^{1}H NUCLEAR MAGNETIC RESONANCE STUDIES OF MELANOSTATIN: DEPENDENCE OF THE CHEMICAL SHIFTS OF NH PROTONS ON TEMPERATURE AND CONCENTRATION

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1. Introduction

Melanostatin, formerly called melanocyte-stimulating-hormone release inhibiting hormone, has been found in hypothalamic extracts and its chemical structure has been identified as L-prolyl-L-leucylglycine amide [1]. An X-ray crystallographic study has shown that this molecule has a β-turn structure with a weak intramolecular hydrogen bond (3.04 Å) between Pro (0) and Gly-NH_2 [2]. The same structure has been suggested from a ^{1}H NMR study in deuterated dimethyl sulfoxide (DMSO-d_6) [3]. On the other hand, measurements of the ^{13}C chemical shifts and spin-lattice relaxation times in D_2O and DMSO-d_6 have indicated that the melanostatin molecule undergoes a solvent-induced conformational change and that it aggregates in DMSO-d_6 [4].

We have observed the temperature and concentration dependences in DMSO-d_6 of the chemical shifts of NH protons in two forms of melanostatin, one with the unprotonated proline ring and the other with the protonated proline ring. Since melanostatin has the protonated proline ring at the physiological pH value of 7.3–7.4, it seems important to study the conformation and molecular interactions of the protonated melanostatin as well as those of the unprotonated form.

2. Materials and methods

Melanostatin used in this study was synthesized by Dr H. Sugano of Tanabe Seiyaku Company. An aqueous solution (ca. 0.4 M) of melanostatin as synthesized showed a pH value of 10.4. The NMR titration of the chemical shifts of the proline C_α and C_δ protons gave a pK_a value of 8.9 for the ionization of the Pro imino group. Therefore, melanostatin as synthesized must have an unprotonated proline ring [hereafter called melanostatin (0)]. The protonated melanostatin [melanostatin (+)] was prepared by lyophilizing an aqueous solution at pH 7.0.

All ^{1}H NMR spectra were recorded on a Hitachi R-22 spectrometer (90 MHz for ^{1}H) with a variable-temperature accessory. The solvent DMSO-d_6 was purchased from the Commissariat a l'Energie Atomique and was dried with Zeolite A-4. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal standard.

3. Results and discussion

The ^{1}H NMR spectra of melanostatin (0) and melanostatin (+) (ca. 0.4 M in DMSO-d_6) are shown in fig. 1 along with the peak assignments confirmed by the usual spin-decoupling technique. The spectrum and assignments for melanostatin (0) are consistent with the results reported by other workers [3,5]. Upon protonation all the peaks due to the prolyl ring protons showed downfield shifts. Interestingly, the Leu NH proton resonances also shifted downfield, indicating that the protonation of the prolyl ring induced a change in the leucyl moiety.

The temperature dependence of NH proton resonances has been utilized to discriminate free and intramolecularly hydrogen-bonded NH groups in peptides [6]. This technique was applied to the two forms of melano-

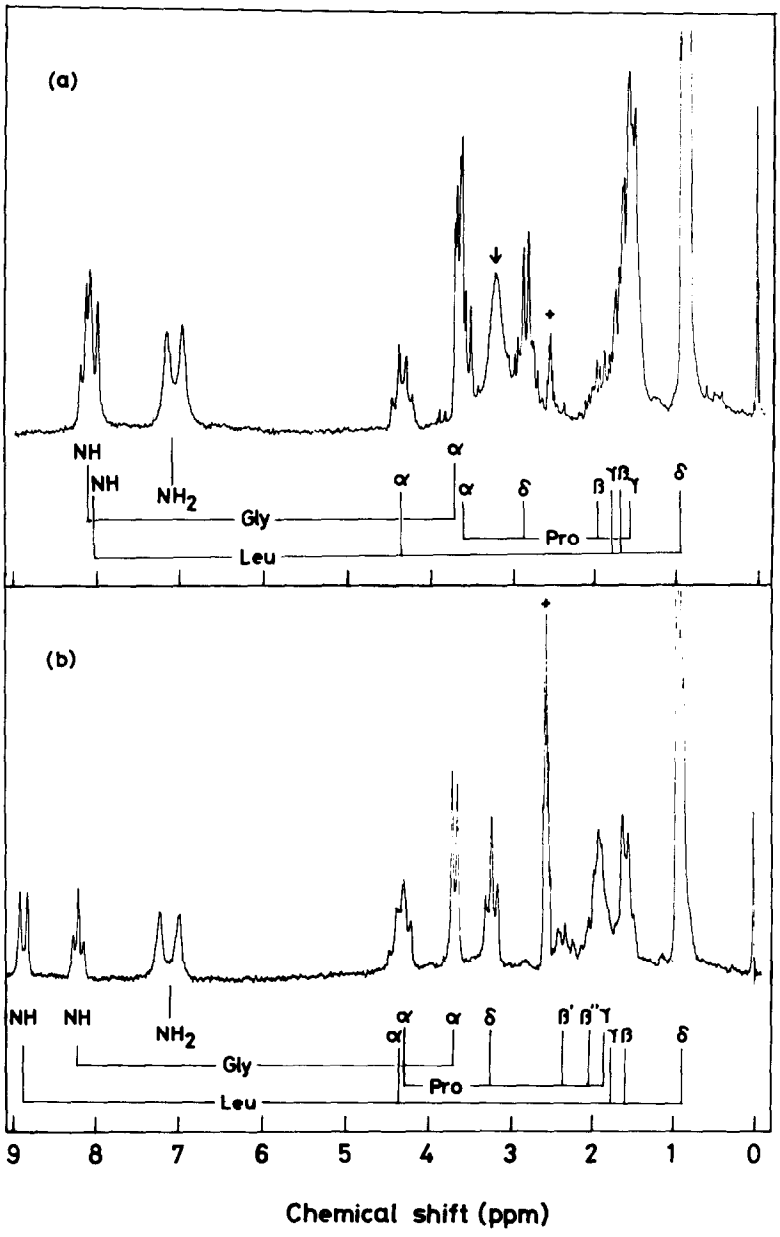

Fig.1. The ^{1}H NMR spectra (90 MHz) of two forms of melanostatin (ca. 0.4 M in DMSO-d_6, 37°C). (a) Melanostatin (0) [unprotonated]; (b) melanostatin (+) [protonated]. The arrow and plus symbols indicate, respectively, the resonances due to residual water and partially deuterated DMSO.

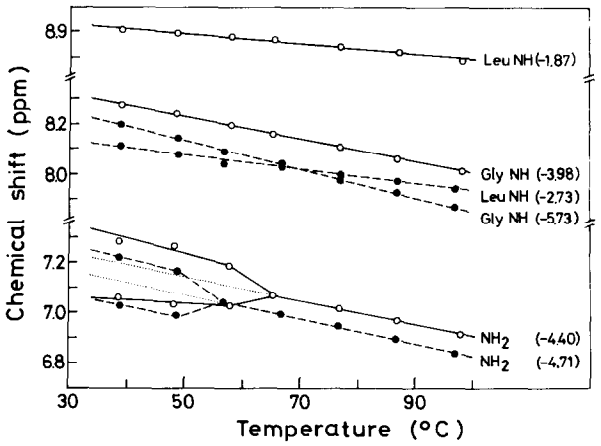

Fig.2. The temperature dependences of the NH proton resonances from two forms of melanostatin (ca. 0.4 M in DMSO-d_6). (- - -●- - -), Melanostatin (0) [unprotonated]; (—○—), melanostatin (+) [protonated]. The numbers in parentheses indicate the temperature coefficients in units of 10^{-3} ppm/°C.

statin in order to gain a deeper insight into their conformations. The results are shown in fig. 2. The Leu NH proton resonance of melanostatin (+) showed a small temperature dependence of −0.00187 ppm/°C, a typical value for an intramolecularly hydrogen-bonded (or buried) proton [7,8], whereas the corresponding resonance of melanostatin (0) exhibited a larger value (−0.00273 ppm/°C). The temperature coefficients of the chemical shifts of the Gly NH and carboxamide protons were much larger for both melanostatin (0) and melanostatin (+). These results seemed to indicate that, in DMSO-d_6, neither melanostatin (0) nor melanostatin (+) had a β-turn structure as found in a crystal. Rather, the Leu NH group of melanostatin (+) was likely to be involved in a hydrogen bonding.

In order to obtain more information on the hydrogen bonding associated with the Leu NH group, the NH proton resonances were observed at various concentrations. As shown in fig. 3, the chemical shifts of the NH proton resonances of melanostatin (+) exhi-

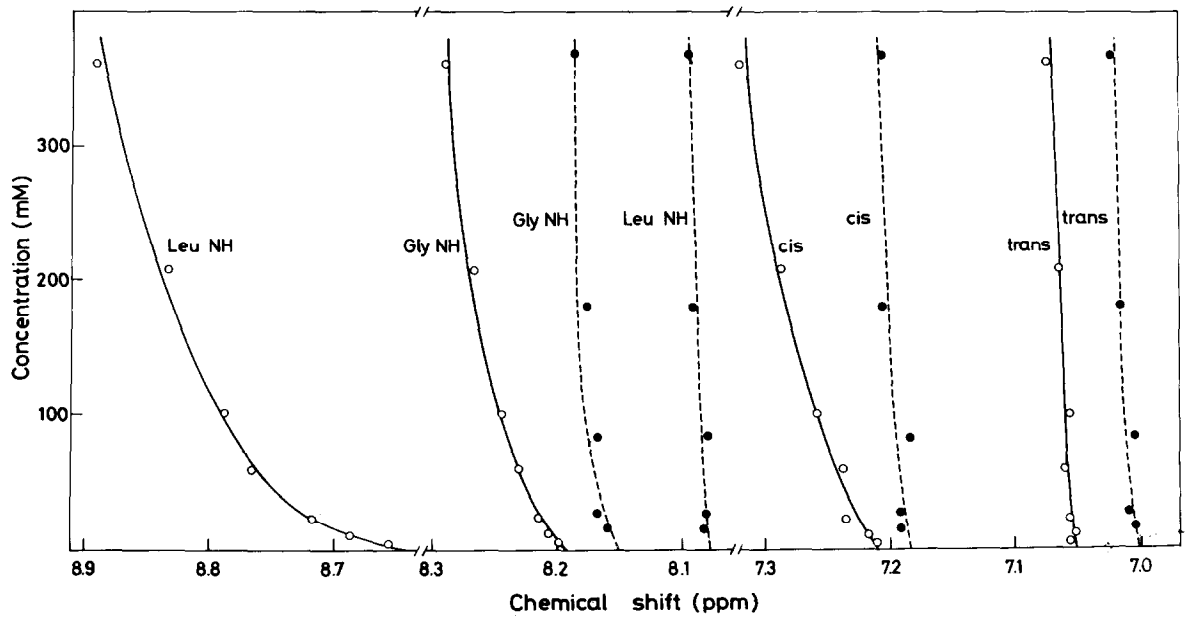

Fig.3. The chemical shifts of the NH protons as functions of concentration in DMSO-d_6 at 37°C. (- - -●- - -), Melanostatin (0) [unprotonated]; (—○—), melanostatin (+) [protonated].

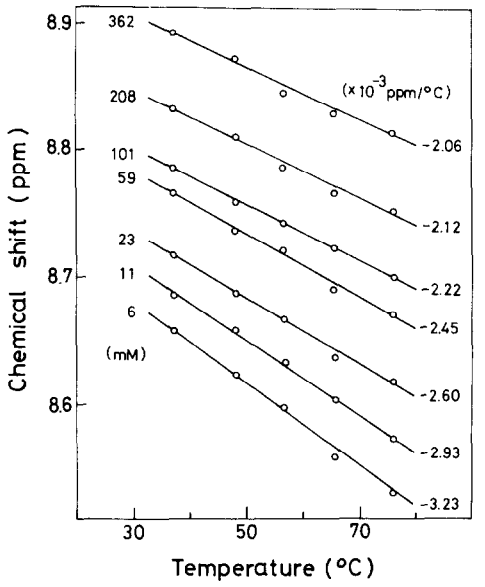

Fig.4. The chemical shift of the Leu NH proton of melanostatin (+) [protonated] as functions of temperature and concentration in DMSO-d_6.

bited considerable concentration dependences, whereas those of melanostatin (0) depended little on the concentration. No concentration dependences were observed for the CH protons in these experiments. The marked concentration dependence in DMSO-d_6 of the Leu NH proton resonance of melanostatin (+) suggested that the Leu NH group was involved in a self-aggregation through the formation of intermolecular hydrogen bonding. Such a view was substantiated by measuring the temperature coefficient of the Leu NH chemical shift at various concentrations. As shown in fig. 4, the Leu NH proton resonance showed a larger temperature dependence with decreasing concentration. This indicated clearly that the melanostatin (+) molecule became increasingly free as the concentration was lowered. In contrast to melanostatin (+) the effect of aggregation was less apparent for melanostatin (0).

It was noted that the chemical shifts of the *trans* carboxamide protons of melanostatin (+) as well as of melanostatin (0) were nearly constant over a wide concentration range (fig.3). Then, it seemed necessary to re-examine the possibility of the β-turn model in which the *trans* carboxamide NH proton was intramolecularly hydrogen-bonded to the Pro Co oxygen.

As far as we know, the concentration dependence of the NH proton chemical shift has not been examined extensively in the NMR studies of biologically important peptides. However, the present results do show that the measurements of the NH chemical shifts as functions of concentration provide useful information on the intra- and intermolecular hydrogen bonds and possibly on the molecular conformations as well. On the other hand, the importance of the measurements at various concentrations has been well recognized in the infrared spectroscopic studies of a number of model peptide molecules [9,10]. Since recent developments in the pulse-Fourier transform technique have facilitated the NMR measurements at low concentrations, it should be interesting and rewarding to follow the concentration dependences of the NH proton chemical shifts for other peptides also.

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