# Determination of $^{15}N$ Isotope Effects on the Acid–Base Equilibria of Amino Groups in Amino Acids by $^{13}C$ NMR

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*Received April 12, 1993*

## Introduction

$^{15}N$ kinetic isotope effects are used extensively to elucidate the mechanisms of enzyme-catalyzed reactions involving amino acids.[1,2] Quantitative analysis of $^{15}N$ kinetic isotope effects in terms of a chemical mechanism often requires the use of a value for the isotope effect on the acid–base equilibria of the ammonium group of the amino acid.[3–7] However, the only $^{15}N$ equilibrium isotope effect that has been reported for an amino acid is for phenylalanine,[8] and thus this value is generally used in such analyses.

$^{14}N/^{15}N$ isotope effects on acid–base equilibria are also of interest in view of the recent demonstration that $^{14}N/^{15}N$ isotopomers can be separated by reverse-phase liquid chromatography.[9,10] The separation is based on isotope effects on the acidity or basicity of functional groups. For example, it was demonstrated that the two $^{14}N/^{15}N$ isotopomers of aniline can be separated based on the small difference in basicity of the amino groups of $C_6H_5{}^{14}NH_2$ and $C_6H_5{}^{15}NH_2$. To design chromatographic conditions for the efficient separation of isotopomers, it is necessary to optimize the mobile phase not only for retention and peak shape but also for maximum difference between the $pK_a$s of the isotopomers.[11]

Nuclear magnetic resonance (NMR) spectroscopy provides a direct method for determination of equilibrium isotope effects on acid–base equilibria.[12] The method is based on the simultaneous measurement of chemical shift titration curves for the isotopically substituted compounds. This method has been used to measure $^{2}H$ and $^{18}O$ equilibrium isotope effects on the acid–base chemistry of formic acid,[12] the $^{18}O$ isotope effect on deprotonation of several dicarboxylic acids,[13] and the $^{18}O$ isotope effect on the acid–base chemistry of the phosphate groups of glycerol phosphate and adenosine triphosphate.[14,15]

In the present study, we have investigated the use of this method for the direct measurement of $^{15}N$ equilibrium isotope effects for deprotonation of the ammonium groups of amino acids. It is shown that, although the equilibrium isotope effect cannot be obtained from chemical shift titration curves for the alkyl carbon directly attached to the amino nitrogen, precise values can be obtained from chemical shift titration curves for the carboxyl carbons. The $^{14}N/^{15}N$ equilibrium isotope effect is reported for glycine.

## Experimental Section

Glycine was obtained from Aldrich. $^{15}N$-enriched (99%) glycine was supplied by MSD Isotopes. Solutions were prepared in 90% $H_2O$/10% $D_2O$ and 1,4-dioxane was added as an internal chemical shift reference. To minimize variations in ionic strength, the solution pH was initially adjusted to ~12 with NaOH. The $^{13}C$ NMR spectrum was measured, and then the pH was decreased by addition of concentrated HCl and $^{13}C$ NMR spectra measured at appropriate pH values. $^{13}C$ NMR spectra were measured at 125.7 MHz with WALTZ decoupling. The solution temperature was 25 ± 0.1 °C.

## Results and Discussion

The determination by NMR of equilibrium isotope effects on acid–base reactions is based on the dependence of chemical shifts on the protonation state of proximate acidic groups. Proton exchange is rapid on the NMR time scale, and thus the chemical shifts are weighted averages of the chemical shifts of the resonances for the protonated and deprotonated species:

$$\delta = f_p\delta_p + f_d\delta_d \quad (1)$$

where $\delta$ is the observed chemical shift and $f_p$ and $f_d$ the fractional concentrations and $\delta_p$ and $\delta_d$ the chemical shifts of the protonated and deprotonated species. Since the acid dissociation constants of isotopomers are different, $f_p$ and $f_d$ and thus the pH dependence of the chemical shift titration curves for isotopomers are different. The equilibrium isotope effect can be determined most precisely from the difference $\Delta$ between the chemical shifts of a pair of resonances for the two isotopomers when they are partially titrated.[16] The equilibrium isotope effect is related to $\Delta$ by eq 2:

$$\Delta = \Delta_d + \frac{Rn(\Delta^{15})}{Rn - n + 1} - n(\Delta^{14}) \quad (2)$$

where $\Delta_d = \delta_d^{14} - \delta_d^{15}$, $\Delta^{14} = \delta_d^{14} - \delta_p^{14}$, $\Delta^{15} = \delta_d^{15} - \delta_p^{15}$, $R = K_a^{14}/K_a^{15}$ and $n$ is the fractional concentration of the

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(8) A value of 1.016 ± 0.002 was obtained for the $^{15}N$ equilibrium isotope effect for the deprotonation of the ammonium group of phenylalanine ($^{15}K_a/^{14}K_a$) from the pH dependence of the $^{15}N$ kinetic isotope effect on the phenylalanine ammonia-lyase catalyzed elimination of ammonia.[3]
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(10) This method offers an attractive alternative to the chemical synthesis of isotopically-enriched compounds which cannot conveniently be prepared by biosynthesis e.g. unnatural amino acids.
(11) Equilibrium isotope effects, and thus $\Delta pK_a$, are a function of solvent composition. For example, the $^{16}O/^{18}O$ equilibrium isotope effect for formic acid decreases as the acetone content of the solvent increases.
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(16) Acid dissociation constants for the isotopomers can be obtained directly from the chemical shift–pH titration curves. In principle, the equilibrium isotope effect can be obtained from the $pK_a$ values; however, such values lack the necessary precision, primarily due to errors associated with the pH measurement.

0022-3263/93/1958-4487$04.00/0 

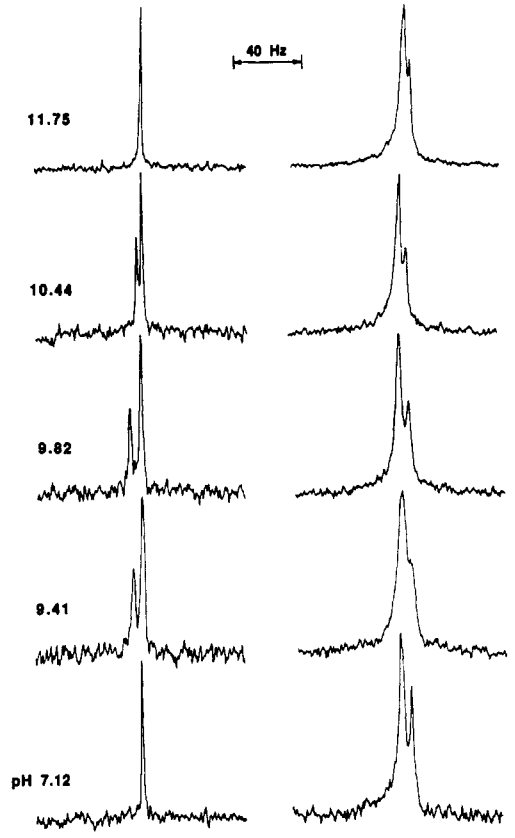


**Figure 1.** $^{13}C$ NMR spectra of a solution containing 0.1 M [$^{14}N$]-glycine and 0.2 M [$^{15}N$]glycine. The resonances on the left are for the carboxyl carbons; those on the right are for the methylene carbons. The chemical shifts of the resonances (left to right) are: pH 7.12, 173.304, 42.554, and 42.505 ppm; pH 9.41, 175.915, 175.872, 43.446, and ~43.404 ppm; pH 9.82, 177.908, 177.859, 44.153, and 44.104 ppm; pH 10.44, 180.938, 180.908, 45.228, and 45.192 ppm; and pH 11.75, 182.219, 45.690, and 45.659 ppm.

protonated form of the $^{14}N$ compound; n is determined from the chemical shift of the resonance for the $^{14}N$ compound using the relationship $n = (\delta - \delta_d)/(\delta_p - \delta_d)$.

$^{13}C$ NMR spectra were measured as a function of pH for solutions containing both $^{14}N$- and $^{15}N$-glycine. Representative spectra are presented in Figure 1 for a solution which contained 0.1 M [$^{14}N$]glycine and 0.2 M [$^{15}N$]glycine. The series on the left is for the carboxyl carbon; that on the right is for the methylene carbon. For both carbons, the spectra are plotted so that the resonances for [$^{15}N$]-glycine line up; however, the resonance for the carboxyl carbon actually shifts to high frequency by 9.0450 ppm over this pH range, while that for the methylene carbon shifts to higher frequency by 3.1634 ppm.[17] The chemical shifts for both pairs of resonances at each pH are given in the figure legend.

With $^1H$-decoupling, the resonance for the methylene carbon of [$^{14}N$]glycine is a singlet while that for $^{15}N$-glycine is a doublet. The $^{13}C$–$^{15}N$ coupling constant for [$^{15}N$]-glycine changes from 6.8 Hz at pH 6.21 to 4.5 Hz at pH 11.75. The singlet for [$^{14}N$]glycine overlaps one component

(17) Although the methylene carbon is closer, the chemical shift of the carboxyl carbon changes more upon deprotonation of the ammonium group. This is generally the case for amino acids. For example, the average change in chemical shift of the resonance for $C_\alpha$ of a series of amino acids is 1.57 ppm, while that for the carboxyl carbon is 8.77 ppm (Rabenstein, D. L.; Sayer, T. L. *J. Magn. Reson.* **1976**, *24*, 27–39).

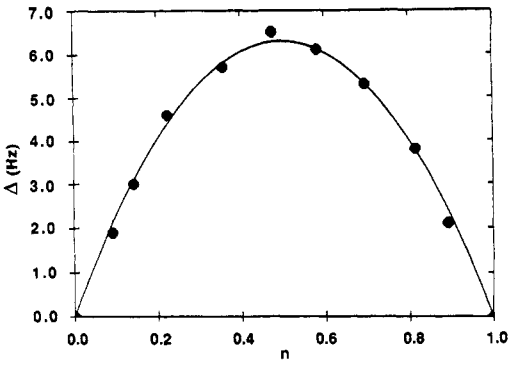


**Figure 2.** The difference between the chemical shifts of the carboxyl carbon resonances of the [$^{14}N$]- and [$^{15}N$]glycine as a function of the fractional protonation of the [$^{14}N$]glycine. The smooth curve through the points is the theoretical curve calculated using the value obtained for $R$ from a nonlinear least squares fit of the data to eq 3.

of the [$^{15}N$]glycine doublet, rather than being located at the center of the doublet, due to a nitrogen isotope effect on the chemical shift of the methylene carbon ($\delta$([$^{14}N$]-glycine) – $\delta$([$^{15}N$]glycine) = 3.1 Hz at pH 6.21 and 2.0 Hz at pH 11.75). Because of overlap and broadening of the methylene carbon resonances over the pH range where the ammonium group is titrated and the relatively small change in the chemical shift of the methylene carbon upon titration of the ammonium group,[17] the chemical shift difference $\Delta$ could not be measured with high precision and consequently attempts to determine the equilibrium isotope effect by fitting the data to eq 2 were unsuccessful.

In contrast, a precise value was obtained for the equilibrium isotope effect from the chemical shift data for the carboxyl carbon. The carboxyl carbon resonances for both [$^{14}N$]glycine and [$^{15}N$]glycine are singlets. At pH 7.12 and 11.75, where the glycine nitrogen is in the ammonium and amino forms, respectively, the two singlets overlap, indicating no $^{14}N/^{15}N$ isotope effects on the carboxyl carbon chemical shifts. However, over the pH range where the ammonium groups are titrated, the two singlets separate due to differences in the acid dissociation constants for [$^{14}N$]- and [$^{15}N$]glycine. Specifically, the resonance for [$^{14}N$]glycine shifts downfield more rapidly than that for [$^{15}N$]glycine as the pH is increased from 7.12, as expected since the acid dissociation constant for [$^{14}N$]glycine is expected to be larger.[3] The chemical shift difference $\Delta$ is plotted as a function of the fractional protonation of [$^{14}N$]glycine in Figure 2. Since $\delta_p^{14} = \delta_p^{15}$ and $\delta_d^{14} = \delta_d^{15}$ for the carboxyl carbon, eq 2 simplifies to:

$$\Delta = \frac{Rn\Delta^{14}}{Rn - n + 1} - n\Delta^{14} \qquad (3)$$

A value of $R = 1.0224 \pm 0.00026$ was obtained by fitting the data in Figure 2 to eq 3,[18] which corresponds to $pK_a$-($^{15}N$) – $pK_a$($^{14}N$) = 0.00962 ± 0.00011. The smooth curve through the points is the theoretical curve calculated using this value for $R$.

$pK_a$ values of 9.767 ± 0.009 and 9.776 ± 0.009 were obtained for [$^{14}N$]glycine and [$^{15}N$]glycine, respectively, from separate fits of the same carboxyl carbon chemical

(18) The curve-fitting procedure involved first fitting the data to eq 3 by the simplex method, followed by nonlinear least squares fitting of the data using the simplex values as initial estimates.

shift vs pH data.[19] The precision of the $pK_a$ values is considerably less than that of the $R$ and $\Delta pK_a$ values calculated above from the same data, because the precision of the $pK_a$ values is affected by the precision of the pH measurements.

The results presented here demonstrate that a precise value can be obtained for the $^{14}N/^{15}N$ equilibrium isotope effect on the acid–base chemistry of the amino group of glycine and presumably other amino acids, by NMR. Although the equilibrium isotope effect could not be determined from chemical shift titration data for the carbon directly attached to the amino group, a precise value was obtained from the carboxyl carbon data. Since resonances for the carboxyl carbons of other amino acids also shift by similar amounts,[17] it is expected that this will be a general method for the precise measurement of $^{14}N/^{15}N$ equilibrium isotope effects on the acid–base chemistry of the ammonium groups of amino acids.

(19) The $pK_a$ values were calculated by nonlinear least squares fits of the carboxyl carbon chemical shift vs pH data to the equation $\delta = \delta_p + (K_a/([H^+] + K_a))(\delta_d - \delta_p)$ where $\delta$ is the observed chemical shift and $\delta_p$ and $\delta_d$ the chemical shifts of $H_3N^+CH_2CO_2^-$ and $H_2NCH_2CO_2^-$.

**Acknowledgment.** This research was supported in part by the University of California, Riverside Committee on Research.