Nuclear Magnetic Resonance Study of the Protolysis Kinetics of the Peptide Hydrogens of Triglycine[1a]

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Abstract: The rates of exchange of the peptide hydrogens of triglycine were measured, using the nuclear magnetic resonance technique, over a range of peptide and hydrogen ion concentrations. It was found that the exchange rate of the hydrogen of the peptide group adjacent to the carboxylic group (α) is much slower than the rate of exchange of the hydrogen of the second peptide group, adjacent to the terminal amino group (β). The difference in the exchange rates of the protons of the α- and β-peptide groups is interpreted in terms of the influences of the neighboring charged groups, NH_3^+ and COO^-, on the peptide hydrogens. A correlation between the rate of exchange of a peptide hydrogen of RCONHR′ and the ionization constant of the carboxylic group of RCOOH (where R is common for both substances) is proposed and discussed.

Investigation of the rates of proton exchange of compounds in solution can yield valuable information on their structure and on their interactions with the solvents. Comparison of rates of exchange of protons in a series of related compounds having a common functional group may provide information on the acidity of this functional group and on the nature and extent of its solvation.[2]

In the case of peptides, the chemical nature of a peptide bond probably depends on its location in the molecule in relation to other funct onal groups and on the conformation of the peptide molecule. Partial clarification of these matters may be obtained from the knowledge of the rates of exchange of peptide hydrogens.

The hydrogen–deuterium exchange of peptide hydrogens of some tripeptides have been studied. Linderström-Lang[3] reported that the rates of exchange of deuterated triglycine at 0° and at pH 3 with water is very fast. He found that within 0.8 min of dissolution, 80% of the peptide hydrogen had exchanged. Nielsen, Bryan, and Mikkelsen[4] measured the H–D exchange of some tripeptides (Gly-Gly-Gly, Ala-Gly-Gly, Leu-Gly-Gly) at 22° using infrared spectroscopy. According to these authors the rate of exchange of the two peptide groups in each of the tripeptide molecules proceeds according to different first-order rates. In the case of Ala-Gly-Gly, values of the rates of exchange at different pD are given. The experimental results given by the above authors show that the exchange is acid catalyzed and that the exchange of the C-terminal peptide (denoted in this paper as α) is faster than the exchange of the N-terminal peptide (denoted here as β). However, the authors did not extend their studies to the basic range and they only succeeded in measuring relatively slow rates.

In this paper we report measurements of rates of exchange of the peptide hydrogens of triglycine (GGG) in aqueous solutions as measured by the nuclear magnetic resonance technique. Using the above technique it is possible to measure fast rates of exchange where the mean lifetime of the exchanging group between successive exchanges is in the range of $1–10^{-3}$ sec. Thus we could extend the range of exchange measurements as compared with other techniques. However, low rates of exchange like the one reported by Nielsen, *et al.*,[4] cannot be determined by this method.

We designate the peptide and methylene groups of GGG as follows

	NH_3^+ CH_2	CONH CH_2	CONHCH_2COO^-
peptide group		β	α
methylene group	γ	β	α

Experimental Section

The nmr spectrometer used in this study has been described previously.[5] Chromatographically pure triglycine was supplied by Mann Research Laboratories. Stock solutions of GGG were prepared by a standard analytical procedure. The pH of the solutions was varied by addition of concentrated base or acid, so that there was practically no change in the concentration of the peptide. The pH of the solutions was measured with a Radiometer pH meter (Model TTT1B) with scale expander.

Results

Interpretation of the Spectra. At different pH values the spectrum of an aqueous solution of GGG undergoes two types of pH-dependent change: shifts of the spectral lines and changes of the line shapes. These effects are illustrated in Figures 1 and 2. Changes in the positions of the spectral lines are due to the different ionized species of triglycine molecules which are present at different pH values. The line shapes of the methylene groups depend on the rate of exchange of the peptide hydrogen and the exchange of the protons of the terminal NH_3^+ group.

The spectrum of the γ-methylene group should be a quadruplet, due to the spin–spin interaction with the protons of the NH_3^+ group. However, unless the solutions of GGG are very acidic (pH <0), the protons of the NH_3^+ group exchange rapidly;[6] consequently, the γ-methylene spectrum will appear as a single line.

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(2) See, *e.g.*, M. T. Emerson, E. Grunwald, M. L. Kaplan, and R. A. Kromhout, *J. Am. Chem. Soc.*, **82**, 6307 (1960).

(3) K. Linderström-Lang in Symposium on Protein Structure, London, 1958, p 23.

(4) S. O. Nielsen, W. P. Bryan, and K. Mikkelsen, *Biochim. Biophys. Acta*, **42**, 550 (1960).

(5) M. Sheinblatt, *J. Am. Chem. Soc.*, **87**, 572 (1965).

(6) Presumably the exchange of the protons of the NH_3^+ group of GGG is similar to the exchange of the NH_3^+ of glycine: M. Sheinblatt and H. S. Gutowsky, *J. Am. Chem. Soc.*, **86**, 4814 (1964).

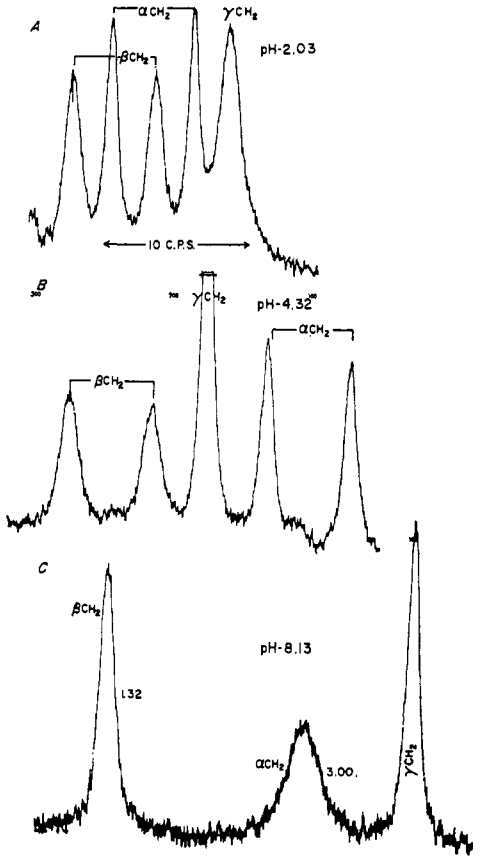

Figure 1. Typical shifts of the spectral lines of methylene groups of triglycine as a function of pH. The magnetic field increases from left to right. The spectral lines of the α- and γ-methylene shift toward lower and higher fields at pH $\cong$ pK_{COOH} and pH $\cong$ p$K_{NH_3^+}$, respectively. The three curves are drawn on a common abscissa; the external water peak was taken as a common reference.

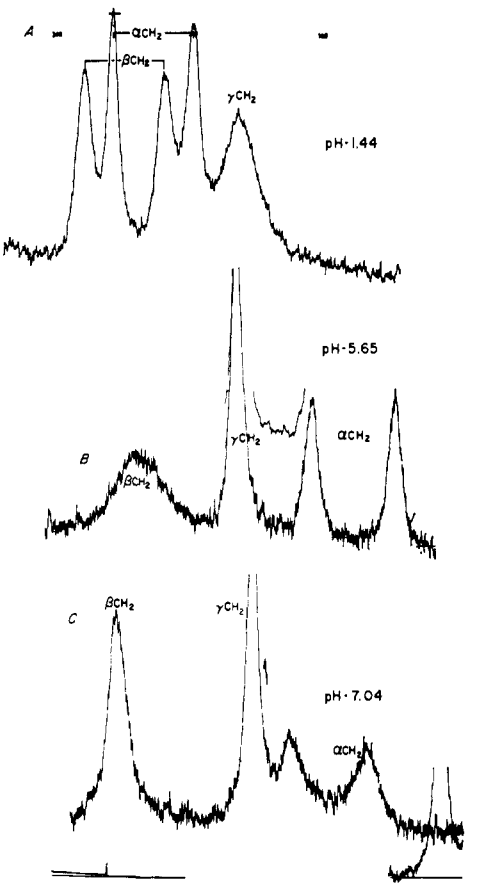

Figure 2. Typical changes in shape of the spectral lines of the methylene groups of triglycine as a function of pH.

The spectrum of each of the other two methylene groups should be a doublet due to the spin–spin interaction with the hydrogen of the peptide groups. As a result of the exchange of the peptide hydrogens, the lines of the doublet broaden, and at high rate of exchange each doublet collapses into a single line. Thus the spectrum of an α- or β-methylene group should be a single line or a doublet, depending on how fast the peptide hydrogens exchange. If we assume that the peptide hydrogens of GGG exchange in the same pH range as that of glycylglycine[5] we would expect that by acidifying the solution of GGG we would reach a stage where the spectrum would consist of two doublets due to the α- and β-methylenes and a single line due to the γ-methylene. This, in fact, is observed (Figures 1A and 1B). Furthermore, neutralization of the NH_3^+ group by addition of NaOH causes the γ-methylene line to shift toward higher field[7] (Figure 1C). Thus the spectrum of the γ-methylene is readily assigned.

In order to identify the lines due to the α-methylene group, we used the fact that protonation of the carboxylate (COO^-) ion by addition of HCl to GGG solution causes the above line to shift toward lower field,[5] while the lines of the γ and β groups should be affected very little. This shift is illustrated in Figures 1B and 1A and tabulated in Table I.

(7) E. Grunwald, A. Loewenstein, and S. Meiboom, *J. Chem. Phys.*, 27, 641 (1957).

In Table I the chemical shifts, δ, in cycles per second, as referred to an external water sample, are given at different pH values. It is shown that one of the lines shifts toward lower field with the lowering of pH. A plot of δ as a function of pH gives a sigmoid curve with an inflection at pH 3.2, which equals the pK_{COOH} of GGG. Thus we were able to assign the spectral line of the α-methylene. Knowing the spectral line of α and γ we could assign the line due to the β-methylene group.

Table I. The Chemical Shifts, δ, of the Methylene Lines as Referred to an External Water Sample

		Chemical shifts, cps		
	pH	α_{CH_2}	β_{CH_2}	γ_{CH_2}
1	2.03	43	46	52
2	2.56	43	46	51
3	2.88	50	44	52
4	3.33	53	43	52
5	4.32	58	44	51
6	5.65	60	44	51

The following changes in line shape were observed. In very acidic solutions of GGG, pH $\simeq$2.00, the spectrum of the γ-methylene group broadens, as compared to its width in higher pH's, indicating a decrease in the rate of exchange of the NH_3^+ protons (Figure 2A). The changes in the shapes of the β- and α-methylene lines due to the exchange of the corresponding peptide protons are illustrated in Figures 2B and 2C, respec-

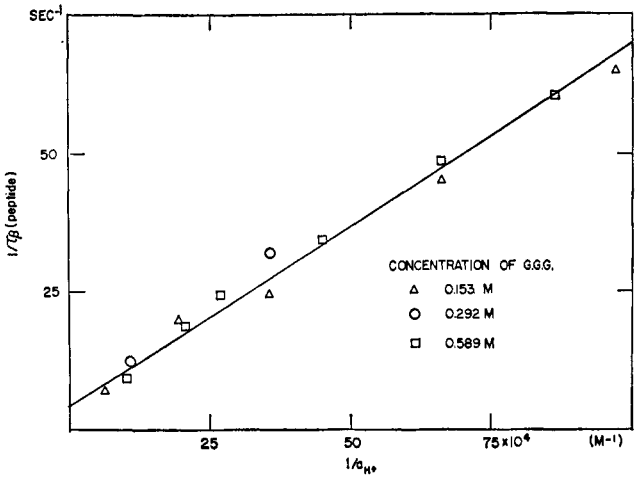

Figure 3. Values of the reciprocal of the mean lifetime of the β-peptide hydrogen, $1/\tau_B$, for various peptide concentration as a function of $1/a_{H^+}$ (23 ± 1°).

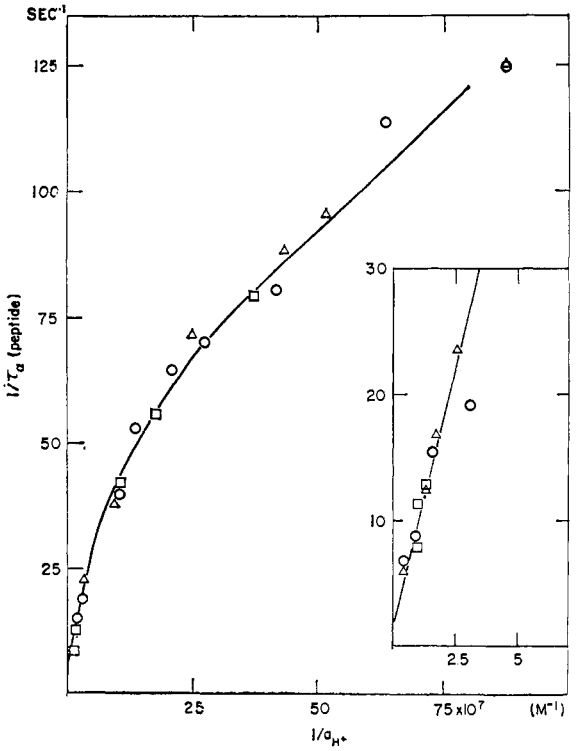

Figure 4. Values of the reciprocal of the mean lifetime of the α-peptide proton, $1/\tau_\alpha$, for various peptide concentrations as functions of $1/a_{H^+}$ (23 ± 1°). The solid curve represents calculated values of $1/\tau_\alpha$ using eq 2. The acidic range is given in an enlarged scale at the right side of the figure.

tively. These figures show that the exchange of the proton of the β-peptide is faster than that of the α-peptide.

Calculation of Rates of Exchange. The reciprocal mean lifetimes, $1/\tau$, of the peptide hydrogens of GGG were calculated from the line shape of the methylene groups by the usual procedure.[8] The experimental results for the β- and α-methylenes are summarized in Figures 3 and 4, respectively. In these figures, values of $1/\tau$ for various concentrations of GGG are given as a function of $1/a_{H^+}$. These figures show that values of $1/\tau$ for both peptide hydrogens are independent of GGG concentration. However, while $1/\tau$ of the β-peptide group depends linearly on $1/a_{H^+}$, a nonlinear relation between $1/\tau$ and $1/a_{H^+}$ is observed for the α-peptide group. The value of $1/\tau$ varies rapidly at low $1/a_{H^+}$ values, and less in more basic solutions.

Discussion

Exchange Reactions and Calculation of Rate Constants. The exchange mechanisms of the peptide hydrogens of GGG are probably the same as those proposed for glycylglycine (GG)[5] and N-methylacetamide (NMA).[9] The exchange reaction of each of the peptide protons can therefore be described as

$$\mathrm{RCONHR' + H_2O \underset{}{\overset{k_I}{\rightleftharpoons}} RCO\bar{N}R' + H_3O^+} \qquad \text{(I)}$$

$$\mathrm{RCONHR' + OH^- \overset{k_{II}}{\rightleftharpoons} RCO\bar{N}R' + H_2O} \qquad \text{(II)}$$

The reciprocal of the mean lifetime of the peptide hydrogen, $1/\tau$, is therefore given by[10]

$$1/\tau = k_I[H_2O] + k_{II}[OH^-] = k_I[H_2O] + k_{II}\frac{K_w}{[H^+]} \qquad (1)$$

According to eq 1, $1/\tau$ should be independent of the peptide concentration, and the plot of $1/\tau$ against $1/[H^+]$ should give a straight line with an intercept of $k_I[H_2O]$ and a slope of $k_{II}K_w$. It is also expected, by comparison with previous cases,[5,9] that the exchange with OH^- ion is dominant, while the exchange with water molecules contributes very little to $1/\tau$.

The experimental results for the exchange of the β-peptide proton agree with the above-proposed exchange reactions. From Figure 3 the two rate constants were calculated at 23 ± 1°; $k_{I\beta}[H_2O] = 5$ (sec^{-1}) and $k_{II\beta} = 6.7 \times 10^9$ (M^{-1} sec^{-1}).

The experimental results for the exchange of the proton of the α-peptide group do not agree in one respect with the proposed exchange reactions; namely, we do not observe a linear relation between $1/\tau$ and $1/a_{H^+}$. However, values of $1/\tau$ are independent of the peptide concentration in accordance with the proposed exchange mechanism. In fact, the exchange rate can be explained in terms of the above reaction if we consider the following arguments. The $pK_{NH_3^+}$ of the terminal NH_3^+ group is 7.9.[11] Thus this group is titrated in the pH range (7 < pH < 9) in which the exchange of the peptide hydrogen was measured. Consequently two different ionic species of GGG are present in the measured solutions, the zwitterion ($GGG^\pm$) and the triglycinate anion (GGG^-). The two ionic species probably have two different rates of exchange. The rate of exchange of the α-peptide proton of $GGG^\pm$ can be calculated from the initial slope of Figure 4 ($1/a_{H^+} < 3 \times 10^7$), which is given in an enlarged scale at the right side of the Figure 4; $k_{II\alpha}$ was found to be 8.4×10^7 (M^{-1} sec^{-1}). The negative deviation from the pre-

(8) A. Loewenstein and S. Meiboom, *J. Chem. Phys.*, 27, 1067 (1957).
(9) A. Berger, A. Loewenstein, and S. Meiboom, *J. Am. Chem. Soc.*, 81, 62 (1959).
(10) A detailed discussion of the relation between τ and rate of exchange is given in ref 6.
(11) C. Long, "Biochemist's Handbook," E. Spon and F. N. Spon, Ed., 1961, p 43.

dicted linearity between $1/\tau$ and $1/[H^+]$ indicates that the rate of exchange of the α-peptide of GGG^-, $k_{II\alpha}'$, is smaller than $k_{II\alpha}$.

In the pH range where considerable concentrations of both ionic species are present, each of the two ionic species of GGG contributes to the exchange. Thus we get

$$1/\tau = \frac{K_w}{[H^+]}[(1 - P)k_{II\alpha} + Pk_{II\alpha}'] \quad (2)$$

where P is the fraction of GGG^- out of the total peptide. Using eq 2 and substituting P as calculated from the $[H^+]$ and $pK_{NH_3^+}$ of GGG, $k_{II\alpha}'$ was found to be 0.8×10^7 (M^{-1} sec^{-1}), so as to give the best agreement to the experimental results. Values of $1/\tau$ as a function of $1/[H^+]$ calculated from eq 2 are presented as a solid line in Figure 4.

Correlation between Rates of Exchange and the Acidity of the Exchanging Groups. A significant observation in this study is that, although the base-catalyzed exchange mechanisms are identical for both peptide protons, the exchange of the β-peptide proton is by far faster than that of the α-peptide ($k_{II\beta}/k_{II\alpha} = 80$) and that $k_{II\alpha} > k_{II\alpha}'$. These differences in rate may be attributed to the difference in the acidity of the two peptide protons which in turn arises from the presence of the charged group in the peptide molecule. The oppositely charged groups of the peptide molecule (NH_3^+ and COO^-) have different influences on the exchange rates of the peptide hydrogens. Whereas the positively charged group tends to make the peptide hydrogen more acidic, thus accelerating its exchange, the negatively charged group has the opposite effect. It is expected also that the strength of these effects depends on the distance between the charge and the peptide groups.

The functional groups of the peptide molecules can be expected to influence other properties of the peptide and related molecules. Thus one may anticipate that the effect of a functional group on the ionization constant of a carboxylic group[12] of RCOOH and on the rate of exchange of a peptide hydrogen of RCONHR′ should be related provided the R's are identical and that the effect of the functional groups present in R′ is taken into account when considering the exchange rate of the peptide hydrogen.

The rate of exchange of the peptide hydrogen of N-methylacetamide (NMA) is chosen as a reference for the calculation of the effect of functional groups on the rate of exchange of the peptide hydrogen, since this molecule lacks functional groups. For the same reason the pK_{COOH} of acetic acid (AcOH) is chosen as a reference for the calculation of the effect of a functional group on the ionization constant of the carboxylic group.

It has been shown previously[5] that the effect of the NH_3^+ group on the base-catalyzed rate of exchange of its adjacent peptide hydrogen is given by

$$\frac{k_{II}(GG)}{k_{II}(NMA)} = 1.5 \times 10^2 \quad (3)$$

Similarly the effect of the NH_3^+ group on the ionization of the adjacent carboxylic group is given by

$$\frac{K_{COOH}(\text{glycine})}{K_{COOH}(AcOH)} = 2.5 \times 10^2 \quad (4)$$

(12) J. G. Kirkwood and F. H. Westheimer, *J. Chem. Phys.*, **6**, 506 (1938).

In the calculation of the rate of exchange it is necessary to consider the presence of the carboxylic group of GG which will tend to decrease the rate of exchange of the peptide hydrogen. Thus eq 3 represents the net effect of both the NH_3^+ and COO^- groups.

The effect of the carboxylate group on the rate of exchange of a neighboring peptide hydrogen can be estimated from eq 3 and 4. According to these equations the carboxylate group should decrease the rate by a factor of 1.7.

It was found[13] that the rate of exchange of the peptide hydrogen of diglycine methyl ester is greater by a factor of 1.5 than that of diglycine. The result is in good agreement with the predicted value of 1.7.

We may extend the calculation of the effects of functional groups on the exchange rates of peptide hydrogens and the corresponding correlation with ionization constants of related substances utilizing the experimental data given in this paper.

The rate of exchange of the β-peptide hydrogen is influenced by the effects of its adjacent NH_3^+ group and the α-peptide group. The effects of both functional groups tend to increase the acidity of the peptide hydrogen and hence increase its rate of exchange. If we assume that the effect of the NH_3^+ group on the exchange of the β-peptide proton is equal to that given in eq 4, it is possible to calculate the effect of a right adjacent peptide on rates of exchange. In the case of triglycine the rate of exchange of the β-peptide increases by a factor of 6 owing to the presence of the α-peptide group.[14]

The exchange of the α-peptide hydrogen is governed by the presence of the carboxylic group, the β-peptide group, and the remote NH_3^+ group. While the first functional group tends to decrease the rate of exchange, the other two groups increase it.

The effect of the three functional groups on the exchange of the α-peptide can be determined from

$$\frac{k_{II\alpha}(GGG)}{k_{II}(NMA)} = 16 \quad (5)$$

On the other hand, the effect of the NH_3^+ group and the β-peptide group on the ionization of the carboxylic group can be calculated from

$$\frac{K_{COOH}(GG)}{K_{COOH}(AcOH)} = 41 \quad (6)$$

The ratio of eq 5 and 6 represents the upper limit[15] of the retarding effect of the COO^- group on the exchange of the α-peptide proton.

The effect of the NH_3^+ group on the rate of exchange of its next nearest neighbor peptide group (α) is given by the ratio $k_{II\alpha}/k_{II\alpha}' = 10$.

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(13) M. Sheinblatt, in preparation.

(14) The increase in the acidity of a group due to its right-adjacent peptide group can be seen by comparing the pK_{NH} of CH_3NH_2 (10.6) and the pK_{NH} of $NH_2CH_2CONH_2$ (7.9): D. D. Perrin, "Dissociation Constants of Organic Bases in Aqueous Solutions," Butterworths, London, 1965.

(15) The formation of an intrahydrogen bond between the proton of the α-peptide group and the carboxyl of the β-peptide group can have a retarding effect on the exchange of the α-peptide proton.