most of the data from Wolfenden et al.[26] (pressure was not measured, but we estimate that the highest pressure was not more than the water stream pressure at a given temperature) were estimated from their graphs, but are not listed in Table 1 because they are less accurate than those obtained here. It can be seen from Figure 1 that the present results agree very well with Qian's data at high pressure (265 bar) when extrapolated to high temperatures, while the low-pressure results from Qian et al. and Wolfenden et al. had similar activation energies but different frequency factors. The Arrhenius parameters were therefore calculated with a correlation coefficient of 0.99 based on the present work and that of Qian et al. at high pressure. A similar result was obtained when the present data were compared with those of Wolfenden

TABLE 1: Hydrolysis Rate Constants k_h of Glycylglycine to Glycine and Equilibrium Constants K_{eq} between Diketopiperazine and Glycylglycine at 275 bar

temp/°C	k_h/s^{-1}	K_{eq}
310	1.9×10^{-4}	0.01762
320	2.2×10^{-4}	0.01332
330	2.8×10^{-4}	0.01152
100	1.10×10^{-6} [a]	
120	3.28×10^{-6} [a]	
160	8.94×10^{-6} [a]	
220	3.72×10^{-5} [a]	

Arrhenius plot		van't Hoff plot	
E_a/kJ·mol^{-1}	ln(A,s^{-1})	$\Delta_r H°$/kJ·mol^{-1} [c]	$\Delta_r H°$/kJ·mol^{-1} [d]
43.53 ± 1.13 (96.23)[b]	0.47 ± 0.29	−62.32 ± 10.74	−14.62

[a] From Qian et al. at 265 bar. [b] From Wolfenden et al. [c] This work at 275 bar. [d] Based on Wolfenden's data (unknown pressure).

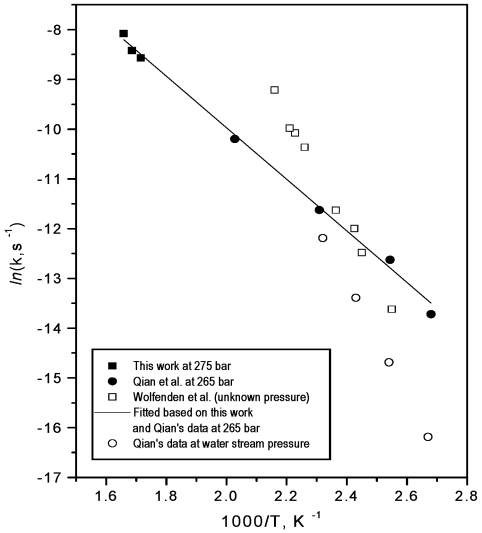


Figure 1. Arrhenius plot of glycylglycine hydrolysis to glycine.

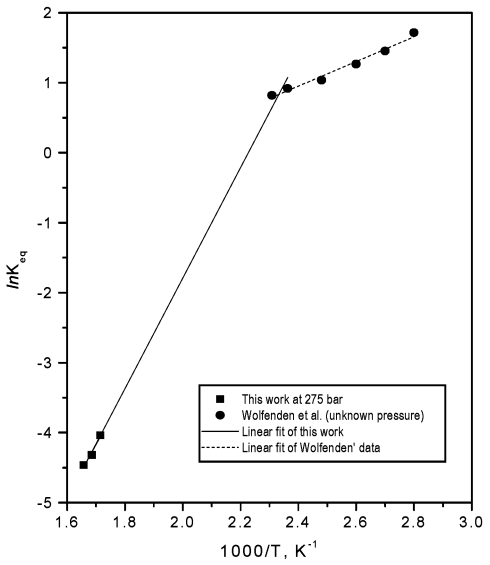


Figure 2. Van't Hoff plot of cyclization of glycylglycine to diketopiperazine.

in Figure 2. Interestingly, the intersections in Figures 1 and 2 occur at about 150 °C with respect to low and high pressures. Pressure has an obvious effect on the hydrolysis and cyclization of the dipeptide.

The effects of temperature and pressure studied theoretically by Shock[37] show that increasing temperature and decreasing pressure would increase the thermal stability of the peptide bond in hydrothermal solutions and that the decrease of thermal stability of the peptide bond at high pressure was offset by the increase in stability at high temperature. An activation energy of about 90 kJ/mol is needed to hydrolyze a peptide bond at low pressure,[25,26,45,46] while increasing the pressure to 275 bar lowers the activation energy to about half that at low pressure. At the same time, the standard enthalpy change of cyclization of the dipeptide increases about 50 kJ/mol as pressure increases. It should be noted that pressure had no effect on the hydrolysis of an ester[47] or the decarboxylation of amino acids,[32,33] and the peptide bond dealt with (including the thermodynamic calculations by Shock) is only in the dipeptide. It is not known whether pressure has a similar effect on the peptide bonds in polypeptides and proteins. But if the observations by Bada et al.[18−20] that the hydrolysis of polypeptide occurs via the formation of diketopiperazine and by Takaoka et al.[22,23] and Rode[27] that the formation of polypeptide is mediated by diketopiperazine hold, then more kinetic data at high pressure are needed to explore its role in prebiotic conditions.

When the kinetic method here was applied to alanine anhydride, rate and equilibrium constants could not be obtained because the decarboxylation rate of alanine was so slow that the CO_2 concentration could be determined only at the upper limit of temperature and the longest residence time of the flow reactor. However, this method of data analysis is general and can be applied to other dipeptides provided the decomposition (decarboxylation and deamination) of the free amino acid product is monitored.

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References and Notes

(1) Brown, R. S.; Bennet, A. J.; Slebocka-Tilk, H. *Acc. Chem. Res.* **1992**, *25*, 481.
(2) Fife, T. H. *Acc. Chem. Res.* **1993**, *26*, 325.
(3) Konuklar, F. A.; Aviyente, V.; Ruiz Lopez, M. F. *J. Phys. Chem. A* **2002**, *106*, 11205.
(4) Antonczak, S.; Ruiz Lopez, M. F.; Rivail, J. L. *J. Am. Chem. Soc.* **1994**, *116*, 3912.
(5) Krug, J. P.; Popelier, P. L. A.; Bader, R. F. W. *J. Phys. Chem.* **1992**, *96*, 7604.
(6) Jensen J. H.; Baldridge K. K.; Gordon, M. S. *J. Phys. Chem.* **1992**, *96*, 8340.
(7) Chalmet, S.; Harb, W.; Ruiz Lopez, M. F. *J. Phys. Chem. A* **2001**, *105*, 11574.
(8) Lawrence, L.; Moore, W. J. *J. Am. Chem. Soc.* **1951**, *73*, 3973.
(9) Brewerton, A. A.; Long, D. A.; Truscott, T. G. *Trans. Faraday Soc.* **1970**, *66*, 2297.
(10) Hartmann, H.; Fernandez, J.; Heidberg, J.; Jung, H. *Z. Naturforsch.* **1963**, *18B*, 269.
(11) Zhu, L.; Qin, L.; Parac, T. N.; Kostic, N. M. *J. Am. Chem. Soc.* **1994**, *116*, 5218.
(12) Hegg, E. L.; Burstyn, J. N. *J. Am. Chem. Soc.* **1995**, *117*, 7015.
(13) Sayre, L. M.; Reddy, K. V.; Jacobson, A. R.; Tang, W. *Inorg. Chem.* **1992**, *31*, 935.
(14) Boreham, C. J.; Buckingham, A. D.; Keene, F. R. *J. Am. Chem. Soc.* **1979**, *101*, 1409.
(15) Martin, R. B. *Biopolymers* **1998**, *45*, 351.
(16) Purdie, J. E.; Benoiston, N. L. *J. Chem. Soc., Perkin Trans. 2* **1973**, *1845*, 13.
(17) Kawamura, K.; Yukioka, M. *Thermochim. Acta* **2001**, *375*, 9.
(18) Gaines, S. M.; Bada, J. L. *J. Org. Chem.* **1988**, *53*, 2757.
(19) Steinberg, S. M.; Bada, J. L. *J. Org. Chem.* **1983**, *48*, 2295.
(20) Steinberg, S. M.; Bada, J. L. *Science* **1981**, *213*, 544.
(21) Imai, E.; Honda, H.; Hatori, K.; Brack, A.; Matsuno, K. *Science* **1999**, *283*, 831.
(22) Nagayama, M.; Takaoka, O.; Inomata, K.; Yamagata, Y. *Origins Life Evol. Biosphere* **1990**, *20*, 249.
(23) Takaoka, O.; Yamagata, Y.; Inomata, K. *Origins Life Evol. Biosphere* **1991**, *21*, 113.
(24) Watanabe, H.; Kozai, Y. *Bull. Inst. Chem. Res., Kyoto Univ.* **1972**, *50*, 318.
(25) Qian, Y.; Engel, M. H.; Macko, S. A.; Carpenter, S. C.; Deming, J. W. *Geochim. Cosmochim. Acta* **1993**, *57*, 3281.
(26) Radzicka, A.; Wolfenden, R. *J. Am. Chem. Soc.* **1996**, *118*, 6105.
(27) Rode, B. M. *Peptides* **1999**, *20*, 773.
(28) Bryant, R. A. R.; Hansen, D. E. *J. Am. Chem. Soc.* **1996**, *118*, 5498.
(29) Smith, R. M.; Hansen, D. E. *J. Am. Chem. Soc.* **1998**, *120*, 8910.
(30) Kahne, D.; Still, W. C. *J. Am. Chem. Soc.* **1988**, *110*, 7529.
(31) Pantoja, S.; Lee, C.; Marecek, J. F. *Marine Chem.* **1997**, *57*, 25.
(32) Li, J.; Brill, T. B. *Int. J. Chem. Kinet.*, in press.
(33) Li, J.; Brill, T. B. *J. Phys. Chem. A* **2003**, *107*, 5987.
(34) Miller, S. L.; Bada, J. L. *Nature* **1988**, *334*, 609.
(35) Bada, J. L. *Philos. Trans. R. Soc. London B* **1991**, *333*, 349.
(36) White, R. H. *Nature* **1984**, *310*, 430.
(37) Shock, E. L. *Geochim. Cosmochim. Acta* **1992**, *56*, 3481.
(38) Kieke, M. L.; Schoppelrei, J. W.; Brill, T. B. *J. Phys. Chem.* **1996**, *100*, 7455.
(39) Schoppelrei, J. W.; Kieke, M. L.; Wang, X.; Klein. M. T.; Brill, T. B. *J. Phys. Chem.* **1996**, *100*, 143439.
(40) Maiella, P. G.; Schoppelrei, J. W.; Brill, T. B. *Appl. Spectrosc.* **1999**, *53*, 351.
(41) Butler, J. N. *Ion Equilibrium, Solubility and pH Calculation*; John Wiley & Sons: New York, 1998; p 368.
(42) Lindsay, W. T. *Proc. Int. Water Conf. Eng. Soc. W. Pa.* **1980**, *41*, 284.
(43) Marshall, W. L.; Franck, E. U. *J. Phys. Chem. Ref. Data* **1981**, *10*, 295.
(44) Uematsu, M.; Franck, E. U. *J. Phys. Chem. Ref. Data* **1980**, *9*, 1291.
(45) Hare, P. E. *Carnegie Inst. Wash. Yearb.* **1976**, *75*, 801.
(46) Robinson, B. A.; Tester, J. W. *Int. J. Chem. Kinet.* **1990**, *22*, 431.
(47) Li, J.; Brill, T. B. *J. Phys. Chem. A* **2001**, *105*, 6171.