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Carbon-13 Nuclear Magnetic Resonance Studies of the Binding of Selectively ^{13}C-Enriched Oxytocins to the Neurohypophyseal Protein, Bovine Neurophysin II†

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ABSTRACT: Complex formation between bovine neurophysin II and oxytocin molecules containing 85% ^{13}C enrichment in specific amino acid residues was studied using ^{13}C nuclear magnetic resonance spectroscopy. Chemical shift and relaxation time values of the analogue [^{13}C-Leu^3]oxytocin, [^{13}C-Gly^9]oxytocin, and the doubly labeled [^{13}C-Ile^3 Gly^9]oxytocin were obtained for the hormones in the absence and presence of neurophysin. The results showed that certain ^{13}C nuclear magnetic resonance parameters of residue 3 but not of residue 9 of oxytocin are altered upon binding to neurophysin. These observations suggest that residue 3 but not residue 9 is involved in the protein–hormone interaction, and they demonstrate the general applicability of selective ^{13}C enrichment for the study of peptide–protein interactions.

Nuclear magnetic resonance (NMR) spectroscopy can give detailed molecular information about biochemical systems in solution (Roberts and Jardetzky, 1970; Dwek, 1973; e.g., Lasker and Milvy, 1973). With the recent availability of signal enhancement by Fourier transform techniques, ^{13}C NMR studies of biomolecules became feasible and several observations of individual carbon resonances in natural abundance spectra of proteins have been reported (Allerhand et al., 1973; Oldfield et al., 1974; Shindo and Cohen, 1976). ^{13}C NMR studies have also provided new information about peptides and peptide hormones (Deslauriers and Smith, 1975). The studies of selectively ^{13}C-enriched oxytocins reported here were undertaken, first, to demonstrate the utility of ^{13}C enrichment in the study of peptide hormones themselves since the natural abundance of ^{13}C is only 1.1% and, second, to explore the value of selective ^{13}C enrichment in studying the molecular interactions between oxytocin and the neurohypophyseal hormone binding protein, bovine neurophysin (e.g., Walter, 1975). Complex formation between neurophysin and the neurohypophyseal hormones provides a model system for the study of

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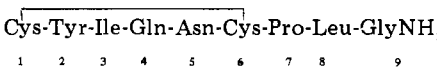
protein–peptide hormone interactions and it is hoped that methods used here may eventually be applicable to study interactions between peptide hormones and their receptors. Neurophysin is a well-characterized protein with a molecular weight of 10 000 and with a known sequence (Walter et al., 1971; Schlesinger and Walter, 1972; Wuu and Crumm, 1976; Chauvet et al., 1976). The complex stoichiometry and mechanism of the hormone binding reaction have been recently defined such that a dimer of neurophysin (20 000 molecular weight) normally binds two oxytocin molecules (Camier et al., 1973; Nicolas et al., 1976). Proton NMR studies have shown that oxytocin exchanges rapidly between its bound state and solution (Cohen et al., 1972; Alazard et al., 1974) and, therefore, that the observed NMR signals of the hormone in the presence of a limited amount of neurophysin are the weighted average of the signals representing the bound and the free states (Sykes and Scott, 1972). This fact, combined with the selective ^{13}C enrichment of oxytocin reported here, allows observation of ^{13}C NMR spectra of hormone–protein complexes at 1 mM concentration.

The structure of oxytocin is



Initially, ^{13}C enrichment of residues 3 and 9 of oxytocin were undertaken, and this report presents some of our observations on the binding of [85% ^{13}C-Leu^3]oxytocin, [85% ^{13}C-Gly^9] oxytocin, and [85% ^{13}C-Ile^3, Gly^9]oxytocin to bovine neurophysin II.

Materials and Methods

Uniformly 85% ^{13}C enriched amino acids were prepared at the CEN, Saclay, by biosynthesis using algae grown on 85% ^{13}C-labeled sodium bicarbonate followed by hydrolysis of the extracted protein and isolation of each amino acid (Tran-Dinh et al., 1974). The synthesis of oxytocin was made using the solid phase peptide synthesis method (Merrifield, 1965) with the modifications previously described (Griffin et al., 1975). The ammonolytic cleavage of the crude peptide from the solid phase support, the HF deblockage, and the ferricyanide oxidation have been described as well as the purification of the crude peptide on Sephadex G-15 columns according to Manning et al. (1970). A final additional purification step was necessary to separate oxytocin from a minor contaminant. The contaminant migrated with a relative mobility of 0.60 on cellulose plates in BuOH–acetic acid–H_2O (15:2:5) compared with reference oxytocin with a relative mobility of 0.53. The separation was effected on a 2.5 × 50 cm column of cellulose (Whatman CM31) with elution by BuOH–acetic acid–H_2O (15:2:5) at a flow rate of 12 mL/h. Analysis of each oxytocin preparation by thin-layer chromatography yielded a single spot upon staining with ninhydrin or a specific disulfide stain (Griffin et al., 1975). Acid hydrolysis of each oxytocin preparation followed by amino acid analysis using a Technicon analyzer TSM gave the expected amino acid composition. Pharmacologic assays (kindly performed by Prof. Jard, Collège de France) of each oxytocin showed the full expected biologic activity ± 10% when tested using the pig kidney medulla adenylate cyclase assay (Pradelles et al., 1972). In hydroosmotic assays, each ^{13}C-enriched peptide possessed 450 to 480 international units of activity per mg (Pradelles et al., 1972).

Bovine neurophysin II was prepared and its purity was assessed as previously described (Camier et al., 1973).

^{13}C NMR spectra were obtained on a Varian XL-100 12WG spectrometer operating at 25.5 MHz interfaced with a Varian 620 computer operating in Fourier transform mode. Ten-microsecond (45°) pulses were used with 0.8-s acquisition time and a spectral width of 5000 Hz. The probe temperature was 30 ± 1 °C. Proton noise decoupling was employed. The hormone was dissolved in 1.5 mL of D_2O–0.1 M NaCl, pH 6.8 (uncorrected pH meter reading), and placed in a 10-mm NMR tube with a Teflon anti-vortex plug. If pD = pH meter reading + 0.4, then the pD was 7.2. The D_2O provided a deuterium lock signal. Five microliters of dioxane was added to the sample to provide an internal reference peak. Chemical shift values downfield from external Me_4Si were calculated assuming the dioxane peak to be 67.4 ppm downfield from external Me_4Si.

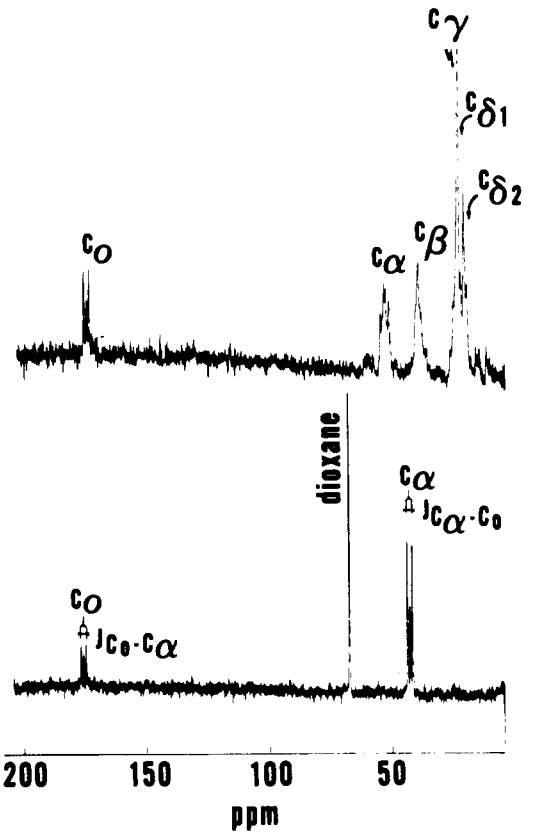


FIGURE 1: ^{13}C NMR spectra of 85% ^{13}C-enriched peptides. [^{13}C-Leu^3]-Oxytocin at 5 mM (45 000 transients) is shown in upper spectrum. [^{13}C-Gly^9]Oxytocin at 5 mM (40 000 transients) is seen in the lower spectrum.

Spin–lattice relaxation times, T_1, were obtained using the inversion recovery technique (Freeman and Hill, 1970). The delay time between each set of pulses was at least six times longer than the longest T_1 value being studied.

^{13}C NMR spectra of mixtures of hormone and neurophysin were obtained by adding neurophysin to hormone solutions whose spectrum had just been recorded (Alazard et al., 1974). In some cases the peptide solution was added directly to a weighed amount of dry, lyophilized neurophysin and the sample was stirred with a small magnet while microliter amounts of 1 N NaOH were added to maintain pH 6.8.

Results

[^{13}*C-Leu*^{3}]*Oxytocin.* An analogue of oxytocin containing 85% ^{13}C-labeled leucine in residue 3 was synthesized and its ^{13}C NMR spectrum is seen in the upper part of Figure 1. The spectrum is similar to that reported previously for 85% ^{13}C-labeled leucine alone (Tran-Dinh et al., 1974). The multiplicity

of lines for each carbon atom is due to the presence of ^{13}C–^{13}C coupling and to the fact that the ^{13}C enrichment is only partial, as discussed by Tran-Dinh et al. (1974). The ^{13}C–^{13}C coupling constant values observed for the hormone are very similar to those reported for the amino acid (Tran-Dinh et al., 1974). The chemical shift values for the carbonyl, α, β, γ, δ_1, and δ_2 carbons, respectively, are 175.0, 53.5, 39.8, 23.7, 21.9, and 20.6 ppm. The portion of the spectrum between 20 and 25 ppm is also complicated by its deviation from first order. In spite of these complexities, the molecule increases the sensitivity of ^{13}C NMR measuresments and affords the opportunity to study individual carbon atoms of residue 3 of the peptide complexed with neurophysin.

Since the peptide is in rapid exchange between the bound and free states, the observed NMR signals are the weighted average of the bound and free species (Cohen et al., 1972; Alazard et al., 1974). Therefore, various increasing amounts of neurophysin II from 0.05 to 0.35 molar equivalents were added to a 5 mM solution of peptide and the ^{13}C NMR spectra were obtained after each addition. The only observed change in chemical shift values involved the α carbon of residue 3 whose chemical shift moved downfield with increasing neurophysin. Under the conditions employed, the peptide dissociation constant is approximately 5 μM (Camier et al., 1973). Extrapolation of the displacement to a 1 to 1 molar complex (Camier et al., 1973; Nicolas et al., 1976) showed that the resonance of the α carbon of the bound [^{13}C-Leu^3]oxytocin is 0.9 ± 0.2 ppm downfield of that of the free peptide. No other change in the chemical shift (±0.3 ppm) of the other carbon resonances of Leu^3 was observed.

Measurements of T_1 values of the peptide in the absence and presence of 0.35 equiv of neurophysin II were made in order to assess the relative mobility of residue 3 in the bound and free states. It has been shown that in the extreme narrowing limit, the longer NT_1 is (where N is the number of directly bound protons), the greater is the mobility of the carbon atom (Allerhand et al., 1971; Allerhand and Komoroski, 1973). In the absence of neurophysin, the values of NT_1 (±20%) for the α, β, γ, and combined δ_1 and δ_2 carbons of [85% ^{13}-Leu^3]oxytocin were 0.095, 0.15, 0.28, and 1.74 s, respectively. The value for each individual methyl carbon, δ_1 and δ_2, could not be determined due to their overlapping peaks at 21.3 ppm, so the value of 1.74 reflects the average mobility of both δ carbons. In the presence of 0.33 equiv of neurophysin II, the values of NT_1 (±20%) for the α, β, γ, and combined δ_1 and δ_2 carbons were 0.08, 0.15, 0.24, and 0.58 s, respectively. The largest change in NT_1 occurred for the δ_1 and δ_2 methyl groups of the tail of residue 3. Extrapolation of this observed change in NT_1 to a one-to-one complex suggests that the value of NT_1 decreases from 1.74 to 0.24 s due to binding. Thus, the binding of the peptide to neurophysin II results in a sevenfold decrease in the mobility of the terminal δ-methyl groups of residue 3.

[^{13}C-*Gly*^{9}]*Oxytocin.* Oxytocin containing 85% of [^{13}C]-glycine at residue 9 was synthesized and its ^{13}C NMR spectrum is seen in the lower panel of Figure 1. The spectrum resembles that of the free amino acid (Tran-Dinh et al., 1975) and exhibits sharp peaks for the carbonyl and α carbons. The chemical shift values for the carbonyl and α carbons are 174.9 and 42.9 ppm, respectively, and the C_o–C_α one bond coupling constant is 50.5 Hz (Griffin et al., 1975).

The effects of neurophysin II on the glycine residue in oxytocin was assessed by recording ^{13}C NMR spectra of the hormone in the presence of increasing amounts of neurophysin II from 0 to 0.30 equiv. No change (±0.04 ppm) in the chemical shift values of the carbonyl or α carbons was observed.

The relaxation time, T_1, of the α carbon of Gly^9 in oxytocin

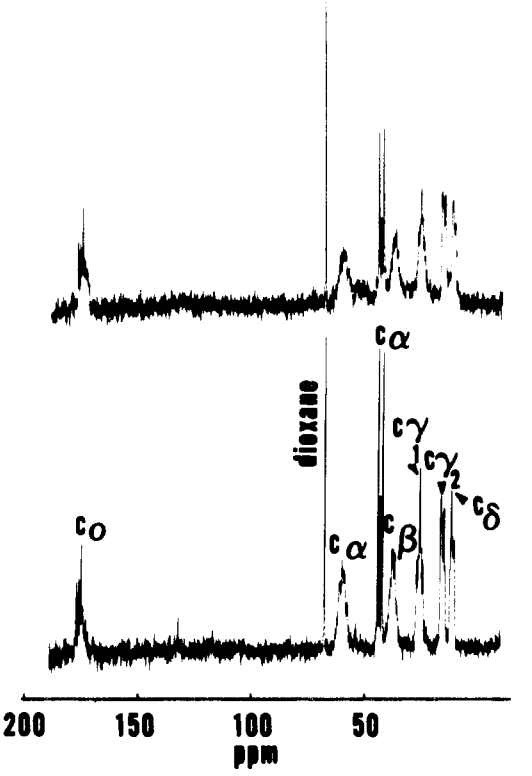


FIGURE 2: ^{13}C NMR spectrum of [85% ^{13}C-Ile^3, Gly^9]oxytocin at 5 mM in the absence (lower panel) and presence (upper panel) of 1.5 mM neurophysin II, based on 152 000 and 259 000 transients, respectively.

was measured in the absence and presence of neurophysin II. The observed value of NT_1 for the hormone alone was 0.42 ± 0.08 s which agrees well with the reported value of 0.37 s (Deslauriers et al., 1974). In the presence of 0.24 equiv of neurophysin II, the observed value of NT_1 was 0.46 ± 0.08 s, showing that the presence of neurophysin II did not affect measurably this parameter of the mobility of residue 9. The line width of the sharp peaks of the α carbon of glycine 9 in oxytocin was 7.5 Hz and did not change (±1.4 Hz) in the presence of up to 0.3 equiv of neurophysin II. In order to verify that [^{13}C-Gly^9]oxytocin was bound to neurophysin II, a control experiment was performed in which it was shown that the mixture of this hormone with neurophysin II produced a UV absorbance difference spectrum which is typical of hormone binding (Haar et al., 1975). Consequently, the failure of neurophysin to perturb markedly any of the ^{13}C NMR parameters of residue 9 cannot be ascribed to an inability of the hormone to bind to the protein.

[^{13}C-*Ile*^{3}-*Gly*^{9}]*Oxytocin.* Since the above ^{13}C NMR results from separate experiments indicated that hormone binding to neurophysin selectively perturbs residue 3 but not residue 9, a doubly labeled hormone was made in order to demonstrate the selective and specific nature of the spectroscopic perturbations in the same molecule in a single experiment. [85% ^{13}C-Ile^3, Gly^9]Oxytocin was synthesized and its spectrum is seen in the lower half of Figure 2. Each individual carbon resonance is visible except for the overlapping carbonyl carbons. The peaks for the carbons of isoleucine 3 are less sharp than those for glycine 9, presumably reflecting the greater mobility of the tripeptide "tail" of the hormone (Deslauriers et al., 1974). The splitting pattern seen in Figure 2 for the carbons of isoleucine-3 due to carbon–carbon coupling resembles that which has been presented and analyzed for the free amino acid (Tran-Dinh et al., 1974). The chemical shift

values for glycine-9 in Figure 2 were identical (± 0.04 ppm) with those seen in Figure 1 for [^{13}C-Gly^9]oxytocin. The chemical shift values of isoleucine-3 in oxytocin for the carbonyl α, β, γ_1, γ_2, and δ carbons, respectively, were 173.9, 59.1, 37.2, 25.4, 15.5, and 11.2 ppm.

The effects of 0.3 equiv of neurophysin II on the ^{13}C NMR spectrum of [^{13}C-Ile^3, Gly^9]oxytocin is seen in the upper panel of Figure 2. This spectrum shows how the resonances of the enriched carbons continue to dominate the spectrum even in the presence of added protein. The major observed change in chemical shift values due to neurophysin addition involved the α carbon of isoleucine-3 which shifted downfield 0.36 ± 0.1 ppm. The δ_2 carbon moved downfield 0.2 ± 0.1 ppm and the δ_1 carbon shifted downfield by 0.1 ± 0.1 ppm. For a one-to-one complex, the extrapolated downfield chemical shift of the α carbon of isoleucine-3 is 1.2 ± 0.3 ppm. No other changes (±0.08 ppm) in chemical shift values of the other carbon resonances due to neurophysin addition were observed.

Discussion

In the course of our studies of interactions between neurophysin and the neurohypophyseal hormones, the use of ^{13}C NMR spectroscopy and specifically ^{13}C-enriched hormones has proven particularly valuable (Griffin et al., 1975). The ^{13}C enrichment extends the sensitivity of ^{13}C NMR spectroscopy by more than an order of magnitude and enrichment of specific amino acids in peptides allows the study of each individual carbon atom in the molecule (Griffin et al., 1975; Haar et al., 1975). The results presented here show that hormone binding to neurophysin results in both chemical shift changes and shortened relaxation times of residue 3 of oxytocin. These observations add to a number of other indications reported by Breslow and colleagues (see Breslow, 1975, for review) and by our laboratories (Griffin et al., 1973; Cohen et al., 1975) that residue 3 is involved in the hormone binding site on neurophysin. In contrast, the ^{13}C NMR parameters of the carbon atoms of Gly^9 are unaffected by hormone binding and this strongly suggests that residue 9 is not directly involved in the hormone binding reaction. Related to this point is the report that the deuterium line width of [^{2}H-Gly^9]oxytocin is only slightly altered in the presence of bovine neurophysin, although these studies were done at pH 2.4, 0.4 M KCl (Glasel et al., 1973).

The observations made using ^{13}C enrichment of either residue 3 or residue 9 alone were confirmed in studies using the doubly labeled [^{13}C-Ile^3, Gly^9]oxytocin (Figure 2). This particular compound offers the advantage of observing simultaneously one perturbed residue and one unperturbed residue in the very same reaction mixture.

The chemical shift of the α carbon of residue 3 of oxytocin moves downfield upon binding to neurophysin by 1.2 ± 0.3 ppm for [^{13}C-Ile^3]oxytocin and by 0.9 ± 0.2 ppm for the (Leu^3) analogue. Interpretation of this large perturbation is complicated by the observation that the protonation of the α amino group of Cys^1 of oxytocin causes a 0.7-ppm downfield chemical shift change of the α carbon of Ile^3 (Deslauriers et al., 1974). Under the solution conditions used for these experiments with pD ~ 7.2, most of the oxytocin molecules in solution had the α amino group unprotonated. Thus, the large downfield chemical shift change is compatible with the suggestion that the α amino group of the peptide is protonated in the neurophysin–hormone complex (Camier et al., 1973; Cohen et al., 1975; e.g., Breslow, 1975). However, the data of Deslauriers et al. (1974) show that the observed downfield chemical shift change for protonation of a solution of oxytocin at pD 7.2 would be 0.5 ppm. Consequently, the large downfield change of 0.9 to 1.2 ppm seen for complex formation must also reflect changes in the conformation or electromagnetic microenvironment of the α carbon of residue 3 due to complex formation. It is not possible at present to distinguish the relative importance of through-bond or through-space contributions to this large perturbation.

The value of NT_1 for the α carbon of Gly^9 in oxytocin is 0.42 s, and this value does not shorten due to complex formation. As discussed by Deslauriers et al. (1974), the C-terminal tripeptide tail of oxytocin is less rigid than the cyclic portion of the molecule based on studies of NT_1 values. Consequently, the observation reported here that complex formation does not alter the NT_1 value of Gly^9 suggests that those elements of segmental and rotational motion which determine the NT_1 value of Gly^9 are not affected by complex formation, and that the C-terminal tail of oxytocin seems rather mobile whether or not oxytocin is bound to neurophysin.

Previous ^{13}C NMR studies of 85% ^{13}C-enriched amino acids (Tran-Dinh et al., 1974, 1975) and thyrotropin releasing factor (Haar et al., 1975) showed that three-bond ^{13}C–^{13}C coupling constants could be obtained for conformation analysis. Unfortunately, the line widths of the ^{13}C-enriched carbons of oxytocin were always ≥7 Hz and did not allow a determination of any three-bond ^{13}C–^{13}C coupling constants. Further studies are warranted to see whether ^{13}C enrichment above 20% ^{13}C in peptides larger than 400 molecular weight is worthwhile or, in fact, disadvantageous due to possible complex splitting.

The data reported here for the binding of [^{13}C-Gly^9]oxytocin to neurophysin agree with and extend two recent preliminary reports. Chaiken et al. (1975) showed that hormone binding caused no chemical shift changes for Gly^9 while Blumenstein and Hruby (1976) described how precipitation of neurophysin–hormone complexes at high concentrations leads to large ^{13}C line width values. Neither of these studies reported T_1 values of Gly^9 which are most useful in measuring changes in local mobility.

The approach of using specific ^{13}C enrichment of particular residues in peptides is of general applicability for sensitivity enhancement, specific assignments, and ligand binding studies. [^{13}C-Pro^7]Oxytocin and [^{13}C-Leu^8]oxytocin have been synthesized in our laboratories and further ^{13}C NMR studies on these and other analogues should provide more information about neurophysin–hormone interactions.

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Pulsed Electron Paramagnetic Resonance Studies of Types I and II Copper of *Rhus vernicifera* Laccase and Porcine Ceruloplasmin†

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ABSTRACT: Electron spin-echo decay envelopes for types I and II copper of *Rhus vernicifera* laccase and for type II copper of porcine ceruloplasmin have been studied. Nuclear modulation patterns show that imidazole is a ligand for all of them. The linear electric field effect (LEFE) in EPR was studied for type I copper in a laccase preparation from which type II had been removed. The symmetry of the site is near tetrahedral and the magnitude of the LEFE is correlated with the intensity of blue color.

It has recently been shown that pulsed EPR[1] techniques provide a useful means for assigning ligands (Peisach and Mims, 1973; Mims and Peisach, 1976a) and for investigating the symmetry of metal sites (Mims and Peisach, 1974, 1976b; Peisach and Mims, submitted) in proteins. In one kind of experiment (Mims and Peisach, 1976a) a recording is made of the decay envelope for electron spin-echo signals. Periodicities in the envelope indicate the presence of electron nuclear coupling. This effect is termed the "nuclear modulation effect". A second type of experiment, also involving the generation of electron spin-echoes, is concerned with the measurement of the g shifts induced by the application of an external electric field (Peisach and Mims, 1973; Mims and Peisach, 1974, 1976b). These "linear electric field effect" experiments yield information about the odd symmetry part of the ligand field

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[1] Abbreviations used are: EPR, electron paramagnetic resonance; LEFE, linear electric field effect.