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^{17}O NMR and FT-IR study of the ionization state of peptides in aprotic solvents

Application to Leu-enkephalin

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The ionization state of Leu-enkephalin in DMSO and MeCN/DMSO (4/1) solution was studied by the combined use of ^{17}O NMR and FT-IR spectroscopy. After lyophilization of an aqueous solution at nearly neutral pH, Leu-enkephalin essentially exists in the uncharged state in MeCN/DMSO (4/1) solution. In pure DMSO, only 40% of the Leu-enkephalin molecules are in the zwitterionic state under the same conditions.

FT-IR; Ionization state of peptide; Leu-enkephalin; ^{17}O NMR

1. INTRODUCTION

Many conformational studies of peptide hormones, and particularly of enkephalins (Tyr-Gly-Gly-Phe-Leu/Met), have been carried out in the organic DMSO (Me_2SO) solvent [1]. In practically all the cases, it was assumed that, after lyophilization of a nearly neutral aqueous solution, enkephalins (and more generally peptides with ionizable termini) retain the zwitterionic state after dissolving in DMSO. However, this hypothesis was already questioned on the basis of ^{13}C NMR data and IR and Raman spectroscopy [2,3] and it is known that the pK scale in DMSO differs drastically from that in water [4]. In particular, and in contrast with aqueous solutions of carboxylic acids and primary amines, the pK values for acetic acid and butylamine are not very different in DMSO (12.6 and 11.1, respectively) [5].

In view of the importance of establishing the ionization state of peptides prior to conformational analysis, we report here on combined results of an ^{17}O NMR and FT-IR study of Leu-enkephalin in pure DMSO and in MeCN/DMSO (4/1) solution.

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2. MATERIALS AND METHODS

Leu-enkephalin with selectively ^{17}O-enriched C-terminial carboxyl group, Tyr-Gly-Gly-Phe-[^{17}O]Leu-OH, was obtained by saponification of the methyl ester with sodium ethanolate in $H_2{}^{17}O$ at 40% enrichment [6,7]. For spectroscopic experiments, aqueous Leu-enkephalin solutions were adjusted to three pH values (1.9, 5.8 (isoelectric point) and 9.2), lyophilized to dryness, and the residual solid was taken up in the organic solvent.

In order to consider the influence of the dielectric constant and polarity on the spectroscopic data, we have added MeCN to DMSO to a composition compatible with peptide solubility, i.e. the MeCN/DMSO (4/1) mixture [8].

^{17}O NMR spectra were run at 40°C on a Bruker AM-400 spectrometer (54.48 MHz) under the following experimental conditions: concentration 0.01 M; 10 nm sample tubes; spectral width 50 kHz; 90° pulse 30 μs; in quadrature phase detection. Acoustic ringing effects were alleviated by using either a pre-acquisition delay or special pulse sequences [9]. Chemical shifts (ppm) are reported relative to 1,4-dioxane.

The experimental conditions for the FT-IR experiments on a Brüker IFS-85 spectrometer were as follows: room temperature; concentration 0.01 M; cell path length 100 μm; 512 scans; subtraction of the solvent spectrum from that of the solution.

3. RESULTS AND DISCUSSION

The ^{17}O resonance of Leu-enkephalin, Tyr-Gly-Gly-Phe[^{17}O]Leu-OH, in the MeCN/DMSO (4/1) mixture is illustrated in Fig. 1 as a function of the original pH of the aqueous solution. The chemical shift value resulting from lyophilization of an acid aqueous solution (pH ≈ 1.9), δ = 259.4 ppm, is in excellent agreement with that for AcProOH or AcSarOH in acetone [10], confirming the neutral form of the Leu-carboxyl group under these conditions. Raising the pH of the aqueous solution to

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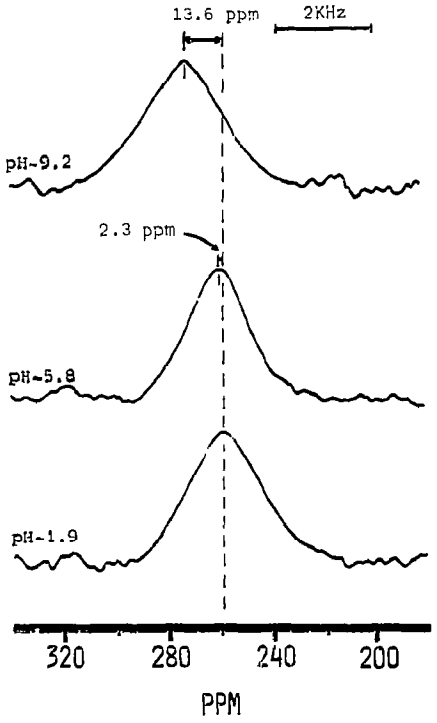

Fig. 1. 54.48 MHz ^{17}O NMR spectra of Leu-enkephalin (Tyr-Gly-Gly-Phe-[^{17}O]Leu-OH) in CH_3CN/DMSO (4/1) solution at three different pH values. Temperature 40°C; concentration (10^{-2} M, number of scans ≈300,000, exponential multiplication of the FIDs with a line broadening factor LB = 300 Hz, pre-acquisition delay Δt = 130 μs.

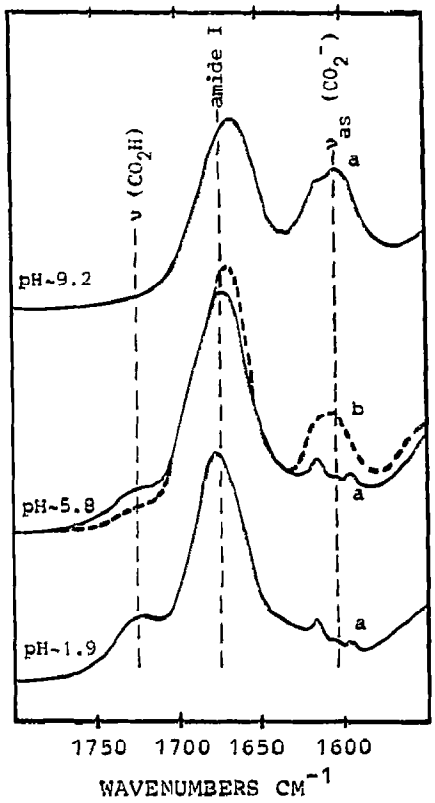

Fig. 2. FT-IR spectrum of Leu-enkephalin in (a) MeCN/DMSO (4/1) solution for three different pH values of the lyophilized aqueous solution, and (b) in DMSO for pH ≈5.8.

the isoelectric point of Leu-enkephalin (pH ≈ 5.8) induces a shift of its ^{17}O resonance to high frequency by only 1.8–2.3 ppm depending on temperature [6]. This small value contrasts with the titration shift to high frequency of about 19–20 ppm for Leu-enkephalin [7] and AcProOH or AcSarOH [10] upon deprotonation of the carboxyl group in water and 21–23 ppm for Ala and Pro in DMSO [11]. Leu-enkephalin lyophilized at pH ≈ 9.2 effectively exhibits a more significant chemical shift to high frequency by 10.0–13.6 ppm, thus probably illustrating a significant deprotonation of the Leu-carboxyl group under these basic conditions. This was confirmed by IR absorption which is more sensitive to the protonation state of the carboxylic group (see below).

Fig. 2 illustrates the influence of the pH of the lyophilized Leu-enkephalin aqueous solution on the IR spectrum in the MeCN/DMSO (4/1) mixture. The spectrum obtained in DMSO from Leu-enkephalin lyophilized at pH ≈ 5.8 is also represented for comparison. Under acidic conditions, both IR spectra in the organic solutions are identical and exhibit a strong amide I absorption band at ≈ 1670 cm^{-1}, and a weak contribution at ≈ 1725 cm^{-1} due to the carboxylic C=O stretching vibration [12]. In both solvents this latter absorption decreases a little for lyophilized neutral enkephalin while an absorption, overlapping the Tyr aromatic contributions at 1600 cm^{-1}, appears only in DMSO. This region is expected to contain the antisymmetric carboxylate stretching vibration [12] and we have verified on Tyr at different pHs, that the N-terminal amine group has no contribution in this domain. The absorption at 1600 cm^{-1} progressively increases in both organic media for higher pH values while the carboxylic contribution at ≈ 1725 cm^{-1} vanishes.

IR spectroscopy confirms that Leu-enkephalin in organic solvents effectively undergoes a transition from the carboxylic to the carboxylate state when increasing the pH of the lyophilized aqueous solution. However, a nearly neutral pH does not result in the pure zwitterionic state as in water. This would give a stronger shift of the ^{17}O resonance (Fig. 1) and a stronger IR contribution at 1600 cm^{-1} (Fig. 2) than those observed experimentally. Furthermore, it appears that practically all the Leu-enkephalin molecules are in the uncharged state in the MeCN/DMSO (4/1) mixture after lyophilization of a nearly neutral aqueous solution. The percentage of this uncharged form decreases in the DMSO solvent, as shown by the stronger asymmetric carboxylate contribution with respect to the above mixture (Fig. 1). This can be attributed not only to the higher dielectric constant [8] but also to the stronger base and hydrogen bond acceptor (solvation) properties of DMSO compared with CH_3CN. On the basis of the

molar extinction coefficient of this absorption band measured on the prototype Ac-Leu-O^- Cs^+ molecule (ε = 900 $M^{-1} \cdot cm^{-1}$), it can be concluded that only 40% of the Leu-enkephalin molecules are in the zwitterionic state in DMSO.

Similar IR observations have been carried out on a variety of linear peptides containing 3–5 amino acid residues (i.e. Gly-Phe-Leu, Gly-Gly-Phe-Leu, [D-Ala^2, Leu^5]enkephalin and [D-Ala^2, D-Leu^5]enkephalin), indicating that the above conclusions are probably of general value for linear oligopeptides in aprotic media. Therefore, the two transitions with pH titration in DMSO, which are generally attributed to the carboxylic and ammonium deprotonation for increasing pH values, could rather correspond to the opposite deprotonation order.

The result is that the ionization state of linear peptides in non-aqueous solvents should be determined prior to concluding on their conformational properties from titration effects on their NMR data.

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