UNEQUIVOCAL ASSIGNMENTS OF NH PROTON MAGNETIC RESONANCE BANDS IN OXYTOCIN USING ^{2}H- AND ^{15}N-SUBSTITUTED MOLECULES

A. F. BRADBURY, A. S. V. BURGEN, J. FEENEY, G. C. K. ROBERTS and D. G. SMYTH
National Institute for Medical Research, Mill Hill, London NW7 1AA, UK

Received 18 March 1974

1. Introduction

During the last few years, considerable efforts have been made to obtain conformational information on hormonal peptides in aqueous solution by the use of high-resolution nuclear magnetic resonance spectroscopy. Particular attention has been devoted to the measurement of the coupling constant, J_{NC}, between the αCH and NH protons, since this can be used as a measure of the dihedral angle, ϕ, about the αC–N bond [1,2]. This coupling constant is measured as a splitting on the absorption band of the NH proton. If meaningful conformational information is to be obtained, it is clearly essential that unambiguous assignments of the NH resonances be made.

Hitherto, NH assignments have depended on the use of double resonance experiments to connect NH and αCH, and αCH and βCH resonances, the assignments being made by comparison of the βCH chemical shifts with those in free amino acids. This procedure is straightforward for small peptides, but for more complex molecules such as oxytocin, where some of the βCH_2 protons have similar chemical shifts, and where the conformational effects on the βCH_2 shielding are unknown, ambiguities can arise. A further complication arises in studies in aqueous solution, since the αCH resonances are obscured by the large H_2O resonance.

Two reports of assignments of the ^{1}H NMR spectra of oxytocin in aqueous solution have appeared [3,4]. Walter and his colleagues [3] took the assignments made by double resonance experiments in dimethylsulphoxide (DMSO) solution [5], and attempted to transfer them to aqueous solution by measuring the NH chemical shifts in a series of DMSO/water mixtures. The extensive overlap of the NH resonances (notably at about 80% water, 20% DMSO) and the lack of any a priori information on the anticipated direction and magnitude of the changes in chemical shift lead to considerable ambiguities (apart from any ambiguities in the original assignments in DMSO solution [5]). More recently Brewster and Hruby [4] reported assignments based on double resonance experiments in aqueous solution. The connection between αCH and βCH resonances was made in D_2O solution; some ambiguities were resolved by the use of oxytocin analogues containing fully deuterated cystine or proline residues. Assignment of the NH resonances required the use of H_2O solution, and 'blind' irradiation of the αCH resonances underneath the H_2O absorption band. The assignments of Brewster and Hruby [4] are in substantial disagreement with those of Walter and coworkers [3].

In our earlier study of the NH proton resonances of oxytocin in aqueous solution [6] we did not attempt detailed assignments. We now report unambiguous assignments of the NH resonances of the tyrosine, isoleucine and leucine residues of oxytocin in aqueous solution, by observing the effects of selective ^{2}H or ^{15}N substitution on the NH resonances directly, thus eliminating the necessity for double-resonance experiments.

2. Materials and methods

2.1. *Isotopically substituted amino acids*

[^{15}N] L-isoleucine and [^{15}N] L-tyrosine (both

95% enriched) were obtained from Schwarz-Mann. [α-^{2}H]-DL-leucine was obtained by a modification of the method of Abbot and Martell [7], by exchange of L-leucine in D_2O in the presence of pyridoxal and aluminium sulphate. The [α-^{2}H]DL-leucine was resolved by acetylation followed by treatment with hog renal acylase I [8]. It was purified by recrystallisation and characterised as >90% deuterated at the α-position by proton NMR spectroscopy.

2.2. *Synthesis of ^{2}H- and ^{15}N-labelled oxytocins*

In each case the protected nonapeptide amide, BOC-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucyl-glycinamide was synthesised by the solid phase method of Merrifield [9–11] with the modifications introduced by Corley et al. [12] for the washing and deprotection steps.

Isotopically labelled amino acids were converted to their t-BOC derivatives according to the method of Schnabel [13]. Coupling of unlabelled amino acids was carried out in two stages, separated by a methanol wash; a 3-fold excess of t-BOC amino acid was used. For the coupling of labelled amino acids the resin peptide in the amino form was dried and transferred to a siliconised flat-bottomed glass tube. The labelled amino acid, in an amount equivalent to the free amino groups on the resin, was introduced in the minimum volume of methylene chloride that would allow for stirring. After 10 min, a 1.5-fold excess of dicyclohexylcarbodiimide was introduced, and the resin was stirred for 5 hr at room temperature.

After the coupling of each t-BOC amino acid, the resin peptide was examined by transesterification and chromatography of the peptide methyl esters. A sample (2–5 mg) was removed washed with methanol and stirred for 30 min at 50°C in a mixture of dry methanol (200 μl) and triethylamine (10 μl). The solution of peptide methyl ester was chromatographed in chloroform/acetic acid (95:5 v/v) on silica gel (Merck 60 F_{254}). Peptide methyl esters with free amino groups remained at the origin. In addition a semi-quantitative measure of the degree of coupling was established by applying a direct ninhydrin test to the beads.

Unreacted amino groups were acetylated by shaking for 2 hr in a methylene chloride solution of *N*-hydroxysuccinimide acetate (4-fold excess over the glycine substitution of the resin).

The protected nonapeptide amide was liberated from the resin by ammonolysis in a mixture of dimethylformamide and ethanol (1:1 v/v) saturated with ammonia at −5°C, stirring the suspension at room temperature for 48 hr [14].

The resin was removed by filtration and washed with dimethylformamide. The combined filtrates were evaporated in vacuo to small volume and chromatography was carried out on a column of Sephadex LH 20 (2.5 cm × 150 cm) in dimethylformamide. The fractions from the main peak were combined and evaporated in vacuo. On the addition of ether, the protected nonapeptide amide precipitated.

The protected peptide was treated with trifluoracetic acid for 20 min to remove the t-BOC group, and after isolation by the addition of ether, was exposed to sodium in liquid ammonia according to the method of du Vigneaud et al. [15]. The remaining free thiol groups were oxidised by air at pH 6.8 [14]. The solution was acidified to pH 4 with acetic acid and freeze-dried.

Purification was carried out by column chromatography on Sephadex G15 according to Manning et al. [16].

Oxytocic activity of the final products was measured on the isolated rat uterus under standard conditions employing Munsick solution [17]. The uterus was obtained from rats in which oestrus had been induced by prior injection of stilboesterol.

2.3. *8[α-^{2}H-leucine]oxytocin*

From 60 mg [^{2}H]leucine, 30 mg of 8[α-^{2}H-leucine]oxytocin was obtained with a biological activity of 350 units $\mu mole^{-1}$. Amino acid analysis gave 0.9 glycine, 1.0 leucine, 1.1 proline, 1.0 aspartic acid, 1.1 glutamic acid, 1.1 isoleucine, 1.1 tyrosine residues per mole. The material showed one spot on TLC (Merck 60 F_{254}) $R_f = 0.27$.

2.4. *3-[^{15}N-isoleucine]oxytocin*

From 25 mg [^{15}N]isoleucine, 9 mg of 3-[^{15}N-isoleucine]oxytocin was obtained with a biological activity of 300 units $\mu mole^{-1}$. Amino acid analysis gave 1.0 glycine, 1.0 leucine, 0.9 proline, 0.9 aspartic acid, 1.0 glutamic acid, 0.9 isoleucine and 0.9 tyrosine residues per mole.

2.5. *2-[^{15}N-tyrosine]oxytocin*

From 25 mg [^{15}N]tyrosine, 20 mg 2-[^{15}N-tyrosine]oxytocin was obtained with a biological activity of 350 units μmole^{-1}. Amino acid analysis gave 1.0 glycine, 1.1 leucine, 1.1 proline, 0.9 aspartic acid, 1.0 glutamic acid, 1.0 isoleucine and 0.9 tyrosine residues per mole. The material showed one spot on TLC, $R_f = 0.27$.

2.6. *NMR measurements*

Samples were dissolved in H_2O at concentrations in the range 9–14 mg/0.5 ml. The pH of the solution was adjusted to 3.0–3.5 with HCl. All spectra were obtained on a Varian HR-220 spectrometer equipped with a C-1024 time-averaging computer at a probe temperature of 22 ± 1°C. The oxytocin spectrum was referenced with respect to a hexamethyldisiloxane (HMS) external reference and the data were converted to an internal DSS reference scale (DSS, sodium-2, 2-dimethyl-2-sila pentane-5-sulphonate). We are most grateful to Dr C. A. Baker, PCMU, Harwell, for much valuable assistance in obtaining these spectra.

3. Results

Two distinct methods of assignment by isotopic substitution have been used in the present work.

In the first method, the peptide nitrogen is replaced by ^{15}N, which has a spin quantum number I = $\frac{1}{2}$. Coupling between the NH proton and the ^{15}N nucleus gives rise to an additional large doublet splitting ($^1J_{^{15}N-H} = 93$ Hz) of the NH proton absorption band. (Coupling between ^{14}N and the directly bonded proton is not usually observed due to the rapid relaxation of ^{14}N nucleus). Thus a comparison of the spectra of 2-[^{15}NTyr]oxytocin (fig. 1a) and oxytocin (fig. 1d) shows clearly that it is the NH proton whose resonance appears at 8.99 ppm which is coupled to the ^{15}N. This resonance can therefore be assigned unambiguously to the tyrosine NH proton. This is in agreement with the results of both Walter and coworkers [3] and Brewster and Hruby [4]. However, both these groups assigned this resonance essentially by elimination, since the double-resonance experiment in H_2O was inconclusive [4], and the resonance disappeared in DMSO/water mixtures containing between 30% and 80% water [3].

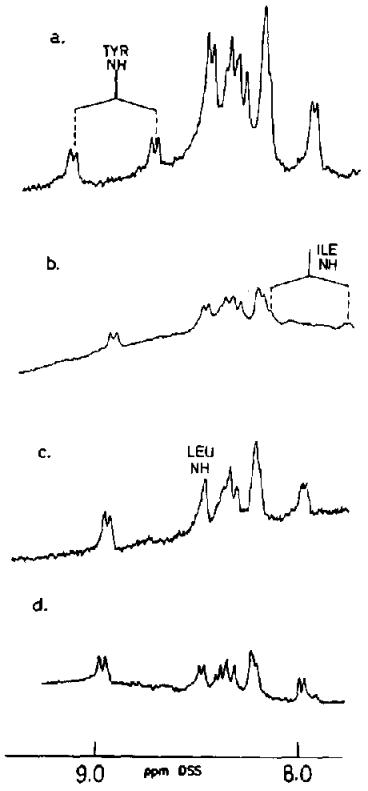

Fig. 1. The peptide NH proton resonances at 220 MHz (aqueous solution at 22°C) of: a) 2-[^{15}N-Tyr]oxytocin, b) 3-[^{15}N-Ile]oxytocin, c) 8-[α-^{2}H-Leu]oxytocin, and d) Oxytocin.

Similarly, examination of the spectrum of 3-[^{15}NIle]-oxytocin (fig. 1b) shows clearly that the NH resonance at 7.94 ppm in oxytocin is that of isoleucine. This assignment is in agreement with that of Brewster and Hruby [4], but not with that of Walter et al. [3].

The second type of isotopic substitution we have employed for assignment purposes is exemplified by 8-[α-^{2}H-Leu]-oxytocin (fig. 1c). Deuterium has a spin quantum number I = 1, so that replacement of the α-hydrogen by deuterium will convert the doublet NH resonance to a triplet. However, because $J_{HD} = \frac{\gamma_D}{\gamma_H} J_{HH} = 0.15\, J_{HH}$, the triplet splitting will be very small, and in practice a broad singlet resonance is observed. Comparison of fig. 1c and fig. 1d shows that deuteration at the α-position of the leucine residue leads to the collapse of the doublet NH resonance at 8.47 ppm to a singlet, thus unambiguously assigning

this resonance to the NH of leucine. Again this is in agreement with Brewster and Hruby [4], but not with Walter et al. [3].

4. Discussion

The methods of isotopic substitution which we have used lead to unequivocal assignments of some of the oxytocin NH resonances since they do not depend on double-resonance experiments or on the use of solvent mixtures, both of which can lead to ambiguities (as indicated in the introduction). These methods are generally applicable to the assignment of peptide NH resonances, and will be a valuable adjunct to nmr studies of the conformation of biologically active peptides.

The assignments which we have made for oxytocin (Tyr NH 8.99 ppm, J_{NC} 7.5 Hz; Leu NH 8.47 ppm, J_{NC} 6.5 Hz and Ile NH 7.94 ppm, J_{NC} 6.5 Hz) are in agreement with those of Brewster and Hruby [4], but not those of Walter et al. [3]. It is clear from these results that the conformation proposed by Walter et al. [7] for oxytocin in DMSO does not exist in aqueous solution [4,6]; indeed it is likely that in aqueous solution oxytocin does not have a unique conformation, but is conformationally flexible [4,6]. In addition, Walter et al. [3] stated that (in contrast to our earlier conclusion [6]) the conformations of oxytocin and lysine vasopressin in aqueous solution are quite different. Their conclusion was based on assignments which are now shown to be incorrect, and the data available at present is consistent with the conclusion that the conformational distributions of these two closely similar molecules are indeed very similar.

References

[1] Bystrov, V. F., Portnova, S. L., Tsetlin, V. I., Ivanov, V. T., and Ovchinnikov, Yu A. (1969) Tetrahedron, 25, 493.
[2] Gibbons, W. A., Nemethy, G., Stern, A., and Craig, L. C. (1970) Proc. Natl. Acad. Sci. U.S., 67, 239.
[3] Glickson, J. D., Urry, D. W., and Walter, R. (1972) Proc. Natl. Acad. Sci. U.S., 69, 2566.
[4] Brewster, A. I. R., and Hruby, V. J. (1973) Proc. Natl. Acad. Sci. U.S., 70, 3806.
[5] Johnson, L. F., Schwartz, I. L., and Walter, R., (1969) Proc. Natl. Acad. Sci. U.S., 64, 1269.
[6] Feeney, J., Roberts, G. C. K., Rockey, J. H., and Burgen, A. S. V. (1971) Nature, 232, 108.
[7] Abbott, E. H., and Martell, A. E. (1968) Chem. Commun., 1501.
[8] Greenstein, J. P., and Winitz, M. (1961) Chemistry of the amino acids, Vol. 3., p. 2094, Wiley, New York.
[9] Merrifield, R. B., (1963) J. Am. Chem. Soc., 85, 2149.
[10] Merrifield, R. B. (1964) Biochemistry, 3, 1385.
[11] Merrifield, R. B. (1965) Science, 150, 175.
[12] Corley, L., Sachs, D. H. Anfinsen, C. B. (1972) Biochem. Biophys. Res. Comm., 47, 1353.
[13] Schnabel, E., (1967) Liebig's Ann. Chem., 702, 188.
[14] Meienhofer, J., Trzeciak, A., Harran, R. T., Naikr, R., (1970) J. Am. Chem. Soc., 92, 2199.
[15] du Vigneaud, V., Ressler, L., Swan, J. M., Roberts, C. W., Katsoyannis, P. G., Gordon, S. (1953) J. Am. Chem. Soc., 75, 4879.
[16] Manning, M., Nun, T. C., Baxter, J. N. M. (1968) J. Chromatog. 38, 396.
[17] Munsick, R. A. (1960) Endocrinology, 66, 451.