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Nitrogen-15 Chemical Shifts and $^1J_{^{15}N^1H}$ of Some Tripeptides Measured at the Natural Abundance Level†

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ABSTRACT: An indirect method combining double-resonance and difference spectroscopy has been used in order to determine ^{15}N chemical shifts and $^1J_{^{15}N^1H}$ in glutathione (in H_2O at pH 3 and under the same conditions with urea added) and in a series of tripeptides of the type Gly-Gly-L-X (with X = Glu, His, Val, Leu, and Ile) in H_2O and at two different pH values. This method has proved to be very efficient as long as the NH proton is not in exchange. The chemical shifts are shown to depend on the considered sequence and especially on the substituent in the γ position. One-bond couplings show some systematic trends which have been tentatively interpreted in terms of the *s* character of the N–H bond. Although these latter parameters seem of potential utility in structural determinations, additional data will be needed in order to rationalize their variations.

Nitrogen-15 chemical shifts have proved shifts to be valuable in the study of small peptides. These parameters provide information about the order of the residues in the sequence (Posner et al., 1975; Markowski et al., 1977; Gattegno et al., 1976; Hawkes et al., 1975). They furthermore allow the study of solvent and/or pH effects (Posner et al., 1975; Gattegno et al., 1976). In a general way, it may be safely assumed that, owing to the importance of nitrogen in molecules of biological

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interest, ^{15}N spectroscopy will in the near future tend to complement NMR results obtained via other nuclei. A major difficulty with ^{15}N spectroscopy arises from its poor sensitivity, relatively long relaxation times, and, to a lesser extent, negative nuclear Overhauser factors. Several instrumental improvements (use of wide-bore superconducting magnets and large sample tubes) nowadays allow one to perform nitrogen-15 chemical shift measurements under reasonable conditions (Witanowski et al., 1977). However, everyone does not have a superconducting magnet spectrometer at his disposal; furthermore, the above-mentioned experiments are performed with proton decoupling in order to enhance the signal to noise ratio. As a result, the J coupling information is lost. We wish to show here that an indirect method (AISEFT), previously published (Canet et al., 1974; Marchal & Canet, 1978), allows the measurements of both ^{15}N chemical shifts and $^1J_{^{15}N^1H}$ in peptides, using medium-size sample tubes (8-mm diameter) and thus small amounts of products, the total experimental time being generally smaller than that necessitated by direct observation. Especially, owing to the concentrations involved in the present work (i.e., 0.5 M at the natural abundance level), measurement of labeled compounds at a concentration as low as 2 mM becomes possible and should consequently be useful for tracer experiments, for example, in metabolism studies. The only condition of the applicability of the method is the existence of a nonexchanging hydrogen directly bonded to the nitrogen atom. This latter condition is precisely met in peptides, except for the terminal NH_2 residue.

Experimental Section

Except reduced glutathione (Merck, Darmstadt), the following tripeptides were purchased from Bachem: Gly-Gly-L-Glu, Gly-Gly-L-Val, Gly-Gly-L-Leu, Gly-Gly-L-Ile, and Gly-Gly-L-His. They were used without purification and dissolved in H_2O at a concentration of approximatively 0.5 M and studied in an 8-mm sample tube. H_2O was used rather than D_2O to avoid the replacement of the NH proton by deuterium in the case of an exchange which would be slow enough to allow the observation of the ^{15}N–^1H coupling. The 8-mm tube was centered in a 10-mm tube containing D_2O which provides the lock signal. For glutathione, a buffer solution was used in order to perform all measurements at pH 3. The other tripeptides were studied at two pH values given in the following tables. pH was adjusted by addition of HCl and measured with a Tacussel pH meter.

NMR measurements were carried out with a Bruker HX90 interfaced to a Nicolet 1080 computer (Centre régional de measures physiques de l'Academie de Nancy-Metz), modified as described previously (Marchal & Canet, 1978). The method has been described in detail elsewhere (Canet et al., 1974; Marchal & Canet, 1978) and only its main features are recalled here. It consists of observing ^{15}N satellites in the proton spectrum by subtracting ^{15}N decoupled spectra from ^{15}N coupled spectra. When ^{15}N irradiation is off resonance, the shifted satellites allow the determination of the ^{15}N chemical shift.

A 90-MHz proton pulse width of 15 μs which results in a flip angle of 20° and a repetition rate of 0.416 s (corresponding to a dwell time of 416 μs, the FID being stored in a 1K array) was used. The ADC input filter is set to 300 Hz in order to reduce the amplitude of the water signal. Fourier transformation is performed after zero filling up to 8K memory words. The power of the ^{15}N irradiation around 9.12 MHz, expressed as γH_2, is equal to 35 Hz. Noise modulation with ^{15}N frequency on resonance may be employed in order to determine solely one-bond coupling constants. At least two experiments with ^{15}N continuous wave irradiation at two different frequencies are needed for simultaneously measuring chemical shifts and coupling constants. The exact frequencies are measured with a Schlumberger frequency counter.

Usually 7 h is sufficient in order to obtain a satisfactory signal to noise ratio. Typical spectra are shown in Figure 1. These spectra can be compared with recently published results obtained by Yavari & Roberts (1979), who managed to measure the ^{15}N–^1H coupling in some lactams by direct observation of nitrogen-15. By taking into account the difference in sample size and concentration, we can estimate a 200-fold increase in sensitivity by the method used in the present work. It should be further mentioned that our spectrometer has no quadrature detection and does not seem to be peculiarly optimized for proton observation. Concerning the presence of an intense signal (the one arising from H_2O), we have recently shown (Marchal et al., 1979) that under certain conditions, surely met with here, the small signals are measured properly provided the computational procedures are considered as ideal, in spite of a relatively low resolution analogue to the digital converter.

Table I

Gly-Gly-L-X	pH	$^1J_{^{15}N^1H}$ (Hz) central Gly	$^1J_{^{15}N^1H}$ (Hz) X	chemical shift (Hz)[b] central Gly	chemical shift (Hz)[b] X
Gly-Gly-L-Glu	4.0	93.6	91.6	9 120 616 (270.7)	9 120 750 (256.0)
	1.9	94.8	93.5	9 120 625 (269.7)	9 120 721 (259.2)
Gly-Gly-L-His	3.5	95.1	93.2	9 120 618 (270.5)	9 120 733 (257.9)
	1.2	95.9	93.9	9 120 629 (269.3)	9 120 702 (261.3)
Gly-Gly-L-Val	5.2	[a]	92.4	[a]	9 120 744 (256.7)
	2.3	95.1	93.3	9 120 615 (270.8)	9 120 708 (260.6)
Gly-Gly-L-Leu	5.0	[a]	92.6	[a]	9 120 784 (252.3)
	2.7	94.8	93.9	9 120 616 (270.7)	9 120 749 (256.1)
Gly-Gly-L-Ile	5.4	[a]	92.1	[a]	9 120 756 (255.4)
	2.0	94.8	93.0	9 120 616 (270.7)	9 120 720 (259.3)

[a] NH proton in exchange. [b] The values in parentheses are in parts per million.

Table II

	glutathione in H_2O[a] Cys	glutathione in H_2O[a] Gly	glutathione in 5 M urea soln[a] Cys	glutathione in 5 M urea soln[a] Gly
chemical shift (Hz)[b]	9 120 748 (256.2)	9 120 657 (266.2)	9 120 748 (256.2)	9 120 657 (266.2)
$^1J_{^{15}N^1H}$ (Hz)	93.9	94.5	92.7	93.6

[a] pH 3.0. [b] The values in parentheses are in parts per million.

Results

Experimental results are displayed in Tables I and II. Because of exchangeable protons, ^{15}N chemical shifts of the NH_2-terminal residue could not be determined. In some cases, the same situation prevailed for the proton of central Gly, thus preventing any measurement on this residue.

The uncertainty of J values is of the order of 0.3 Hz, whereas it reaches 2 Hz (0.2 ppm) for resonance frequencies.

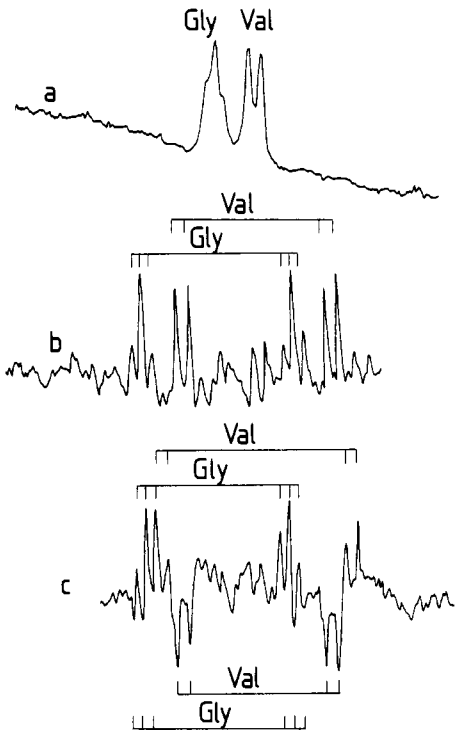

FIGURE 1: (a) Portion of the conventional ^{1}H spectrum relative to the NH resonances of Gly-Gly-L-Val in H_2O; pH 2.3; 16 scans. (b) AISEFT spectrum with ^{15}N irradiation on resonance, noise modulated (113 000 scans; experiment time = 14 h), showing the one-bond NH couplings in the Gly and Val peptidic fragments. Notice the quasi-elimination of resonances due to ^{14}N-containing molecules. (c) AISEFT spectrum with CW ^{15}N irradiation off resonance (56 000 scans; experiment time = 7 h). Normal satellites appear as lines down. Lines up correspond to the off-resonance irradiation and enable chemical shift determination.

The ^{15}N frequency resonance is given either in hertz or in parts per million. In the first case, the results are consistent with Me_4Si resonating at exactly 90 MHz. ^{15}N chemical shifts in parts per million are relative to that of pure nitromethane. This scaling is accomplished in two steps. The resonance frequency of *N*-methylacetamide (NMA) has first been determined under our standard experimental conditions (i.e., Me_4Si resonance at exactly 90 MHz). Since the NMA chemical shift with respect to that of pure nitromethane is known (Srinivasan & Lichter, 1977), it is then possible to report all our data according to this latter reference. The chemical shifts are given here according to the screening constant scale.

Discussion

We will first discuss chemical shifts. A prominent feature is the constancy of the central Gly chemical shift in the Gly-Gly-L-X series. This is in contrast with the results of Markowski et al. (1977), who studied an X-Gly-Gly series. In fact, this different behavior can be easily explained by the γ effect (Gattegno et al., 1976). In the tripeptides under investigation here, the side chain in the γ position with respect to the ^{15}N is the same, whereas in the X-Gly-Gly series it is different from one molecule to the other.

Concerning the glycyl residue in glutathione, we find a value similar to those reported by Markowski et al. (1977) (if we recalibrate their results to our chemical shift reference) involving tri- and dipeptides containing a COOH-terminal glycyl. This result confirms their conclusions regarding the insensitivity of this shift as long as nitrogen exists in a protonated form.

The chemical shifts of COOH-terminal residues follow qualitatively the parent amino acid variations. The pH effect is systematic irrespective to the considered residue and results in an upfield shift of 4 ppm when decreasing the pH by about 3 units. This behavior is in agreement with the conclusion of Gattegno et al. (1976).

We turn now to the one-bond nitrogen-15 proton coupling constants. Although the relative variation is not very important, some systematic trends are observed. The data presented here show a range of 91.6–95.9 Hz. Several salient features are noticeable: $^1J_{^{15}NH}$ is systematically larger in the Gly residue than in any other residue. For all peptidic NH bonds, $^1J_{^{15}NH}$ decreased with increasing pH. Upon the addition of urea to glutathione, a similar lowering is observed. The first effect, concerning the magnitude of $^1J_{NH}$ in other residues than glycyl, arises probably from the influence of the side chain. This could be thought as being due to a decrease in the *s* character of the NH bond. This already has been suggested by Binsch et al. (1964) and Bourn & Randall (1964) and is further supported by previous results obtained on simple amides (Marchal & Canet, 1975).

It is unlikely that $^1J_{NH}$ variations with pH result from a structural change in spite of the value obtained at pH 4–5 which at first glance could be interpreted as a cis–trans isomerism (Sogn et al., 1973). As a matter of fact, it is well-known that the amide fragment remains in a trans form, irrespective of the pH value. We would rather attribute this effect to a protonation of the peptidic C=O which probably results in an increase of the *s* character and, consequently, of the NH coupling. It must, however, be outlined that this type of argument is only valid in the limit of the assumption of an unmodified structure (Blanchard, 1977).

The $^1J_{NH}$ variation observed upon addition of urea to the solution is somewhat more difficult to explain. It presumably depends on a modification of the intramolecular hydrogen bond.

Conclusion

In the present communication, we have shown that determination of ^{15}N parameters in polypeptides is quite feasible with a conventional electromagnet spectrometer, provided a one-bond coupling exists between the nitrogen and proton. It can be inferred that the same technique, used in conjunction with wide-bore superconducting magnets, would allow one to perform measurements at much lower concentrations. Furthermore, one-bond coupling constants are obtained at the same time, and it is likely that the trends observed in the present work merit confirmation and rationalization from experimental as well as theoretical points of view. They presumably would lead to complementary information regarding structural modification and/or electronic distribution in each peptidic residue.

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Pulsed Electron Paramagnetic Resonance Studies of the Copper(II) Site in Galactose Oxidase†

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ABSTRACT: Two-pulse and three-pulse electron spin–echo decay envelopes for the nonblue copper protein galactose oxidase have been studied. Analysis of the modulation patterns indicates that the Cu(II) is coordinated to at least one and possibly two protein histidine imidazoles. Fourier cosine transforms of three-pulse data yield superhyperfine frequencies 0.55, 1.0, 1.53, and 4.1 MHz at a magnetic field H_0 = 3170 G. Computer simulations of the superhyperfine spectrum for a ^{14}N nucleus coupled to an electron spin indicate that these frequencies are due to ^{14}N in a quadrupolar field characterized by frequencies ν_+, ν_-, and ν_0 = 1.54, 1.0, and 0.54 MHz and coupled to the electron spin by a term $A\mathbf{I}\cdot\mathbf{S}$ where A/h = 1.8 MHz. Comparison of the two-pulse echo envelope for the native enzyme with the envelopes for CN^--, F^--, and imidazole-coordinated derivatives suggests that galactose oxidase contains a ligand other than imidazole which is readily displaced by any of these three. Linear electric field effect (LEFE) measurements were also made in order to compare the behavior of galactose oxidase with that of blue copper proteins. The form of the LEFE curves and the magnitude of the shifts were similar to those observed for nonblue copper centers and for a number of complexes where Cu(II) is coordinated by N or O. There was no resemblance to LEFE results obtained for blue copper proteins.

The nuclear modulation patterns observed in the electron spin–echo decay envelope have proved to be a useful means of identifying the ligands of paramagnetic ions in metalloproteins and of determining the coupling between nuclei belonging to these ligands and the unpaired electron spin (Mims & Peisach, 1976a, 1978, 1979; Mims et al., 1977; Zweier et al., 1979; Peisach et al., 1979). Electron spin–echo measurements of a different type in which measurements are made of the electronic field induced g shifts have also been useful for examining the symmetry properties of the complexes concerned (Peisach & Mims, 1973; Mims & Peisach, 1974, 1976b; Peisach et al., 1977) and for detecting the effects of charge transfer between a metal ion and its ligands (Peisach & Mims, 1978a).

We report here a series of studies on the single Cu(II) site of the "low-" or "nonblue" copper protein (Vänngård, 1972) galactose oxidase (Kosman et al., 1974; Ettinger, 1974; Giordano & Bereman, 1974; Giordano et al., 1974; Bereman et al., 1977). The experiments have been designed to investigate the Cu(II) coordination site in the native protein and to complement EPR studies (Giordano & Bereman, 1974; Giordano et al., 1974; Bereman & Kosman, 1977) and pulsed NMR[1] studies (Marwedel et al., 1975) which have focused on the coordination chemistry of this metal site.

Materials and Methods

Galactose oxidase (EC 1.1.3.9) was isolated as described (Kosman et al., 1974). Copper(II) diethylenetriamine cyanide complexes were prepared by optical titration of the copper amine complex in a 1:1 glycerol–H_2O mixture at pH 8.3 with partially neutralized KCN. Imidazole (Sigma Chemical Co.) was twice recrystallized from benzene following an initial treatment with Norite. $KC^{15}N$ (99 atom % ^{15}N) was purchased from Stohler Isotope Chemicals. KF was a zone-refined sample from Bell Laboratories.

Measurements of the electron spin–echo envelope were made as described by Mims & Peisach (1976a), and the LEFE measurements were made as described by Peisach & Mims (1973) and Mims (1974). The echo envelopes obtained in three-pulse experiments were Fourier transformed as described previously (Shimizu et al., 1979; Mims & Peisach, 1979).

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[1] Abbreviations used: EPR, electron paramagnetic resonance: LEFE, linear electronic field effect; NMR, nuclear magnetic resonance; shfs, superhyperfine structure.

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