Nuclear Magnetic Resonance Spectrum of Lysine-Vasopressin and Its Structural Implications

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ABSTRACT The NMR spectra at 220 MHz of lysine-vasopressin and its precursors were measured in dimethyl sulfoxide, and the peaks were assigned to specific protons. Information about hydrogen bonding was obtained from the temperature coefficients of the chemical shifts. With these data, and with several chemical-shift positions and coupling constants, structural information was derived for this polypeptide hormone.

High-resolution nuclear magnetic resonance (NMR) studies offer the possibility of determining the conformations of macromolecules in solution (1). This method is applied here to the well-known octapeptide hormone lysine-vasopressin (LysVP) in deuterated dimethyl sulfoxide [1H]6Me2SO solution. The interpretation of the NMR spectrum of this polypeptide was aided materially by the availability of the blocked precursors (dipeptide to nonapeptide) used in the synthesis of LysVP, i.e., the changes in the spectrum in the progression from dipeptide to nonapeptide to LysVP provided information on the chemical shift of specific protons, as well as on changes in conformation as each amino acid residue is added.

LysVP (I) has the following structure, in which the numbers indicate the positions of the individual amino acid residues:

\[ \text{Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH}_2 \]

The half-cystine residues in positions 1 and 6 are referred to as Cys-1 and Cys-6, respectively.

MATERIALS AND METHODS

LysVP was a purified synthetic preparation (2, 3) that possessed about 250 U/mg of rat pressor activity.† The following protected peptide intermediates were also used in this study: Z-Lys(Tos)-Gly-OEt (II) (4), Z-Pro-Lys(Tos)-Gly-OEt (III) (4), Z-Cys(Bzl)-Pro-Lys(Tos)-Gly-NH2 (IV) (3, 4), Z-Asn-Cys(Bzl)-Pro-Lys(Tos)-Gly-NH2 (V) (5), Z-Gln-Asn-Cys(Bzl)-Pro-Lys(Tos)-Gly-NH2 (VI) (5), Z-Phe-Gln-Asn-Cys(Bzl)-Pro-Lys(Tos)-Gly-NH2 (VII) (5), Z-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Pro-Lys(Tos)-Gly-NH2 (VIII) (5), Z-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Pro-Lys(Tos)-Gly-NH2 (IX) (3).

Abbreviations for amino acid residues and protecting groups are given in accordance with the IUPAC-IUB Tentative Rules [J. Biol. Chem., 24, 2491 (1966)]. All optically active amino acids are of the \( \alpha \)-configuration. LysVP, lysine-vasopressin.

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† Pressor assays were performed on anesthetized male rats, as described in The Pharmacopeia of the United States of America (Eaton, Pa.: Mack Publishing Co., 1970) 18th revision, p. 771.

The proton NMR spectra were recorded on a Varian HR-220 spectrometer, and on a Varian HA-100 spectrometer using an internal lock and frequency sweep mode. The sample concentrations were 4–10 g/100 ml in (100 mol %) [1H]6Me2SO, that had been stored over molecular sieves. When the polypeptide concentration was varied in this range, no change in the spectra was detected. All chemical shifts are downfield from the internal standard tetramethylsilane. The temperature of the sample was controlled to ±2°C. Homonuclear spin-decoupling on the HR-220 spectrometer was done by the field sweep method, using a General Radio Co. 1107-A inter-polation oscillator, and on the HA-100 by the frequency sweep method. The spectra were calibrated by the side-band technique.

After the NMR measurements were completed, the LysVP solutions were bioassayed. No appreciable inactivation of the LysVP was detected.

RESULTS AND DISCUSSION

Assignment of peaks

The peaks of the NMR spectrum of LysVP were assigned by making use of the spectra of the blocked precursors, from the di- to the nonapeptide, together with information on the spectra of other oligopeptides and of the isolated amino acids. The latter could not be used by themselves because of the influence of charges on the \( \alpha \)-NH\( ^+ \) and \( \alpha \)-COO\(^- \) groups. However, an amino acid residue in a blocked precursor oligopeptide, in which the residue is bordered by peptide bonds and charged groups, is in a magnetic environment more typical of that in LysVP, and therefore provides better information for use in assigning the peaks.

Consider first the spectrum of the blocked dipeptide (II) in [1H]6Me2SO solution. The glycine residue, with two \( \alpha \)-CH protons, gave a distinctive triplet in the NH region, while the lysine (as well as all other amino acids in the higher oligomers) gave a doublet. The \( \alpha \)-CH peaks were assigned by spin decoupling, generally by irradiating the \( \alpha \)-CH and observing the change in the NH region. The spectrum of the protected tripeptide (III) exhibited new peaks in the \( \alpha \)-CH and \( \beta \), \( \gamma \), \( \delta \)-CH regions, characteristic of the added proline. The relation between, and identification of, the newly-added \( \alpha \)-CH and \( \beta \)-CH was verified by decoupling. This procedure was continued through the nonapeptide to provide the assignments for each proton in all of the amino acid residues.

The benzyloxy carbonyl group influenced the chemical shift of the \( \alpha \)-CH and NH protons of the N-terminal amino acid in all peptides, causing them to appear upfield (by 0.2 and 0.4 ppm, respectively) from their (later) positions when another
amino acid was added to the chain. For example, in the tetrapeptide (IV) (with N-terminal Cys-6), the \( \alpha\)-CH resonance occurred at 4.45 ppm. However, in the pentapeptide (V) (with N-terminal Asn), the resonance of the \( \alpha\)-CH proton of Cys-6 moved to 4.68 ppm, and remained there in the longer peptides. Once a residue was removed by one amino acid from the Z group, the chemical shifts (but not necessarily the coupling constants) of the \( C'\), \( C''\), \( C'\), and \( C''\) protons remained remarkably constant, as shown in Fig. 1. This behavior was of considerable value in assigning the peaks of LysVP.

The 220 MHz spectrum of LysVP in \([\text{H}]_6\text{MeSO}\) (7 g/100 ml) is shown in Fig. 2. From the spectra at several temperatures, and from measurements of the areas of the peaks, it was possible to locate seven NH-peaks in the amide region, as expected for the nine amino acids, since proline has no NH group and the \( \alpha\)-NH\(^+\) of Cys-1 appears elsewhere. Eight of the nine \( \alpha\)-CH peaks were visible; the peak for the ninth (Cys-1) will be discussed below. The peak assignments were made as follows. The triplet in the NH region (8.13 ppm) distinguished the glycine residue, and the corresponding \( \alpha\)-CH resonance at 3.62 ppm was located by spin decoupling. The remaining NH peaks were assigned by decoupling from the \( \alpha\)-CH peaks; the decouplings of all but the Tyr-NH are shown in Fig. 2.

The \( \alpha\)-CH peaks were generally assigned by decoupling from the \( \beta\)-CH peaks; thus, the \( \beta\)-peaks provide the bases for many of the assignments. All the \( \alpha\)-CH peaks, except the three grouped at about 4.30 ppm, will decouple an NH proton: hence, the proline \( \alpha\)-CH peak lies near 4.30 ppm. The \( \beta\), \( \gamma\), and \( \delta\) CH\(_2\) protons in lysine characteristically resonate between 1.30 and 1.60 ppm, as was found for the precursors of LysVP, for lysine peptides (6), and for poly(\( \gamma\)-lysine) (6). In LysVP, the ratio of the areas of the 1.50 to the 1.35 ppm peaks is 2:1.

Since the peak at 4.02 ppm, in the \( \alpha\)-CH region, could be decoupled from the 1.50 ppm peak (implying that the \( \beta\)-CH\(_2\) protons resonate at this position), and since a different downfield CH\(_2\) group at 2.72 ppm (presumably the \( \epsilon\)-CH\(_2\)) is also coupled to the 1.50 ppm peak (implying that the \( \epsilon\)-CH\(_2\) protons resonate at this position), the \( \beta\) and \( \delta\) CH\(_2\) resonances of lysine were assigned to the 1.50 ppm peak, the \( \epsilon\)-CH\(_2\) to the 2.72 ppm peak, the \( \alpha\)-CH to the 4.02 ppm peak, and, by difference, the \( \gamma\)-CH\(_2\) to the 1.35 ppm peak. The \( \alpha\)-CH at 4.17 ppm is coupled, 490-Hz upfield, to a region corresponding both to the \( \beta\)-CH\(_2\) of Gln and one \( \beta\)-CH of Pro; since this \( \alpha\)-CH is also coupled to an NH, it must be that of Gln. Of all the decouplings mentioned in this paper, the most difficult to see were those of the \( \alpha\)-CH to \( \beta\)-CH of Lys and Gln. The possibility exists that the \( \alpha\)-CH and, therefore, the NH peaks assigned to these two residues may be reversed. However, the structural information contained in this paper is not affected by this uncertainty.

The protons on the \( \beta\) carbons of amino acids that do not have a CH\(_2\) group at the \( \gamma\) position, such as Asn, Phe, Tyr, and Cys, resonate in the interval from 2.45 to 3.20 ppm. The peaks at 2.55 ppm have a position and splitting pattern similar to those found for the nearly equivalent \( \beta\)-CH\(_2\) protons of Asn in the precursors and were assigned to that residue; these decouple from the \( \alpha\)-CH at 4.46 ppm, which is therefore assigned to Asn. The \( \alpha\)-CH resonance of Cys-6 was assigned to the peak at 4.78 ppm, since this peak appeared at 4.68 ppm in the
benzylated precursors, and would be expected to shift downfield when the more electronegative disulfide of LysVP replaces the benzyl-S group; this peak appeared at 4.77 ppm in oxytocin and at 4.78 ppm in deamino-oxytocin (7).

In order to assign the $\alpha$-CH and $\beta$-CH$_2$ peaks of Phe and Tyr, the normal procedure would have been to observe changes in the $\alpha$-CH region upon spin decoupling. However, this approach did not work here because one of the four $\alpha$-CH protons could not be located (Cys-1), and two of the other three $\alpha$-CH protons resonate together at the position of the large peak at 4.30 ppm, thus preventing observation of any visible change upon spin decoupling. The $\beta$-CH$_2$ protons in the 2.45-3.20 region are not equivalent. The resulting ABX splitting of the eight $\beta$-CH$_2$ protons (Cys-1, Cys-6, Phe, and Tyr) and the $\epsilon$-CH protons of Lys form a complex pattern in this region of the spectrum. Therefore, it was also difficult to observe changes in the complex $\beta$-CH region while field-sweep irradiation was performed; hence, an HA-100 spectrometer was used to perform frequency sweep decoupling in order to decouple both $\beta$-CH$_2$ protons at the same time. Thus, irradiation at 4.27 ppm resulted in sharpening of the peaks at 3.00 and 3.16 ppm; irradiation at 4.35 ppm resulted in sharpening at 2.69 and 2.83 ppm; while irradiation at 4.78 ppm produced no visible change elsewhere. In the precursors, the $\beta$-CH$_2$ protons of Phe appear downfield from those of Tyr; we assume that this relative position is preserved in LysVP, and assign the $\beta$-CH$_2$ peaks at 3.00 and 3.16 ppm to Phe, and the $\beta$-CH$_2$ peaks at 2.69 and 2.83 ppm to Tyr, and, hence, the $\alpha$-CH peaks at 4.27 ppm and 4.35 ppm to Phe and Tyr, respectively.

The aromatic region of the spectrum shows that all five ring protons of Phe resonate at 7.27 ppm, and that the usual AA'XX' pattern of two doublets (one at 6.61 ppm and one at 6.91 ppm) arises from Tyr. The six protons of the primary-amide groups of Asn, Gln, and glycine amide also resonate in this region. Four peaks are readily visible at 6.82, 7.08, 7.42, and 7.45 ppm. The remaining two are hidden, one each, under the 6.91 ppm doublet and 7.27 ppm peak, respectively, as shown by area measurements and observations of the spectrum at various temperatures. From several studies (8, 9), the non-equivalent protons of amides in $-CONH_2$ groups appear as separate peaks, the upfield and downfield ones corresponding to protons that are cis and trans, respectively, to the carbonyl group. Thus, the NH peak at 6.82 ppm in LysVP (6.82 ppm in all Gln-containing precursors) is assigned to the cis NH of Gln, that at 6.93 ppm (6.94 ppm in all Asm-containing precursors) to the cis form of Asn, and that at 7.06 ppm (7.07 ppm in all glycine amide-containing precursors) to the cis form of glycine amide.

The broad peak at 5.33 ppm is the site of the resonances of the more rapidly exchangeable protons, namely, those of the $\epsilon$-NH$_2$ group of Lys, the $\alpha$-NH$_2$ of Cys-1, the OH of Tyr, and residual H$_2$O. This peak sharpens with increasing temperature, which indicates that the measurements were performed above its coalescence temperature. The position of this peak is one place where one might find the missing $\alpha$-CH peak for Cys-1. On deuterium exchange (10% D$_2$O in [H]$\times$Me$_2$SO), the broad peak at 5.3 ppm disappeared, leaving a flat baseline. The absence of a peak for Cys-1 in this, or in the usual $\alpha$-CH region, is also apparent from the spectrum of oxytocin (7).

In LysVP the Tyr-NH peak was broader than the other NH peaks present. This peak becomes even broader with increasing temperature, whereas the peaks of the other amide protons become sharper. In oxytocin (7) (which contains an NH$_3^+$ group as does LysVP) the Tyr peak also appears to be broadened, although in deamino-oxytocin (in which the NH$_3^+$ group is replaced by a proton) the Tyr peak is sharp. This suggests that the NH proton of Tyr in LysVP, though four bonds removed from the $\alpha$-NH$_2$ group, might be influenced by it. This could arise from an electric field gradient effect that would alter the coupling of the nitrogen quadrupole to the NH proton, or through a relaxation broadening (10, 11), which would also explain the absence of a Cys-1 peak. Further experiments may clarify the origin of the broadening.

Evidence for hydrogen bonding and the structure of the ring

By study of the temperature dependence of the chemical shifts of NH protons of LysVP (Fig. 3), and comparison of the shifts with those of N-methyl acetamide (about 7 $\times$ 10$^{-4}$ ppm/$^\circ$C in MeOH and 6 $\times$ 10$^{-4}$ ppm/$^\circ$C in [H]$\times$Me$_2$SO) (12), and comparison also with the shifts of other NH groups in LysVP (since traces of acid or base in the solution might alter the absolute values of the temperature coefficients), it is possible to determine which NH groups may be hydrogen bonded. For example, in gramicidin SA in methanol (12), the hydrogen-bonded and nonhydrogen-bonded NH groups have temperature coefficients of 3 to 3.5 $\times$ 10$^{-4}$ ppm/$^\circ$C and 7 to 7.5 $\times$ 10$^{-4}$ ppm/$^\circ$C, respectively. In cyclo-pentaglyceryltyrosine in [H]$\times$Me$_2$SO (13), the corresponding values are 2 $\times$ 10$^{-4}$ ppm/$^\circ$C and 5 $\times$ 10$^{-4}$ ppm/$^\circ$C, respectively; in valinomycin in [H]$\times$Me$_2$SO (12), they are 4.3 $\times$ 10$^{-4}$ ppm/$^\circ$C and 8.4 $\times$ 10$^{-4}$ ppm/$^\circ$C, respectively; these are average values for two interconverting structures, each having the same hydrogen-bonded NH group.

In LysVP, the backbone NH groups of Asn and Lys or Gln have temperature coefficients of 0.2 $\times$ 10$^{-4}$ and 4 $\times$ 10$^{-3}$
ppm/°C, respectively, which are half, or less, of the values obtained for other NH protons in this molecule; these values compare well with those obtained for hydrogen-bonded groups in other molecules. This suggests that the Asn and Lys or Gln backbone amides are hydrogen-bonded or buried. The Asn-NH peak in LysVP appears upfield from all other NH peaks, whereas this is not the case with this proton in the precursors. The presence of this hydrogen-bonded NH at high field suggests the existence of a β-turn (an i to i + 3 hydrogen bond that is shielded by the amide group between residues i + 1 and i + 2), similar to that observed in other cyclic structures (13–15). In such a β-turn, the NH of Asn is hydrogen-bonded to the CO of Tyr, as previously suggested for oxytocin and deaminooxytocin (15). The Tyr-NH group is probably not hydrogen-bonded, since its temperature coefficient is 5 × 10^{-3} ppm/°C; hence, there appears to be only one hydrogen bond across the ring.

To determine the C-S-S-C dihedral angle, use is made of circular dichroism (CD) studies (16, 17) of compounds containing this group. Since the CD spectra of LysVP and oxytocin are qualitatively the same (16), and since the right-handed screw sense was assigned to the C-S-S-C group in oxytocin (17), we use this same dihedral angle for LysVP, pending further evidence for the handedness in [\textsuperscript{2}H\textsubscript{4}]Me$_{2}$SO.

Comparison of spectra of LysVP and precursors: side-chain conformations

A comparison of the chemical shifts of the protons of LysVP with those of the protected nonapeptide (IX) in the same solvent gave some structural insights. Some of the α-CH resonances were the same for both compounds (e.g., Gly at 3.60 ppm and Pro at 4.30 ppm), or varied very little (e.g., Lys at 4.08 ppm in the blocked nonapeptide shifts to 4.02 ppm in LysVP); this suggests that the magnetic environment of the α-CH protons of these residues is similar in both of these compounds. However, in the conversion of the protected nonapeptide (IX) to LysVP, the α-CH proton of Phe shifts upfield by 0.24 ppm, while its NH proton shifts downfield by 0.6 ppm. It is suggested that the Phe ring is rotationally averaged about the C-C bond in the nonapeptide (IX), while one of the rotamers is preferred in LysVP. In this orientation, the α-CH proton lies below the plane of the Phe ring and is somewhat anisotropically shielded. The deshielding effect on the NH proton of Phe may be rationalized in terms of the C\textsuperscript{α}-NH dihedral angle (which could be about ±30° to about ±145°, on the basis (18, 19) of an observed coupling constant of J = 6.5-7.0; however, the positive angles may be ruled out for steric reasons when the proposed β-turn obtains). With either negative value of the C\textsuperscript{α}-NH dihedral angle, the dihedral angle χ can be varied to place the NH proton of Phe in the plane of the ring, thereby deshielding it. However, the observed deshielding is too large to be explained solely by the orientation of the NH proton with respect to the Phe ring (see below).

In LysVP, the α-CH resonance of Tyr is shifted slightly upfield from its position in the nonapeptide (IX), while its NH resonance is shifted downfield by 0.4 ppm. Since the Tyr-NH peak is too broad, the coupling constant for the C\textsuperscript{α}-NH dihedral angle could not be determined; however, values between 0° and ±90° are sterically forbidden (with the assumed conformation of the C-S-S-C group). If this angle is >90°, the α-CH proton lies below the tyrosine ring at a relatively neutral position between a shielding and a deshielding effect; however, the NH proton of Tyr is then in the deshielding plane of the Tyr ring. This same orientation of the Tyr ring places the NH proton of Phe in the deshielding regions of both the Tyr and the Phe rings, producing the strong deshielding effect described in the previous paragraph.

The above structural features of LysVP have been based primarily on a consideration of the NMR spectra of this compound and of its blocked precursors. The NMR spectra of other hormones (and their precursors) are also being examined (7, 15, 20). We hope that as more information becomes available on the structures of polypeptide hormones, it may be possible to relate structural features to biological properties.

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