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^{1}H NMR CONFORMATIONAL STUDY OF SULFATED AND NON-SULFATED CHOLECYSTOKININ FRAGMENT CCK_{27-33} : INFLUENCE OF THE SULFATE GROUP ON THE PEPTIDE FOLDING

Christiane DURIEUX[1], Joël BELLENEY[1], Jean-Yves LALLEMAND[2], Bernard P. ROQUES[1] and Marie-Claude FOURNIE-ZALUSKI[1*]

[1] *Département de Chimie Organique, ERA 613 CNRS et SC 21 INSERM U.E.R. des Sciences Pharmaceutiques et Biologiques, 4 avenue de l'Observatoire, 75006 Paris, France*

[2] *Institut de Chimie des Substances Naturelles C.N.R.S., 91190 Gif sur Yvette, France*

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^{1}H NMR study of cholecystokinin fragment (CCK_{27-33}) in $(C^2H_3)_2SO$ and in ^2H_2O at different pH shows that sulfated (CCK_7) and non sulfated (NS-CCK_7) peptides are under preferentially folded conformations characterized by a β-turn including the sequence Gly-Trp-Met-Asp with a H-bond between the CO of Gly and the NH of Asp. This structure is probably stabilized by an ionic interaction between Tyr and Asp. Moreover, the N-terminal part of CCK_7 forms a C_7 structure with a weak H-bond between the CO of Gly and the NH of Trp. In this model all CCK_7 hydrophobic side chains are in close vicinity, far from the hydrophilic sulfate group. Full interaction with brain CCK_8 receptors could require both the sulfate group and the maintening of conformational constraints.

Cholecystokinin is a 33 aminoacid peptide originally isolated from the gastrointestinal tract (1) where it produces gallblader contraction (2) and pancreatic enzyme secretion (3). Recently high concentration of the cholecystokinin C-terminal octapeptide (CCK_{26-33} or CCK_8) has been found in the brain (4-6) and various experiments suggest that this peptide behaves as a neuromodulator in the central nervous system (7). The structural characteristic of the CCK_{26-33} fragment Asp-(SO_3H)Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2 is the presence of an unusual sulfated tyrosine in position 27. Structure activity studies have shown that neither the N-terminal Asp^{26} residue nor a free ammonium group are required for activity since CCK_{27-33} (CCK_7) and its N-protected derivatives (acetyl, terbutyloxycarbonyl or benzyloxycarbonyl) have the same potency than the CCK_8 fragment in peripheral or central bioassays (8-9). On the contrary, the biological activity of these cholecystokinin-related peptides strongly depends on the presence of the sulfate group, since its removal leads to about a 100 fold loss of potency (9). Moreover, the CCK_5 fragment (penta-

*To whom correspondence should be addressed.

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gastrin) displays only a ten times lower activity than CCK_8 for the displacement of ^{125}I CCK_{33} binding to guinea pig brain but is unable to displace this iodinated cholecystokinin from pancreas receptors (10). Taking into account all these features it was of great theoretical interest to study the conformational behaviour of sulfated and non-sulfated cholecystokinin. As we have shown in the case of enkephalins (11), such a structural investigation is a prerequisite for a rational design of potent agonists or antagonists. At this time only the non-sulfated CCK_7 ($NS\text{-}CCK_7$) has been studied by fluorescence transfer energy (12). In this paper, we report preliminary results on the first ^1H NMR conformational study of the potent sulfated CCK_7 and its inactive analog $NS\text{-}CCK_7$.

MATERIAL AND METHODS

Sulfated and non-sulfated CCK_7 were synthesized following previously described procedures (13). The purity of both compounds was checked by HPLC on a C_{18} μ-bondapak column using triethylamine-phosphoric acid buffer (pH 6.6) / acetonitrile as solvents. The NMR samples were prepared by dissolution of the peptides in H_2O or ^2H_2O. The solutions were adjusted to the appropriate pH value by HCl (or ^2HCl) or NaOH (or NaO^2H) and then lyophilized. The dried peptides were dissolved in ^2H_2O or $(C^2H_3)_2SO$ at a concentration of $5.10^{-3}M$. The pH values of aqueous solutions were measured with a microelectrode (Ingold 405.M3) using a pH meter Taccusel pHN 75 without correction for the deuterium effect. pKa values were determined from titration curves. Spectra were run in the Fourier transform mode at 270 MHz on a Brüker WH 270 spectrometer and at 400 MHz on a Brüker WM 400 spectrometer, both equipped with aspect 2000 computer and Brüker temperature controller. Resolution enhancement were achieved using a gaussian multiplication of the F.I.D. Chemical shifts are given in ppm ± 0.01 ppm from tetramethylsilane (Me_4Si) as internal reference in $(C^2H_3)_2SO$ solutions and from Me_4Si in CCl_4 as external reference in ^2H_2O solutions. Complete assignment of each spectrum was performed by selective irradiation experiments and comparison with the spectra of the related CCK_5 and CCK_6 fragments.

RESULTS

^1H NMR study of CCK_7 and $NS\text{-}CCK_7$ in $(C^2H_3)_2SO$ solutions.

The spectra of CCK_7 (Fig. 1) and $NS\text{-}CCK_7$ were done after lyophilization of aqueous solutions adjusted at pH 5. In these conditions, it can be assumed that for both peptides, the amino group of Tyr^{27} is under its protonated form whereas the Asp^{32} β-carboxy group is deprotonated. According to its strong acidity the sulfate group is negatively charged. The protons chemical shifts of CCK_7 and $NS\text{-}CCK_7$ are reported in Table 1. A great similarity occurs between both spectra for the tetrapeptide fragment Trp-Met-Asp-Phe-NH_2, while significant differences are observed in the N-terminal tripeptide Tyr-Met-Gly. So, as compared to $NS\text{-}CCK_7$, the NH of Gly is deshielded (0.10 ppm) in CCK_7 but the NH of Met^{28} and Tyr α, β-protons are upfield shifted (Table 1). This latter effect cannot be related to the O-sulfatation of tyrosine since, owing to its electroattractive effect, the sulfate group should induce a deshielding of Tyr α and β-protons as it appears on aromatic signals (Table 1). Likewise,

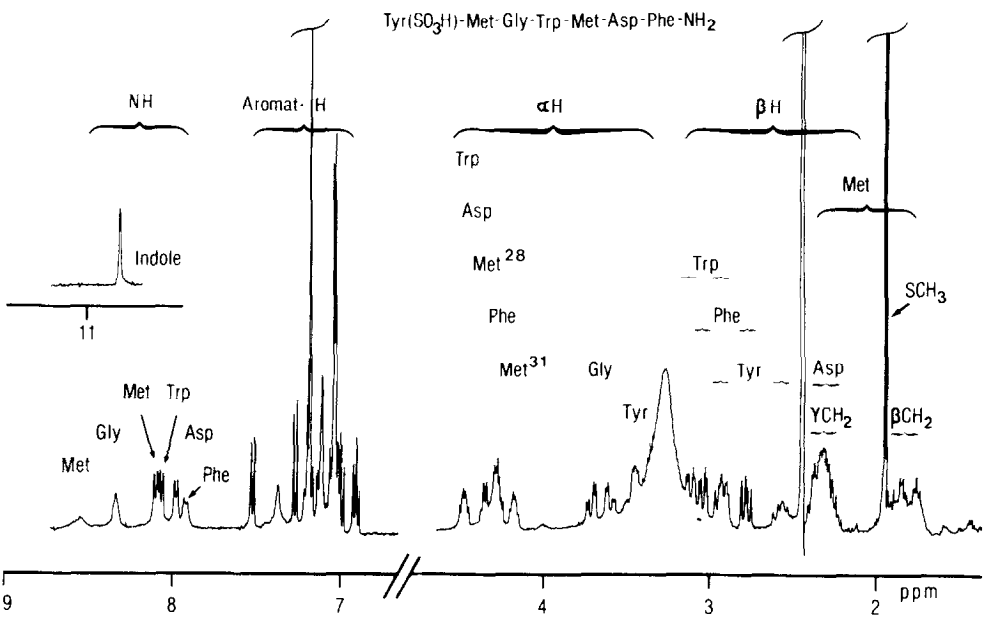

Figure 1. *^1H NMR spectra of sulfated CCK_7 in $(C^2H_3)_2SO$ solution ($\sim 5 \times 10^{-3}$M) at 400 MHz.*

a modification of the pKa value of Tyr ammonium group in CCK_7 as compared to NS-CCK_7 cannot be put forward to explain the shielding of CCK_7 Tyr protons since as shown by the titration curves in D_2O (Fig. 2) both peptides display very similar pKa values. Therefore, chemical shift differences occuring at the Tyr-Met-Gly level between CCK_7 and its non sulfated analog are probably due to conformational differences between both peptides. This assumption is reinforced by examination of the amide protons temperature dependency.

Indeed, in CCK_7 and NS-CCK_7 a relatively weak variation is observed for Asp-NH resonances (-3.4 and -3.6 ppm/°C, respectively) but the temperature coefficient of the Trp-NH chemical shift is significantly smaller in CCK_7 (-4.4 ppm/°C) than in NS-CCK_7 (-7 ppm/°C). These results strongly suggest a similar kind of folding for the C-terminal peptide backbone in both peptides but the presence of an additional bending in the N-terminal tripeptide moiety of CCK_7. These assumptions are supported by the values of $^3H_{NH-\alpha}$ coupling constants which are related to the backbone ϕ-angles (14). So, a set of ϕ and ψ angles, consistent with a β_I-turn can be derived from the value of 7.5 Hz measured for $^3J_{NH-\alpha}$ in Trp and Met residues which are located at the corner of the chain reversal. Likewise, the $^3J_{NH-\alpha}$ coupling constants determined in CCK_7 for Met^{28} (7 Hz) ; Gly (5.5 Hz) and Trp (7.5 Hz) seem in favour of the involvement of these three residues in a C_7 γ-turn. These proposed models are strengthened by the aminoacids side-chains conformations since for all residues measurements of the $^3J_{\alpha-\beta}$ indicate a great preference for a tg^- conformer

Table 1. Chemical shifts of the sulfated and non-sulfated CCK_7 protons and amide temperature dependency in $(C^2H_3)_2SO$.

residue	NS-CCK_7			CCK_7		
	NH [a]	H_α [a]	H_β [a]	NH [a]	H_α [a]	H_β [a]
Tyr [c]	--	3.65	2.66 2.88	--	3.49	2.62 2.85
Met	8.79 [b] *(-16.0)* [b]	4.26	1.71 1.89	8.67 [b] *(-12.0)* [b]	4.25	1.76 1.91
Gly	8.17 [b] *(-6.3)* [b]	3.58 3.68		8.27 [b] *(-6.6)* [b]	3.57 3.70	
Trp	8.06 [b] *(-7.0)* [b]	4.45	2.92 3.08	8.05 [b] *(-4.4)* [b]	4.45	2.94 3.10
Met	8.10 [b] *(-5.8)* [b]	4.15	1.74 1.82	8.09 [b] *(-5.2)* [b]	4.16	1.73 1.82
Asp	7.96 [b] *(-3.6)* [b]	4.34	2.36 2.47	7.95 [b] *(-3.4)* [b]	4.34	2.31 2.36
Phe	7.74 [b] *(-4.4)* [b]	4.25	2.77 3.00	7.81 [b] *(-5.0)* [b]	4.28	2.77 3.02

a) *The chemical shifts are expressed in ppm using TMS as internal references.*

b) *Slope of the chemical shift variation in function of the temperature in* $\times 10^3$ *ppm per °C.*

c) *The chemical shifts of Tyr aromatic protons are respectively 6.94 ppm and 6.61 ppm in NS-CCK_7, and 7.03 ppm and 7.00 ppm in CCK_7.*

(50 to 60 %). This feature corresponds to a favourable disposition of hydrophobic lateral chains around the folded backbone.

pH Titrations of CCK_7 and NS-CCK_7 in ^2H_2O.

The spectra of both sulfated and unsulfated peptides have been studied in the 2 to 10 pH range. The CCK_7 spectrum at pH 5 is shown on Fig. 2. At this pH, chemical shifts of CCK_7 and NS-CCK_7 protons are similar except for those of Tyr, Met^{28} and Gly residues which are more shielded in the non sulfated cholecystokinin. It can be observed that in $(C^2H_3)_2SO$, the reverse effect is observed for α and β-Tyr signals. Furthermore, the titration curves of CCK_7 and NS-CCK_7 in ^2H_2O show very interesting results. Hence, titration of the carboxy group of Asp leads to very weak effects on the α and β protons of Phe, Tyr and Met (28 and 31) in NS-CCK_7 whereas significant shifts are observed on all CCK_7 residues unless Phe β-protons and those belonging to Trp and Met^{31} residues. Likewise, titration of the N-terminal Tyr (pKa $\sim$ 7.4) in CCK_7 is clearly felt on all α and β protons but not on those of Trp and

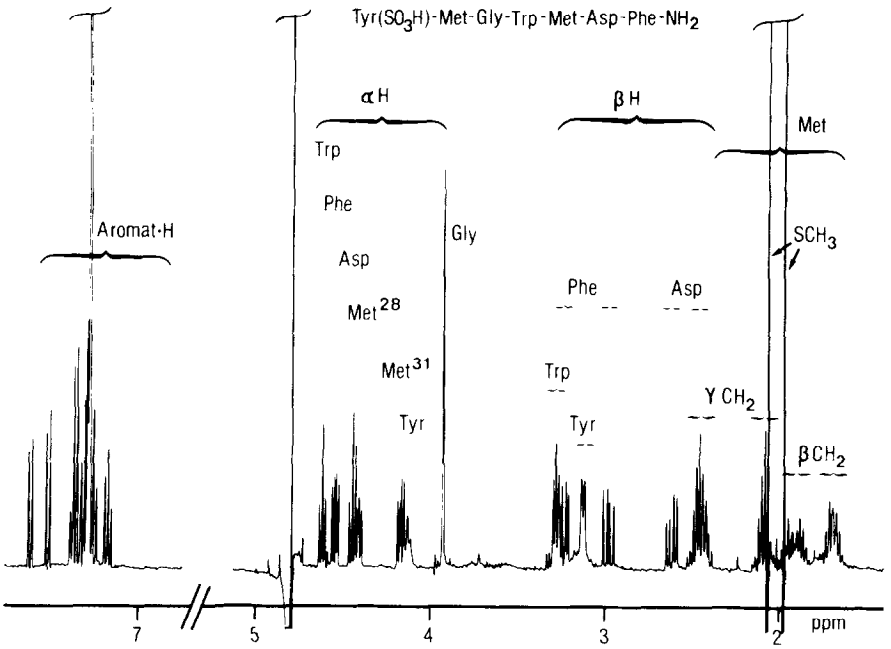

Figure 2. *^1H NMR spectrum (400 MHz) of sulfated CCK_7 ($\sim 5.10^{-3}M$) in ^2H_2O solution (pH $\sim$ 5).*

Phe while in NS-CCK_7 measurable effects are induced only on Gly and Met^{28} protons (Fig. 3). These findings reflect through space effects related to peptide puckering (15,16). So, it seems that in ^2H_2O the two peptides exhibit preferential folded structures which appear more defined in CCK_7 than in NS-CCK_7. As in $(C^2H_3)_2SO$ the conformers population of lateral chains is largely in favour of the tg^- orientation.

DISCUSSION

The different results obtained in this NMR study clearly show that CCK_7 and NS-CCK_7 exist preferentially in solution under folded conformations, which seem more puckered in the sulfated peptide than in the non-sulfated one. It is very interesting to notice that similar conformations are found in $(C^2H_3)_2SO$ and in ^2H_2O. Indeed, it is unusual for linear peptides to have well defined structures in aqueous solutions (16). A Dreiding model of CCK_7 proposed conformation is shown in Figure 4. This folded conformation, probably induced by the high content of hydrophobic side chains in this peptide, may be stabilized by an ionic interaction between the charged Tyr and Asp residues. A β-bend including the sequence Gly-Trp-Met-Asp with an hydrogen bond between the NH of Asp and the CO of Gly is observed in the C-terminal part of both peptides. Occurrence of such a folded structure is reinforced by low temperature dependency of Asp amide proton. Moreover according to the derived ϕ, ψ angles of Trp and Met residues, the β-bend is probably of type I. However some differences appear

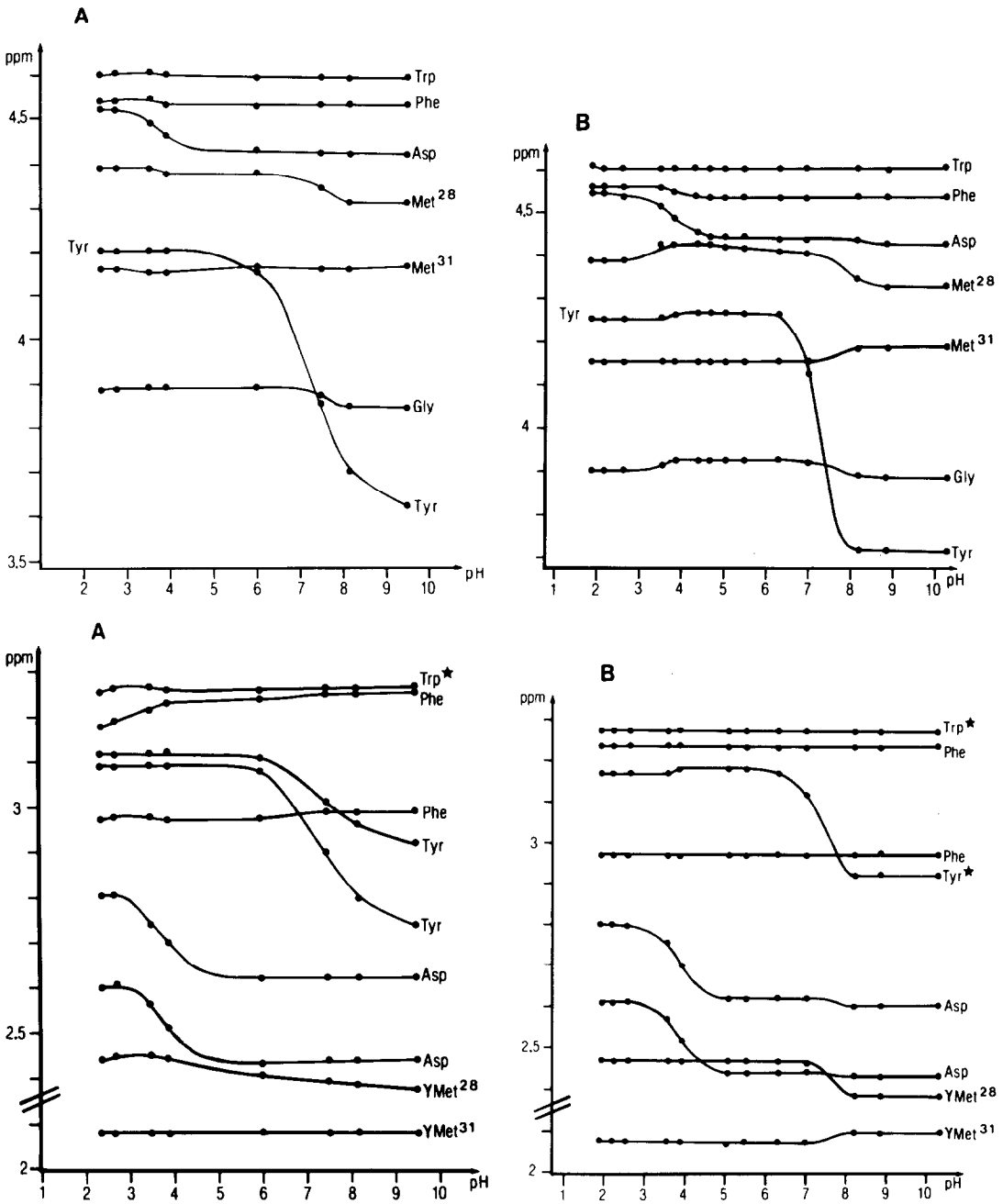

Figure 3. *^1H NMR titration curves in ^2H_2O for NS-CCK$_7$* **A** *and CCK$_7$* **B** .
Upper curves : chemical shift variations of α-protons.
Lower curves : chemical shift variation of β and γ-protons.
★ *At 270 MHz, the signals of the two β protons are overlapped.*

in the conformation of CCK$_7$ and NS-CCK$_7$ N-terminal part. In the sulfated peptide, rather weak temperature variation of Trp-NH, proximity between Gly-CH$_2$, Met^{28}-H$_\alpha$ and Asp-carboxyl group evidenced through titration experiments lead

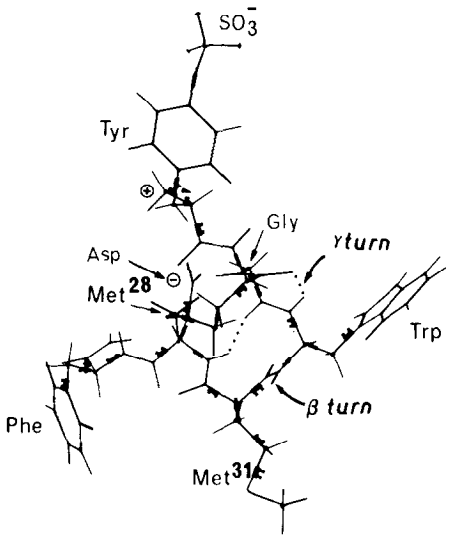

Figure 4. Dreiding model of the proposed folded conformation of sulfated CCK_7 in solution.

to propose a γ-turn for the sequence Met^{28}-Gly-Trp with a hydrogen bond between the CO of Met and the NH of Trp. This C_7 structure is favoured by the presence of a flexible glycine as central residue.

In relation with this highly puckered backbone, the lateral chains are directed away in their preferential tg^- conformation leading to a hydrophobic surrounding of the β-turn. In CCK_7 the presence of a γ-turn leads to a particular orientation of the sulfated tyrosine at the opposite of the hydrophobic part of the molecule, whereas in NS-CCK_7, the peptide N-terminal moiety remains more flexible. This sulfate induced folding could be accounted for both by the hydrophilic properties and the steric hindrance of this group. In aqueous solution, it can be assumed that organisation of water molecules around the charged sulfate produces a very important solvatation sphere which increases the folding tendency of the remaining residues.

In conclusion, the folded conformation of NS-CCK_7 supports the assumption of a β-type structure proposed by Schiller et al. (12) from fluorescence energy transfer. The highly folded conformation of sulfated CCK_7 in aqueous solution could explain its ability to cross the blood-brain barrier and therefore its pharmacological activity in CNS after systemic administration. Finally, affinity for brain receptors increases from CCK_4 to sulfated CCK_7 (10). This could indicate that a full interaction with a single type of receptors requires not only the sulfate group but also the conformational constraints observed in CCK_7. Such an hypothesis may be tested using appropriate cyclic cholecystokinin analogues, which synthesis is now in progress.

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