Nuclear Magnetic Resonance Study of the Protolysis Kinetics of the Peptide Hydrogen of Glycylglycine

M. Sheinblatt[1]

Contribution from the National Institute of Arthritis and Metabolic Diseases, National Insitutes of Health, Bethesda, Maryland. *Received July 16, 1964*

The rate of exchange of the peptide hydrogen of glycylglycine (GG) was measured using the n.m.r. technique. It was found that the main exchange of the peptide hydrogen occurs with OH^- ions. The rate constant for this reaction was calculated to be $k = 7.8 \times 10^8$ $(M \text{ sec.})^{-1}$ at 23°. A comparison of the rates of exchange of the peptide hydrogen of GG to that found for N-methylacetamide is given.

Introduction

Hydrogen–deuterium exchange between water and polypeptides or proteins has been measured using several techniques.[2-6] It is customary to divide the exchangeable hydrogens into two groups: (1) hydrogen which exchanges "instantaneously" (within 30 sec.), and (2) hydrogen which exchanges "slowly." The measurements by the isotope-labeling technique cover only the latter group. The slow rate of exchange is usually attributed to the formation of internal hydrogen bonds or to steric effects. In short peptides, where these conditions do not exist, the rates of exchange are usually very fast.[7] Rates of exchange of some di- and tripeptides were determined recently under such conditions where the rates were "slow."[8]

The rate of exchange of the peptide hydrogen of glycylglycine (GG) as measured by nuclear magnetic resonance technique is reported in this paper. Using the above method we were able to measure, as in previous cases,[9] "fast" rates of exchange and thus extend the range of measurements.

The protons of two groups of GG are exchangeable: (i) the peptide proton, and (ii) the protons of the NH_3^+ group. While the exchange of the first group resembles very much the exchange found in N-methylacetamide,[10,11] the behavior of the latter group is probably similar to that found in simple amino acids.[12,13]

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Experimental

All spectra used for kinetics studies were obtained with a Varian A-60 spectrometer and high-resolution 12-in. magnet system. The temperature in the probe was 23 ± 1°. The homogeneity of the magnetic field was such that $1/T_2$ of pure water (not degassed) was in the range 0.95–1.25 sec.$^{-1}$. Spin-decoupling measurements were conducted with a suppressed carrier, single side-band generator operating at 60 Mc. (manufactured by Space Avionic Inc.). The decoupling experiments were conducted with a Varian H-R-60 spectrometer and the usual field sweep.

Chromatographically pure glycylglycine was supplied by Mann. Volumetric solutions of GG were prepared by standard analytical procedure. The pH was adjusted to the desired values by addition of concentrated base or acid, so that the change in volume was negligible. The pH of solutions was measured with a Radiometer pH meter with an expanded scale.

Interpretation of Spectra and the Calculation of the Rate of Exchange

The n.m.r. spectra of aqueous solutions of GG (pH 5.2) are given in Figure 1. The lines in order of increasing magnetic field are (1) the broad line of the peptide hydrogen, (2) a sharp line due to the fast exchanging NH_3^+ and water protons, and (3) an unequal doublet due to the two methylene groups.

The spectrum of the β-methylene should be a quadruplet due to the spin interaction with the protons of the NH_3^+ group. However, at the pH range where the kinetic measurements were carried out, pH >5, the exchange of the protons of the NH_3^+ group is fast; as a result the multiplet collapses into a single narrow line whose width is ~1 c.p.s. When the pH of the solution is lowered to approximately pH 2, it is possible to observe a significant broadening of the methylene line indicating a decrease of the rate of exchange of the NH_3^+ protons. The spectrum of the α-methylene should be a doublet or a single line depending on the rate of exchange of the proton of the peptide group. It seems, therefore, that the unequal doublet in Figure 1 is really an overlap of the sharp line due to the β-methylene on the lower field line of the doublet due to the α-methylene. The above assignment was proved by the following spin-decoupling experiment.

Irradiation of the NH protons should cause the doublet of the α-methylene group to coalesce into a single line. Hence, the spectrum should consist of two lines of equal height, one due to the α-methylene and the second due to the β-methylene. The spin-decoupling results are shown in Figure 2. We get optimum decoupling when the difference between the frequencies of the strong irradiating side band and the center band is

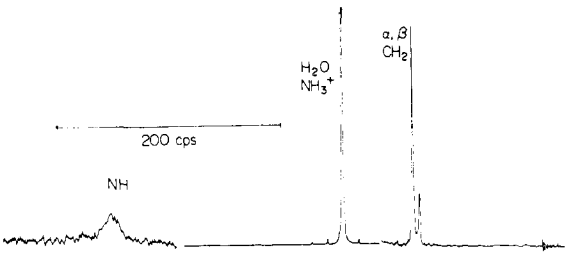

Figure 1. Typical n.m.r. spectrum of aqueous solution of glycylglycine (pH 5.2). The magnetic field increases from left to right. The lines of the spectrum were recorded in different gains (23 ± 1°).

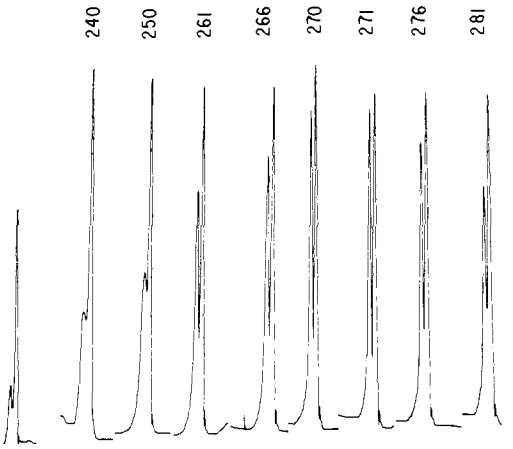

Figure 2. The effect of strong radiofrequency irradiation of the proton of the NH peak on the spectrum of the two methylene groups. The number above each spectrum represents the difference in c.p.s. between the strong radiofrequency irradiation and the normal one. The optimum collapsing occurs when the difference between the radiofrequency values is approximately equal to the chemical shifts between the lines of the methylene groups and the peptide hydrogen. The spectrum of the methylene groups without applying the strong radiofrequency irradiation is given at left. The frequency scale of the last spectrum is different from the one describing the double resonance experiment.

approximately equal to the chemical shift between the NH and the α-methylene peaks.

The positions of the lines of the two methylene groups change with the pH of the solution. This can be very helpful in identification of the spectral lines. Neutralizing the NH_3^+ group ($pK = 8.2$) should cause the spectral line of the adjacent methylene group to shift toward higher field, the plot of which shows a sigmoid shape, similar to a titration curve,[14] with an inflection point at pH = pK. On the other hand, it is expected that protonation of the carboxylate COO^- ion should cause the spectra line of the α-methylene group to shift in the opposite direction. Figure 3 shows the shifts measured from an external water reference of the center of the doublet of the α-methylene and of the single line of the β-methylene groups as a function of pH. While the line due to the β-methylene shifts very little, the spectrum of the α-methylene shifts in a sigmoid curve fashion toward lower field, with an inflection point at pH 3.1 which equals $pK_{COOH} = 3.1$.

The rate of exchange of the peptide hydrogen increases with the addition of base to GG solution. This is shown in the changes of the line shape of the α-

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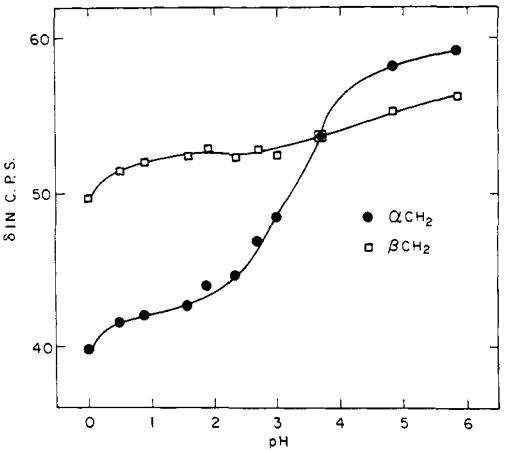

Figure 3. The chemical shifts, δ, in c.p.s. of the α and β spectral lines referred to an external water sample as a function of pH (23 ± 1°).

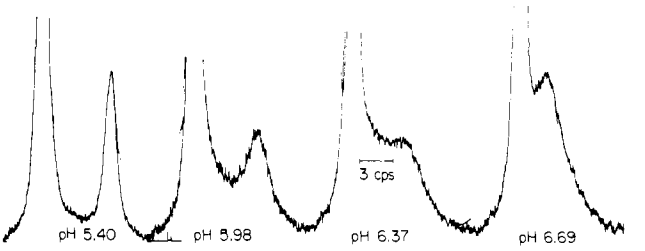

Figure 4. Changes in the line shape of the α-methylene group with pH. The doublet (of which only the higher field part of it is seen) broadens with increasing pH and ultimately collapses into a single line.

methylene spectrum (Figure 4). At low rate of exchange the observable half of the doublet broadens and with increasing rate of exchange the doublet collapses into a single line.

The mean life time, τ, of the proton of the peptide group was calculated from the line shape of the α-methylene spectrum using the theoretical curve for the case of a doublet.[15] Because of the overlapping of the spectral lines, we could not calculate τ for every spectrum; our measurements therefore were concentrated in two small regions where the spectrum of the α-methylene is either a doublet or a sharp line (the last region is less accurate).

Results and Discussion

The experimental results of the exchange of the peptide proton are summarized in Figure 5. In this figure the reciprocal of the mean life time $1/\tau$ is given as a function of $1/a_{H^+}$. This figure shows that $1/\tau$ increases linearly with $1/a_{H^+}$ and is independent of GG concentration. The intercept indicates the existence of a reaction which is independent of hydrogen ion concentration. The peptide hydrogen can participate in exchange reactions I and II.

$$NH_3^+CH_2CONHCH_2COO^- + H_2O \overset{1}{\rightleftharpoons} NH_3^+CH_2CO\bar{N}CH_2COO^- + H_3O^+ \quad (I)$$

$$NH_3^+CH_2CONHCH_2COO^- + OH^- \overset{k_{II}}{\rightleftharpoons} NH_3^+CH_2CO\bar{N}CH_2COO^- + H_2O \quad (II)$$

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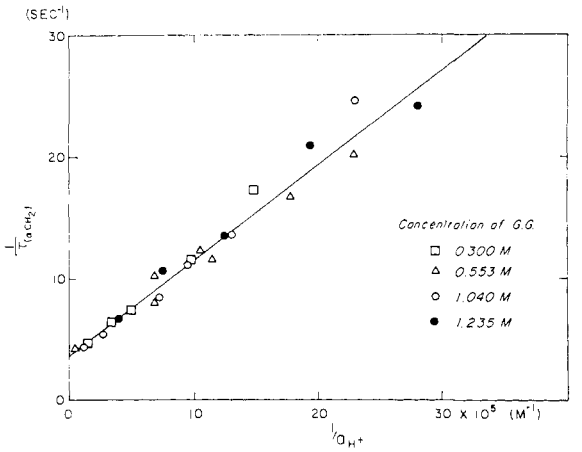

Figure 5. The reciprocal of the mean life time of the peptide hydrogen ($1/\tau$) as measured from the line shape of the α-methylene spectra as a function of $1/a_{H^+}$ (23 ± 1°).

The exchange rate is given by the sum of the exchange reactions

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

Thus the value of the intercept in Figure 3 equals $k_I[H_2O]$ and the slope $k_{II}K_w$. It was found that $k_I[H_2O] = 3.5$ (sec.$^{-1}$)[16] and $k_{II} = 7.8 \times 10^8$ (M sec.)$^{-1}$.

(16) Another possibility to be considered is the intramolecular reaction

$\langle NH_3^+CH_2CONHCH_2COO^- \rightleftarrows NH_3^+CH_2CO\bar{N}CH_2COOH \rangle$

Since this reaction, as measured by us, must involve a water molecule (see discussion for reaction III for sarcosine in the zwitterion form),[12] we cannot distinguish between the intramolecular reaction and reaction I. The value of the rate constant, k_I, should be looked upon as a sum of the two reactions.

The mechanism and rate of exchange of N-methylacetamide is considered to represent the exchange behavior of short peptides where there are no internal hydrogen bonds. It is natural, therefore, to compare our results to that reported by Berger, Loewenstein, and Meiboom.[10] The rate of exchange reaction of the peptide hydrogen with OH^- ion, k_{II} for GG, is 1.5×10^2 times greater than the corresponding rate constant for N-methylacetamide. This is due probably to the presence of the adjoining positively charged NH_3^+ which will make the peptide hydrogen of GG more positive. A similar behavior of the influence of the NH_3^+ can be drawn when we compare the ionization constant of the carboxylic group of glycine to that of acetic acid.[17] The presence of the positively charged

$$k_{COOH}(\text{glycine})/k_{COOH}(\text{AcOH}) = 2.6 \times 10^2$$

NH_3^+ in glycine causes the proton of the carboxylic group to become more positive and increases the acid dissociation.

The exchange of GG is different, however, from that of N-methylacetamide in one respect. We did not observe any exchange in the acidic range although we went down to pH 0.0. This result is also in contrast to acid-catalyzed exchange reported for glycylglycine.[8]

Acknowledgement. The author wishes to express his gratitude to Dr. E. D. Becker for his valuable discussions and to Mr. R. B. Bradley for maintaining the n.m.r. spectrometer in top condition.

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Mass Spectrometry in Structural and Stereochemical Problems. LX.[1] The Electron Impact Induced Fragmentation of Steroidal Dimethylamines[2]

Z. Pelah, D. H. Williams, H. Budzikiewicz, and Carl Djerassi

Contribution from the Department of Chemistry, Stanford University, Stanford, California. *Received September 4, 1964*

The mass spectra of a number of dimethylaminoandrostanes and dimethylaminocholestanes have been measured. The most abundant ions in the spectra correspond to immonium species, whose formation is readily understandable, and even predictable, in terms of charge localization on nitrogen in the molecular ion and subsequent fragmentation by rational bond homolyses and hydrogen transfers. Dimethylamino compounds, like ethylene ketals, should be regarded therefore as highly desirable derivatives for mass spectrometric purposes.

Introduction

The ability of a dimethylamino function at C-3 or C-20 in the steroid nucleus to direct electron impact induced fragmentation in such compounds has previously been noted.[3-5] All 3-dimethylamino steroids that have so far been investigated, and which do not

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