^{1}H-NMR of Met-enkephalin in AOT reverse micelles

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^{1}H-NMR spectra of Met-enkephalin dissolved in AOT/isooctane reverse micelles are reported at various temperatures. The NH temperature coefficients are compared with those obtained for the same peptide in normal SDS micelles, in bulk water, and in DMSO. The results show that the opioid molecule undergoes the greatest folding in the reverse micellar system. Shift reagents selectively dissolved in the water pools or in the hydrophobic matrix affect the Phe-4 or the Tyr-1 aromatic resonances, respectively. This suggests that the phenylalanyl sidechain is spatially close to the carboxyl group at the C-terminus, whereas the tyrosyl ring points towards the outer region of the AOT micelles.

Met-enkephalin *SDS micelle* *AOT reverse micelle* *Shift reagent* *NMR*

1. INTRODUCTION

In recent years there has been an increasing interest in studies of micellar systems as models for conformational studies of peptides in membrane-mimetic media. This effort is motivated by the fact that many biological processes, such as enzymatic reactions, or even recognition phenomena, occur at the interface between peptides and membranes. NMR has long been known as a highly suitable approach for studies of membrane-like systems. A number of papers has appeared in the last ten years on studies of vesicular systems, although they present several disadvantages, such as slow tumbling of the vesicles themselves, and high association of the solutes [1]. Normal micelles, such as those formed by phosphatidylcholine or SDS in water, usually yield well-resolved spectra, and a considerable amount of work has been done on the micelles themselves, and on peptide–micelle interactions [2–4].

Abbreviations: Ek, Met-enkephalin; AOT, bis(2-ethylhexyl)sulfosuccinate, sodium salt; Pr $(dpm)_3$, tris-(2,2,6,6-tetramethylheptane-3,5-dionato)-praseodymium(III); DMSO, [$^2H_6$]dimethyl sulfoxide; TSP, 3-trimethylsilyl [2,2,3,3-$^2H_4$]propionate, sodium salt; TMS, tetramethylsilane

In the so-called 'reverse' micelles the surfactant aggregates in non-polar solvents take up water in pools formed at the interior of the micelles. The size and shape of the micelles, their mobility and many other physico-chemical properties are strongly dependent on the water content. Reverse micellar systems constitute a simple and efficient way of solubilizing peptides and proteins in apolar solvents, opening a full range of possible studies of enzymatic activities, catalytic reactions, and, in general, all processes which occur at the interface of biological membranes.

Although a number of NMR studies have already been published, centered on the spectroscopic properties of water [5–9] or on the surfactant resonances [8–12], little has been accomplished to date on signals from polypeptides in reverse micellar systems [12,13]. We here report a variable temperature study of the aromatic and amide region of Met-enkephalin in AOT reverse micelles in isooctane.

Met-enkephalin is an endogenous pentapeptide, which acts on opiate receptors in the brain, the main interactions occurring at the level of cerebroside sulfates [14,15]. Owing to its small size and to the presence of Tyr, Phe and Met side chains, which usually give well-resolved resonances in

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characteristic spectral regions, Met-enkephalin is an ideal model peptide for ^{1}H-NMR studies of protein–lipid interactions.

2. EXPERIMENTAL

The sample of Ek was purchased from Bachem Feinkemicalien, and used without further purification. AOT (Serva) was purified by HPLC [16]; isooctane (Merck) was used without further purification; the solution of AOT in isooctane was prepared as in [16]; H_2O was bidistilled. To prepare the sample of Ek in reverse micelles, approx. 0.5 mg peptide and 5 μl water (pH 7) were shaken with 0.3 ml of 50 mM AOT in isooctane, until the solution became perfectly clear [17]. ^{1}H-NMR spectra were recorded with a Bruker HX-270 spectrometer, equipped with an Aspect-2000 computer, in single channel detection. The carrier frequency was set at 3000 Hz from the TMS line (internal) using a cutoff filter bandwidth of 1700 Hz. This, combined with simultaneous suppression of the isooctane methyls and of the H_2O resonance (via gated irradiation between scans) helped to improve the dynamic range of the experiment, making it possible to collect the peptide resonances with a good signal-to-noise ratio in a reasonable time. The deuterium signal for the lock was provided by $(C^2H_3)_2SO$ in an internal capillary, coaxial with the NMR tube. Spectra were resolution-enhanced via the Gaussian convolution [18] Typically, 500 scans were accumulated for each experiment. $LaCl_3$ anhydrous was from Aldrich (USA); $Pr(dpm)_3$ was from Merck, Sharp and Dohme (Canada). Perdeuterated SDS was from CEA (France). Chemical shifts are referred to internal TSP (H_2O, SDS) or TMS (AOT, DMSO).

3. RESULTS AND DISCUSSION

Fig.1 shows the aromatic and amide spectrum of Ek in H_2O (A), SDS micelles in H_2O (B), AOT/H_2O reverse micelles in isooctane (C) and DMSO (D), at 21°C. From the peptide sequence Tyr-Gly-Gly-Phe-Met, in this region we expect – for fast flipping aromatic side chains – two doublets from the Tyr-1 ring protons, and a more complex resonance pattern for Phe-4. The spectrum should also include two doublets from Phe-4 and Met-5 amide protons, and two triplets from Gly-2 and Gly-3 NH's; for a peptide of this size the NH_2 protons from the N-terminal group exchange too fast with H_2O to be detected as separate signals. All the above resonances are found in spectra A–D in fig.1. The Tyr-1 four-line pattern does not change from bulk water (A) to normal (B) and reverse micelles (C), except for minor linewidth effects. In DMSO (D) the two Tyr doublets resonate at fields

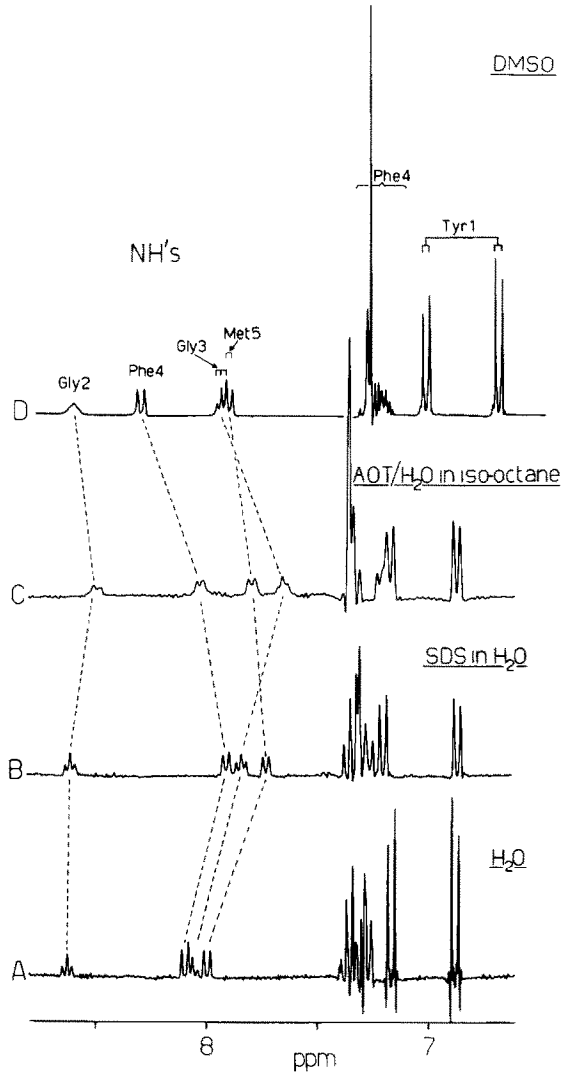

Fig.1. ^{1}H-NMR of Ek: aromatic and amide region. (A) 6 mM solution in H_2O, pH 4.1, (B) 3 mM solution in H_2O, in the presence of SDS, molar ratio (SDS)/(EK) = 20, pH 4.1, (C) in AOT/isooctane reverse micelles (see section 2), molar ratio (AOT)/(EK) = 20, W_o = $(H_2O)/(AOT)$ = 16, (D) 6 mM in DMSO. Temperature, 21°C.

higher than in the other three solvents; this can be simply interpreted as being due to the medium, as it is known that in random-coiled model peptides ϵ and δ tyrosyl protons resonate at 6.86 and 7.15 ppm in water, and at 6.64 and 7.02 ppm in DMSO, respectively [19,20]. The effect of a less polar environment on the phenylalanyl aromatic resonances is manifested as an increase of the spectral degeneracy: in H_2O the resonances from the δ, ϵ and ζ protons scatter over more than 0.2 ppm and are well-separate in at least two groups of signals (fig.1A). In the presence of SDS micelles there is some tendency to coalesce (fig.1B). In DMSO and AOT micelles the Phe resonance pattern is quite similar: the δ and ϵ signals are almost degenerate in a narrow multiplet, while the ζ signal is separate, at higher fields, especially in AOT (fig.1C,D), partially overlapping with the δ-resonances from Tyr-1. To check the correctness of this assignment, the spectrum was computer-stimulated (not shown).

The amide resonances in fig.1 are connected by dotted lines through the four spectra. In H_2O and DMSO, assignments were published previously [21,22]. In SDS and AOT the present assignments are based, besides the signal multiplicity, on the selective line broadening caused by increasing temperature: it is known that in linear, flexible peptides inductive effects resulting from the positively charged N-terminal ammonium group generate a progressive reduction of the exchange rate between NH and water protons along the peptide backbone [23].

The amide resonances were followed *vs* temperature in the four solvents; the temperature coefficients are reported in table 1. Fig.2 shows selected

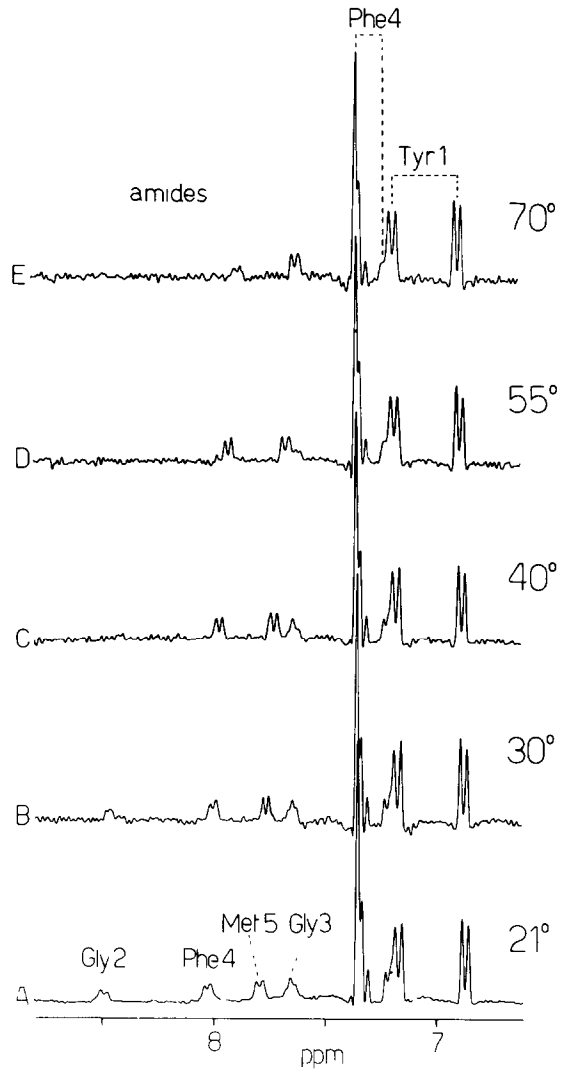

Fig.2. ^{1}H-NMR of Ek in AOT reverse micelles. Spectra at selected temperatures, as indicated on the right. Sample characteristics, as in fig.1.

Table 1

Temperature coefficients for amide protons in Ek ($\Delta\delta/\Delta T$), as measured in the spectra in H_2O, SDS micelles in H_2O, AOT reverse micelles in isooctane, and DMSO

	$-\Delta\delta/\Delta T$ (ppb/°C)			
	Gly-2	Gly-3	Phe-4	Met-5
H_2O	6.3	5.3	5.5	6.1
SDS	6.3	3.6	2.9	1.9
AOT	3.9	0.8	3.1	3.4
DMSO	8.3	3.5	7.4	0.8

spectra of Ek in AOT reverse micelles, between 21°C and 70°C. Tyr and Phe resonances are fully temperature-independent. The Gly-2 amide resonances, i.e. those from the residue nearest to the N-terminus, are the first to disappear with increasing temperature [23], followed by Gly-3 at the next site. As observed [21,24], the temperature coefficients in H_2O monitor a full exposure of the NH groups, whereas in DMSO the Met-5 NH looks strongly hindered, at least below 50°C, and so does the Gly-3 amide proton, although to a lesser ex-

tent. While the former phenomenon was interpreted as being due to an intramolecular H-bond involving the Met-5 amide, the latter was ascribed to a screening effect on the Gly-3 NH, produced by a preferred orientation of the Phe-4 side chain in DMSO [22].

In SDS the Phe-4 NH also becomes less exposed, as indicated by the increase of its temperature coefficient from −7.4 (DMSO) to −2.9 ppb/°C (table 1). In AOT all coefficients are above −4 ppb/°C, including that from Gly-2, which was −8.3 in DMSO and −6.3 ppb/°C in H_2O and SDS. However, the most surprising result from the variable temperature experiment in reverse micelles is that the NH group whose chemical shift is essentially temperature-independent is that from Gly-3, rather than that from Met-5, as observed in normal SDS micelles and DMSO. Hence, the conformation of Ek seems to be quite different, not only in water and DMSO, as already known [22,25], but also in normal and reverse micelles.

In particular, in the AOT system, the low temperature coefficients suggest the presence of a highly compact structure, not easily accessible to the water molecules.

The concentration of Ek inside the water pools in the interior of the reverse micelles is considerably higher than in the bulk water or in the SDS solutions used for the NMR experiments (~30 times), and this could induce aggregations which may alter the exposure of the amide groups. However, the temperature coefficients were also derived for a sample of Ek in AOT 4-times more diluted than the first one; the values were the same as in the previous experiment within 0.4 ppb/°C.

To obtain more information on the geometry of Ek in reverse micelles, two experiments were performed, which made use of shift reagents soluble in the aqueous and in the organic phase, i.e., $LaCl_3$ and $Pr(dpm)_3$, respectively.

Titration curves for the shifts induced by lanthanide ions (Pr^{3+}, Eu^{3+} and La^{3+}) on the Ek protons in aqueous solution ([25,26], unpublished) indicate that the binding occurs at the carboxylate group and does not significantly change the peptide conformation (neither a variation of the $^3J_{NH-CH}$, nor a change in the NH temperature coefficients is observed upon addition of shift reagents). Fig.3B again shows the aromatic region of the spectrum of Ek in reverse micelles: as pointed out

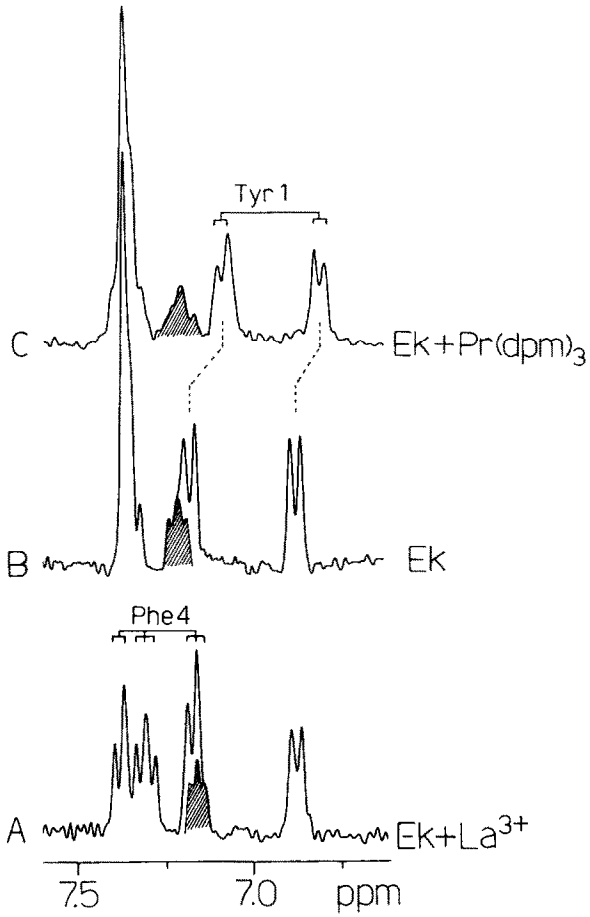

Fig.3. ^{1}H-NMR of Ek in AOT reverse micelles. (B) reference spectrum, (A) in the presence of La^{3+}, molar ratio $(La^{3+})/(Ek)$ = 2, (C) in the presence of $Pr(dpm)_3$, molar ratio $(Pr(dpm)_3)/(Ek)$ = 0.1. The Tyr-1 resonances are indicated in (C), those from Phe-4 in (A). The ζ triplet from Phe-4 has been shadowed in all spectra.

above, Phe-4 gives a strongly degenerate signal at ~7.4 ppm, originating from δ and ϵ protons, and a triplet at 7.2 ppm from the ζ proton (dashed). Tyr-1 exhibits the typical AA′ and XX′ doublets at ~6.9 and 7.2 ppm, from the ϵ and δ protons, respectively. Upon addition of a 2:1 excess of $LaCl_3$ (fig.3A), an induced shift is observed on the aromatic signals of Phe-4, whose resonances assume the characteristic pattern of an AA′ BB′C spin system: a two-proton doublet at 7.38 ppm from the δ protons, a two-proton triplet at 7.30 ppm from the ϵ protons, and a one-proton triplet at 7.17 ppm from the ζ proton (dashed). Converse-

ly, the Tyr-1 resonances are totally unperturbed by the presence of La^{3+}. Addition of a small amount of $Pr(dpm)_3$ (molar ratio 0.1:1) causes a general broadening of the aromatic signals and a net shift on both doublets from Tyr-1 ring protons (fig.3C).

Although Met-enkephalin is likely to be mostly in the water phase, the shift observed on the tyrosyl resonances upon addition of $Pr(dpm)_3$ suggests that the N-terminal part of the peptide points towards the lipid matrix, in proximity to the praseodymium chelate, soluble only in the organic phase. On the other hand, the large shifts experienced by the Phe-4 ring resonances in the presence of $LaCl_3$ (which is soluble only in the water phase, i.e., inside the micelles) are not observed in an aqueous solution of Met-enkephalin (unpublished). This confirms that the peptide conformation is different in water and AOT. Besides possible aggregation effects, in the reverse micelles a re-orientation must occur, which brings the Phe-4 side chain much closer to the C-terminal carboxylate group than in water. As reported above, it is in fact known that the COO^- constitutes the primary binding site for the lanthanides in the aqueous phase [25,26]. Finally, the shift experienced by the Tyr-1 proton resonances in the presence of $Pr(dpm)_3$ indicates that the tyrosyl hydroxyl group provides an alternative, but efficient binding site in the hydrophobic matrix.

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