^{13}C NUCLEAR MAGNETIC RESONANCE STUDIES OF THE INTERACTIONS OF BOVINE NEUROPHYSINS WITH (1-HEMI-[1-^{13}C]CYSTINE)OXYTOCIN AND (1-HEMI-[1-^{13}C]CYSTINE, 8-ARGININE)VASOPRESSIN

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Received 20 June 1977

1. Introduction

In the pituitary gland of most mammals, the neurohypophyseal hormones, oxytocin and arginine vasopressin (AVP) are found in neurosecretory granules complexed to a group of proteins called neurophysins. Several studies [1–5] indicate that the α-amino group of the hormones is intimately involved in the interaction with the proteins. Binding studies of neurohypophyseal hormone–neurophysin interaction have indicated that the binding constant is pH-dependent [2,5–7] and a plot of binding constants versus pH [5] yields a bell shaped curve, with pK_a values of about 4.5 and 7. The pK_a of the α-amino group of the free hormones is about 6.3 [4] and the pK_a of 7 observed in the presence of neurophysin was suggested to represent the pK_a of this group in the protein–hormone complex. On the other hand, the pK_a of about 4.5 was suggested to be due to a negatively charged group on the neurophysin which interacts with the hormone.

In order to directly monitor the ionization state of the α-amino group of oxytocin and arginine vasopressin when complexed to the neurophysins, we have synthesized hormones specifically enriched with ^{13}C (90%) in the carbonyl carbon of the half-cystine-1 residue of the hormones, and observed the ^{13}C NMR spectra of the protein–hormone complexes. The chemical shift of this carbonyl carbon in the hormones is very sensitive to the ionization state of the adjacent amino group [8]. We have found the ^{13}C resonance of the enriched carbon to be easily observed in the 1:1 (molar ratio) hormone–protein complex and this has enabled us to monitor the ionization state and pK_a value of the hormone's α-amino group in the hormone–protein complex.

2. Materials and methods

The synthesis of (1-hemi-[1-^{13}C]cystine)oxytocin (i), (1-hemi-[1-^{13}C]cystine, 8-arginine)vasopressin (ii), (1-hemi-D-[1-^{13}C]cystine)oxytocin (iii) and [1-hemi-D-[1-^{13}C]cystine, 8-arginine)vasopressin (iv) will be described in detail elsewhere (Hruby, V. J., Yamamoto, D., Blumenstein, M. and Yang, Y. in preparation). The bovine neurophysins were isolated and purified as described elsewhere [7,9].

The samples for NMR study were prepared as previously [9]. The quoted pH values are direct readings (Radiometer 26) in D_2O. Spectra were recorded on a Bruker WP-60 FT spectrometer operating at 15.1 MHz. Spectral conditions were as follows: spectral width, 5000 Hz; memory, 4 K or 8 K; repetition rate, 2.5 s; pulse width, 16.5 μs (~90°); number of scans, 9000–36 000 (6–24 h); exponential multiplication (artificial broadening), about 4 Hz.

3. Results

The far downfield region of the ^{13}C NMR spectra of (ii) in the absence and presence of neurophysin II (NPII) (1:1 molar ratio), as well as a spectrum of NPII in the presence of unlabeled AVP is shown in fig.1. The peak at about 168 ppm in the complex is due to the enriched carbon. In free AVP, the position of this resonance is pH-dependent, but in the protein–hormone complex the position is independent of pH.

The linewidth of the bound resonance is 5–10 Hz. This width is more than an order of magnitude less than that observed for the labeled carbon of (1-hemi-[2-^{13}C]cystine)oxytocin when the hormone is bound to NPII [16]. The decreased linewidth is due to the absence of directly bonded protons [10] and has been previously observed in studies of enriched carbonyl carbons in molecules ligarded to proteins [11–14].

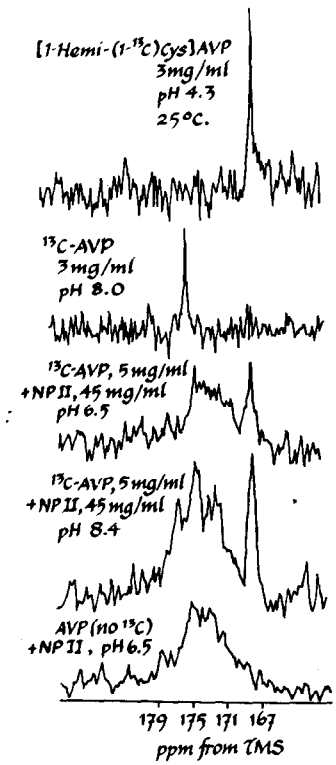

Fig.1. ^{13}C NMR spectra: of (1-hemi-[1-^{13}C]cystine, 8-arginine)vasopressin (ii), at pH 4.3 and 8.0 (no added protein); of (ii) and bovine neurophysin II, at pH 6.5 and 8.4; and of unlabeled AVP in the presence of neurophysin II, at pH 6.5. All spectra were run at 25°C.

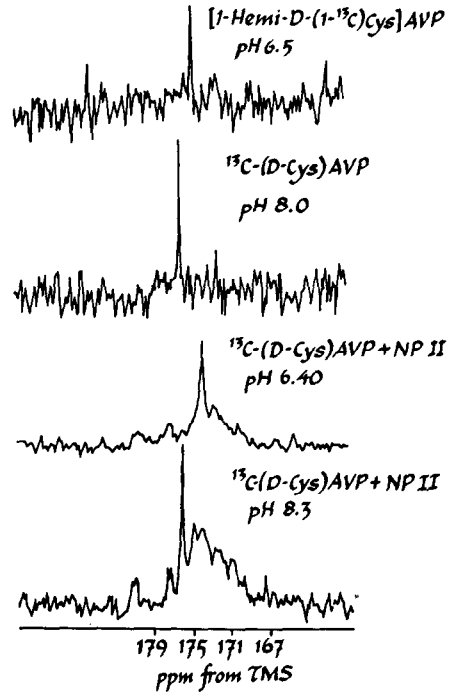

Fig.2. ^{13}C NMR spectra: of (1-hemi-D-[1-^{13}C]cystine, 8-arginine)vasopressin (iv), at pH 6.5 and 8.0 (no protein added – top 2 spectra); and of (iv) 4.5 mg/ml in the presence of bovine neurophysin II (45 mg/ml) at pH 6.4 and 8.3. All spectra were taken at 25°C.

Spectra of (1-hemi-D-[1-^{13}C]cystine, 8 arginine)-vasopressin (iv) in the absence and presence of NPII are shown in fig.2. This diastereoisomer apparently does not significantly bind to NPII, and the position of the resonance due to the enriched carbon is unaffected by the presence of protein.

Figure 3 shows the titration of (ii) and (iv) in the absence and presence of NPII. In the presence of protein, data could not be acquired below pH 6.5 due to the insolubility of the hormone–protein complex. The chemical shift of the carbonyl carbon of bound AVP from pH 6.5 to pH 9 is almost identical to that observed in the protonated form of free AVP. For the 1-hemi-D-cystine derivative (iv) the pK_a is different than in AVP, but the same pH dependence of the chemical shift is seen with our without NPII. Similar titration curves were performed

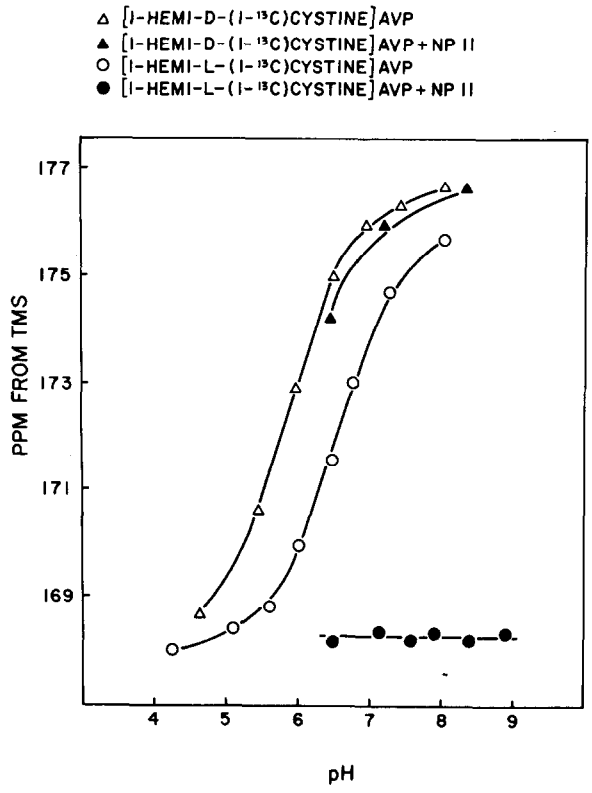

Fig.3. Plot of the chemical shift of the labeled carbon in (1-hemi-[1-^{13}C]cystine, 8-arginine)vasopressin in the absence (○) and presence (●) of bovine neurophysin II, and of (1-hemi-D-[1-^{13}C]cystine, 8-arginine)vasopressin in the absence (△) and presence (▲) of neurophysin II as a function of pH.

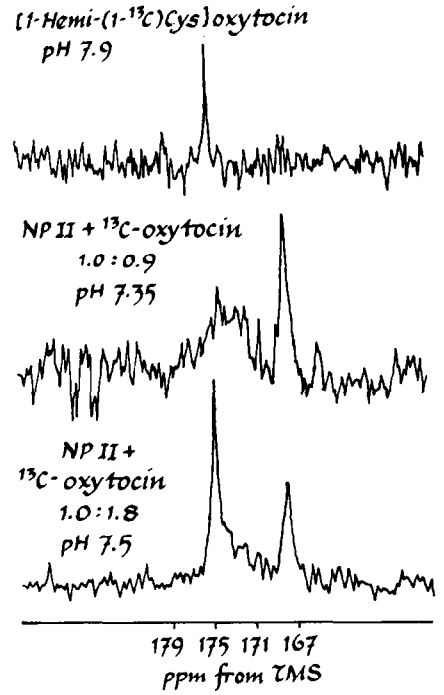

Fig.4. ^{13}C NMR spectra: of (1-hemi-[1-^{13}C]cystine)oxytocin (i) at pH 7.8 (top); of (i) and bovine neurophysin II in a 0.9:1.0 molar ratio (middle) at pH 7.35; and of excess (i) in the presence of neurophysin II (1.8:1.0 molar ratio) at pH 7.5.

for free and bound (i) (and for (iii)) and the data obtained were essentially identical to those obtained with AVP derivatives (ii) (and (iv)).

Figure 4 shows the spectra of free oxytocin derivative (i), of (i) in the presence of equimolar NPII and of excess (i) in the presence of NPII. The spectrum with excess hormone is a superposition of the spectra of the free and fully bound hormone. Therefore, the rate of exchange of the hormone between the neurophysin and bulk solution is slow, and we can put an upper limit of 5 s^{-1} on this exchange rate. Similar results were obtained with NPI and (i). The addition of excess AVP derivative (iii) to neurophysins is more complicated and will not be discussed here.

4. Discussion

The concentrations of hormone and protein used in these NMR studies are well above the dissociation constants of the hormone–neurophysin complexes under the same conditions. Therefore, by mixing the hormones and proteins at a 1:1 molar ratio, we are able to observe directly the resonance due to the enriched carbon of the bound hormone.

The position of the bound resonance of the labeled carbonyl in (i) strongly indicates that the α-amino group of half-cystine-1 is in the charged form throughout the observed pH range. Thus the α-amino group in the bound hormone has a $pK_a > 9.5$, in contrast to the value of 6.3 observed in free hormone. This raised pK_a is consistent with a strong interaction of the amino group with a negatively charged moiety on the neurophysin, and the magnitude of the pK_a increase supports the hypothesis [4] that this interaction occurs in a hydrophobic region which is rather inaccessible to solvent. The apparent pK_a of about 7

which influences the protein–hormone dissociation constant [5] is most likely due to the ionization of the α-amino group of the free hormone, as no protein ionization which affects the carbonyl chemical shift occurs in this pH region. The different value of the pK_a of the free hormone as compared to the pK_a which affects binding, may be due to the different conditions under which these pK_a values were determined. Also the actual pK_a which influences the binding constant is best determined as described by Dixon [17] i.e., plotting log K versus pH, rather than K versus pH [5].

The finding of a relatively slow rate of exchange of oxytocin between neurophysin and bulk solution is in general agreement with our previous work [9,16], as well as the work of Balaram et al. [18] and Pearlmutter and McMains [19]. The reasons for the disagreement of the above studies and thos Alazard et al. [20] and Glasel et al. [21] have been discussed previously [16]. The upper limit of 5 s^{-1} which we find is less than the rate of 18 s^{-1} determined by Pearlmutter and McMains [18]. However, the off rate will likely be affected by protein concentration as well as buffer and salt conditions, and these conditions all differ between our NMR studies and the temperature jump studies.

Our results indicate that the interactions of the amino residue of oxytocin and AVP with the neurophysins are identical in a hormone–protein ratio of 1:1. We have found a near equivalence of oxytocin and AVP to be present for the methylene carbon of the glycinamide-9 [9,16] and the α-carbon of tyrosine-2 (Blumenstein and Hruby, unpublished). At greater than 1:1 hormone to protein ratio, the behavior of oxytocin and AVP appear to differ with AVP possibly interacting with a second binding site [22]. NMR experiments with compound (ii) as well as with hormones labeled in other positions are in progress to shed additional light on the interaction of AVP with neurophysins at greater than 1:1 stoichiometries.

Acknowledgements

This work was supported by the US Public Health Service AM-17420 (VJH) and HD-10616 (MB), The National Science Foundation and Abbott Laboratories (VJH).

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