CARBON-13 NUCLEAR MAGNETIC RESONANCE STUDIES ON (85% ^{13}C-ENRICHED GLY^{9}) OXYTOCIN

John H. GRIFFIN*, Robert ALAZARD, Carlo DIBELLO**, Ernest SALA, R. MERMET-BOUVIER and Paul COHEN

Service de Biochimie, Centre D'Etudes Nucléaires de Saclay, 91190 Gif-sur-Yvette and Laboratoire de Biochimie, UER 58, Université Pierre et Marie Curie, Paris, France

Received 18 November 1974
Revised version received 11 December 1974

1. Introduction

Carbon-13 nuclear magnetic resonance (^{13}C nmr) spectroscopy has only recently been applied to the study of peptides and peptide hormones [1,2]. A major obstacle to such studies has been the very low sensitivity of ^{13}C nmr. Fourier transform nmr techniques have extended the useful sensitivity of the method to study solutions containing peptide concentrations of 0.05 M to 0.5 M. Since the natural abundance of ^{13}C is only 1%, isotopic enrichment is capable of further enhancing the sensitivity of ^{13}C nmr spectroscopy. Selective enrichment of peptide hormones offers the additional advantage of giving the absolute assignment of the observed chemical shift values to particular carbon atoms in the peptide as recently shown in studies on the ^{13}C resonances of gramicidin-S(3). Such definite assignments can then be used in the interpretation of ^{13}C experiments which utilize the ^{13}C natural abundance spectra. ^{15}N enrichment of oxytocin has been used to define the assignment of NH protons in the proton nmr spectrum of the hormone [4]. Moreover, ^{13}C and ^{15}N enriched hormone molecules offer a new approach for characterizing conformational and microdynamical properties of neurohypophyseal peptides bound to their physiological carrier proteins, the neutophysins [5–8]. We describe here the synthesis and the ^{13}C nmr spectra of (85% ^{13}C enriched Gly^{9}) oxytocin. These results demonstrate the usefulness of selective ^{13}C enrichment of oxytocin for extending the working sensitivity of ^{13}C nmr spectroscopy to the millimolar concentration range and for assigning unambiguously the ^{13}C resonances which can be observed in the natural abundance spectrum [9–12].

2. Materials and methods

Uniformally 85% ^{13}C enriched amino acids were prepared in our laboratory in large scale by biosynthesis using algae, *Spirulina maxima*, grown on 85% enriched $^{13}CO_3HNa$ [13]. The synthesis of (85% ^{13}C enriched Gly^{9}) oxytocin was made following the general methods of solid phase peptide synthesis [14,15]. The following protected amino acids were obtained from Fox Chemical Co., Los Angeles: Boc–Leu, Boc–Pro, Boc–Ile, Boc–(S–p–MeO–Bzl)Cys, Boc–(O–Bzl)Tyr, Boc–Asn–ONp, and Boc–Gln–ONp. Boc–(85% ^{13}C enriched) Gly was prepared from the enriched amino acid using the method of Schnabel [16]. Methylene chloride was dried over $CaCl_2$ for 8 hr prior to its use. Triethylamine and DMF were purified by distillation. *N,N'* dicyclohexylcarbodiimide was used in methylene chloride for all couplings except that the protected Asn and Gln were incorporated into the growing peptide chain by reaction of the paranitrophenyl ester

* Supported in part by NIH Special Fellowship. Present address: Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, CA 92037, USA

** Centro di Studio per la Fisica delle Macromolecole del CNR, Istituto di Chimica Generale, Università di Padova, via Loredan 4, 35100 Padova, Italy.

derivatives in DMF. The ^{13}C enriched Boc–Gly was coupled to the chlorobenzyl-resin (Bio-Beads SX-1, 0.75 eq. per gm, BioRad Co., Richmond, Calif.) in refluxing ethanol in the presence of triethylamine for 24 hr. Deprotection of the Boc group was made with 25% TFA in methylene chloride. Neutralization was performed using 10% triethylamine in methylene chloride. For each residue two cycles of coupling were made, each one lasting at least 2 hr in the case of diimide coupling and at least 8 hr in the case of active paranitrophenyl esters. Cleavage of the final protected peptide chain from the resin was effected by ammonolysis according to Manning et al. [17] to give the protected peptide amide. Deblocking of the protected peptide amide was accomplished using anhydrous HF containing anisole as described by Sakakibara [18]. Oxidation of the deprotected oxytocin was made using potassium ferricyanide [19]. The product mixture was further purified according to Manning [17] using successively two Sephadex G-15 columns (1.2 X 140 cm) with elution by 50% acetic acid and 0.2 N acetic acid, respectively. Pharmacological assays of the final product (kindly performed by Professor Jard, Collège de France) showed the product gave 90% of the rat blood pressor activity and 100% of the hydroosmotic tonic activity of reference oxytocin (gift of Dr Guttmann, Sandoz, Basel. Thin layer chromatography of the final product on cellulose plates in BuOH:acetic acid:H_2O 75:10:25 followed by staining with either ninhydrin or a stain specific for disulfide groups showed a single spot with the mobility of reference oxytocin, $R_f = 0.53$. Acid hydrolysis of the product for 24 hr in sealed evacuated tubes containing constant boiling HCl followed by amino acid analysis using a Technicon amino acid analyzer TSM gave: Cys, 2.08; Tyr, 0.89; Ile, 0.97; Glu, 1.00; Asp, 0.88; Pro, 1.03; Leu, 1.05; Gly, 0.97. Also the synthetic hormone was shown to bind to neurophysin II with an affinity similar to that of oxytocin as measured by ultraviolet absorbance difference spectroscopy [8,20].

^{13}C nmr spectra were obtained on a Varian XL-100 12WG spectrometer operating at 25.5 MHz which was interfaced to a Varian 16K 620 f computer. An acquisition time of 0.8 sec was used for a spectral width of 5000 Hz with a resolution of 1.25 Hz. Proton noise decoupling was made with a Varian Gyrocode spin decoupler. Spectra were obtained at 30°C using 10 mm sample tubes containing 5 mM hormone solutions in D_2O, 0.1 M NaCl, pH 6.8 (uncorrected meter reading) or in DMSO-d_6. The deuterated solvent provided an internal deuterium lock signal. Either DMSO-d_6 itself or dioxane added to each sample containing D_2O provided an internal reference. Chemical shift values relative to internal tetramethylsilane (TMS) were calculated on the basis that the chemical shift of dioxane is 67.4 ppm downfield from external TMS and that the difference between external and internal TMS is 0.9 ppm, and that the central DMSO-d_6 resonance is 39.6 ppm downfield from internal TMS.

3. Results and discussion

The ^{13}C NMR spectrum of (85% ^{13}C enriched Gly^9) oxytocin at 5 mg/ml, i.e. 5 mM, in DMSO-d_6 is seen in fig. 1. Such a spectrum represents an extension of the working sensitivity of ^{13}C nmr of peptide hormones by at least an order of magnitude above natural abundance studies [2]. The carbonyl region of the spectrum between 167 and 177 ppm exhibits three resonance peaks as seen for 85% ^{13}C enriched free glycine [21]. The center peak at 171.0 ppm represents the chemical shift of the Gly^9 carbonyl carbon and it arises from molecules which contain a ^{13}C carbonyl carbon but a ^{12}C alpha carbon. The two sidepeaks represent a doublet which arises from ^{13}C–^{13}C coupling in those Gly^9 residues containing ^{13}C isotopes at both the carbonyl and alpha positions. The observed value of $^1J_{^{13}CO-^{13}C\alpha}$ is 50.4 Hz. For the Gly^9 alpha carbon of oxytocin, three peaks centered at 42.1 ppm appear in fig. 1 which partially overlap the DMSO-d_6 solvent peaks. The confident assignment of the alpha carbon peaks was aided by the expected observation that $^1J_{^{13}CO-^{13}C\alpha} = {}^1J_{^{13}C\alpha-^{13}CO} = 50.4$ Hz. For the ^{13}C enriched hormone in D_2O [5], the ^{13}C nmr spectrum similarly contained three simple peaks for each of the carbonyl and alpha carbons with the chemical shift values shown in table 1 and a ^{13}C–^{13}C coupling constant of 50.5 Hz. The existence of a single chemical shift value for each of the Gly^9 carbons of the enriched hormone shows that the glycine residue of oxytocin either exists in a single conformation or in multiple conformations which rapidly interconvert to give the single observed peaks.

The data in table 1 show that in changing solvent

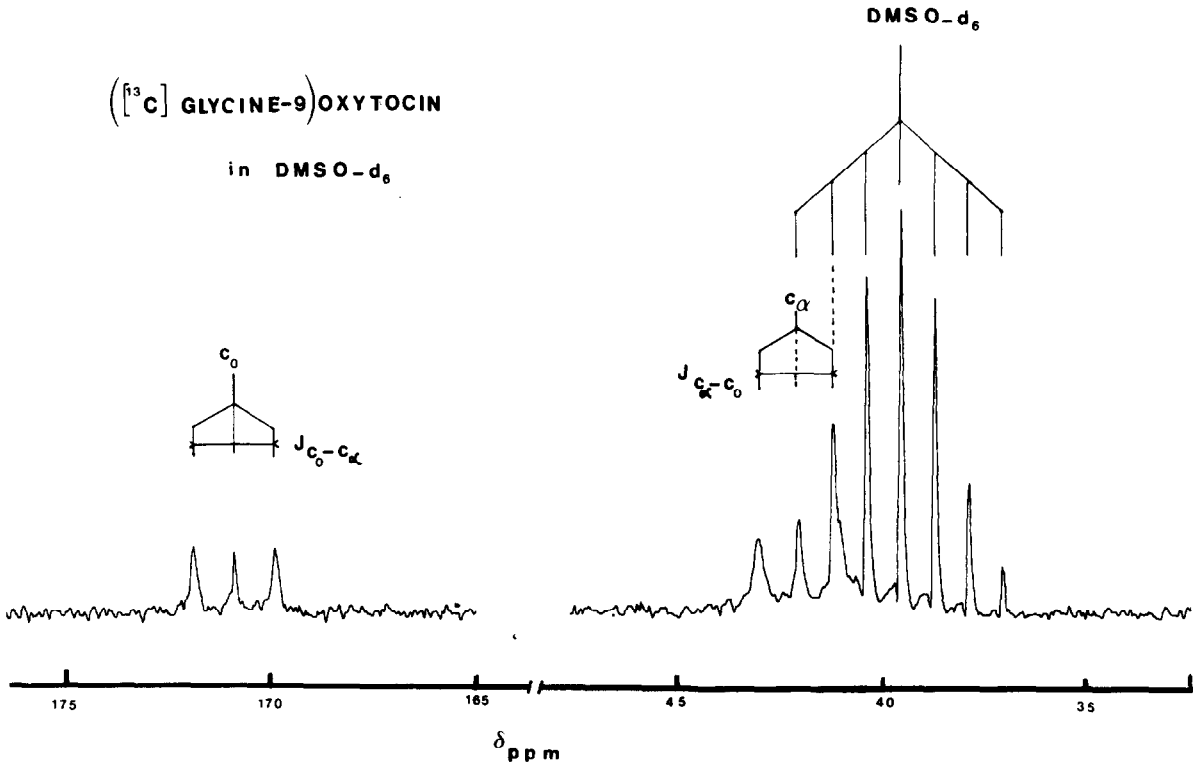

Fig. 1. The ^{13}C nmr spectrum of 5 mM (85% ^{13}C enriched Gly^9) oxytocin in DMSO-d_6. Spectrum obtained from 16,309 transients. Chemical shift values are given as downfield from TMS.

Table 1
^{13}C chemical shift values for oxytocin (ppm downfield from internal TMS)

Compound in DMSO-d_6	Gly^9 C_O	Gly^9 C_α	Reference
(^{13}C enriched Gly^9) Oxytocin	171.0	42.1	This study
Oxytocin, ^{13}C natural abundance	not given	42.3	Deslauriers et al. [9][a]
Oxytocin, ^{13}C natural abundance	170.6	44.0	Brewster et al. [10][a]
Compound in D_2O[c]			
(^{13}C enriched Gly^9) Oxytocin	174.0	42.0	This study, pH 6.5
Oxytocin, ^{13}C natural abundance	169.5	42.2	Lyerla and Freedman [11][b], pH 5.8
Oxytocin, ^{13}C natural abundance	174.2	42.4	Walter et al. [12], pH 6.6

[a] Chemical shift values calculated on the basis that reference CS_2 was reported at 192.8 ppm downfield from internal TMS (10), or that DMSO-d_6 was at 40.9 ppm from external TMS [9, personal communication].

[b] Chemical shift values calculated on basis that reference dioxane was reported at 126.1 ppm downfield from external TMS [11].

[c] Chemical shift values are given with respect to internal TMS assuming that the difference between external and internal TMS is 0.9 ppm.

from DMSO-d_6 to D_2O the carbonyl carbon resonance moves downfield by 3.0 ppm while the alpha carbon resonance does not change. Such large solvent effects on the chemical shift values of carbonyl carbon resonance have been previously reported for Pro–Leu–Gly NH_2 [22] and gramicidin-S [3]. Unfortunately, no clear correlation between the magnitude of these solvent-induced chemical shift changes and the presence of intramolecular H-bonds has yet been established.

The ^{13}C assignments established here by isotopic enrichment of Gly^9 in oxytocin are compared in table 1 with those previously proposed on the basis of natural abundance ^{13}C spectra [9–12]. The value of 42.1 ppm seen here for the alpha carbon of Gly^9 of oxytocin in DMSO-d_6 seems to confirm the value proposed by Deslauriers et al. [9] but disagrees with that of Brewster et al. [10]. The erroneous α-carbon assignment probably arose from the employed method of assignment [10] which consisted of studying the chemical shifts of the various CBZ-protected intermediate peptides used in the synthesis of oxytocin and comparing these values to those of oxytocin. Unfortunately, large changes in chemical shift values, including the beta carbon resonances of Cys^1 and Cys^6 between 40 and 45 ppm, were observed when the linear nonapeptide was oxidized to form the disulfide linked 20-membered ring, and this may have contributed ambiguity to the analyses [9,10]. Interestingly, Brewster et al. [10] showed using [^{2}H]Gly that the alpha carbon of Gly^9 in the protected nonapeptide has a chemical shift of 42.0 ppm, i.e. the same value (± 0.1 ppm) as established here using ^{13}C enriched (Gly^9) oxytocin. Brewster et al. then suggested that ring closure at Cys^1 and Cys^6 causes a 1.9 ppm downfield shift of the alpha carbon of Gly^9, a rather large shift which is not easily rationalized. For the carbonyl carbon of Gly^9 of oxytocin in D_2O, the proposed tentative assignment of Lyerla and Freedman [11] based on comparison to amino acid chemical shifts is in disagreement by 4.5 ppm with the chemical shift value established here (table 1). Consequently, it is clear that unambiguous assignments of nmr resonances are best made in the case of peptide hormones of the size of oxytocin by specific ^{13}C isotopic substitution.

Acknowledgements

We should like to thank Dr. P. Fromagoet for his continous interest and helpful support.

We gratefully acknowledge the kindness of Drs Tran-Dinh, Haar, Fermandjian, and Toma in making available the Varian XL-100 spectrometer.

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