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Binding of Oxytocin and 8-Arginine-vasopressin to Neurophysin Studied by ^{15}N NMR Using Magnetization Transfer and Indirect Detection via Protons†

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ABSTRACT: NMR was used to monitor the binding to neurophysin of oxytocin and 8-arginine-vasopressin, ^{15}N labeling being used to identify specific backbone ^{15}N and ^{1}H signals. The most significant effects of binding were large downfield shifts in the amino nitrogen resonance of Phe-3 of vasopressin and in its associated proton, providing evidence that the peptide bond between residues 2 and 3 of the hormones is hydrogen-bonded to the protein within hormone–neurophysin complexes. Suggestive evidence for hydrogen bonding of the amino nitrogen of Tyr-2 was also obtained in the form of decreased proton exchange rates on binding; however, the chemical shift changes of this nitrogen and its associated proton indicated that such hydrogen bonding, if present, is probably weak. Shifts in the amino nitrogen of Asn-5 and in the –NH protons of both Asn-5 and Cys-6 demonstrated that these residues are significantly perturbed by binding, suggesting conformational changes of the ring on binding and/or the presence of binding sites on the hormone outside the 1–3 region. No support was obtained for the thesis that there is a significant second binding site for vasopressin on each neurophysin chain. The behavior of both oxytocin and vasopressin on binding was consistent with formation of 1:1 complexes in slow exchange with the free state under most pH conditions. At low pH there was evidence of an increased exchange rate. Additionally, broadening of ^{15}N resonances in the bound state at low pH occurred without a corresponding change in the resonances of equilibrating free hormone. The results suggest significant conformational alteration in neurophysin–hormone complexes at low pH possibly associated with protonation of the carboxyl group of the hormone–protein salt bridge.

The sensitivity of ^{15}N chemical shifts in peptidic amides to hydrogen bonding of the amide proton or to the carboxamide carbonyl has been well estabished in model systems (Saito et al., 1971) and in peptides (Live et al., 1979, 1984a). However, conventional NMR methods for detecting ^{15}N have found little applicability to peptides and proteins, even in enriched systems. This is due to the low intrinsic sensitivity of direct detection and the futility of using the nuclear Overhauser effect to enhance sensitivity; for large molecules the NOE[1] is either very small or may cause a nulling of the ^{15}N signal. New NMR methods for enhancement of sensitivity in studies of peptides in water via magnetization transfer (Morris & Freeman, 1979), or indirect detection via protons (Bax et al., 1984; Live et al., 1984b) have now dramatically increased sensitivity, offering nominal enhancements in favorable cases of 10- and 1000-fold, respectively. Chemical shift information for ^{15}N from indirect detection via protons is obtained with a two-dimensional experiment (Aue et al., 1976; Bax et al., 1983), and the shift of the directly bonded amide proton is simultaneously obtained. In the present study, we have applied ^{15}N NMR spectroscopy to the interaction of bovine neurophysin

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[1] Abbreviations: NOE, nuclear Overhauser effect; AVP, 8-arginine-vasopressin; FT, Fourier transform; HPLC, high-performance liquid chromatography.

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I with ^{15}N-substituted oxytocin and 8-arginine-vasopressin. Neurophysins are small proteins (M_r 10000, dimerizing to M_r 20000) that interact noncovalently with oxytocin and vasopressin within the neurosecretory granules of the hypothalamo–neurohypophyseal tract [for reviews, see Breslow (1984), Chaiken et al. (1983), and Cohen et al. (1979)]. ^{13}C NMR studies involving labeled hormones and neurophysin have also been reported [e.g., Blumenstein et al. (1979, 1984)]. In this system, the ^{13}C lines are much broader than those of ^{15}N due to the greater gyromagnetic ratio of the nucleus; thus, the latter nucleus can provide a more sensitive measure of changes in chemical shift.

Bonding interactions involved in neurophysin–hormone complex formation have been extensively investigated, but significant questions remain. One issue is the role of hormone backbone hydrogen bonding in stabilization of the complex. The major stabilizing bonds between hormone and protein are a salt bridge between the protonated hormone α-amino group and a protein carboxyl group and apolar bonding interactions involving the side chains of hormone residues 1–3 and the protein [e.g., Breslow (1984)]. The large negative enthalpy change associated with the reaction has additionally suggested a hydrogen-bonding contribution (Carlson & Breslow, 1981). Thermodynamic studies of the binding of systematically modified dipeptide amides to the hormone site on the protein have provided specific evidence that the peptide bond between residues 2 and 3 of small ligand peptides is hydrogen-bonded to the protein (Carlson & Breslow, 1981; Whittaker et al., 1985). This hydrogen bonding accounts for some, but not all, of the negative enthalpy change associated with neurophysin–peptide and neurophysin–hormone interaction (Whittaker et al., 1985). The question arises as to whether comparable interactions are found in complexes of neurophysin with the intact hormone and whether other elements of the hormone backbone also participate in hydrogen bonding. The latter issue is significant since the affinity of neurophysin for the intact hormones is greater than 10-fold that for tripeptides representing the hormone amino terminus. Weak interactions of neurophysin with hormone residues beyond the 1–3 region are therefore possible (Breslow et al., 1973; Breslow, 1984). ^{15}N NMR is ideally suited for investigating hydrogen-bonding interactions. We have studied the effects of neurophysin on the ^{15}N resonances of oxytocin and vasopressin specifically enriched with ^{15}N in selected backbone N atoms and have determined the binding-induced chemical shift changes of protons attached to ^{15}N.

The number of hormone binding sites per neurophysin chain is uncertain. In addition to the dominant binding site, to which either oxytocin or vasopressin or small peptide analogues of their amino termini can bind, evidence of a second site, with affinity approximately $^1/_{10}$ that of the strong site at pH 6, has been reported [e.g., Cohen et al. (1979)]. This site has been reported to bind vasopressin under physiological conditions and oxytocin only in the presence of approximately 1.5 M LiCl (Cohen et al., 1979). In contrast, Bothner-By et al. (1980) have presented evidence that the vasopressin-specific site, if present, must have an affinity much lower than the reported value of 10^4 M^{-1}. In the present study, we have probed the existence of a second vasopressin site by ^{15}N NMR. Studies were carried out at both neutral and very acid pH to allow for the possibility that the reported difference in pH dependence of the two sites (Nicolas et al., 1980) might lead to a change in distribution between sites with pH. In the course of this study, no second site of the affinity reported by Cohen et al. (1979) was found. However, we have discovered significant changes in neurophysin–hormone complexes at very low pH, probably associated with protonation of the amino–carboxylate salt bridge.

Materials and Methods

Proteins and Peptides. Bovine neurophysin I was purified as described previously (Virmani-Sardana & Breslow, 1983). The preparations of ^{15}N-enriched peptides used are those described elsewhere (Live et al., 1979; Fischman et al., 1980; Cowburn et al., 1983). ^{15}N enrichment was estimated as greater than 90% in the final products. Recovery of neurophysin from its complexes with peptide was achieved by chromatography on Sephadex G-25 in 0.2 M acetic acid, followed by lyophilization of the protein peak. Concentrations of peptide and protein were determined by lyophilized weights or absorbance coefficients [e.g., Bothner-By et al. (1980)]. Molar equivalency is per neurophysin monomer (M_r 10000). Peptides were routinely examined by proton NMR before addition of neurophysin. Occasionally oligomeric peptide material was detected; this was removed by gel filtration (Live et al., 1977).

NMR Spectroscopy. NMR spectra were obtained in 10% D_2O–90% H_2O. A Nicolet 300WB was used for all studies. Conventional single-pulse proton spectra were acquired with 90° pulses of 6–8 μs. Selective excitation for amide ^1H spectra in H_2O were performed with the sequence

$$(\text{delay},(\text{pulse } 2_x,1_{-x},4_x,1_{-x},2_x,\alpha_x),\text{acquire}) \quad (1)$$

A single period of the pulse was approximately equal to [10 × (transmitter frequency − water resonance frequency)]$^{-1}$, and α was 10–20% of the single period. This is a trivial extension of the widely used Redfield sequence (Redfield et al., 1975), and the α period compensates for phase distortion of gating on and off. Five-millimeter or 12-mm sample tubes were used in appropriate probes.

Magnetization-Transfer Direct-Detection Pulse/FT ^{15}N NMR. A standard Nicolet 20-mm $^{15}N[^1H]$ probe was used. The ^{15}N transmitter was attenuated 6 dB to reduce probe arcing, leading to a 90° (^{15}N) pulse width of 110 μs. Several sequences based on the INEPT experiment (Morris & Freeman, 1979; Morris, 1980) were used. Proton-decoupled spectra were obtained by the sequence

$$(\text{delay},90_x°(^1H),4J^{-1},180_y°(^1H);180_x°(^{15}N),4J^{-1},90_y°(^1H;\ ^{15}N),4J^{-1},180_x°(^1H);180_y°(^{15}N),4J^{-1},\text{acquire with } ^1H \text{ decoupling}) \quad (2)$$

for $J \simeq 92$ Hz. In sequences 1 and 2 all phases and acquisition were rotated 90° on each acquisition to suppress quadrature artifacts.

Proton-Detected ^{15}N Multiquantum Two-Dimensional Detection via Protons. Probes designed for proton detection were modified by addition of orthogonal coils tunable to the ^{15}N frequency. Pulse lengths used were, for 90° ^1H, 8 (5 mm) and 15 (12 mm) μs and, for 90° ^{15}N, about 130 μs in both probes. The pulse sequence used (Bax et al., 1983) was

$$(\text{delay},\theta(^1H),2J^{-1},90_\phi°(^{15}N),t_1,90°(^{15}N),\text{acquire}(^1H)) \quad (3)$$

The phase ϕ and the acquisition phase were rotated 90° on each acquisition. The proton pulse θ was the lengthened Redfield pulse described in (1). The evolution period t_1 was generally incremented through 16 or 32 steps equivalent to the multiquantum dispersion (f_1) of 2000 Hz. The transmitter frequencies were normally centered at the position equivalent to ca. 8 ppm (^1H) and ca. 125 ppm (^{15}N). Further details have been published previously (Live et al., 1984; Ortiz-Polo et al., 1986).

Table I: Effects of Binding and pH on Apparent Chemical Shifts of Hormone in Hormone–Neurophysin Mixtures

	chemical shift (ppm)							
	unbound hormone[a]		hormone–neurophysin mixtures					
			bound			free		
	pH 3	pH 6	pH 6	pH 2.6–3	pH 2	pH 6	pH 2.6–3	pH 2
[[^{15}N]Asn^5]oxytocin	116.1	116.1	114.2	114.4	115.1	116.1	116.1	116.0
[[^{15}N]Tyr^2]oxytocin	123.5		125.0	124.7	124.6		123.5	123.7
[[^{15}N]Asn^5]AVP	115.9	115.9	115.1	[115.7][b]	[115.9][b]	115.9	[115.7][b]	[115.9][b]
[[^{15}N]Tyr^2]AVP	123.1		124.7	124.4			123.1	
[[^{15}N]Phe^3]AVP	120.8	120.2	123.9	123.4	123.0	120.2	120.8	121.2
[[^{15}N]Cys^6]AVP	119.7		120.9					

[a] With the exception noted, the chemical shifts of the free hormones represent those separately determined in the absence of protein. The chemical shift of unbound [[^{15}N]Phe^3]AVP at pH 6 was determined from two-dimensional ^{1}H[^{15}N] spectra in the presence of protein. The proton shift of the unbound hormone (determined in the absence of protein) was used to compute the chemical shift of the ^{15}N with which it was associated in hormone–protein mixtures. [b] Bound and free peaks could not be resolved. The chemical shift shown represents the average peak position.

Proton spectra were referenced to 2-methyl-2-propanol. Directly detected ^{15}N spectra are referenced indirectly via proton references (Live et al., 1984) to liquid $^{15}NH_3$ (Levy & Lichter, 1979). Temperatures of samples were directly read from the spectrometer's controller and are accurate to ca. 1.5° and reproducible to better than 1°. Proton-detected ^{15}N two-dimensional spectra are displayed with frequency scales in hertz, since the f_1 dimension measures the sums or differences of the offset frequencies for ^{1}H and ^{15}N.

Spectra were analyzed by the curve fitting routine NMCCAP supplied by the spectrometer manufacturer. The precision of measurement was limited by the available signal to noise ratio and possible phasing errors. Repeated manual analysis of the same data gave results reproducible to better than 10%.

Equilibrium Dialysis. Equilibrium dialysis was performed by modification of the procedure described earlier (Bothner-By et al., 1980), with 10 mg/mL protein and 0.16 M KCl, pH 3, as solvent. All components were adjusted to pH 3 prior to dialysis; the pH was unchanged at the end of the run. Analysis at equilibrium of total protein and peptide components in the two dialysis chambers was performed by quantitative analytical HPLC with a Zorbax CN column attached to an IBM HPLC equipped with a UV absorption monitor and integrator. Elution was performed according to the procedure of Chaiken and Hough (1980); the protein–hormone complexes were demonstrated to be completely dissociated by the elution conditions, permitting each component to be measured in the presence of the other.

Results

[[^{15}N]Tyr^2]oxytocin. Figure 1 shows the ^{15}N spectrum of oxytocin, enriched with ^{15}N in the amino nitrogen of Tyr-2, under varying conditions of pH and temperature in the presence of 1 equiv of neurophysin. The results show the effects of binding on this nitrogen and additionally demonstrate that the behavior of the oxytocin complex, as monitored by ^{15}N spectroscopy, parallels that demonstrated by other techniques.

At pH 6, 25 °C, in the presence of neurophysin, a single peak is seen centered at 125.0 ppm and is assignable to oxytocin bound to the principal hormone binding site. The spectrum of unbound oxytocin labeled at this position cannot be observed because of rapid exchange of the proton of the ^{15}N–H under these conditions. The chemical shift of 125.0 ppm of the complex compares with the value for free oxytocin at pH 3 of 123.5 ppm (Table I). The completely protonated free hormone is the correct reference point for the bound state since the hormone is completely protonated in the bound state at all pH values (Blumenstein et al., 1979).

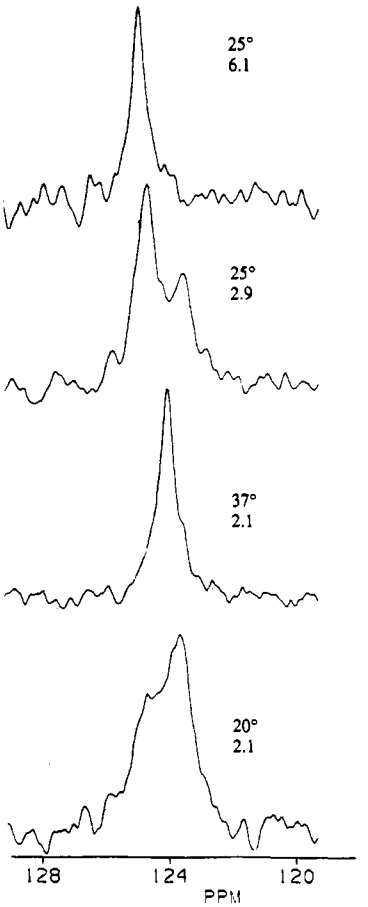

FIGURE 1: INEPT ^{15}N spectra of [[^{15}N]Tyr^2]oxytocin, 1.77 mM, with 1.66 mM neurophysin, at temperature and pH values shown. Each spectrum was obtained by averaging approximately 15 000–20 000 transients in about 3 h, in this and other figures.

At a 1:1 oxytocin:neurophysin ratio, lowering the pH to 2.9 at 25 °C (Figure 1) results in the appearance of a second peak with the chemical shift of free oxytocin (123.5 ppm), indicating increased dissociation at low pH as has been well documented previously [e.g., Breslow and Gargiulo (1977)]. There is also an apparent slight shift of the "bound" peak to higher field (124.7 ppm), which is not due to exchange with free hormone, since unbound hormone has a normal chemical shift. The reality of the shift is supported by the behavior of the other labeled hormones at low pH (vide infra). Accordingly, the shift is attributed to a change in the environment of bound hormone associated with increased protein protonation. Slow exchange between bound and free hormone is then indicated under these conditions, as observed earlier at neutral pH by proton and ^{13}C NMR (Blumenstein et al., 1978) and by

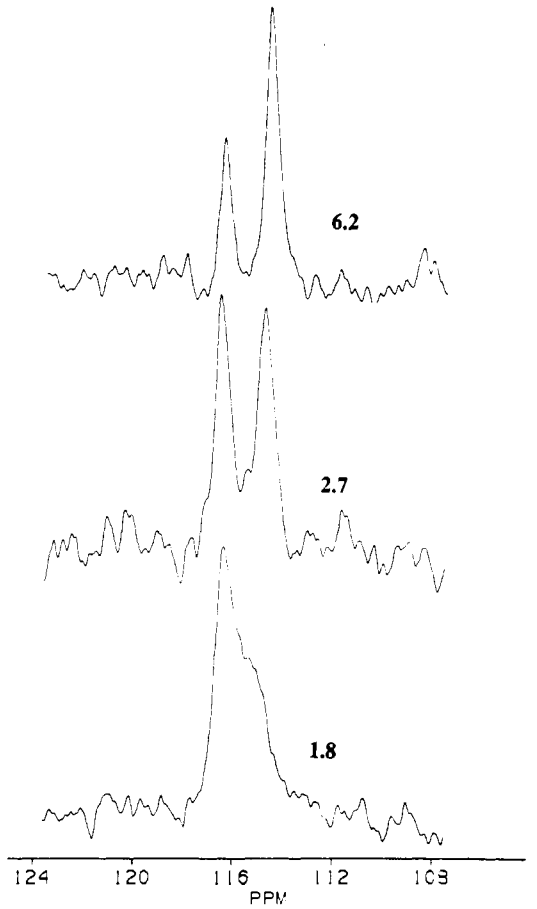

FIGURE 2: INEPT ^{15}N spectra of [[^{15}N]Asn^5]oxytocin, 1.9 mM, with 1.4 mM neurophysin, at the pH values shown and 25 °C.

temperature-jump kinetics (Pearlmutter & McMains, 1977). From the chemical shift difference between free and bound states, the exchange rate is estimated as <5 per second under these conditions.

The relative intensities of the "bound" and "free" hormone peaks at pH 2.9 were determined by curve fitting as 65:35. With use of the concentrations given (1.7 mM NP, 1.8 mM oxytocin), a binding constant of 4×10^3 M^{-1} at pH 2.9 was calculated from the data. At pH 6.0, the equivalent constant is 10^5 M^{-1}; the reduction in binding strength at low pH is in agreement with expectation (Breslow & Gargiulo, 1977).

When the pH is further lowered to 2.1 (20 °C) at a 1:1 hormone:protein ratio (Figure 1), there is a further increase in the ratio of free:bound hormone, in accord with expectation. This is accompanied by a slightly increased merger of free and bound peaks, suggestive of an increased exchange rate at this pH, as demonstrated earlier (Blumenstein et al., 1979). At 37 °C, pH 2.1, a single dominant peak is present, with a chemical shift intermediate between that of the free state and that of the bound state, indicating a further increase in exchange rate with increased temperature at this pH, also in agreement with earlier work (Blumenstein et al., 1979).

[[^{15}N]Asn^5]oxytocin. The effects of 0.7 equiv of neurophysin on the spectrum of the peptide nitrogen of [[^{15}N]-Asn^5]oxytocin are shown in Figure 2 as a function of pH. At pH 6, two peaks are present, representing the free hormone, as can also be measured in the absence of protein (116.1 ppm), and bound hormone (114.2 ppm) in slow exchange. The calculated ratio of the areas of bound to free hormone is 7:3, in excellent agreement with that expected for 1:1 binding and 0.3 equiv of excess hormone. The direction of the chemical shift of this nitrogen on binding is opposite to that seen above

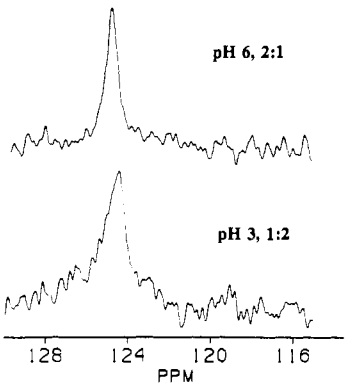

FIGURE 3: INEPT ^{15}N spectra of [[^{15}N]Tyr^2]AVP at 25 °C and the pH values and ratios (hormone:protein) indicated. Concentration of neurophysin is 1.33 mM in each case.

for the nitrogen of Tyr-2. On lowering the pH to 2.7, the intensity of the free peak increases relative to that of bound as expected (vide supra), and there is a 0.2 ppm shift in the spectrum of the bound form, as observed also for the nitrogen of Tyr-2 but opposite in direction. There are no changes in the chemical shifts of the free hormone at these pH values. At pH 1.8, there is a further increase in free hormone and, again, a partial merger of free and bound hormone peaks. It appears that a probable pH dependence of exchange rates (Blumenstein et al., 1979) is insufficient to explain this, since curve fitting indicates that the peak for free hormone is shifted upfield by 0.1 ppm while that for bound is shifted downfield by 0.7 ppm and broadened slightly more than the free peak (data not shown). This suggests a significant pH dependence of the nature of the bound form below pH 3.

[[^{15}N]Tyr^2]AVP. At pH 6.2, 25 °C, in the presence of approximately 0.5 equiv of neurophysin (7.5 μmol of hormone and 4 μmol of protein in 3 mL), this hormone showed a single line at 124.7 ppm (Figure 3). This position compares with 123.07 ppm for the free hormone at low pH (Table I). (Again, the free hormone cannot be seen at pH 6 because of fast proton exchange at this position.) The bound position for this ^{15}N in AVP is almost identical with that in bound oxytocin (vide supra). At pH 3.1, also as with oxytocin, the bound peak shifted to 0.3 ppm to higher field (124.4 ppm). This is seen in Figure 3 from the pH 3 spectrum obtained in the presence of 2 equiv of protein. At higher hormone:protein ratios at pH 3, there is the appearance of a second peak near the position of free hormone at this pH (data not shown). To determine whether any of the low-pH phenomena in our AVP studies reflected the presence of additional sites, we carried out equilibrium dialysis studies of AVP and protein at pH 3 using similar protein concentrations as used for the NMR studies, 2.5 equiv of hormone. At an equilibrium AVP concentration of 9×10^{-4} M, only 0.8 mol of hormone was bound per mole of neurophysin, and a binding constant was calculated for a single site of 4×10^3 M^{-1}. This value is in good agreement with earlier results at this pH using 8-lysine-vasopressin (Bothner-By et al., 1980), with the binding constant for oxytocin at this pH calculated from spectral data above and with the value for AVP calculated from spectral data below.

[[^{15}N]Asn^5]AVP. Figure 4 shows the spectrum of this hormone in the presence of neurophysin at a hormone:protein ratio of 1.5:1 at pH 6; the curve resolution of the spectrum is also shown. Two principal peaks are present with chemical shifts of 115.14 and 115.87 ppm, corresponding to bound and free hormone, respectively, the latter independently determined in the absence of protein. A smaller peak at 118.4 ppm was shown to be an impurity or artifact.[2] Curve resolution in-

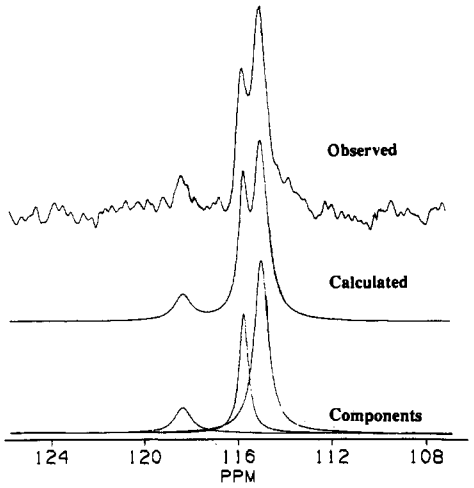

FIGURE 4: INEPT ^{15}N spectra of [[^{15}N]Asn^5]AVP, 2.1 mM, in the presence of 1.4 mM neurophysin at 25 °C, at pH 6, with curve fitting results.

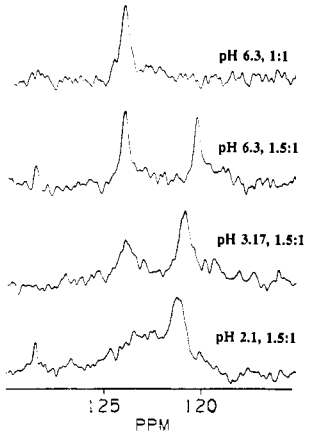

FIGURE 5: INEPT ^{15}N spectra of [[^{15}N]Phe^3]AVP in the presence of 1.4 mM neurophysin at the hormone:protein ratios and pH values indicated at 25 °C.

dicates a ratio of bound to unbound hormone of 2.5:1, in reasonable agreement with the value of 2:1 calculated for a 1:1 complex plus excess unbound hormone.

Upon lowering the pH of solutions of [[^{15}N]Asn^5]AVP and neurophysin (1.2:1 ratio) to pH 3 and 2, decreased binding is evidenced by a progressive shift in the average peak position of two poorly resolved peaks such that only a single peak, with the chemical shift of free hormone, is discernible at pH 2 (see Table I). The failure to resolve free and bound peaks at low pH in part reflects the relatively small chemical shift separation between free and bound forms of this nitrogen but probably also reflects an increased exchange rate between free and bound AVP at pH 2, as evidenced from studies below.

[[^{15}N]Phe^3]AVP. The chemical shift of the ^{15}N of this hormone in the absence of protein is 120.2 ppm at pH 6 and 120.8 ppm at pH 3 (see also Table I). Figure 5 shows the spectrum of the hormone at pH 6 at hormone:protein ratios of 1:1 and 1.5:1. At the 1:1 ratio, a single peak, representing

[2] The extra small peak seen in the presence of [[^{15}N]Asn^5]AVP and neurophysin is essentially invariant under different conditions and, accordingly, is not assigned to a bound form of the hormone. However, if the view is taken that it represents a second site on neurophysin for AVP, its binding affinity for hormone can be calculated. From the data obtained in the presence of excess AVP at pH 6, for example, where the free AVP concentration can be obtained from the spectra, the relative intensities of the extra peak and that of free hormone give a calculated binding constant of 4×10^2 M^{-1}. This is markedly less than the value 10^4 M^{-1} calculated for a second AVP site by Cohen et al. (1979).

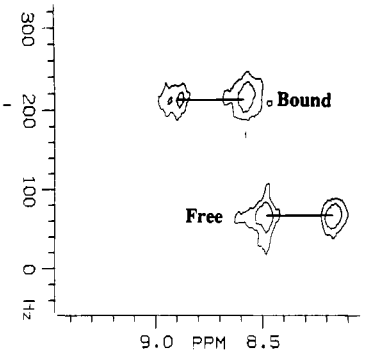

FIGURE 6: Heteronuclear multispin coherent proton-detected NMR $^1H[^{15}N]$ spectra of [[^{15}N]Asn^5]AVP, 2.1 mM, in the presence of 1.4 mM neurophysin, at pH 3.0, 25 °C.

Table II: Chemical Shifts of ^{15}N–H Protons in Free and Bound Hormone

hormone	chemical shift (ppm) free[a]	chemical shift (ppm) bound (pH 6)
[[^{15}N]Asn^5]oxytocin	8.32 (pH 3) 8.33 (pH 6)	8.69
[[^{15}N]Asn^5]AVP	8.35 (pH 3) 8.32 (pH 6)	8.71
[[^{15}N]Phe^3]AVP	8.13 (pH 3) 8.03 (pH 6)	8.89
[[^{15}N]Tyr^2]AVP	8.90 (pH 3)	9.00
[[^{15}N]Cys^6]AVP	8.16 (pH 3) 8.18 (pH 6)	7.70

[a] Determined in the absence of protein.

bound hormone, is present at 123.9 ppm. At a 1.5:1 ratio, both bound (123.9 ppm) and free (120.2 ppm) hormones are evident. The relative areas of bound and free hormone peaks are 60:40, just slightly less than the expected 2:1 ratio for a 1:1 complex. Under these conditions, there is again no evidence of a second site for AVP.

Upon lowering the pH of the hormone–protein system, two phenomena are apparent (Figure 5). First, there is an increase in free hormone. At pH 3.1, at a hormone:protein ratio of 1.5, for example, the calculated ratio of bound:free has decreased to 46:54. The binding constant calculated from this ratio, 2×10^3 M^{-1}, is in very good agreement with that calculated above under these conditions by equilibrium dialysis. Second, there is a 0.5 ppm upfield shift in the position of bound hormone and an approximately 0.6 ppm downfield shift in the free hormone position. The shift in free hormone is accounted for by the effects of α-NH_2 protonation ($pK_a = 6.3$) on the chemical shift of this nitrogen in the unbound state. As with ^{15}N-substituted oxytocin (vide supra), the spectrum of the bound form therefore shifts at low pH without comparable effect on the unbound hormone. At pH 2.1, the peak corresponding to free hormone has shifted downfield by an additional 0.1 ppm, suggesting an increased exchange rate. The peak corresponding to bound hormone, however, has broadened without a comparable broadening of the free hormone peak, indicating a further change in the nature of the bound species at this pH. This is the same observation made with [[^{15}N]-Asn^5]oxytocin at pH 2 (vide supra). The width of the bound peak prevents accurate determination of its area or position. It is relevant that the broadening of the bound hormone peak at low pH is unaffected by the presence of EDTA, arguing that it is not induced by paramagnetic metal ions.

[[^{15}N]Cys^5]AVP. The chemical shift of the ^{15}N in the unbound state is 119.75 ppm. A 1:1 mixture of hormone and protein at pH 6.3 showed a single peak at 120.9 ppm that broadened at pH 3 and shifted to a position (average of free

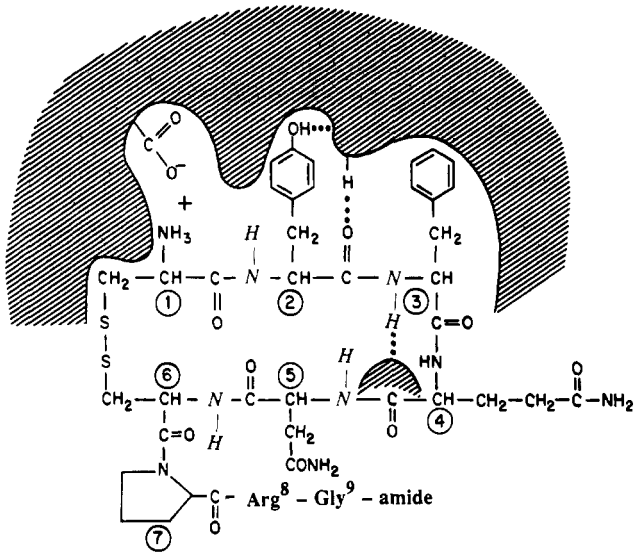

FIGURE 7: Proposed bonding interactions between AVP and neurophysin (Breslow, 1984; Whittaker et al., 1985). The nitrogen atoms and associated protons investigated in this study are italicized.

and bound) of 120.6 ppm (data not shown).

Summary of ^{15}N Chemical Shifts. Table I summarizes the binding-induced changes in chemical shifts of the different ^{15}N-substituted positions at pH 6 and the effects of pH on the observed chemical shifts of selected neurophysin–hormone mixtures. The significance of these shifts is discussed below.

Proton Shifts. Figure 6 shows the two-dimensional $^{1}H[^{15}N]$ spectra from which the chemical shift of the ^{15}N–H proton of [[^{15}N]Asn^5]AVP was obtained. Table II summarizes the proton shifts associated with binding of this and other ^{15}N-enriched hormones. The proton at position 3 undergoes the largest of the shifts (0.76 ppm downfield), but significant downfield shifts are also seen in the protons of [^{15}N]Asn-5-labeled hormones and a significant upfield shift is seen in the proton associated with ^{15}N at position 6.

Discussion

The most recent view of proposed bonding interactions between hormone and neurophysin [e.g., Breslow (1984) and Whittaker et al. (1985)] is shown in Figure 7. The present results support the concept that elements of the hormone backbone are hydrogen-bonded to the protein within its complex with neurophysin as proposed. This is most evident for the amino nitrogen of residue 3, in agreement with predictions based on the binding of modified dipeptides to neurophysin (Carlson & Breslow, 1981; Whittaker et al., 1985). The ^{15}N of Phe-3 shifts 3.2 ppm downfield in the complex, the direction associated with increased hydrogen bonding (Witanowski et al., 1977) and the largest of the binding-induced shifts. This shift is accompanied by a large (0.76 ppm) downfield shift in the associated proton; downfield proton shifts of this magnitude are also characteristic of the formation of strong –N–H hydrogen bonds (Pardi et al., 1983). Significantly, Blumenstein et al. (1984) found a 4.2 ppm binding-induced downfield shift in the α-carbon resonance of position 3 of oxytocin, also the greatest of the backbone carbon shifts observed, but attributed this to a change in side-chain conformation. We cannot preclude changes in conformation as contributing to the chemical shift change at position 3, particularly since recent studies (D. Cowburn and J. Glushka, unpublished data) suggest that unusual backbone conformations might influence chemical shift. The side chain of residue 3 is not highly fixed in the binding pocket however [e.g., Breslow (1979) and Whittaker et al. (1985)], making it unlikely that changes in its conformation per se would produce the chemical shift seen. Ring currents can also, in principle, contribute to shifts at position 3, but the observed effects are too large to arise solely from this source; the large downfield shifts of ^{15}N (and ^{13}C) necessitate distances from an aromatic ring that are shorter than van der Waal's distances (Johnson & Bovey, 1958). Therefore, although effects of conformation remain to be further explored, the single explanation that suffices for both the ^{15}N and proton shifts and the effect of peptide modification on binding to neurophysin (Carlson & Breslow, 1981) is that the peptide bond between hormone residues 2 and 3 is hydrogen-bonded to neurophysin in the complex.

Evidence for hydrogen bonding of other hormone backbone segments is less clear. Binding of hormone to neurophysin reduces the proton exchange rate at the nitrogen of Tyr-2, as evidenced by the fact that ^{15}N at this position can be observed only in the bound state. The reduced proton exchange rate is consistent with a shielded and/or hydrogen-bonded environment for this nitrogen in the bound state. The binding-induced change in chemical shift of this nitrogen is, however, only 1.5–1.6 ppm downfield, and the change in chemical shift of its associated proton is only 0.1 ppm. Thus, any hydrogen bonding of this –NH to the protein is likely to be weak [cf., Witanowski et al. (1977) and Pardi et al. (1983)]. It is also possible that the change in proton exchange rate at this position reflects the formation in the bound state of a salt bridge between the hormone α-NH_3^+ and a protein carboxyl [e.g., Breslow (1984)]; the α-NH_3^+ is a potential contributor to the rate of exchange of the –NH proton at Tyr-2 (Krauss & Cowburn, 1981).

Binding-induced changes at positions 5 and 6 demonstrate that segments of the ring outside of the 1–3 sequence are perturbed by binding, probably both by conformational change as suggested by Blumenstein et al. (1984) and by direct interaction with the protein as we suggest. The binding-induced upfield shift of the peptide nitrogen of Asn-5 indicates that the abnormal upfield position of this nitrogen relative to model compounds in the unbound state (Live et al., 1979) becomes even more abnormal in the bound state. The abnormal position of Asn-5 in the free hormone reflects conformational factors arising from the disulfide cyclization of the hormone ring. This is demonstrated by the change in the Asn-5 shift toward a more normal value upon disulfide reduction. Disulfide reduction leads to a 4.4 ppm downfield shift in the ^{15}N of Asn-5 while shifting the ^{15}N of Tyr-2, which has a normal value in the hormone, only 0.3 ppm upfield (data not shown). Possible explanations of the upfield shift of Asn-5 in the free hormone include conformational strain and/or abnormal solvation. The additional upfield shift upon complex formation therefore indicates a still more abnormal state for this region in the bound state.

In addition to binding-induced changes in ^{15}N shifts outside the 1–3 region of the hormones, the 0.4 ppm downfield shift in the peptide proton of Asn-5 and the 0.5 ppm upfield shift in the –NH proton of Cys-6 are sufficiently large to warrant attention. These effects should be viewed within the context of the greater affinity of neurophysin for oxytocin and vasopressin than for tripeptides representing the amino-terminal region of the hormones (introduction). This has been interpreted as indicating weak interactions of the protein with regions of the hormone beyond residues 1–3 or a conformation of the free hormone that is more similar to that of the bound state than is that of free tripeptide (Breslow et al., 1973; Breslow, 1984). The significant chemical shifts at Asn-5 and

Cys-6 therefore may represent direct interactions of this segment of the hormone backbone with neurophysin. Alternatively, if these particular shifts arise from conformational change (by definition energetically uphill), the increased affinity of neurophysin for the intact hormone relative to tripeptides probably reflects compensating interactions with other parts of the molecule outside the 1–3 sequence. A potential interaction involves the side chain of Cys-6, since this is directly bonded to the involved side chain of Cys-1.

The present results argue against a second binding site for vasopressin of the pH-independent affinity ($\sim 10^4$ M^{-1}) reported by Cohen and co-workers [e.g., Cohen et al. (1979)]. For example, in the presence of excess [[^{15}N]Asn^5]- or [[^{15}N]Phe^3]vasopressin at neutral pH, only two significant[2] bands are present, one assignable to hormone at the principal binding site as judged by saturation of this site in the presence of a molar equivalent of hormone and a second with the chemical shift of free vasopressin. Moreover, the ratio of the two bands is in good agreement with that predicted for 1:1 interaction. Additionally, only two proton peaks are seen in the two-dimensional spectra of these hormones, one with the chemical shift of free hormone and the other representing bound hormone (e.g., Figure 6). A second site of any significant affinity ($\geq 10^3$ M^{-1}) can only then be present if, at both ^{15}N and ^{1}H positions, it is associated with a chemical shift identical with that of unbound hormone. Observation of the ring protons of Tyr-2 of 8-lysine-vasopressin in the presence of neurophysin and equilibrium dialysis studies at high protein concentration also failed to produce any evidence of a significant second binding site for vasopressin (Bothner-By et al., 1980). Some potential sources of this discrepancy have been discussed elsewhere (Breslow, 1984).

Effects of pH on hormone binding as observed by ^{15}N NMR were essentially the same as those found by other techniques [e.g., Blumenstein et al. (1979)]. A surprising feature of the pH data, however, is the shift and broadening of the bound state at low pH in the absence of comparable broadening of the free hormone resonance. This suggests that bound hormone is equilibrating between two different states at low pH. The pH region in which this occurs is compatible with the concept that it is associated with protonation of the salt bridge carboxyl, which has a pK of ~ 2 in neurophysin–dipeptide complexes (Breslow & Gargiulo, 1977; Blumenstein et al., 1979). Given this, the different substituent positions that are affected at low pH (e.g., Asn-5 and Phe-3) suggest that the conformation or positioning of the entire hormone within the complex is altered by salt bridge protonation. Proton NMR data suggest that bound tripeptides may exhibit similar pH effects (Balaram et al., 1973).

In summary, this study demonstrates that ^{15}N NMR spectra of labeled peptides in complexes of M_r 22 000 are readily observable by magnetization-transfer methods (INEPT) or indirect-detection multispin coherence. While on one hand this method is limited because of possible rapid exchange of the ^{15}N–^{1}H proton (exchange of the order of $2J^{-1}$ prevents Z magnetization transfer or transverse multispin coherence), the method can be used to detect changes in this exchange rate, as seen here for ^{15}N at position 2. Additionally, changes in ^{15}N shifts due to hydrogen bonding can be relatively large compared to probable ring current shifts, facilitating the interpretation of such shifts as in the case here of ^{15}N at position 3. In the present system, ^{15}N studies of enriched peptides have allowed the observation of binding-induced changes in hormonal peptides that could not be seen by other techniques. The usefulness of ^{15}N enrichment for distance measurements via proton–proton nuclear Overhauser effects (Weiss et al., 1986) should also find potential application in this and related systems. We have, as well, demonstrated that the proton-detected two-dimensional experiment provides a particularly practical means of detecting specific –NH protons in a complex such as this. The significant proton shifts observed suggest that this measurement should prove valuable in future studies of such complexes.

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Hydrodynamic Properties of the Gonadotropin Receptor from a Murine Leydig Tumor Cell Line Are Altered by Desensitization

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ABSTRACT: The murine Leydig tumor cell line 1 (MLTC-1) contains gonadotropin receptors (GR) that are coupled to adenylate cyclase through the stimulatory guanine nucleotide binding protein (G_s). The binding of human choriogonadotropin (hCG) causes MLTC-1 cells to accumulate cAMP. With time, the ability of MLTC-1 cells to respond to hCG is attenuated by a process called desensitization. The hydrodynamic properties of GR from control and desensitized MLTC-1 cells were studied. Sucrose density gradient sedimentation in H_2O and D_2O and gel filtration chromatography were used to estimate the Stokes radius (a), partial specific volume (v_c), sedimentation coefficient ($s_{20,w}$), and molecular weight (M_r) of the detergent-solubilized hormone–receptor complex (hCG–GR). [^{125}I]hCG was bound to MLTC-1 cells under conditions that allow (37 °C) or prevent (0 °C) desensitization, and hCG–GR was solubilized in Triton X-100. In the absence of desensitization, control hCG–GR had a M_r of 213 000 (a = 6.2; v_c = 0.76; $s_{20,w}$ = 7.3), whereas desensitized hCG–GR had a M_r of 158 000 (a = 6.1; v_c = 0.71; $s_{20,w}$ = 6.6). Deglycosylated hCG (DG-hCG) is an antagonist that binds to GR with high affinity but fails to stimulate adenylate cyclase or cause desensitization. [^{125}I]DG-hCG was bound to MLTC-1 cells and DG-hCG–GR solubilized in Triton X-100. The hydrodynamic properties of DG-hCG–GR (M_r 213 000; a = 5.8; v_c = 0.77; $s_{20,w}$ = 7.6) were the same as that for control hCG–GR. There was no evidence for the association of adenylate cyclase or G_s with GR in Triton X-100 solubilized preparations. When hCG was cross-linked to GR and solubilized with sodium dodecyl sulfate (SDS), the M_r was found to be 116 000 (a = 4.9; v_c = 0.75; $s_{20,w}$ = 5.2), which was similar to that determined by SDS–polyacrylamide gel electrophoresis and less than that of the Triton X-100 solubilized control hCG–GR.

Chemical or photoaffinity cross-linking of radiolabeled gonadotropin to its receptor in conjunction with SDS–PAGE[1] has been used to determine the molecular weight and possible subunit structure of the gonadotropin receptor. Studies employing these techniques have not led to agreement on either point. Conflicting results may reflect differences in the tissue source and in experimental technique. A number of studies contend that the hormone-binding subunit of the GR is in the range of M_r 70 000–90 000 (Dattatreytamurty et al., 1983; Rapoport et al., 1984; Hwang & Menon, 1984; Ascoli & Segaloff, 1986; Bruch et al., 1986; Wimalasena et al., 1986). Some studies indicate that this is the only polypeptide that makes up the GR (Metsikko & Rajaniemi, 1982; Metsikko, 1984; Kusuda & Dufau, 1986). Other studies suggest that the GR is heterooligomeric (Hwang & Menon, 1984; Ji et al., 1985; Bruch et al., 1986). Further complicating the issue are results suggesting that the heterooligomeric nature of the receptor seen by some investigators is the result of proteolysis (Kellokumpu & Rajaniemi, 1985; Ascoli & Segaloff, 1986).

Hydrodynamic studies on the gonadotropin receptor, though fewer in number, have been less conflicting in their results. Although these studies have not approached the subject by rigorous application of hydrodynamic techniques, they do indicate that the receptor is larger than the 70–90 kDa suggested for the hormone-binding subunit of GR by most of the SDS–

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[1] Abbreviations: hCG, human chorionic gonadotropin; DG-hCG, deglycosylated hCG; EGS, ethylene glycol bis(succinimidyl succinate); GR, gonadotropin receptor; G_s, stimulatory guanine nucleotide binding protein; M_r, molecular weight; PAGE, polyacrylamide gel electrophoresis; PBS, Dulbecco's calcium–magnesium-free phosphate-buffered saline; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; cAMP, adenosine cyclic 3′,5′-phosphate; ATP, adenosine 5′-triphosphate; NAD, nicotinamide adenine dinucleotide.