Carbon-13 Chemical Shifts on Oxytocin as a Consequence of Its Interaction with Neurophysins†

Michael Blumenstein,* Victor J. Hruby,* V. Viswanatha, and D. Chaturvedi

ABSTRACT: Carbon-13 NMR was used to study the interaction of the hormone oxytocin with neurophysin (NP). Oxytocins specifically enriched to 90% ^{13}C in the α-carbons of Leu-3 (in [3-leucine]oxytocin), Gln-4, and Leu-8 and in the carbonyl carbon of Cys-6 were synthesized, so that the effect on these positions of binding to NP could be monitored. The α-carbons of residues 3 and 4 experienced shifts of −4.2 and −1.5 ppm (negative shifts are downfield), respectively, upon binding of the hormone to NP. The carbonyl carbon of residue 6 underwent a shift of +0.7 ppm, while the α-carbon of residue 8 displayed no shift. For each enriched residue, the hormone diastereoisomer in which this residue had the D configuration was also synthesized. NMR was then used to determine the binding affinity of the various diastereoisomers to NP, as well as to measure the NMR parameters of the bound peptides. When position 3 had the D configuration, the binding affinity for NP was 10–20% that of the native hormone. For positions 4, 6, and 8, the D diastereoisomers bound with the same affinity as oxytocin. The α-carbons of D residues of positions 3 and 4 shifted by −2.5 and +0.4 ppm, respectively, the carbonyl carbon of D-Cys-6 shifted by +1.4 ppm, and the α-carbon of D-Leu-8 was unshifted on binding to NP. The shift and diastereoisomer binding data, combined with previous results involving enriched carbons and/or diastereoisomers of residues 1, 2, and 9, support the conclusion that residues 1 and 2 are most crucial for binding of oxytocin to NP, residue 3 is less important, and residues 4–9 are of only slight significance. An unequivocal interpretation of the chemical shift effects was not possible, but side chain conformation was deduced to contribute significantly. It appears that the conformations of several side chains of oxytocin bound to NP differ significantly from the predominantly averaged side chain conformations present in solution. For Cys-1, Tyr-2, and Leu-3, the change in side chain conformation is caused by direct interaction of the side chain with NP. For Gln-4, the side chain does not directly interact with NP, but its bound conformation differs from its free conformation due to changed interactions with the rest of the oxytocin molecule. The overall dissociation rate constant of oxytocin from NP was found to be 2–4 s^{-1}, in good agreement with results from rapid kinetic experiments. Substitution of a D residue in position 3, which resulted in a decreased binding affinity for NP, also led to an increased dissociation rate constant, while the association rate constant for the D diastereoisomer was the same or somewhat higher than that of the unmodified peptide. Thus, the weaker binding of the D diastereoisomer is due to a weaker complex rather than to an unfavorable conformation of the free hormone.

The neurohypophyseal hormones oxytocin (H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2) and arginine-vasopressin (H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2) bind in vivo, in neurosecretory granules, to a group of related proteins known as neurophysins (NP). This protein–hormone interaction has been extensively studied in vivo and the findings have been reviewed (Breslow, 1979; Chaiken et al., 1983). Since NP can be isolated in gram quantities and the hormones are available by total synthesis, the system has proven to be particularly amenable to study by nuclear magnetic resonance (NMR) (Blumenstein, 1984). A major conclusion from work involving the binding of hormone analogues to NP (Breslow & Abrash, 1966; Breslow et al., 1971, 1973), as well as from NMR studies (Balaram et al., 1973; Alazard et al., 1974; Glasel et al., 1973; Chaiken et al., 1975; Griffin et al., 1977; Blumenstein & Hruby, 1977; Blumenstein et al., 1978), has been that residues 1 and 2, and to a lesser extent residue 3, are important in NP–hormone interaction, while residues 7–9 play little, if any, role in the binding. Our experimental approach has been to specifically enrich hormones to 90% ^{13}C in various positions and then to use ^{13}C NMR to study the hormone–neurophysin interaction (Blumenstein et al., 1979, 1980, and references cited therein). We have previously reported experiments utilizing labels in the α-carbons of residues 1, 2, and 9, the carbonyl carbon of residue 1, and the aromatic side chain of residue 2. In this paper we describe experiments with labels in the α-carbons of residues 3, 4, and 8, and the carbonyl carbon of residue 6. For each of these positions we have also examined the diastereoisomeric hormone in which the labeled amino acid has the D configuration. These studies have yielded new information about the hormone–NP complex and also about the relationships between observed ^{13}C chemical shifts and polypeptide structure.

Experimental Procedures

Materials. Ethyl acetamido[2-^{13}C]cyanoacetate was purchased from Kor Isotopes (Cambridge, MA). N^{α}-Boc-amino acids were purchased from Vega Biochemicals (Tucson, AZ) or Chemical Dynamics Corp. (South Plainfield, NJ) and were checked for purity before use by thin-layer chromatography (TLC) in three solvent systems, by melting point determination, and by the ninhydrin test (Kaiser et al., 1970). The polystyrene resin, 1% cross-linked with divinylbenzene and chloromethylated to an extent of 1.07 mmol/g of resin, was purchased from Lab Systems, Inc. (San Mateo, CA). N^{α}-Boc-Gly was attached to the resin by the method of Gisin (1973). N^{α}-Boc-Gly-NH-benzhydrylamine-resin was prepared as previously reported (Hruby et al., 1977). Solvents used for partition chromatography and TLC were purified as before (Hruby & Groginsky, 1971). The synthesis of the labeled

† From the Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111 (M.B.), and the Departments of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721 (V.J.H., V.V., and D.C.). *Received September 21, 1983.* This research was supported by grants from the U.S. Public Health Service, AM-17420 (V.J.H.) and HD-10616 (M.B.). Experiments on the Bruker HX-270 were performed at the Francis Bitter National Magnet Laboratory of the Massachusetts Institute of Technology, which is supported by National Science Foundation Contract C-670 and National Institutes of Health Grant RR-00995.

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amino acid derivatives N^{α}-Boc-DL-[2-^{13}C]leucine (Viswanatha et al., 1979) and N^{α}-Boc-*S*-benzyl-DL-[1-^{13}C]cysteine (Hruby et al., 1980) was performed as previously described. The bovine neurophysins (NP I and NP II) were isolated and purified as previously described (Blumenstein & Hruby, 1976) by a slight modification of the methods of Breslow et al. (1971). Neurophysin I mononitrated on Tyr-49 was prepared according to Furth & Hope (1970).

Analytical Methods. Amino acid analyses were obtained by the method of Spackman et al. (1958) on a Beckman 120C amino acid analyzer after hydrolysis in 6 N HCl at 110 °C for 24 h. TLC was performed on silica gel G plates using the solvent system (A) 1-butanol–acetic acid–pyridine–water (15:3:10:12), (B) 1-butanol–acetic acid–water (4:1:5, upper phase only), (C) 1-pentanol–pyridine–water (7:7:6), or (D) ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Amino acid and peptide spots were detected by ninhydrin, iodine, UV light, and fluorescamine. Analytical reversed-phase high-pressure liquid chromatography (HPLC) was performed as previously reported from our laboratory for oxytocin derivatives (Larsen et al., 1978, 1979), and preparative separations followed the procedures of Viswanatha et al. (1979). Under these conditions, diastereoisomers of oxytocin, if present, would be detected and separated from the native hormone (Larsen et al., 1978, 1979). Purification of peptide hormone derivatives by partition chromatography utilized Sephadex G-25 as the solid support as previously reported (Yamashiro, 1964; Hruby et al., 1977; Upson & Hruby, 1976). Optical rotation values were measured at the sodium D line (589) on a Perkin-Elmer 241 MC polarimeter. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected.

[3-[2-^{13}C]Leucine]oxytocin, [3-D-[2-^{13}C]Leucine]oxytocin, [6-Hemi[1-^{13}C]cystine]oxytocin, [6-Hemi-D-[1-^{13}C]cystine]-oxytocin, [8-[2-^{13}C]Leucine]oxytocin, and [8-D-[2-^{13}C]-Leucine]oxytocin. These compounds were prepared by the solid-phase method of peptide synthesis and then purified by high-pressure liquid chromatography and gel filtration chromatography on Sephadex G-25 as previously described (Viswanatha et al., 1979; Hruby et al., 1980). Purity was assessed by HPLC (Viswanatha et al., 1979; Larsen et al., 1979), TLC in three solvent systems, amino acid analysis, optical rotation, and bioassay using the milk ejecting assay (Hruby & Hadley, 1975).

DL-[2-^{13}C]Glutamic Acid Hydrochloride (**3**). To a stirred mixture of ethyl acetamido[2-[^{13}C]cyanoacetate (**1**) (3.8 g, 22 mmol) in 6.6 mL of anhydrous ethanol containing sodium ethoxide (prepared from 11.5 mg, 0.5 mmol, of sodium) at 0 °C was added 1.8 mL (33 mmol) of acrylonitrile. The mixture was stirred for 1 h, and the precipitate was filtered off and washed with anhydrous ethanol to give ethyl 2,4-dicyano-2-acetamido[2-^{13}C]butyrate (**2**) (yield 3.6 g, mp 97–100 °C). A further 0.42 g (mp 98–100 °C) was obtained from the mother liquors. The dinitrile (4.0 g) was heated at reflux with 22 mL of 6 N HCl for 16 h. The solution was evaporated to dryness on a rotary evaporator, and the residue was treated 4 times with 10 mL of water and evaporated to dryness each time to remove excess HCl. A pale cream precipitate of DL-[2-^{13}C]glutamic acid hydrochloride (3.21 g, 98.5%) was obtained. TLC in solvent systems A and B showed the product to be identical with authentic unlabeled glutamic acid.

γ-Methyl-DL-[2-^{13}C]glutamate (**4**). A mixture of 3.21 g of DL-[2-^{13}C]glutamic acid hydrochloride and 42.2 mL of anhydrous methanol was stirred at room temperature for 4 days. Then the mixture was cooled in an ice bath and 3.11 g (31 mmol) of anhydrous triethylamine was added. The mixture was stirred for 15 min and 34 mL of acetone was added. The mixture was stirred for 15 min, and the precipitate was filtered off and washed with chloroform (3 × 10 mL) and anhydrous ether (15 mL). The product **4** was obtained as a white powder, 2.81 g (80%), mp 170–172 °C [lit. mp for L isomer 173.5–174.5 °C (Blomquist et al., 1966)].

N^{α}-Boc-DL-[2-^{13}C]glutamine (**5**). A mixture of 1.4 g (8.7 mmol) of γ-methyl-DL-[2-^{13}C]glutamate, 2.6 g (18 mmol) of *tert*-butyl azidoformate, 5.6 mL of triethylamine, and 28 mL of dimethyl sulfoxide was stirred 48 h at room temperature. Then 100 mL of water was added and the solution washed with three 50-mL portions of ether. The aqueous layer was cooled to 5 °C and acidified to pH 3 with citric acid. The solution was extracted with six 50-mL portions of ethyl acetate. The combined organic layers were washed with two 30-mL portions of water and the solvents removed in vacuo. The oily residue was taken up in 150 mL of chloroform and washed with two 50-mL portions of water. The organic solution was dried over anhydrous Na_2SO_4, and the solvents were removed in vacuo to give a pale yellow semisolid. The solid was dissolved in 225 mL of anhydrous methanol and cooled to 0 °C, and anhydrous ammonia (freshly distilled from sodium) was added to saturation. The solution was stirred for 15 days at room temperature until TLC analysis showed none of the starting material remained (anhydrous ammonia was added every 3–4 days to saturation). The solvent was removed by rotary evaporation at reduced pressure. The residue was dissolved in 25 mL of water, and the solution was saturated with NaCl and then extracted with chloroform (2 × 10 mL) and ethyl acetate (6 × 12 mL). The combined organic solutions were dried over Na_2SO_4 and the solvents were removed to give after crystallization from ethyl acetate–petroleum ether 1.13 g (70%) of **5**, mp 114–116 °C [lit. mp 116–117 °C (Bayer et al., 1968)].

Solid-Phase Peptide Synthesis Methods. Solid-phase peptide syntheses were performed by procedures generally used in our laboratory (Upson & Hruby, 1976; Hruby et al., 1977). *S*-3,4-Dimethylbenzyl protection was used for cysteine sulfhydryl protection (Smith, 1973; Yamashiro et al., 1973) except for labeled cysteine in which case *S*-benzyl protection was used. N^{ϵ}-Tosyl protection was used for arginine. Generally *p*-nitrophenyl ester derivatives of Boc-Gln and Boc-Asn were used for coupling, but in the case of ^{13}C-labeled Gln, the N^{α}-Boc derivative was directly coupled to the growing peptide chain by using dicyclohexylcarbodiimide and *N*-hydroxybenzotriazole as coupling agents. The coupling procedures were monitored for completion by the ninhydrin method (Kaiser et al., 1970).

Preparation of [4-DL-[2-^{13}C]Glutamine]oxytocin and Preparative Separation of the Diastereoisomers by HPLC. Solid-phase syntheses of the protected nonapeptide-resin precursors were done by using 3.5 g of N^{α}-Boc-Gly-NH-benzhydrylamine resin (0.3 mmol/g of resin, 1.0 mmol) following the standard procedures outlined above. Single couplings used a 3-fold excess each of Boc-amino acid and *N*-hydroxybenzotriazole and a 2.4-fold excess of dicyclohexylcarbodiimide, except that for Boc-DL-[2-^{13}C]glutamine coupling only a 1.8-fold excess each of amino acid and dicyclohexylcarbodiimide and a 3.6-fold excess of *N*-hydroxybenzotriazole were used. In the latter case 24 h was needed for completion of the coupling to occur. Following completion of the synthesis, the N-terminal Boc group was removed in the usual manner to give 4.4 g of H-Cys(3,4-DMB)-Tyr-Ile-DL-[2-^{13}C]Gln-Asn-Cys(3,4-DMB)-Pro-Leu-Gly-NH-resin.

A 1.2-g portion of the resin was cleaved with 20 mL of anhydrous HF (freshly distilled from CoF_3) in the presence of 2 mL of anisole at 0 °C for 1 h in the usual manner (Hruby et al., 1977) and the disulfhydryl oxidized by the standard procedures (Hruby et al., 1977). The solution obtained was lyophilized, and the solid was dissolved in 7 mL of 20% HOAc and gel filtered on Sephadex G-15. The fractions corresponding to the peptide products (OD at 280 nm) were pooled and lyophilized to give a pale cream powder (220 mg). This powder was dissolved in 5 mL of the upper phase and 1 mL of the lower phase of the solvent system 1-butanol–3.5% aqueous HOAc containing 1.5% pyridine (1:1) and subjected to partition chromatography on a 2.8 × 58 cm column of Sephadex G-25 (block polymerizate, 100–200 mesh) that had been equilibrated with the upper and lower phases (Yamashiro, 1964). The fractions corresponding to the major peak (R_f 0.26) were pooled and gave 95 mg of the mixture of diastereoisomers. Attempts to design a solvent system for partition chromatgraphy (Yamamoto et al., 1977) that would separate [4-[2-^{13}C]glutamine]oxytocin from [4-D-[2-^{13}C]glutamine]-oxytocin were unsuccessful.

The preparative high-pressure liquid chromatography separation of the diastereoisomers was accomplished on a Partisil 10 M9 ODS column (0.94 × 50 cm) (Whatman, Inc.). A 60-mg sample of the diastereoisomeric mixture was dissolved in 2.0 mL of the HPLC solvent mixture 0.01 M NH_4OAc, pH 4.0, and acetonitrile (82:18 v/v), and 100-μL (~3-mg) samples were injected per run at a flow rate of 6 mL/min [see Viswanatha et al. (1979)]. Three fractions were collected, the first corresponding to the all-L diastereoisomer [4-[2-^{13}C]-glutamine]oxytocin (α = 1.00), a middle fraction corresponding to a mixture of the two isomers, and a third fraction corresponding to [4-D-[2-^{13}C]glutamine]oxytocin (α = 1.20). The pooled fractions from the various runs were lyophilized and desalted by gel filtration on Sephadex G-25 using 20% acetic acid as the eluent solvent. There was obtained 18.5 mg of [4-[2-^{13}C]glutamine]oxytocin, amino acid analysis of which gave the following molar ratios: Asp, 1.00; Glu, 1.02; Pro, 1.07; Gly, 0.98; $^1/_2$-Cys, 1.80; Leu, 0.97; Ile, 1.01; Tyr, 0.88. TLC in solvent systems A–C gave single spots identical with those of authentic oxytocin as previously reported (Larsen et al., 1979). There was also obtained 20.0 mg of [4-D-[2-^{13}C]glutamine]oxytocin, $[\alpha]^{22}_D$ −36.1 (*c* 0.52, 1 N HOAc). Amino acid analysis gave the following molar ratios: Asp, 1.00; Glu, 1.05; Pro, 1.06; Gly, 1.00; $^1/_2$-Cys, 1.82; Leu, 0.96; Ile, 1.02; Tyr, 0.90. TLC in solvent systems A–C gave single spots identical with those of authentic [4-D-glutamine]oxytocin as previously reported (Larsen et al., 1979).

Preparation of [1-Hemi[1-^{13}C]cystine,3-D-leucine]oxytocin. Solid-phase synthesis of the protected nonapeptide was accomplished by using 2.5 g of N^{α}-Boc-glycinate-resin that had a substitution level of 0.39 mmol/g as determined by the modified (Ehler, 1972) aldimine test (Esko et al., 1968). Removal of the N^{α}-Boc protecting group, neutralization of the peptide resin salt, and addition of the next amino acid residue followed as before, except for the addition of Boc-*S*-benzyl-DL-[1-^{13}C]cysteine. In this case, 1.45 g of the octapeptide-protected resin was used for addition of the last Boc-amino acid, the first coupling was done with 0.15 g (0.52 mmol) of the amino acid and 0.10 g (0.48 mmol) of dicyclohexylcarbodiimide for 24 h, and the second coupling employed 0.08 g (0.28 mmol) of the amino acid and 0.05 g (0.24 mmol) of dicyclohexylcarbodiimide for overnight. Following removal of the terminal Boc-protecting group, 1.52 g of H-DL-[1-^{13}C]Cys(Bzl)-Tyr-D-Leu-Gln-Asn-Cys(3,4-DMB)-Pro-Leu-Gly-resin was obtained. The protected peptide was cleaved from the resin by anhydrous methanolic ammonia following the standard procedures (Upson & Hruby, 1976) to give 250 mg of H-DL-[1-^{13}C]Cys(Bzl)-Tyr-D-Leu-Gln-Asn-Cys(3,4-DMB)-Pro-Leu-Gly-NH_2. The protected nonapeptide was dissolved in 100 mL of anhydrous ammonia (freshly distilled from sodium) and treated with a sodium stick until a blue color persisted for 30–45 s. The ammonia was removed by evaporation under a slow stream of nitrogen and the last 10 mL by lyophilization. The white powder was oxidized with $K_3Fe(CN)_6$ in the usual manner. The diastereoisomers were separated from each other and from byproducts by partition chromatography on Sephadex G-25 (block polymerizate, 100–200 mesh) using the solvent system 1-butanol–3.5% aqueous acetic acid in 1.5% pyridine (1:1) (Upson & Hruby, 1976). The [1-hemi[1-^{13}C]cystine,3-D-leucine]oxytocin eluted at R_f 0.20. After gel filtration on Sephadex G-25 using 0.2 N acetic acid, there was obtained 23 mg of the desired compound, which gave a single spot on TLC in solvent systems A–C. Amino acid analysis gave the following molar ratios: Asn, 0.98; Glu, 1.00; Pro, 0.95; Gly, 0.94; $^1/_2$-Cys, 2.06; Leu, 2.06; Tyr, 0.90.

Preparation of [3-D-Phenylalanine,8-arginine]vasopressin. The solid-phase synthesis was carried out by using 1 g (0.3 mmol) of N^{α}-Boc-glycinate-resin following the standard procedures outlined above. Following the coupling of the last amino acid residue to the peptide chain, the N-terminal Boc-protecting group was removed as before to give 1.3 g of H-Cys(3,4-DMB)-Tyr-D-Phe-Gln-Asn-Cys(3,4-DMB)-Pro-Arg(Tos)-Gly-resin. The peptide was removed from the resin by ammonolysis in the manner described above to give 580 mg of H-Cys(3,4-DMB)-Tyr-D-Phe-Gln-Asn-Cys(3,4-DMB)-Pro-Arg(Tos)-Gly-NH_2. The protecting groups from a 300-mg portion of the nonapeptide were removed as before. The product was dissolved in 4 mL of the upper phase and 2 mL of the lower phase of the solvent system 1-butanol–ethanol–water containing 3.5% acetic acid in 1.5% pyridine (4:1:5) and subjected to partition chromatography on a 2.8 × 65 cm column of Sephadex G-25 (block polymerizate, 100–200 mesh) that had been equilibrated with the upper and lower phases. The fractions corresponding to the product (R_f 0.39) were pooled and isolated. The product was further purified by gel filtration on Sephadex G-25 using 20% AcOH as the eluent solvent. The fraction corresponding to the major peak was pooled and lyophilized to give the title compound as a white powder, 65 mg (30%), $[\alpha]^{25}_{547}$ −68° (*c* 0.541, 1 N AcOH). The peptide gave a single spot on TLC in the solvent systems A, B, and D. Amino acid analysis gave the following molar ratios: Arg, 0.93; Asn, 1.06; Glu, 1.09; Pro, 1.00; Gly, 1.07; $^1/_2$-Cys, 1.83; Tyr, 0.91; Phe, 1.04.

Circular Dichroism Methods. Circular dichroism studies of the binding of oxytocin or oxytocin analogues to the modified NP were carried out on a Jasco J-20 spectropolarimeter using known procedures (Breslow et al., 1973).

NMR Methods. Samples were dissolved in a buffer consisting of 0.1 M KCl, 0.05 M potassium phosphate, and 2 mM EDTA in D_2O. About 2 μL of dioxane was generally added per 1.2 mL of sample for use as an internal standard. In certain cases, buffer having a lower EDTA concentration (0.7 mM) was used in order to reduce the intensity of EDTA ^{13}C resonances. NP concentrations were from 15 to 25 mg/mL. NP I was used in virtually all of the experiments reported here, since we have previously found that somewhat narrower resonances were observed with NP I compared with NP II, while chemical shifts of corresponding carbons were identical for

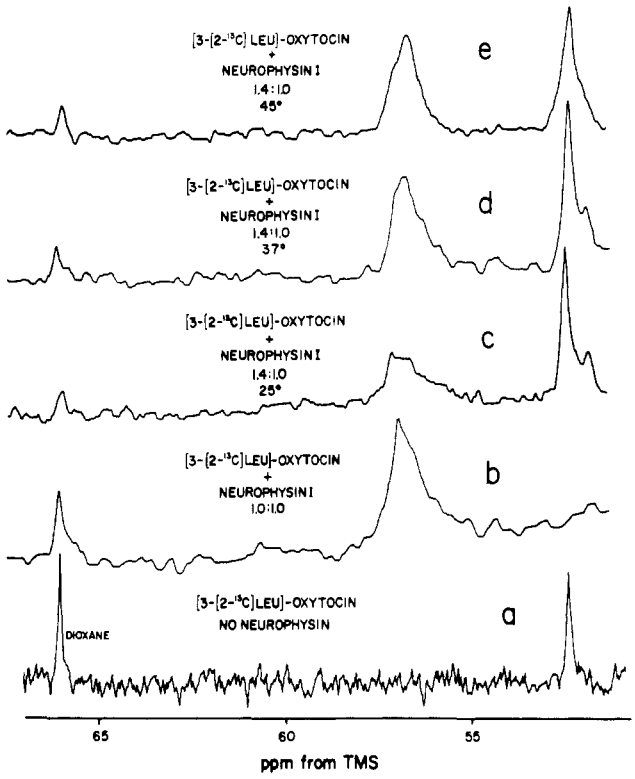

FIGURE 1: ^{13}C NMR spectra of [3-[2-^{13}C]leucine]oxytocin in the absence of NP I (a), in the presence of equimolar NP (b), and in a 1.4:1 peptide:NP ratio at various temperatures (c–e). For (b–e), the NP concentration was 25 mg/mL. The pH for all spectra was 6.6. Spectra were obtained at 67.9 MHz on a Bruker HX-270 spectrometer. The number of pulses was 4000–12000, pulse width 21 μs (about 90°), acquisition time 0.25 s, and pulse delay 0.5 s.

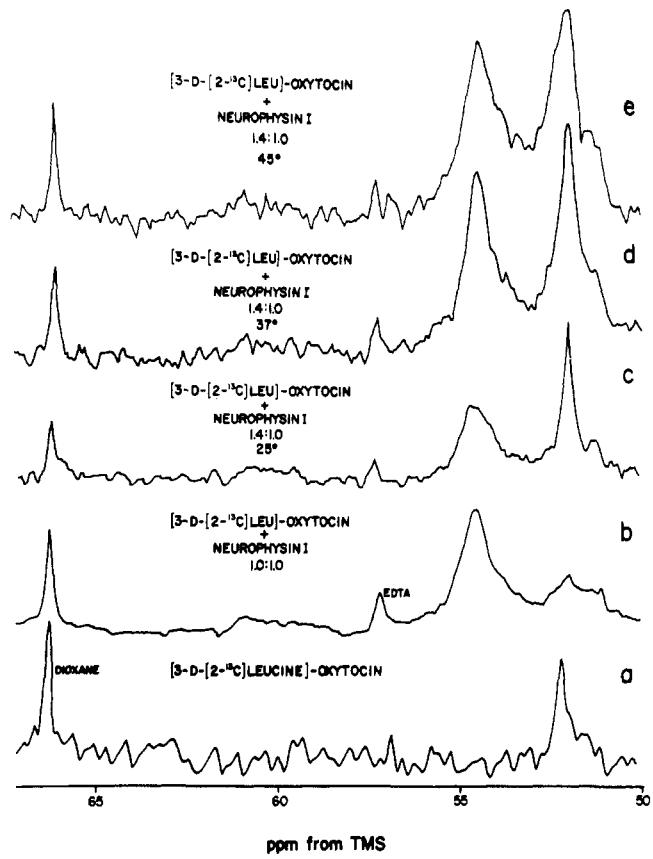

FIGURE 2: Spectra with [3-D-[2-^{13}C]leucine]oxytocin. NP and peptide concentrations and spectral conditions as in Figure 1. Note particularly the progressive broadening of the free resonance in (c–e) and the absence of such broadening in Figure 1c–e.

hormones bound to either protein (Blumenstein & Hruby, 1977; Blumenstein et al., 1978).

Spectra were acquired at 67.9 MHz on a Bruker HX-270 spectrometer. Acquisition times of 0.25 or 0.50 s and repetition rates of 0.5–1.0 s were employed for α-labeled carbons, while repetition rates of 1.0–2.5 s were used for slower relaxing carbonyl carbons. In all cases pulse widths were 20–25 μs (about 90°). The accumulation time for spectra of protein–hormone complexes was 1–4 h. Some spectra of complexes with carbonyl-labeled hormones, as well as some spectra of free hormones, were acquired at 15.1 MHz on a Bruker WP-60 spectrometer using previously described conditions (Blumenstein et al., 1977).

Results

^{13}C NMR spectra of [3-[2-^{13}C]leucine]oxytocin in the presence and absence of NP I are shown in Figure 1. At pH 6.6, there is a 4.5-ppm downfield shift for the labeled carbon of the hormone on complex formation and a line width of about 40 Hz for this carbon in the bound form. In Figure 1c–e are spectra obtained with NP and excess [3-[2-^{13}C]leucine]-oxytocin. Resonances due to both free and bound hormone are present, indicative of a condition of slow exchange. There is no measurable exchange broadening, and since we estimate our line-width measurements to be accurate to ±3 Hz, we can place an upper limit of 10 s^{-1} on k_{off}. The relative binding of [3-[2-^{13}C]leucine]oxytocin and oxytocin to NP was determined by NMR. Known amounts of oxytocin, the ^{13}C-labeled analogue, and NP were mixed, and the amounts of free and bound [3-[2-^{13}C]leucine]oxytocin were determined from the NMR spectrum. In some cases, ^{13}C-labeled oxytocin was also employed, so the free and bound concentrations of both hormones could be directly measured. The affinity of the analogue for NP was found to be 70–80% that of oxytocin, indicating that the mode of binding of the analogue is probably very similar to that of the native hormone.

Our values for the chemical shift, line width, and exchange rate are significantly different than those deduced by Griffin et al. (1977) for the α-carbon of *uniformly (85%) enriched* leucine in [3-leucine]oxytocin bound to NP. These workers employed a 3–10-fold excess of hormone, observed increasing NP-induced shifts of 1–8 Hz as the NP concentration was raised, and ascribed these shifts to a fast exchange averaging of the shifts due to free and bound hormones. The use of a uniformly, rather than a specifically, enriched amino acid led to extensive ^{13}C–^{13}C splittings, and even in free oxytocin, the α-carbon gave rise to a poorly resolved envelope of resonances having a total breadth of about 75 Hz. We believe that the small shift changes observed by Griffin et al. (1977) upon addition of NP were not due to fast exchange but rather to the perturbation of the free hormone envelope caused by the natural abundance spectrum of NP. In our studies the bound resonance is observed directly, and our shift difference between free and bound forms is about 300 Hz for a single 40 Hz wide line.

The ^{13}C NMR spectra of [3-D-[2-^{13}C]leucine]oxytocin are shown in Figure 2. The chemical shift change and line broadening observed on addition of neurophysin indicate that this diastereoisomer binds to NP, in contrast to the diastereoisomers of oxytocin involving hemi-D-Cys-1 and D-Tyr-2, which do not bind (Blumenstein & Hruby, 1977; Breslow & Abrash, 1966; Blumenstein et al., 1978). From NMR competition experiments using labeled oxytocin ([2-[2-^{13}C]tyrosine]oxytocin was employed) and [3-D-[2-^{13}C]leucine]oxytocin, we find that at pH 6.6 the 3-D-leucine diastereoisomer of

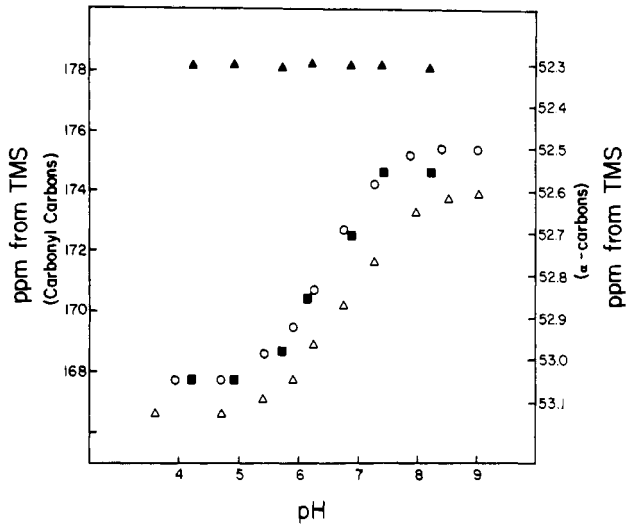

FIGURE 3: Effect of pH on the ^{13}C chemical shifts of the carbonyl carbon of $^1/_2$-Cys-1 in oxytocin (○) and in [3-D-leucine]oxytocin (△) and the α-carbon of residue 3 in [3-D-leucine]oxytocin (▲) and in [3-L-leucine]oxytocin (■). Chemical shifts of carbonyl carbons are given by the scale at the left and of α-carbons by the scale at the right. Chemical shifts were measured at 15.1 MHz on a Bruker WP-60 spectrometer.

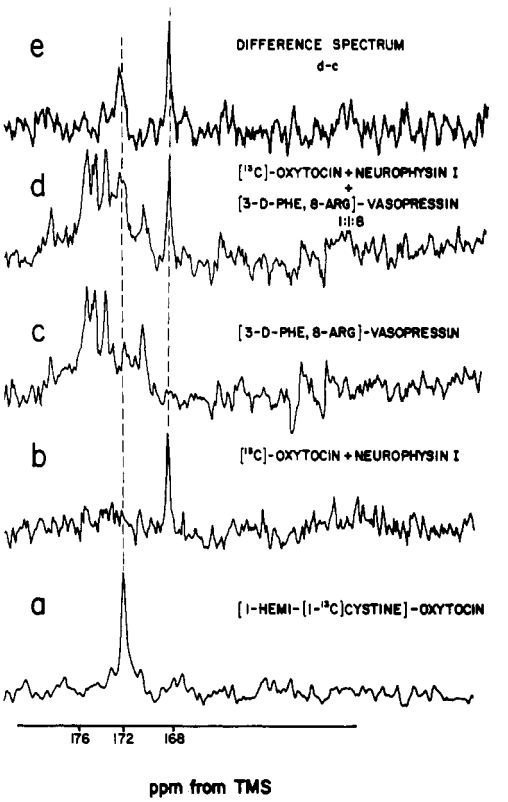

FIGURE 4: Spectra showing that [3-D-phenylalanine,8-arginine]vasopressin displaces oxytocin from NP. (a) [1-Hemi[1-^{13}C]cystine]-oxytocin (no NP); (b) [1-hemi[1-^{13}C]cystine]oxytocin fully bound to NP; (c) spectrum (natural abundance) of [3-D-phenylalanine,8-arginine]vasopressin; (d) excess [3-D-phenylalanine,8-arginine]vasopressin added to an equimolar mixture of [1-hemi[1-^{13}C]cystine]-oxytocin and NP; (e) difference spectrum (d – c); peaks due to both bound and free oxytocin are observed, indicating that the AVP diastereoisomer has displaced previously bound oxytocin from NP. Spectra were obtained at 15.1 MHz, and the difference spectrum was obtained by weighting and subtraction of transformed spectra with a BNC-12 (Nicolet 1080) computer.

oxytocin binds 20–30% as strongly as oxytocin, or about 25–35% as strongly as [3-leucine]oxytocin.

When excess [3-D-[2-^{13}C]leucine]oxytocin and NP are mixed (Figure 2c–e), a broadening of the free resonance is observed on raising the temperature. This broadening, which is observed neither with the L isomer (see Figure 1c–e) nor with other α-labeled hormones studied previously (Blumenstein & Hruby, 1977; Blumenstein et al., 1978), is due to an increased hormone–NP exchange rate at high temperatures. The exchange broadening at 35 °C is about 20 Hz, indicating a dissociation rate constant of about 60 s^{-1}, which is 20 times greater than the dissociation rate constant exhibited by oxytocin (see below). The bound resonance of [3-D-leucine]-oxytocin does not broaden with increasing temperature. A major contribution to the line width of the bound resonance is due to the correlation time of the hormone–NP complex. This correlation time decreases with increasing temperature, thus reducing this contribution and offsetting the increasing contribution due to exchange.

Previously, we have determined that at pH 2.5 the oxytocin–NP exchange rate goes from slow to intermediate and then to fast as the temperature is raised from 15 to 45 °C (Blumenstein et al., 1979). This same effect was seen with [3-leucine]oxytocin. With the [3-D-leucine]oxytocin–NP complex, only a single fairly narrow line, having a shift virtually identical with that of the free hormone, was observed at 15 °C, pH 2.5. This indicates that little, if any, of the hormone was bound to NP, a finding consistent with the greatly reduced binding constant of oxytocin to NP at low pH and the reduced affinity of the 3-D-leucine diastereoisomer as compared to oxytocin.

Experiments with the free peptides showed the 3-D-Leu diastereoisomer to differ from the Leu-3 hormone as well as oxytocin. Walter et al. (1973) have found that the α-carbon of Ile-3 of oxytocin undergoes a downfield shift of 0.7 ppm upon protonation of the hormone's α-amino group. We observed a similar pH-dependent downfield shift for the α-carbon of Leu-3 in [3-leucine]oxytocin (Figure 3). No shift due to pH changes was seen for the α-carbon of the D-Leu-3 in the diastereoisomeric hormone. The pK_a of the α-amino group of oxytocin is 6.3–6.5 (Breslow, 1961; Blumenstein et al., 1977), and the (Leu-3) hormone has the same pK_a, determined from the chemical shift vs. pH profile of the α-carbon of the Leu-3 residue (Figure 3). The D-Leu-3 diastereoisomer, however, has a pK_a of 6.9 ± 0.1, which was measured by monitoring the enriched carbonyl carbon of [1-hemi[1-^{13}C]-cysteine,3-D-leucine]oxytocin (Figure 3). The different pK_a values between oxytocin and its D-Leu-3 analogue must be taken into account when comparing the binding of these compounds to NP, because only the protonated form of the free hormone binds (Stouffer et al., 1963; Blumenstein et al., 1977). At the pH of our binding competition experiments, 6.6, about 70% of the 3-D-Leu hormone derivative would be in the protonated form, as compared to about 45% of oxytocin. The affinity of the protonated D-diastereoisomer for NP is thus 13–20% of the affinity displayed by protonated oxytocin, rather than the 20–30% obtained above without taking into account the different concentration of binding species present.

The D-Leu-3 side chain has the potential for significant internal mobility and conformational rearrangement. In order to judge the effect on NP binding of a more rigid side chain with the D configuration in position 3, we have employed [3-D-phenylalanine,8-arginine]vasopressin. The binding of this analogue was quantitated via NMR competition experiments in which the D diastereoisomer was mixed with ^{13}C-enriched oxytocin and NP (Figure 4). The difference spectrum (Figure 4e) displays resonances due to both free and bound oxytocin and indicates that an 8-fold excess of the D diastereoisomer displaced about 50% of the bound oxytocin. This means that

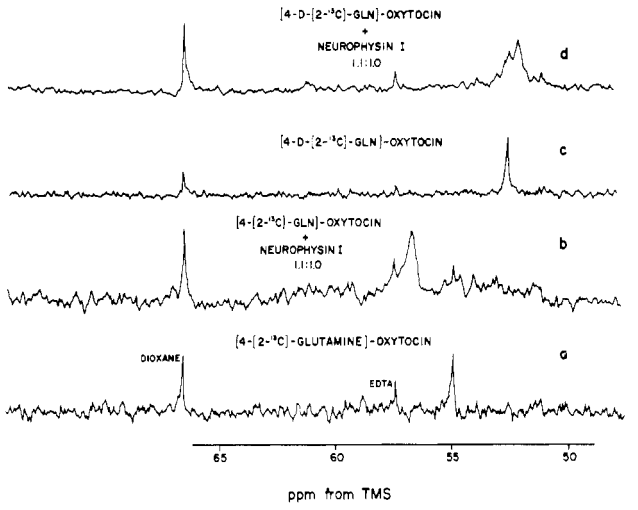

FIGURE 5: ^{13}C spectra at 67.9 MHz of [4-[2-^{13}C]glutamine]oxytocin (a, b) and [4-D-[2-^{13}C]glutamine]oxytocin (c, d) in the absence and presence of NP. The temperature was 30–32 °C, acquisition time 0.5 s, and pulse delay 0.25 s. Other conditions as in Figure 1.

the affinity of the D-Phe peptide for NP is 10–15% of the oxytocin affinity. A similar result was obtained when the binding of the AVP diastereoisomer to NP nitrated on Tyr-49 was monitored by circular dichroism (not shown). The 10–15% affinity is only slightly less than that exhibited by [3-D-leucine]oxytocin and shows that a D residue in position 3 allows binding NP, even if the side chain is rigid.

Experiments with [4-[2-^{13}C]glutamine]oxytocin and [4-D-[2-^{13}C]glutamine]oxytocin are displayed in Figure 5. The α-carbon of Gln-4 of oxytocin experiences a downfield shift of 1.5 ppm upon binding to NP, while the D diastereoisomer shifts upfield by 0.4 ppm. Since the resonance in the (all-L) free hormone is 2.3 ppm downfield relative to that of the free D-Gln-4 diastereoisomer, the difference in bound shifts between the α-carbon atoms of residue 4 in the D and L isomers is 4.2 ppm. Both hormones exhibit slow exchange on binding. NMR competition experiments indicate that the D diastereoisomer binds to NP with an affinity that is 75% that of oxytocin.

Spectra with [6-hemi[1-^{13}C]cystine]oxytocin are shown in Figure 6 (bottom two spectra). The ^{13}C label is in a carbon that does not have covalently bonded protons, and the resonance therefore remains narrow even when the hormone is bound to NP (Oldfield et al., 1975). The bound resonance can be observed readily at 15 MHz, rather than the 67 MHz used for observation of bound resonances due to α-carbons. There is an upfield shift of 0.7 ppm of this labeled carbon when the hormone binds to NP I. In the presence of excess hormone, peaks due to both free and bound forms give lines only a few hertz wide, and this allows us to measure the exchange broadening rather accurately, rather than placing an upper limit on this broadening, as we have done previously. The exchange broadening is 0.7–1.3 Hz for the free resonance at 37 °C when the hormone is present in 50% excess (Figure 7). This indicates an exchange rate of 2–4 s^{-1}. This rate is very similar to the rate of 6 s^{-1} determined by stopped-flow techniques for the $(NP)_2O_x + O_x \rightleftarrows (NP)_2(O_x)_2$ reaction (Pearlmutter & Dalton, 1980). The agreement is noteworthy because the stopped-flow experiments were done with NP concentrations 100 times lower than the NMR experiments, and with a different buffer, yet values obtained by the two methods are essentially identical.

The diastereoisomer [6-hemi-D-[1-^{13}C]cystine]oxytocin also binds to NP I and NP II. An upfield shift of 1.4 ppm is observed on binding (Figure 6, top two spectra). The shift

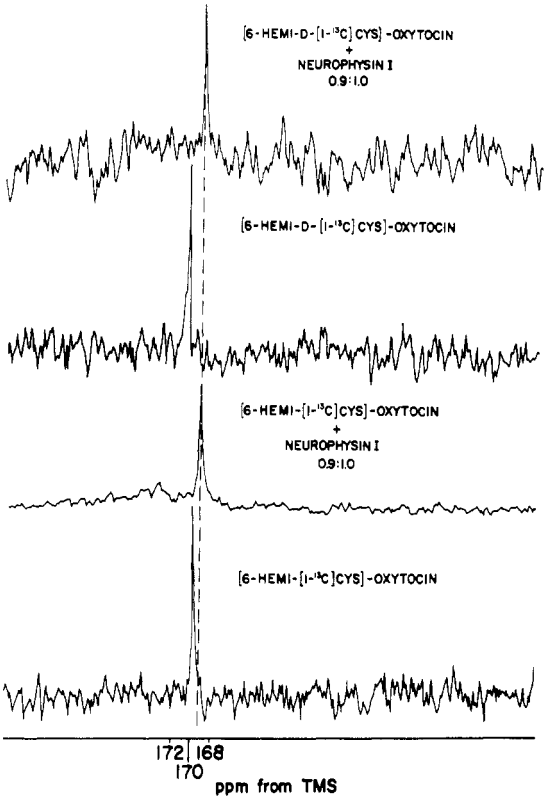

FIGURE 6: ^{13}C spectra of [6-hemi[1-^{13}C]cystine]oxytocin and [6-hemi-D-[1-^{13}C]cystine]oxytocin in the absence and presence of 25 mg/mL NP. The temperature was 28–30 °C, pH 6.6. Spectra were recorded at 15.1 MHz by using 20-μs (about 90°) pulses, an acquisition time of 0.5 s, and a pulse delay of 1.1 s. The number of pulses for (b) was 38 000 and for (d) was 3700.

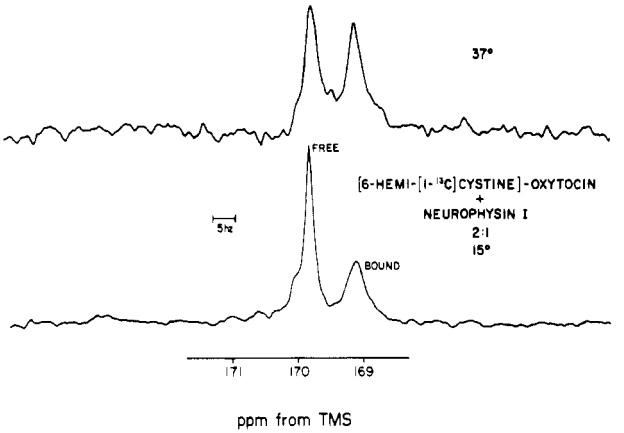

FIGURE 7: ^{13}C NMR spectra of [6-hemi[1-^{13}C]cystine]oxytocin plus NP, 2:1 peptide:protein ratio, pH 6.6, 25 mg/mL NP, at 15 and 37 °C. Spectra were acquired at 15.1 MHz by using 90° pulses, an acquisition time of 2.0 s, and no pulse delay. For the bottom spectrum, 30 000 pulses were accumulated, and for the top spectrum, 10 000 pulses were accumulated.

of the enriched ^{13}C carbon in the free hormone is 0.7 ppm upfield of the corresponding carbon in oxytocin, and the *bound chemical shifts*, relative to Me_4Si, of the D and L diastereoisomers are the same. Competition experiments indicate a very similar binding constant for oxytocin and [6-hemi-D-cystine]oxytocin to NP I.

When [8-[2-^{13}C]leucine]oxytocin binds to NP, there is no chemical shift change, and the broadening is much less than that observed for the α-carbons of residues 1–4. These results are in agreement with those obtained by Griffin et al. (1977) using uniformly enriched [8-[^{13}C]leucine]oxytocin. Competition experiments involving [8-D-leucine]oxytocin showed this

Table I: Chemical Shifts of Enriched Carbons in the Backbone of Oxytocin in the Absence and Presence of Neurophysin (NP)

enriched position in oxytocin	chemical shift: free hormone[a]	chemical shift: bound hormone[a]	shift induced by binding to NP
α-carbons			
1-hemicystine	52.3 (53.5)	50.8	+1.5
1-hemi-D-cystine	(53.9)	does not bind	
2-tyrosine[b]	55.3	57.5	−2.2
2-D-tyrosine	55.5	does not bind	
3-leucine	53.0 (52.7)	57.2	−4.2
3-D-leucine	52.3	54.8	−2.5
4-glutamine	55.1	56.6	−1.5
4-D-glutamine	52.8	52.4	+0.4
8-leucine	52.7	52.7	0
8-D-leucine	52.7	52.7	0
9-glycine[b]	42.7	42.7	0
carbonyl carbons			
1-hemicystine[b]	167.6 (171.6)	167.8	−0.2
1-hemi-D-cystine[b]	168.0	does not bind	
6-hemicystine	169.8	169.1	+0.7
6-hemi-D-cystine	170.5	169.1	+1.4

[a] Shifts are given in ppm downfield from Me_4Si. All shifts were measured relative to internal dioxane, whose shift was defined as being 66.5 ppm. Shifts and shift differences are those observed at low pH, as explained in the text. For resonances of the free hormone whose shifts are pH dependent, the shift at pH 6.6 is given in parentheses. Upfield NP-induced shifts are designated as positive. [b] Corresponding labeled AVP analogues were also observed. In all cases, chemical shifts were identical with those of oxytocin (free and bound).

diastereoisomer to bind to NP with an affinity equal to that of oxytocin. As expected on the basis of results presented above, no chemical shift and a small degree of line broadening were seen in the labeled carbon of the D diastereoisomer on binding to NP. The relative binding of D diasteriosomers of oxytocin (and AVP) to NP is given in Table I.

Discussion

In this and previous work, we have observed the chemical shifts of backbone (C_α and C′) carbons in seven of the nine oxytocin residues when the hormone was bound to NP. In addition, the corresponding shifts in several similar positions of AVP, as well as in several diastereoisomeric hormone analogues, have been observed. These shifts are tabulated in Table II.

The chemical shifts of the C_α and C′ carbons of Cys-1 of free oxytocin and the shift of the α-carbon of Leu-3 of [3-leucine]oxytocin are affected by the protonation state of the hormone's α-amino group. These shifts are therefore pH dependent. The bound shifts of these carbons, as well as of all other hormone carbons, were found to be pH independent. Since only the protonated form of the hormone binds to NP (Stouffer et al., 1963; Blumenstein et al., 1977), the shift difference at low pH, where both free and bound hormones are protonated, represents the "environmental" shift caused by NP. These shifts can be summarized as follows: (1) Most carbons in the ring of oxytocin display relatively large shift differences between free and bound forms. (2) The α-carbons experience larger shifts than do the carbonyl carbons (when the protonation of the α-amino group is taken into account, as discussed above). (3) Shifts observed in carbons of D-amino acid residues of diastereoisomeric hormone analogues that bind to NP differ from the shifts observed for the corresponding carbons of the natural hormone. (4) Binding of the hormone to NP does not affect the chemical shifts of carbons in the tripeptide tail of oxytocin.

Table II: Binding of D Diastereoisomers of Oxytocin (and AVP) to Neurophysin Compared to Binding of Oxytocin[a]

position of D-amino acid in oxytocin analogue	binding of diastereoisomer/ binding of oxytocin
Cys-1	0
Tyr-2	0
Leu-3[b]	0.1–0.2
Phe-3[c]	
Gln-4	0.7–0.8
Cys-6	1.0
Leu-8	1.0

[a] Relative binding was determined by NMR competition experiments at pH 6.6. [b] In [3-D-leucine]oxytocin; binding affinity is corrected for different pK_a value of α-amino group as compared to that of oxytocin (see the text). [c] In [3-D-phenylalanine]AVP.

There are many possible causes for the chemical shifts that occur at the α-carbon or C′ carbon atoms of the peptide hormone when it binds to a neurophysin protein, including the following: (1) ring current effects; (2) electrical charge effects; (3) changes in dielectric constant of the surrounding media; (4) hydrogen-bonding changes; (5) changes in backbone conformation of the hormone; (6) changes in the side chain conformations in the hormone; (7) effects of strain on the hormone in the bound state. Several of these effects are unlikely to be of great importance for our observed shifts. Any shifts of greater than 1 ppm cannot be due to ring current effects, as neither NP I nor NP II contains tryptophan, and shifts due to other aromatic amino acids are rather small (Giessner-Prettre & Pullman, 1970, 1981). Since the observed bound chemical shifts are pH dependent, and most of the peptide is rather lipophilic, electric charge effects are unlikely to be very significant. Hydrogen-bonding and dielectric constant effects of the surrounding medium have been shown to affect the ^{13}C shifts of peptide carbonyl carbons more than the chemical shifts of α-carbons (Urry et al., 1974; Llinas et al., 1977a,b). We, however, observe the opposite situation; i.e., the α-carbons show the largest chemical shift changes on binding (Table II). A change in backbone conformation would also be expected to produce larger carbonyl shifts than we observe. For example, in studies of the binding of ribonuclease S-peptide to S-protein (Chaiken, 1974; Niu et al., 1979) in which the S-peptide undergoes a random coil to α-helix transition, as well as in coil → helix transitions of poly(amino acids) (Paolillo et al., 1972; Boccalon et al., 1972), ^{13}C chemical shift changes of 2 ppm or greater were observed for several carbonyl resonances. Of most probable significance for our observed shifts are strain in the side chain groups of the hormone as well as changes in side chain conformations, effects which would be expected to produce large shifts in the α-carbons.

The former arises from perturbation of sp^3 hydridized orbitals (Llinas et al., 1977a), while the latter comes from differences in conformation-dependent γ effects (Grant & Cheney, 1967; Tonelli, 1980). The α-carbon chemical shifts could also be affected by intermolecular interactions, i.e., proton–proton interactions in the complex between side chain groups on the hormone and side chain groups on the neurophysins, of the same type that gives rise to γ shifts.

If changes in side chain conformation are indeed a significant factor in our observed shifts, it would indicate the side chain conformations present in the free hormone (which are generally averages of rapidly interconverting conformations) differ significantly from the side chain conformations that occur in the hormone–NP complex. This finding, while not

unexpected, is nonetheless significant, since it indicates that studies of side chain conformations of peptides in solution, where side chain conformational averaging is occurring, are unlikely to yield much information concerning biologically active conformations.

The weaker binding of [3-D-leucine]oxytocin to NP compared with [3-L-leucine]oxytocin indicates that the side chain in the D-Leu-3 analogue is likely less constrained in the complex than is the (L) leucine side chain of [3-leucine]oxytocin. While the 3-position of oxytocin and AVP is of some importance in binding to NP, it is not nearly as crucial as positions 1 and 2 (Breslow et al., 1973). Thus, even the rigid D-phenylalanine side chain aromatic ring of [3-D-phenylalanine,8-arginine]vasopressin can be accommodated. Also, the Phe side chain of AVP shows very fast ring flipping when bound to NP (Blumenstein et al., 1981). These observations indicate that the environment of residue 3 of the hormone in the bound complex allows for a wide variety of steric variability in the side chain of that residue, although the proper side chains, L-leucine and L-phenylalanine, still bind more strongly than the D side chains. This contrasts to the constraints at residues 1 and 2, where insertion of a D-amino acid residue precludes any binding from occurring.

The data we obtain with [3-D-leucine]oxytocin and [3-leucine]oxytocin are consistent with our hypothesis that side chain conformation plays a significant role in chemical shifts. Since the side chain of D-leucine-3 is less constrained when bound to NP than is the side chain of L-leucine-3, the bound conformation differs from the free conformation by a lesser amount for the D residue. Its shift on binding to NP is therefore less than the NP-induced shift of L-leucine-3.

The weaker binding of [3-D-leucine]oxytocin is not due to the hormone's adopting a conformation in solution that is less favorable for binding. If this were true, the association rate constant for binding to NP would be expected to be slower for the D diastereoisomer than for oxytocin or [3-L-leucine]-oxytocin. We can ascertain the relative association constants (k_{assoc}) for [D-3-Leu]oxytocin and oxytocin by comparing their dissociation rate constants (k_{dissoc}) and binding constants (K_d), since

$$K_d = k_{dissoc}/k_{assoc}$$

The dissociation rate constants, determined above, are 60 s^{-1} for the D diastereoisomer and 3 s^{-1} for oxytocin, for a ratio of 20, while the ratio of the binding constants is 5–8 (we found the relative binding to be 13–20%). The association rate constant for the D diastereoisomer is, thus, somewhat greater than that of oxytocin, which can be explained by the less stringent conformational restraints on the bound D hormone. The weaker binding of the D diastereoisomer must, therefore, be due to formation of a weaker complex. The fact that weaker binding to NP arises from increased dissociation rate constant, rather than a decreased association rate constant, has also been demonstrated for oxytocin binding to NP's at low pH (Blumenstein et al., 1979) and for small peptide binding to the NP's (Pearlmutter & Dalton, 1980; Virmani-Sardana & Breslow, 1983).

The shift and binding data with [[^{13}C]Gln^4]- and [D-[^{13}C]Gln^4]oxytocin are somewhat peculiar. Since the D diastereoisomer binds to NP with an affinity almost equal to that of the native hormone, it is unlikely that there is a significant direct interaction between the side chain in position 4 and NP. This conclusion is in agreement with previous analogue studies (Breslow & Abrash, 1966). Nonetheless, a 1.5-ppm shift of the α-carbon of Gln-4 occurs on binding to NP. We propose that this shift is due to a changed interaction of this side chain with the rest of the oxytocin molecule, resulting in a different side chain conformation in the bound as opposed to the free peptide. That side chain interactions can affect the shift of the α-carbon in position 4 is clear from the shift difference of 2.3 ppm between these carbons in the free L and D diastereoisomers. Thus, in the case of position 4 of oxytocin, we believe our observed NP-induced shift is an indirect rather than a direct effect.

For the $^1/_2$-Cys-6 and $^1/_2$-D-Cys-6 oxytocin derivatives, the chemical shifts of the enriched carbon for the diastereoisomers in free solution differ. This is expected due to their different stereostructures. However, when bound to neurophysin, these diastereoisomers, which bind with equal affinity, have identical chemical shifts. It is possible that both the native hormone and the $^1/_2$-D-Cys-6 diastereoisomer can assume essentially identical conformations in the neurophsin–hormone complex, though their conformations appear to be somewhat different in solution (Hruby et al., 1979). In this case, it would appear that three-dimensional relationships that are different in the free diastereoisomers become identical or conformation changes occur that render them accidentally in the same chemical environment, in the NP complex.

Our observation of no NP-induced chemical shift change and a small degree of line broadening for the α-carbon of Leu-8 of oxytocin agrees with the results of Griffin et al. (1977) and is consistent with little or no direct interaction between the tripeptide tail of oxytocin and NP (Glasel et al., 1973; Convert et al., 1977; Blumenstein & Hruby, 1977; Griffin et al., 1977). In light of this small degree of interaction, it is not surprising that the 8-D-Leu diastereoisomer binds to NP with the same affinity as does oxytocin.

In summary, we have observed a wide range of chemical shifts for different carbons of oxytocin and oxytocin analogues when the peptides bind to neurophysins. These shifts are influenced by the ionization state of the bound peptide, the conformation of the peptide side chain, the degree of interaction of each particular residue with NP, and the configuration of the observed enriched residue (i.e., D or L). This work illustrates the sensitivity of ^{13}C chemical shifts to noncovalent effects involved in peptide–macromolecule interactions, and the various aspects of this interaction that can be studied by ^{13}C NMR, and provides new insights into the nature of neurohypophyseal hormone–neurophysin interaction.

Acknowledgments

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