HORMONAL INTERACTIONS AT THE MOLECULAR LEVEL: A HIGH RESOLUTION PROTON MAGNETIC RESONANCE STUDY OF BOVINE NEUROPHYSINS AND THEIR INTERACTIONS WITH OXYTOCIN

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1. Introduction

The neurohypophyseal nonapeptides oxytocin and 8-arginine (or 8-lysine) vasopressin are biologically important hormones known to associate non-covalently with the proteins, neurophysins I and II [1–3]. The physical and chemical properties of the neurophysins, whose polypeptides have molecular weight near 10,000, have been investigated by several authors [4,5], and some amino acid sequences for bovine neurophysins I and II have been proposed [6,7]. The study of the molecular interactions between neurophysins and peptide hormones has been approached using classical equilibrium methods [8–12] and optical techniques [13,14].

High resolution proton magnetic resonance (PMR) has proved useful in providing information about selected residues in proteins during the processes of denaturation [15–17] and inhibitor–enzyme interactions [18,19]. A few studies of protein–polypeptide or protein–protein interactions using high resolution magnetic resonance techniques have been reported [20–22]. We present here PMR observations on highly purified bovine neurophysins and the complex formed with oxytocin. In particular, high resolution PMR methods enable us to study the possible involvement of the lone histidine residue of neurophysin I or of the single tyrosyl residue of oxytocin in the hormonal complexes.

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2. Materials and methods

Highly purified neurophysins I and II were obtained by isoelectric focusing and found homogeneous by disc gel electrophoresis. Details of the preparation will be described elsewhere [12]. The synthetic oxytocin peptide was a generous gift from Dr. Guttmann (Sandoz, Basle).

Samples of neurophysins for PMR studies were lyophilized several times from D_2O and were dissolved in 0.1 M NaCl in D_2O (100%, Aldrich Chemical Co.) at a concentration of 30 mg in 0.5 ml.

Spectra were recorded on a Varian HR 220 spectrometer with a probe temperature of 22 ± 1°. Computer time-averaging and pH measurements were carried out as described elsewhere [17]. pH values quoted are direct meter readings. Chemical shift values are quoted in ppm downfield from external 6% tetramethylsilane in carbon tetrachloride.

As a control experiment for viscosity effects, neurohypophyseal proteins of molecular weight ≃ 50,000 obtained during the neurophysin preparation were used. This protein fraction was found to be devoid of hormonal binding ability by equilibrium dialysis experiments carried out with radioactive oxytocin [12]. The PMR spectrum of oxytocin was recorded in the presence of 15 mg of this protein and showed a negligible line broadening effect (< 10%).

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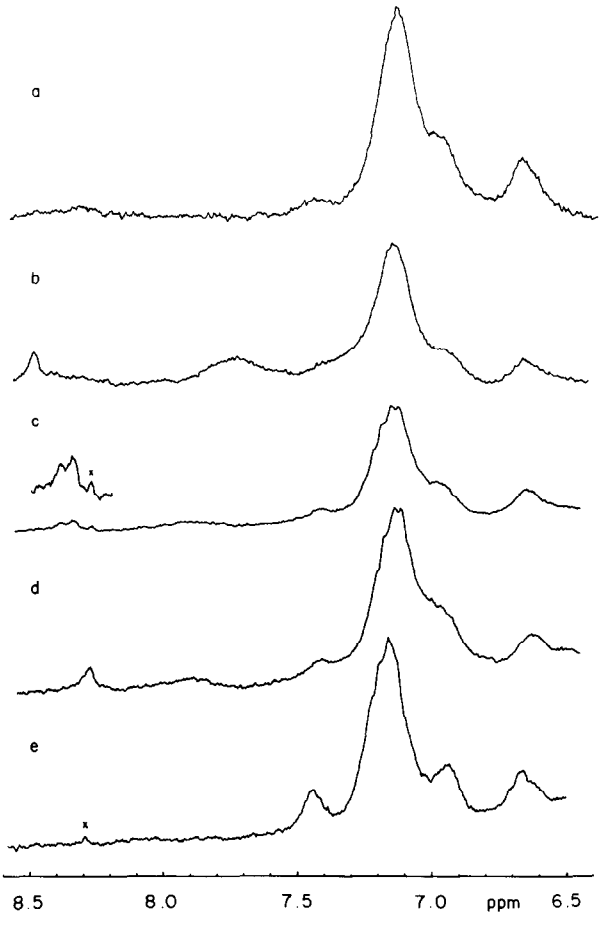

Fig. 1. The aromatic region of PMR spectra recorded in D_2O, 0.1 M NaCl, of a) neurophysin-II at pH 6.52, and of neurophysin-I at pH values b) 2.21 ; c) 5.77 ; d) 6.09 ; e) 8.85. The number of scans varied between 9 and 37. The insert in c) is the same spectrum at higher gain. The peaks marked X are not due to neurophysin.

3. Results and discussion

Some representative PMR spectra of neurophysin-I and II are shown in fig.1. The resonance in the low field region between 8.5 and 7.4 ppm in figs. 1b–e which shifts with pH may be attributed to the C2 imidazole ring proton [20,23,24] of the single histidine residue of neurophysin-I which is absent from neurophysin-II (fig.1a) [25]. Spectrum 1b is taken below the isoelectric point, pH 4.4, of neurophysin-I [5,12]. Near this pH value, the resonance lines broaden due to aggregation and the protein tends to precipate. About pH 5 the protein redissolves and the lines correspondingly sharpen again*.

The assignment of peaks in the downfield region of a spectrum of neurophysin-I (fig.1d) is shown in fig.2 in which the resonance peaks are fitted with Lorentzian functions [24,26].

The pH titration data of the histidine C2 proton resonance of neurophysin-I is shown in fig.3. This fits quite well a theoretical curve based on a simple proton association equilibrium and gives a pK value of 6.9, indicative of a normally titrating histidine residue [24].

The titration curve of the neurophysin-I:oxytocin complex at 1:1 molar ratio (95% oxytocin binding sites filled) [11, 12] is shown in fig. 3. The histidine pKa value is shifted 0.20 pH units to a lower value due to complex formation. This represents a somewhat more positive (less electron dense) environment for the histidine residue in the hormone–neurophysin-I complex. Such a relatively minor pK shift suggests that the histidine residue does not play a significant role in the hormonal interaction. This conclusion might be expected since neurophysin-II binds oxytocin yet lacks a histidine residue.

In the aliphatic region of the PMR spectrum, the protein resonances obscure the oxytocin resonances, even at low protein molar ratios, and it is virtually impossible to distinguish the resonances of the two components. However, oxytocin contains a single tyrosine residue, and it is possible to follow the ef-

* One unusual feature of the titration of the histidine C2 proton resonance in this case was the consistent observation of a doubling of the peak at certain pH values (fig.1c). The analysis of resonances in this region was complicated by the presence of a third small resonance, marked X, which was observed in several spectra and which did not shift with pH. Spectra at three pH values between 5.6 and 5.9 clearly showed the doubling phenomenon of the histidine resonance. It was difficult to obtain spectra at pH values slightly lower than this due to precipitation of the protein [5], and by pH 6.0 the phenomenon was no longer observable. This doubling phenomenon could represent a slow equilibrium of the histidine residue between two environments as previously suggested for similar observations involving histidine resonances [15,17]. Changes in the environment of the histidine residue may possibly be related to aggregation processes known to occur in this pH range [5].

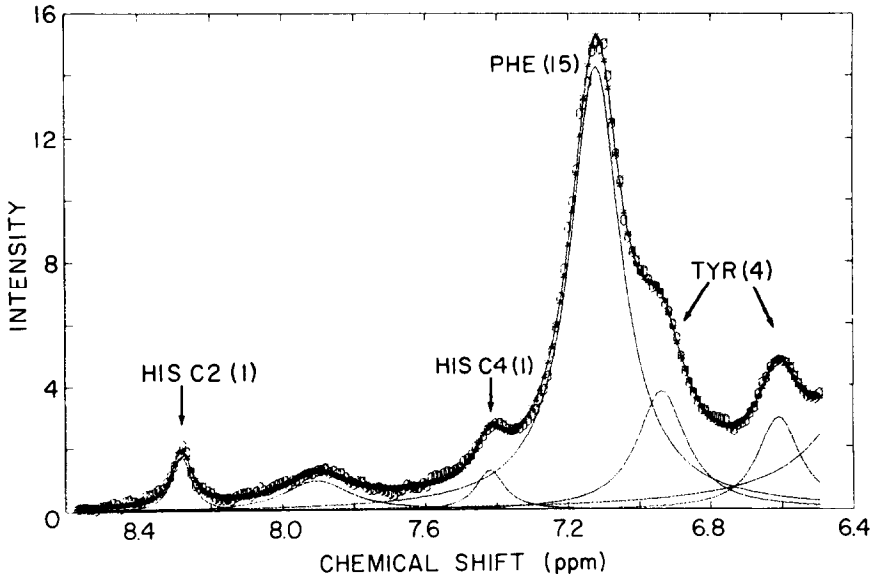

Fig. 2. The PMR spectrum of neurophysin-I from fig. 1d fitted with Lorentzian curves. (o. observed; *, calculated; –, components); The assignments of the peaks are shown in the CALCOMP plot with the relative number of protons in parenthesis. The broad resonance at chemical shift 7.75 ppm is attributed to protons which exchange slowly with water, since it disappears entirely over a 24 hr period due to exchange with deuterium (fig.1).

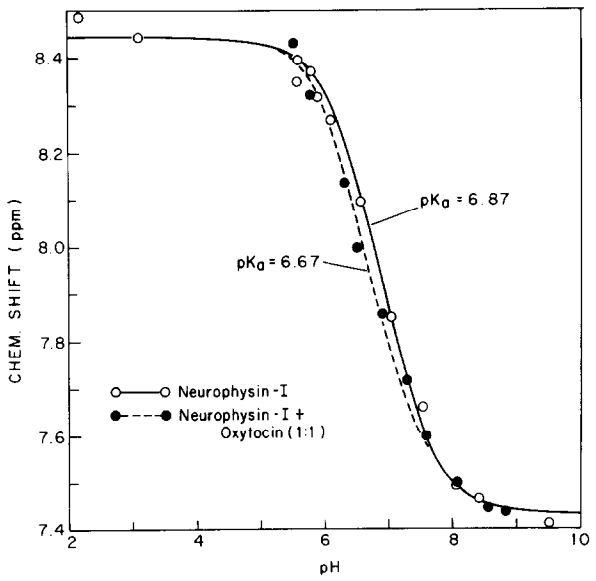

Fig. 3. Titration curves of the C2 proton resonance of the single histidine residue of neurophysin-I and of the neurophysin-I:oxytocin complex. The lines are least squares fits to the data using the theoretical expression for a simple proton.

fects of the addition of neurophysin-II on the tyrosyl aromatic protons. PMR spectra at several molar ratios are shown in fig.4. The mean value of the full width at half height of each of the two tyrosine resonances in the high field doublet at 6.7 ppm is plotted in fig.5 as a function of the molar ratio of the total amount of each species in solution.

The apparent linear increase in line width of the oxytocin tyrosyl C3 and C5 ring proton resonances (fig. 5) could arise from either a slow or a fast exchange process between two states for the tyrosyl residue. For example, in the case of a simple bimolecular process,

$$\text{oxytocin} + \text{neurophysin} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [\text{oxytocin–neurophysin}]$$

the exchange of the tyrosyl residue between free and and bound states can be described by equations derived for either the fast or the slow exchange limit [27]. Work is in progress to measure the line broadening at different temperatures and to determine the longitudinal relaxation time T_1 of the tyrosyl resonances. This should serve to distinguish between the possibil-

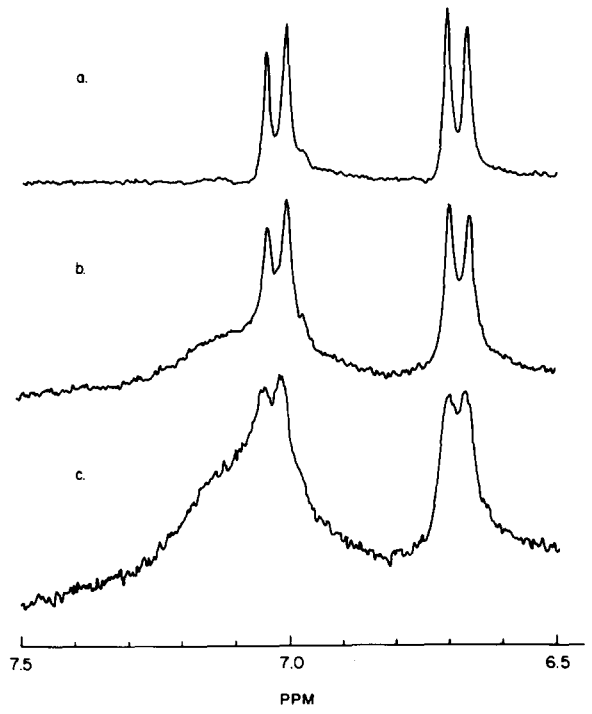

Fig. 4. The aromatic region of PMR spectra of oxytocin a) at pH 6.60, and after the addition of aliquots of neurophysin-II to yield oxytocin:neurophysin molar ratios at pH 6.79 of b) 7:1 and c) 3:1.

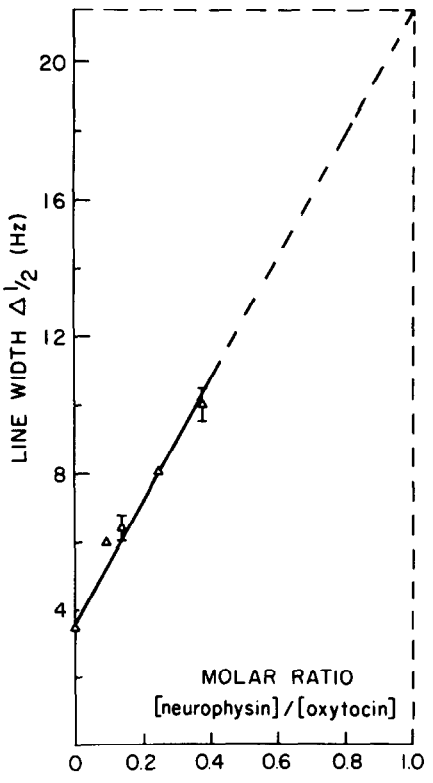

Fig. 5. Plot of the observed full line width at half height of the tyrosyl resonance at 6.7 ppm of oxytocin versus the molar ratio of neurophysin:oxytocin.

ities of slow or fast exchange for the oxytocin tyrosyl binding process.

Since the observed line width is related through $1/T_2$ to the rotational correlation time of the tyrosyl side chain, the large increase in line width upon binding would indicate a significant restriction in motion of the tyrosyl residue if fast exchange obtains. Involvement of the tyrosyl ring of oxytocin in the binding process, compatible with the PMR studies, is consistent with the apparent lack of binding capacity of 2-isoleucine–oxytocin [9] and of oxytocin iodinated on the tyrosyl ring [12]. Experiments carried out with oligopeptides corresponding to the N-terminal amino acid sequence of the hormone also reinforce this idea [5]. Complex formation between neurophysin and oxytocin was suggested to involve hydrophobic or $\pi-\pi$ interactions between neurophysin and the hormone's tyrosyl side chain as well as hydrogen bonding of the tyrosyl phenolic hydroxyl groups. However, $\pi-\pi$ interactions seem unlikely in this case, since the stacking of aromatic rings produces measurable (> 0.1 ppm) upfield shifts [28] and no significant change in chemical shift of the oxytocin tyrosyl resonances is observed at the highest molar ratios studied (fig.4). Additionally, hydrogen bonding of the phenolic hydroxyl may not be essential for hormone binding since 2-phenylalanyl–oxytocin is bound almost as well as oxytocin [9]. In this case, the tyrosyl residue of oxytocin would seem to be bound in the hormonal–neurophysin-II complex through hydrophobic bonding.

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