The Origin of the Anomalous pH Dependence of the ^{15}N-{^1H} Nuclear Overhauser Effect of Amino and Amide Nitrogens

Sir:

Recent ^{15}N NMR studies[1,2] of glycine-^{15}N have shown that the nuclear Overhauser effect (NOE) of the amino nitrogen obtained by proton broad band noise decoupling is pH dependent. The reduction in the ^{15}N-{^1H} NOE of glycine-^{15}N, which is observed as the pH is increased, has been attributed both to scalar relaxation produced by modulation of ^{15}N-^1H spin coupling interaction by pH dependent proton exchange[1] and to spin-rotation relaxation produced by overall and internal rotational correlation times that are related to the charge state of the molecule.[2] These large changes in the ^{15}N-{^1H} NOE of glycine-^{15}N have suggested that the NOE could be used as a convenient probe of proton exchange and rotation of ^{15}N-enriched macromolecules. Furthermore, these studies revealed an anomalous broadening of the amino ^{15}N resonance close to its pK_a, which were attributed to both intermediate rates of exchange of glycine between its zwitterionic and anionic states[1] and to chemical shifts of the amino resonance produced by the coincidence of the proton exchange rate and Larmor frequency of nitrogen, which affect the imaginary part of the relaxation matrix.[2] These effects might be of considerable potential importance in the interpretation of ^{15}N line widths.

We have observed an analogous pH dependence of the ^{15}N-{^1H} NOE of the amide, as well as the amino, nitrogens, of glycyl-^{15}N-glycine-^{15}N and similar anomalous broadening of amino resonance near its pK_a.[3] We here report that all these *pH dependent phenomena observed in glycylglycine, as well as glycine, arise from contamination by paramagnetic impurities.* In order to alert investigators studying the NMR properties of ^{15}N-labeled amino acids and peptides to the effects of minute amounts of paramagnetic impurities, we report the following anomalous pH dependence of spin-lattice relaxation times, NOE enhancements, and line widths observed in standard preparations of glycylglycine. We also report a convenient and efficient method for the removal of these impurities, as well as the NMR properties of purified glycylglycine and glycine and their theoretical implications.

Glycyl-^{15}N-glycine-^{15}N prepared by conventional synthetic methods[4] and used without further purification, displays amino and amide ^{15}N resonances for the cationic, zwitterionic and anionic states at 6.3, 6.3, −5.4 and 94.7, 88.9, 88.9 ppm (downfield from 4 *M* $^{15}NH_4NO_3$ in 2 *M* HNO_3), respectively, when measured at 1 *M* concentrations in H_2O at 9.12 MHz on a Bruker HFX-90 spectrometer.[5] Over the pH range of 0 to 5, the undecoupled amino resonance is initially broadened (50 Hz at pH 2.2) by proton exchange modulation of the ^{15}N-^1H spin coupling, but narrows as the pH is increased, and has a line width of 6 Hz at pH 5.0, and a T_2 time of 0.17 sec (determined by a Carr-Purcell sequence on a Bruker SXP-90). The spin-lattice relaxation (T_1) time of the amino nitrogen was 0.58 sec at pH 5.5, measured on a Bruker SXP-90 spectrometer by a 180°, τ, 90°, pulse sequence at 9.12 MHz, as compared to the T_1 relaxation time of 5.26 sec measured for the amide nitrogen at the same pH by a 90°, τ, 90° pulse sequence on a Bruker HFX-90 spectrometer. Proton broad band noise decoupling eliminates line broadening caused by proton exchange; however, the NOE ($I_{decoupled}/I_{coupled}$) of the amino resonance is pH dependent and decreases from −2.0 at pH 2 to +1 at pH 5.0. The NOE is temperature dependent, being 0 at 34° and −3.1 at 5° at pH 3.4. The NOE is also concentration dependent, being −2.0 for 1 *M* solutions and −2.9 for 0.1 *M* solutions. At pH 7.0 both the coupled and decoupled amino resonance begin to broaden. Between pH 8 and 11 the resonance cannot be observed, but reappears by pH 12. The undecoupled amide resonance, which is a doublet, $^1J_{NH}$ = 94 Hz, below pH 6, is broadened over the pH range of 7-9 (30 Hz at pH 8.5) by base catalyzed proton exchange. The decoupled amide resonance is observed as a narrow signal over the entire range of pH 0-12. However, the NOE of amide resonance, which between pH 0 and 10 was −3.9, decreases as the amino resonance reappears and has a NOE of zero at pH 12.5

Table I. NMR Properties of Glycylglycine

		pH 5		pH 8.3		pH 12.3	
		Non purified	Purified	Non-purified	Purified	Non-purified	Purified
NOE[a]	Amino	+1	−3.5	*b*	−2.8	+1	−3.8
	Amide	−3.9	−3.7	−3.9	−3.9	0	−3.8
T_1 (sec)	Amino	0.58	4.6				
	Amide	5.26	4.7				

[a] NOE = $\eta = I/I_0$; [b] The coupled and decoupled signals are broadened beyond detection.

The attenuation of amino NOE does not appear to arise from proton exchange, since it is completely absent long before the rate of exchange[6] approaches the difference in nitrogen and proton Larmor frequencies, ($\omega_{^{15}N} - \omega_H$).[7] Furthermore, Leipert and Noggle[2] and ourselves[3] have shown that scalar relaxation produced by exchange modulation of the ^{15}N-^1H spin interaction cannot reduce the ^{15}N-{^1H} NOE by more than 0.01%. Although spin rotational relaxation rates of the order of the dipolar relaxation rate have been calculated for ammonia[8] and amino groups[2] and have the same temperature dependence as was observed, there is no reason why rotational correlation times should be so pH dependent in glycylglycine. The behavior of the amide NOE and the amino line width bears a striking resemblance to the behavior of the proton resonances of glycylglycine in the presence of 10^{-5} *M* Cu^{2+}.[9] Below pH 11 only the N-terminal methylene proton resonance is broadened by Cu^{2+}, while at higher pH the N-terminal methylene proton resonance narrows and the C-terminal methylene resonance broadens.[9] The coordination of glycylglycine to Cu^{2+} has a ΔF = −9.1 kcal/mol,[10] which is in agreement with direction of the effect of temperature on the NOE. Concentrations of paramagnetic ion two orders of magnitude less than those required for line broadening have been reported to quench completely ^{13}C-{^1H} NOE.[11] We analyzed our solutions of glycylglycine by atomic absorption spectrometry (Perkin-Elmer 306, 303-6105 M 1856 hallow cathode lamp) and indeed found 10^{-6} to 10^{-7} *M* Cu^{2+} to be present.

The presence of paramagnetic ions in solutions of glycine-^{15}N has been conventionally ruled out by the effect of back-titration[1] or addition of EDTA.[2] Back-titration only establishes the presence of paramagnetic impurities in the acid or base that is used to adjust the pH and neglects the fact that the substance being measured may have been contaminated during its synthesis. The effect of EDTA can be misleading, since EDTA together with glycine or glycylglycine can form ternary complexes with transition metal ions.[12]

We have found the following procedure to be relatively rapid and convenient method for the removal of paramagnetic impurities; Chelex-100 (Biorad) is activated by treatment with NH_4OH, H_2O, HCl, and H_2O by the method of Willard et al.[13] For measurements above pH 5, 1 ml of 1 *M* solutions of glycylglycine or glycine is shaken in a plastic

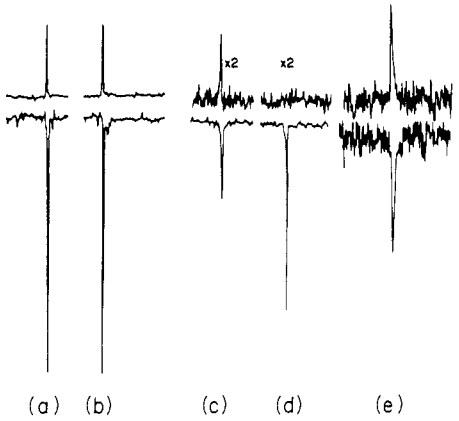

Figure 1. The coupled (upper) and proton broad band noise decoupled (lower) ^{15}N NMR spectra of the glycyl-^{15}N-glycine (a) amino nitrogen, pH 12.3; (b) amide nitrogen, pH 12.3; (c) the amino nitrogen, pH 8.3; (d) the amide nitrogen, pH 8.3; and (e) the glycine-^{15}N amino nitrogen, at pH 9.3. The spectra were obtained from 32 90° pulses collected on 4 K data points over a spectral range of 3000 Hz, using a 10-sec delay between pulses. No exponential time constant was used. NOE enhancements were measured from the integrated intensities.

vial with 0.25 ml of settled Chelex-100 for a few minutes. The suspension is then filtered through a cotton plug directly into an NMR tube. Addition of HCl can be used to adjust the pH of the filtered solution below pH 5 if desired. It should be noted that glycine and glycylglycine bind to the resin to some extent.

The NOE enhancements and T_1 times for non-purified and purified glycylglycine are compared in Table I. Chelex-treated solutions of glycyl-^{15}N-glycine-^{15}N (0.5-1 M) display an amino resonance, whose line width (2 Hz) was pH independent and whose NOE's were −3.5, −2.8, −3.8, at pH 5.1, 8.3 (Figure 1c), and 12.3 (Figure 1a), respectively. The T_1 relaxation time of the amino resonance was 4.6 sec at pH 7.0, compared to 4.7 sec for the amide resonance. The amide resonance displayed NOE enhancements of −3.7, −3.9,[15] and −3.8 at pH 5.0, 8.3 (Figure 1d), and 12.3 (Figure 1b), respectively. The NOE enhancement and line width of a Chelex-100 treated aqueous solution of glycine-^{15}N (~0.5 M) were measured at its pK_a's (pH 9.3) and were found to be −1.7 and 7 Hz, respectively (Figure 1e). The fact that total NOE enhancements of the amino group could not be obtained for glycylglycine and glycine at their pK_a values results, we believe, from the very high affinity of these compounds near their pK_a values for the trace amounts of Cu^{2+} which could not be totally removed by Chelex, rather than from some physical effect associated with the zwitterion-anion transition. This is demonstrated not only by the loss of the NOE but also by the extreme line broadening observed in nontreated samples.

It is interesting to note that dipolar relaxation dominates the amino nitrogen spin-lattice relaxation process, even at pH 12.5, at which the rate of proton exchange is between 10^9 and 10^{10} sec^{-1}.[6] The concept that the $^{15}N\{^1H\}$ NOE is attenuated by proton exchange, which has so permeated the ^{15}N NMR literature,[1,8,14] should be revised.

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(15) Even though the nondecoupled amide resonance at pH 8.3 is considerably broadened by chemical exchange of the amide proton, and is observed (Figure 1d) as a very weak broad signal, its intensity was readily measurable in the integration mode and was the same as the nondecoupled amino resonance.

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A New Type of Base Catalyzed Elimination of Hydrogen Halide from Strained Halocyclopropanes

Sir:

We recently reported a new general synthesis of chloro- and bromospiro[2.4]heptadienes.[1] These compounds are being investigated as part of a project directed toward the synthesis of spiro[2.4]heptatriene for which a ground state stabilization through spiroconjugation has been predicted.[2] Base catalyzed eliminations of hydrogen halide have been reported as general routes to cyclopropenes.[3] We therefore investigated the reaction of the halospiro[2.4]heptadienes with a variety of bases.

Treatment of 1-chloro-2-*tert*-butylspiro[2.4]heptadiene (cis:trans ratio = 20:80) with excess potassium *tert*-butoxide in *tert*-butyl alcohol at 78° or in tetrahydrofuran at 65° for several hours gave in 74% yield the *trans*-1-*tert*-butoxy-2-*tert*-butylspiro[2.4]heptadiene, isolated by column chromatography (Alumina Grade III, hexane) and by vacuum distillation as a colorless oil, $bp_{0.05}$ 42-45°, mp −3 to −7°. Vapor phase chromatography (6 ft, 0.25 in. OV-17 glass column) and NMR indicated the presence of only one isomer. On the basis of the observed 1,2-coupling constants in the cyclopropane ring of J_{12} = 6 Hz, we assign the trans stereochemistry for the *tert*-butoxy compound.[4] The results are summarized in Table I. Tables II (NMR spectra) and III (mass spectra) appear in the microfilm edition; see paragraph at end of paper regarding supplementary material.

The reaction of 1-chloro-2-*tert*-butylspiro[2.4]heptadiene (cis:trans ratio = 20:80) with excess KO-*t*-Bu and $KSCH_2C_6H_5$ (ratio 1:1) in *tert*-butyl alcohol at 78° for 5 hr gave one single isomer of 1-benzylmercapto-2-*tert*-but-