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THE TYROSINE RING OF OXYTOCIN UNDERGOES HINDERED ROTATION WHEN THE HORMONE IS BOUND TO NEUROPHYSIN

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SUMMARY

Tyrosine specifically enriched with ^{13}C in the meta positions has been chemically synthesized and incorporated into oxytocin via solid phase peptide synthesis. The ^{13}C nmr spectrum of a 1:1 mixture of the enriched hormone complexed to neurophysin was obtained. The spectrum consisted of three peaks. The two outer peaks, representing 85% of the total intensity, were of equal area, had shifts of -0.9 and +2.4 parts per million relative to the free peak, and each had a linewidth of 100 hz at 20°C, with increasing linewidths at higher temperatures. These two peaks arise from a binding mode in which tyrosine ring rotation is hindered by interaction with neurophysin. The rotation rate at 20°C is $130s^{-1}$, and at 42°C is $900s^{-1}$. The central peak occurred at the position of the resonance due to free hormone, had a temperature independent linewidth of 30-40 hz, and represented about 15% of the total intensity. We believe this peak is due to a binding mode in which tyrosine ring rotation is rapid, 10^4-10^8s^{-1}.

Rotation rates of aromatic side chains in proteins have been measured (1,2 and references therein). Rapid ($>10^4s^{-1}$), intermediate (10^1-10^4s^{-1}) and very slow ($<10^1s^{-1}$) rates have all been observed. Also, extensive theoretical calculations have been performed on these rotation rates (3). The experimental and the theoretical studies have yielded new insights into the dynamics associated with protein structures. It has been shown that even bulky side chains, located in the interior of proteins and in close contact with other residues, can undergo significant motions. These motions come about due to rapid local fluctuations in the surrounding structure.

This communication presents observation of the phenomenon of aromatic ring rotation, but in a tyrosine residue of a hormone, oxytocin, when bound to a protein, neurophysin (NP). Oxytocin is complexed to NP in vivo, in the posterior pituitary gland, and analog studies have shown that an aromatic residue in position 2 is essential for oxytocin-NP interaction (4,5). We

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have been using ^{13}C nuclear magnetic resonance (nmr) to study the oxytocin-NP system, and have previously reported on work in which the α-carbon at tyrosine 2 has been enriched with 90% ^{13}C, and its spectral characteristics when bound to NP observed (6). Here, we present studies in which the meta carbons of tyrosine-2 of oxytocin have been enriched. These carbons are essentially equivalent in the free hormone, but when the hormone binds to NP they become nonequivalent, a condition which allows tyrosine ring rotation to be measured.

MATERIALS AND METHODS

The synthesis of DL-[3'5'-$^{13}C_2$]-tyrosine, the incorporation of the labeled amino acid into oxytocin, the separation of the resulting oxytocin diastereomers, and the characterization of the labeled hormone as being identical to authentic oxytocin have been described elsewhere (7). The purification of NP, (NP I was used in the experiments described below) as well as the nmr techniques employed, have been described previously (6,8,9). For spectra at 67.9 MHz reported here, the spectral width was ± 3000 hz, the repetition rate 0.75s and the number of pulses 5,000-60,000. Peak positions, areas, and widths were determined by spectral simulation using the Nicolet NUS-530 curve analysis program.

RESULTS

Spectra of [2-[3',5'-$^{13}C_2$]tyrosine]oxytocin in the absence of protein, and in the presence of a slight molar excess of NPI at various temperatures are shown in Fig. 1. As expected, the spectrum of the free hormone consists of one peak, of width 2-3 hz, due to the two equivalent labeled carbons. The chemical shift of the peak is 115.5 parts per million (ppm), on a scale in which the shift of dioxane is defined as 66.5 ppm. In the presence of NPI, three resonances are observed. The central peak has a width of 30-40 hz, and has the same chemical shift as the peak observed in the free hormone. Its area is about 15% of the total due to the enriched hormone. The width of this peak, and the fact that it is observed at a hormone:protein ratio as low as 0.6:1.0, indicate that it cannot be due to unbound hormone. The other two peaks are broader than the central peak, and are of roughly equal area. One peak is shifted downfield 0.9 ppm relative to the central peak, and the other peak is shifted upfield 2.4 ppm. As the temperature is raised,

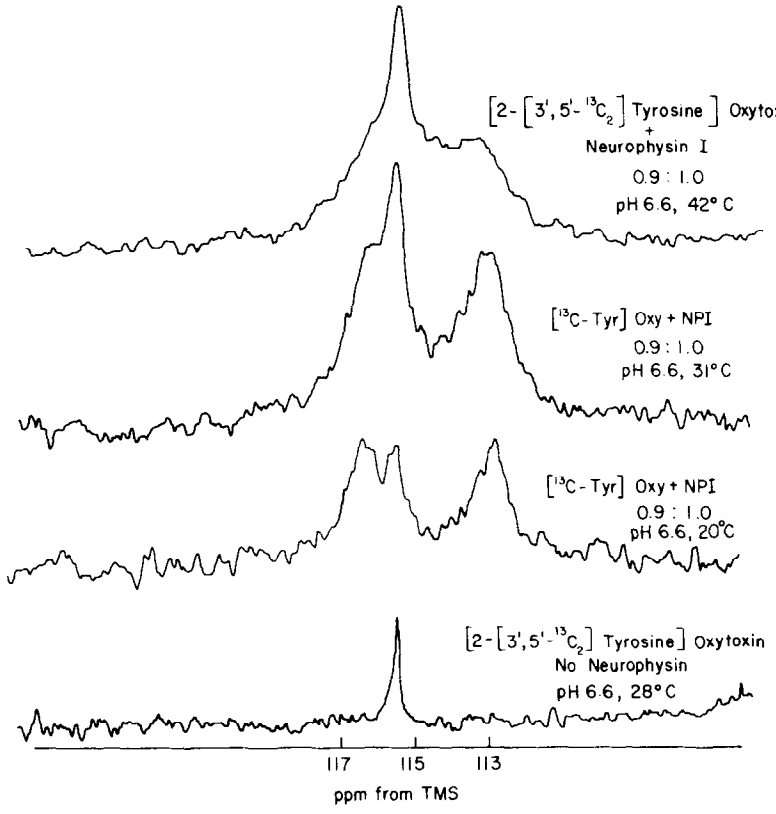

Fig. 1. ^{13}C NMR spectra at 67.9 MHz of [2-[3',5'-$^{13}C_2$]tyrosine]oxytocin in the absence (bottom) and presence of neurophysin I. The repetition rate was 0.75s, the pulse width 21 μsec (∿90°) and the number of pulses (bottom to top) 500, 10,000, 35,000 and 55,000. The protein concentration was 25 mg/ml (2.5 mM).

the outer peaks undergo a marked broadening, while the width of the central peak changes little. A control was performed using [2-D-]3',5'-$^{13}C_2$]tyrosine]-oxytocin, a diastereomer which does not bind NP. The spectrum in the absence of NP was identical to that due to the normal diastereomer, and the addition of NP caused no change in the spectrum.

Addition of excess hormone leads to no change in the broad outer peaks, but to an increased intensity and a gradual narrowing of the central peak. We have determined by spectral simulation that this effect would be observed if a sharp peak was superimposed on a broad peak having an identical shift, or if the broad peak was actually narrowing. Thus, we are unable to distinguish between the cases of fast and slow exchange for the central peak.

However, the outer peaks, representing the majority of the bound hormone, are clearly in slow exchange with respect to the free hormone.

The broadening of the outer peaks observed at pH 6.6 as the temperature is raised is in marked contrast to the behavior of the resonance due to the enriched α-carbon of [2-[2-^{13}C]-tyrosine]oxytocin bound to NPI (6). The α-carbon resonance narrows due to a decreased value of τ_c for the hormone-NP complex, while the broadening observed in the aromatic carbons is due to chemical exchange, which occurs at a faster rate at higher temperatures. The process causing this exchange is almost surely the rotation of the tyrosine ring around the β-γ bond axis. One can calculate this rotation rate by measuring the linewidths at the various temperatures and subtracting the portion of the linewidths due to τ_c effects (20-60 hz, depending on the temperature), ^{13}C-^{13}C coupling ($<$10 hz), and computer broadening (6 hz). These calculations lead to rotation rates ($\pm$ 25%) of 130s^{-1} at 20°C, 300s^{-1} at 31° and 900s^{-1} at 42°C.

For the binding mode (see Discussion) which gives rise to the central peak, only an upper limit can be placed on the rate of rotation of the tyrosine ring. Since the width of this peak is comparable to the width of the labeled α-carbon resonance, the rotation around the β-γ bond cannot be much greater than the overall tumbling rate, $\frac{1}{\tau_c}$, of the hormone-NPI complex (10). The upper limit for tyrosine ring rotation in this binding mode is about 10^8s^{-1}.

DISCUSSION

In free oxytocin, the aromatic ring of tyrosine-2 is rapidly rotating, so the meta carbons are equivalent, and only one resonance is observed for the two enriched carbons. This situation changes when the hormone binds to NP. Most of the added hormone binds in a manner such that rotation of the tyrosine ring is severely hindered, and the two meta carbons display separate resonances due to different interactions with NPI. At present, the direct cause of the observed shifts cannot be determined. From the widths of the

outer resonances, one can estimate a rotation rate about the β-γ bond of $130s^{-1}$ at 20°, and $900s^{-1}$ at 42°. These rates are comparable to those found for some of the aromatic rings in pancreatic trypsin inhibitor (1,2).

Since the aromatic ring in position 2 of neurohypophyseal hormones is essential for interaction with NP (4,5) any rotation of the ring in the bound hormone is somewhat surprising. The most likely explanation for the observed rotation rates is the same as the explanation for the observed rotation of closely surrounded aromatic rings of proteins, i.e., local fluctuations in structure (3). Thus, our results indicate that the peptide chains of oxytocin and neurophysin are undergoing motions relative to one another at rapid enough rates and of sufficient amplitudes to allow the tyrosine ring of oxytocin to flip.

Part of the added hormone, about 15%, binds such that the two meta carbons give rise to only one peak, having a chemical shift identical to that of the free hormone. The two meta carbons remain equivalent, and direct interaction with the protein is probably slight. The equivalence of the meta carbons means that the rate of rotation about the β-γ bond is much greater than the chemical shift difference between those carbons when the hormone binds to NP. Unfortunately, we do not know this chemical shift difference, so we cannot put a lower limit on the rotation rate. If the chemical shift difference were the same as that observed for the major binding mode, 225 hz, a lower limit of $10^4 s^{-1}$ could be put on this rate. An upper limit of $10^8 s^{-1}$ was determined (see results). The shift difference could be much less than 225 hz, and it could even be zero. A small chemical shift difference would also indicate a small degree of direct interaction with the protein, and a rotation rate which would likely be rapid, i.e. $>10^4 s^{-1}$.

There are various possibilities as to the origin of two observed binding modes. A heterogeneous protein preparation, such as is often present with NPI (11) is unlikely, since similar nmr results were obtained with NPII (unpublished results) which generally does not demonstrate the same degree of

heterogeneity. Another possibility is a conformational heterogeneity present in a protein having a single sequence. Such an effect was detected with dihydrofolate reductase (12), although it disappeared upon binding of inhibitor. Another explanation is that the two observed modes represent binding to NP dimer (restricted motion) and NP monomer (faster motion). Preliminary experiments involving varying concentrations make this possibility unlikely, but further experiments of this type must be done. Since both binding modes are observed at hormone:protein stoichiometries of less than 1:1, there is no reason to invoke a weaker hormone binding site, and our experiments with excess hormone indicate that a total of one mole of hormone is bound per mole of protein. The relative intensities of the nmr signals would, in fact, preclude a second site with a binding constant as weak as that postulated for vasopressin and peptide analogues of oxytocin and vasopressin (13,14). Although we observed the peaks due to several enriched carbons of oxytocin bound to NP (6,9,15 and unpublished results), we have not obtained evidence for more than one mode of binding in our other studies. The present case is very favorable for the observation of a minor binding mode, since there are two equivalent carbons in the labeled hormone, and there is virtually no interference from natural abundance background. For the labeled α-carbon of tyrosine-2, and in fact for all labeled α-carbons, a bound peak having an intensity representing 15% of added hormone would most likely not be observable above the natural abundance background.

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