Simolo, K., Stucky, G., Chen, S., Bailey, M., Scholes, C., & McLendon, G. (1985) *J. Am. Chem. Soc. 107*, 2865–2872.
Stern, E. A., & Heald, S. M. (1979) *Rev. Sci. Instrum. 50*, 1579.
Warshel, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1789–1793.

APPENDIX

The following discussion uses the terminology and notation of Bunker (1983). We wish to determine the position and width of a distribution composed of two subdistributions of identical atoms.

$$P(r)^{\text{total}} = P(r) + Q(r)$$

where

$$P(r) = \frac{(P)Np(P)(r)}{r^2} \exp(-2r/\lambda)$$

$$Q(r) = \frac{(Q)Np(Q)(r)}{r^2} \exp(-2r/\lambda)$$

$N^{(P)}p^{(P)}(r)\,dr$ is the probability of finding an atom in the interval $r + dr$ in distribution P, λ is the mean free path, and $\int p^{(P)}(r)\,dr = 1$; analogous expressions hold for distribution Q.

Let $P_n^{\text{total}} = \int P(r)^{\text{total}} r^n\,dr$, $p_n^{\text{total}} = P_n^{\text{total}}/P_0^{\text{total}}$, $P_n = \int P(r) r^n\,dr$, $P_n = P_n/P_0$, $Q_n = \int Q(r) r^n\,dr$, $q_n = Q_n/Q_0$; and $\alpha = Q_0/P_0$. The centroid $\bar{r}_0 P(r)^{\text{total}}$ is

$$\bar{r} = P_1^{\text{total}} = \frac{P_1 + Q_1}{P_0 + Q_0} = \left(\frac{1}{1+\alpha}\right) r^{(P)} + \left(\frac{\alpha}{1+\alpha}\right) r^{(Q)}$$

where $r^{(P)} = P_1$ and $r^{(Q)} = q_1$. Evidently the average position is just the weighted average of the positions of $P(r)$ and $Q(r)$. After some manipulation, the mean square width σ^2 can be expressed as

$$\sigma^2 = p^{\text{total}} - p_1^{\text{total}\,2} = \left(\frac{1}{1+\alpha}\right)^2 [\sigma^{(P)2} + \sigma^{(Q)2} + (r^{(P)} - r^{(Q)})^2 + \alpha^2 \sigma^{(Q)2}]$$

where $\sigma^{(P)2} = p_2 - p_1^2$ and $\sigma^{(Q)2} = q_2 - q_1^2$. These are the desired equations. Note the inequality $|r^{(P)} - r^{(Q)}| \leq [(1 + \alpha)/\alpha^{1/2}]\sigma$.

Conformational Analysis of Cholecystokinin CCK_{26-33} and Related Fragments by ^1H NMR Spectroscopy, Fluorescence-Transfer Measurements, and Calculations†

M. C. Fourniê-Zaluski,‡ J. Belleney,‡ B. Lux,§ C. Durieux,‡ D. Gêrard,§ G. Gacel,‡ B. Maigret,‖ and B. P. Roques*,‡

Département de Chimie Organique, U 266 INSERM et UA 498 CNRS, UER des Sciences Pharmaceutiques et Biologiques, 75006 Paris, France, Laboratoire de Physique UA 491 CNRS, UER des Sciences Pharmaceutiques, 67000 Strasbourg, France, and Laboratoire des Molécules Informatiques, Institut Le Bel, Université de Strasbourg I, 67008 Strasbourg, France

Received October 31, 1985; Revised Manuscript Received February 19, 1986

ABSTRACT: The conformational behavior of CCK_7, Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2, and CCK_8, Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2, in their sulfated and unsulfated forms, was studied both by ^1H NMR spectroscopy in dimethyl-d_6 sulfoxide and water and by fluorescence-transfer measurements at pH 7. In neutral conditions, both experimental methods show that these peptides exist preferentially in folded forms with β and γ turns around the sequence Gly-Trp-Met-Asp and Met-Asp-Phe-NH_2, respectively. The presence of stable folded conformations is supported by through-space effects during the titration of the ionizable groups and by the weak temperature dependency of some amide protons not only in dimethyl sulfoxide but also in water. The folding of the C-terminal part, already shown in CCK_5, seems to be a common conformational characteristic in CCK peptides. The N-terminal part of CCK_8 presents an equilibrium between β and γ turns, whereas this part of the peptide is more flexible in CCK_7. The low quantum yield of Tyr and the large mean distance (R = 15 Å) between Tyr and Trp, determined by fluorescence-transfer measurements, support the occurrence of folded conformations pushing the aromatic rings far from each other. Interestingly, the introduction of the sulfate group enhances the folding tendency even in aqueous medium. The larger amide temperature dependency and the decrease in the R distance at acidic pH suggest that an intramolecular ionic interaction involving the N-terminal amino group and the β-carboxyl groups of Asp^{32} stabilize the folded forms. Metropolis calculations performed on CCK_8 support the existence of stable folded conformations closely related to those deduced from experimental data. The conformationally constrained C-terminal part of CCK_8 and related peptides seems to play a crucial role in the efficient recognition of the brain receptors, whereas the observed external orientation of the sulfated tyrosine ring is probably required to induce the transduction process.

Cholecystokinin CCK (Ivy & Oldberg, 1928), originally described as a gut hormone (Mutt & Jorpes, 1971) and subsequently discovered in the brain (Vanderhaeghen et al., 1975; Rehfeld, 1978), is one of the most abundant peptides in the central nervous system (CNS), where it has recently been implicated as a putative neurotransmitter (Pinget et al., 1979; Dodd & Kelly, 1981). CCK exists in various molecular forms differing among species (Zhou et al., 1985), but the C-terminal octapeptide CCK_8 [Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2], which retains all the activity of the entire hormone in the gastrointestinal tract (Ondetti et al., 1970), is the predominant form in the brain (Dockray, 1976). The struc-

† This research is supported by grants from Université René Descartes, Centre National de la Recherche Scientifique, and Institut National de la Santé et de la Recherche Médicale.
‡ UER des Sciences Pharmaceutiques et Biologiques.
§ UER des Sciences Pharmaceutiques.
‖ Université de Strasbourg I.

0006-2960/86/0425-3778$01.50/0

tural characteristic of CCK_8 is the presence of an unusual sulfated tyrosine that is crucial for the activity of the peptide both in endocrine cells (Gardner et al., 1975) and at the level of peripheral and central neurons (Dodd & Kelly, 1981; Crawley, 1985). In the past few years, specific binding sites have been found for CCK in the brain and in the gut (Hays et al., 1980; Innis & Snyder, 1980; Steigerwalt & Williams, 1984). However, only the sulfated peptides exhibit a high affinity for peripheral receptors while sulfated and unsulfated (NS) forms of CCK_7 and CCK_8, as well as smaller C-terminal fragments, behave as potent ligands for brain binding sites (Gaudreau et al., 1983). These features suggested the existence of multiple CCK receptors, an assumption strongly reinforced by the characterization of entities with different molecular weights for pancreas and brain CCK binding sites (Sakamoto et al., 1984a,b).

Moreover, some recent binding studies suggest the occurrence of different receptor subtypes in the brain (Innis & Snyder, 1980). As shown for the opioid peptides, enkephalins, the unambiguous characterization of a receptor heterogeneity requires the development of selective ligands for each binding site (Zajac & Roques, 1985). This was achieved by deducing the biologically active structures from an extensive conformational analysis of the endogenous peptide, performed by NMR (Roques et al., 1976; Marion et al., 1983) and fluorescence experiments (Guyon-Gruaz et al., 1981) reinforced by conformational calculations (Maigret et al., 1981). With these data, probes were then rationally designed allowing the specific recognition of each receptor subtype. With the aim of extending this approach to neuronal CCK, it was necessary to study the conformational behavior of CCK_7 and CCK_8 in their sulfated and unsulfated forms and to compare their structural characteristics with those of the smaller fragments CCK_4, CCK_5, and CCK_6. For this purpose, three complementary techniques were used: ^1H NMR spectroscopy in dimethyl-d_6 sulfoxide (Me_2SO-d_6) and in aqueous medium, fluorescence-transfer measurements, and conformational calculations.

Experimental Procedures

Materials

The peptides were synthesized in the laboratory following previously described procedures (Bodanszky et al., 1977). The purity of the compounds was checked by high-performance liquid chromatography (HPLC) (Waters Apparatus) on a C_{18} μBondapak column with triethylamine–phosphoric acid buffer (TEAP), 25 mM (pH 6.6)/acetonitrile as eluent. The retention times (R_t) of the various CCK fragments at a flow rate of 1.5 mL/min were as follows: CCK_8, R_t = 9.4 min; CCK_7, R_t = 11.1 min in TEAP/CH_3CN, 74/26; NS CCK_8, R_t = 6.1 min; NS CCK_7, R_t = 13.8 min in TEAP/CH_3CN, 70/30.

Methods

NMR Studies. The NMR samples were prepared by dissolving the peptides in H_2O or D_2O. The solutions were adjusted to the appropriate pH value by addition of HCl (DCl) or NaOH (NaOD) and lyophilized. The dried peptides were redissolved in D_2O, H_2O, or Me_2SO-d_6 at a concentration of 5×10^{-3} M. The pH values of the resultant aqueous solutions were measured with a microelectrode Ingold 405.M3 using a Tacussel pH meter pHN75, without correction for the deuterium effect. pK_a values were determined from titration curves.

Spectra were run in the Fourier-transform mode at 270 MHz on a Brüker WH 270 and at 400-MHz on a Brüker AM 400 equipped with Aspect 2000 and 3000 computers, respectively, and a Brüker temperature controller (±1 °C). Chemical shifts were given in ppm (±0.005 ppm) from tetramethylsilane (Me_4Si) as internal reference in Me_2SO-d_6 solution. In D_2O or H_2O solutions, a 5% solution of Me_4Si in CCl_4 was used as external reference; the chemical shifts were then corrected and given from Me_4Si as internal reference. The coupling constants were determined to ±0.3 Hz. Resolution enhancement was achieved by a Gaussian multiplication of the FID. The study of the NH's chemical shifts in H_2O was performed with the pulse sequence $(1\tau\bar{3}\tau3\tau\bar{1})$ described by Hore (1983). The spectral width was 4000 Hz; τ was chosen to excite the NH region (τ = 0.5 ms). A relaxation delay of 8 s was used between each scan.

COSY Experiment. Two-dimensional (2D) correlation spectroscopy provides connectivities between spins through the scalar couplings. The basic two-pulse Jeener sequence (Jeener, 1971) was used in its improved version known as double quantum filter (DQF) COSY (Piantini et al., 1982):

$$t_0\text{–}90(X)\text{–}T_1\text{–}90(X)90(\psi)\text{–acquisition}(\Psi)$$

This sequence eliminates the major contribution of the dispersive character encountered in the conventional COSY diagonal and thus allows the recording of pure absorption 2D spectra. Elimination of the residual Me_2SO signal was obtained by an inversion–recovery sequence $(180\text{–}D_1\text{–}90)RD$ with D_1 = 8 s and relaxation delay RD = 14 s.

Fluorescence Studies. Absorption spectra were obtained with a Cary 219 spectrophotometer. Fluorescence spectra were recorded on a Fica 55 absolute differential fluorometer. Quantum yields (ϕ) were calculated from areas under the fluorescence spectra, taking as reference ϕ = 0.14 at 25 °C for a solution of L-tryptophan of similar absorbance (Chen, 1967). Peptide solutions were prepared at a concentration of 2×10^{-5} M in Tris(hydroxymethyl)aminomethane (Tris-HCl) buffer, 10 mM, pH 7.5. The samples at pH 4 were obtained by addition of 1 M HCl to the previous buffered solutions.

Fluorescence Energy Transfer. The intramolecular distance between tyrosine or sulfated tyrosine and tryptophan was investigated by quantitative evaluation of the transfer of excitation energy from the phenol ring (donor) to the indole moiety (acceptor) according to the Förster treatment (Förster, 1948). The donor–acceptor separation R is related to the transfer efficiency E by

$$R = R_0(1/E - 1)^{1/6} \quad (1)$$

where R_0 is the Förster critical distance at which E = 50%.

The value of R_0 is given by

$$R_0^6 = (8.75 \times 10^{-25})n^{-4}\phi_D\kappa^2 J_{AD} \quad (2)$$

with

$$J_{AD} = \int_0^\infty F_D(\bar{\nu})\epsilon_A(\bar{\nu})\bar{\nu}^{-4}\, d\bar{\nu} \quad (3)$$

where ϕ_D is the donor emission quantum yield, n is the index of refraction of the medium, and κ^2 is the dipole–dipole orientation factor. J_{AD} is an overlap integral between $\epsilon_A(\bar{\nu})$, the molar extinction coefficient of the acceptor, and $F_D(\bar{\nu})$, the spectral distribution of the donor emission normalized to unity, modified by the wavenumber factor $\bar{\nu}^{-4}$. The efficiency E of such a transfer can be determined by observing sensitized fluorescence of the acceptor or quenching of the donor fluorescence.

Concerning the first method, the fluorescence quantum yield ϕ_p^λ(Trp), when excited at wavelength λ, is given by the equation:

$$\phi_p^\lambda(\text{Trp}) = \phi_{\text{Trp}}[f_{\text{Trp}}(\lambda) + Ef_{\text{Tyr}}(\lambda)]$$

where $f_{\text{Trp}}(\lambda)$ and $f_{\text{Tyr}}(\lambda)$ are the fraction of light absorbed by

Trp and Tyr, respectively, in the peptide. ϕ_{Trp} is the fluorescence quantum yield of Trp in CCK, measured under conditions where only this residue is excited (λ_{exc} = 295 nm).

In the case of the sulfated derivatives, the efficiency of energy transfer E was determined from the quantum yields of the donor evaluated in the absence (ϕ_0) and presence (ϕ_T) of the acceptor, following the equation $E = 1 - \phi_T/\phi_0$. The fluorescence intensity of the donor enclosed in the peptide CCK was compared to that of a mixture of two peptide fragments, one of them containing the donor and the other containing the acceptor. This mixture can be considered as a system in which no radiationless energy transfer occurs. Moreover, one assumes that the donor quantum yield is the same in the CCK peptide and in the peptide fragment and that it is only affected, in presence of the acceptor, by the energy-transfer process itself. This is highly probable since we have shown that the quantum yield of sulfated tyrosine is not greatly perturbed by nearby amino acids (data not shown).

Conformational Calculations. In previous Metropolis calculations on oligopeptide molecules (Prémilat & Maigret, 1977), the usual (ϕ, ψ) dihedral angles used to generate conformations were selected at random in the energetically allowed area of the well-known Ramachandran plots of the constitutive residues in the chain. In such calculations, samples of 10000 conformers give fairly satisfactory results as concerns the statistical behavior of the investigated molecules.

However, if the amino acid side-chain isomeric states are to be introduced and taken into account in the simulation, the resulting increase in the number of degrees of freedom to be considered leads to very low probabilities of obtaining stable conformations among all the possible chain structures. In order to keep the computer time to a reasonable and practical limit for the discovery of the stable conformers in a very complicated hyperspace, the random Metropolis search must be biased to some extent with constraints coming from information of different sources. Several attempts were made to introduce such bias, and the only way to test their validity is to compare the results coming from unbiased simulation, which has proved to converge with experimental relevant data.

A complete simulation obtained with the usual Metropolis procedure performed toward convergence was used in this work. The calculations were done on a CRAY-1 computer and were tested on CCK_8. Two samples of 1 000 000 chains each were obtained and shown to converge the same way. In order to explore the conformational hyperspace as much as possible, the selection of the "improved" (ϕ, ψ, χ) angles in the Metropolis procedure has been modified in these calculations, compared to the previous ones: as usual, an arbitrary chain conformation, defined by a set of (ϕ, ψ) taken at random in a given list, is built. This list is, as usual, set up from the 20° increment of the Ramachandran energy plot of each individual residue. The χ angles are also selected at random from a 20° grid. Starting from each of these initial states (n_1), one pair of (ϕ, ψ) angles randomly selected in the sequence is given other random values coming from the same list of points as above. More or less flexibility of the molecule around the perturbation thus introduced is now added by moving the (ϕ, ψ) angles in a 20° square centered at the new selected grid point. The same is done for the χ values. This perturbation is done n_2 times, as usual, and corresponds to the improvement of the sample toward probable conformations.

RESULTS

^1H NMR Studies

The ^1H NMR study of CCK_7 and CCK_8 was performed in Me_2SO-d_6 and water, in order to compare the conformational properties of these two peptides in two solvents susceptible to mimic the different environments encountered by the peptide in the receptor surrounding. For CCK_7, the sulfated and unsulfated forms can be studied in both solvents, but for the octapeptide the very low solubility of NS CCK_8 precludes its study in aqueous medium. Furthermore, the great sensitivity to hydrolysis of the sulfated peptides in acidic medium prevents the determination of their NH temperature coefficients under these pH conditions. This experiment was performed only with NS CCK_7 and NS CCK_8.

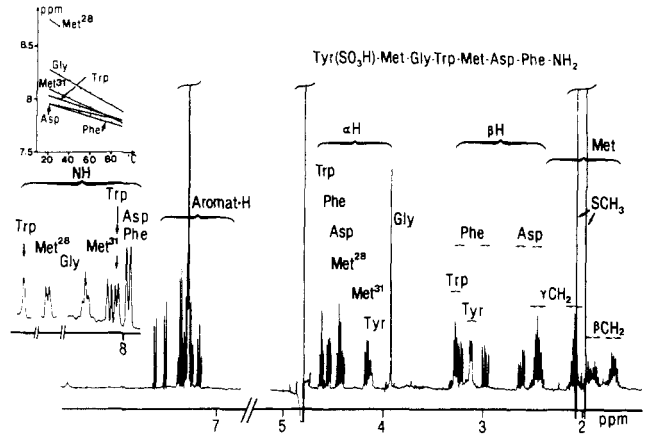

FIGURE 1: ^1H NMR spectrum of CCK_7 in D_2O/H_2O solution at pH 6. (Insert) NH chemical shift dependency as a function of temperature.

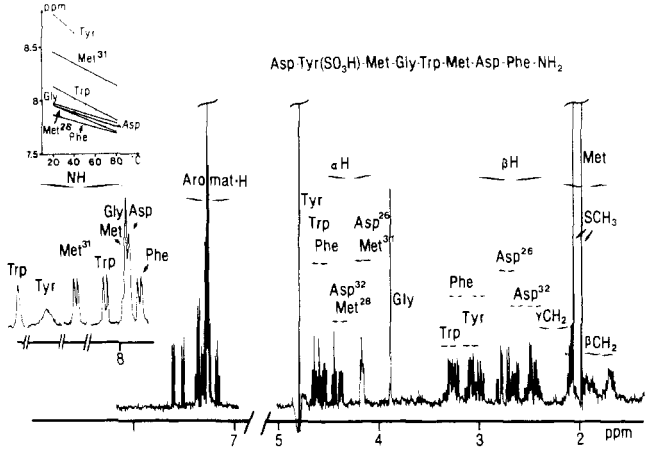

FIGURE 2: ^1H NMR spectrum of CCK_8 in D_2O/H_2O solution at pH 6. (Insert) NH chemical shift dependency as a function of temperature.

^1H NMR Studies in D_2O/H_2O Solutions. In a first series of experiments, the ^1H NMR spectra of CCK_7 and CCK_8 were studied in D_2O solution, as a function of pH. The assignment of the spectra of the two peptides was easily done since there is no overlap of the various proton signals (Figures 1 and 2). Therefore, classical selective irradiations were performed to detect the connectivities between the Hα and side-chain protons. The assignments of the various ABX systems to the corresponding amino acids (Asp, Tyr, Trp, and Phe) were done by comparison with the smaller fragments, which have been previously studied in detail (Fournié-Zaluski et al., 1985). Chemical shift analogies were observed for a given amino acid from CCK_4 to CCK_6, except for the N-terminal residue. These analogies were also observed in CCK_7 and CCK_8 as clearly shown on Figure 3.

The pH increase from 2 to 10 led to the titration of two functional groups in the case of CCK_7: the β-Asp^{32} carboxylic group and the Tyr^{27} ammonium group (Figure 3A). In this

Table I: Chemical Shifts and NH Temperature Dependency for CCK_7 and CCK_8 in H_2O/D_2O Solutions at pH 6

	CCK_7[a]				CCK_8[a]			
	NH[b]	CHα	CH$_2\beta$	others	NH[b]	CHα	CH$_2\beta$	others
Asp^{26}						4.17	2.80–2.69	
Tyr^{27}		4.12	3.12–3.09	7.33–7.26	8.75 (−6.5)	4.65	3.12–3.05	7.27–7.24
Met^{28}	8.75 (−4.0)	4.39	1.93–1.87	γ = 2.45, S–CH$_3$ = 1.96[c]	7.94 (−5.3)	4.38	1.93–1.87	γ = 2.56–2.46, S–CH$_3$ = 1.98[d]
Gly^{29}	8.29 (−5.7)	3.92			7.95 (−2.6)	3.89		
Trp^{30}	8.06 (−3.9)	4.60	3.28–3.23	Ar = 7.58 (d), 7.48 (d), 7.14 (t), 7.23 (t), 7.25 (s)	8.10 (−5.5)	4.60	3.32–3.26	Ar = 7.60 (d), 7.50 (d), 7.16 (t), 7.25 (t), 7.27 (s)
Met^{31}	8.11 (−4.8)	4.16	1.70–1.66	γ = 2.07, S–CH$_3$ = 2.05[c]	8.43 (−4.3)	4.16	1.71–1.67	γ = 2.09, S–CH$_3$ = 2.07[d]
Asp^{32}	7.98 (−2.3)	4.43	2.60–2.43		7.94 (−3.0)	4.44	2.64–2.51	
Phe^{33}	7.98 (−3.1)	4.52	3.21–2.96	~7.25	7.85 (−3.1)	4.55	3.24–2.98	~7.26

[a] Chemical shifts (ppm) are reported to Me_4Si used as internal reference. [b] NH temperature dependencies (ppm/°C × 10^3) are given in parentheses. [c] These values may be reversed. [d] These values may be reversed.

pH range, the sulfated group is always negatively charged. The deprotonation of the Asp^{32} carboxylic group (pK_a ~ 3.85) induced, as expected, a strong shielding of the α- and β-protons of this residue and produced, at the same time, significant effects on the other amino acid signals: a deshielding was observed for Hα Met^{28}, Hα and Hβ Tyr, and CH_2 Gly, whereas Hα Phe was shifted upfield. Spectral modifications were also observed during the titration of the Tyr amino group (pK_a ~ 7.40). The large shielding observed on the α- and β-protons of this amino acid was associated with large and inverse chemical shifts on Met^{28} and Met^{31} residues and significant shielding of Asp^{32} and Gly protons.

For CCK_8, in the pH range 2–10, the α-amino and the β-carboxylic groups of Asp^{26}, as well as the β-carboxylic group of Asp^{32}, were titrated, allowing an unambiguous assignment of Asp^{26} and Asp^{32} signals. The titration curves (Figure 3B) clearly demonstrated that the pK_as of the two aspartyl carboxylic groups are different. A pK_a value of about 4.50 was determined for Asp^{32} from chemical shift variation of its α- and β-protons. For Asp^{26}, a precise determination of the carboxylate pK_a cannot be obtained since the chemical shifts of its own α- and β-protons are influenced by the titration of Asp^{32}. However, a pK_a value of about 3.70 may be deduced from these curves. The deprotonation of the Asp^{26} carboxylic group did not produce very large modifications on CCK_8 proton chemical shifts. The only effects observed were a deshielding of Hα Met^{28}, an upfield shift of the Met^{31} side chain, and a degeneracy of the Gly methylene protons. By contrast, the deprotonation of Asp^{32} is more disturbing, since a displacement of all the α-protons was observed, with a significant downfield shift of Met^{28}, Met^{31}, and Phe and an upfield shift of Hα Trp. Weaker effects were found for α-Tyr, Gly, and side-chain protons. Finally, the titration of the terminal amino group (pK_a ~ 8.5) induced, as expected, a large shielding of Asp^{26} protons but also significant shifts on Tyr, Trp, and Met^{28} α-protons. Very weak effects were observed on CH$_2\beta$ of Tyr and Phe.

The ^{1}H NMR spectra of CCK_7 and CCK_8 were thoroughly analyzed in D_2O and H_2O at pH 6, which corresponded to the ionization state of the peptides under physiological conditions (Figures 1 and 2). The various chemical shifts are reported in Table I while the coupling constants are given in Table IS (see paragraph at end of paper regarding supplementary material).

As shown in Table I, a close analogy exists between the NMR parameters of both peptides with no significant change at the level of the side-chain signals. For α-protons, the single modification corresponds to the tyrosine residue that is more shielded in CCK_7 than in CCK_8 according to its N-terminal position in the former peptide. For the amide protons observed in H_2O/D_2O, 90/10 solution, it was interesting to note that

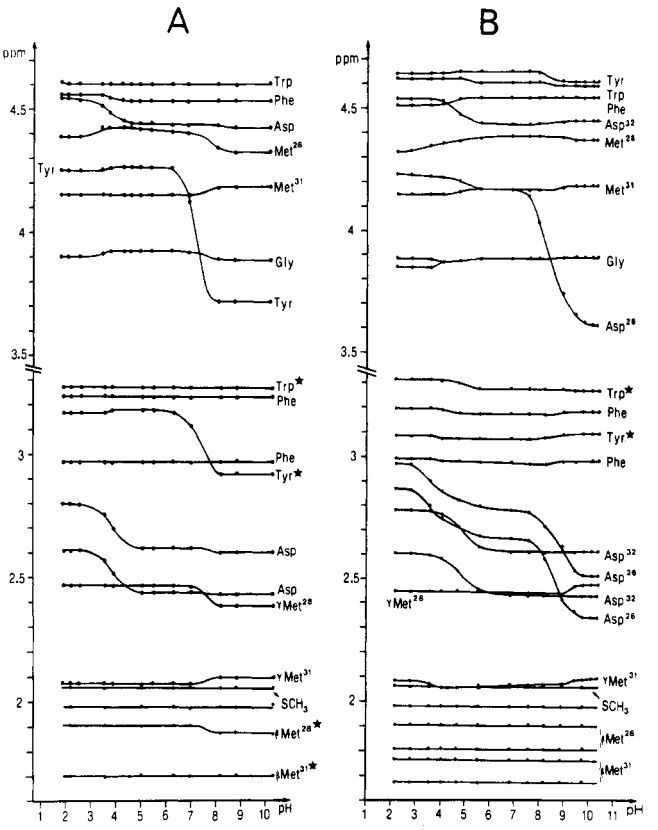

FIGURE 3: ^{1}H NMR titration curves for CCK_7 (A) and CCK_8 (B) in D_2O. (*) At 270 MHz, the signals of the two β-protons overlap.

significant and inverse effects were observed for Gly and Met^{31} NH: in CCK_7, Met^{31} NH is more shielded (0.31 ppm) and Gly NH more deshielded (−0.34 ppm) than in CCK_8. The upfield shift of Gly NH in the octapeptide was associated with a decrease in the NH temperature coefficient, which reflects a burying of this amide group. Conversely, Trp NH is more accessible to the solvent in CCK_8 ($\Delta\delta/\Delta T = -5.5 \times 10^{-3}$ M ppm/°C) than in CCK_7 ($\Delta\delta/\Delta T = -3.9 \times 10^{-3}$ ppm/°C). Moreover, in the two peptides, the temperature coefficients of Asp^{32} NH ($\Delta\delta/\Delta T \simeq -2 \times 10^{-3}$ ppm/°C) and Phe NH ($\Delta\delta/\Delta T \simeq -3.1 \times 10^{-3}$ ppm/°C) are quite low.

The vicinal coupling constants $^3J_{NH-\alpha}$ and $^3J_{\alpha-\beta}$, which are respectively related to the backbone conformation and the mean orientation of the side chains, were in the same range for all the residues in the two peptides. The relative percentage of the three rotamers corresponding to rotation around the C_α–C_β bond was calculated with the Pachler parameters (Pachler, 1964). For Tyr and Asp^{32}, the tg^+ and tg^- conformers were about equally populated, while for the other amino acids the tg^- conformer was largely predominant. The

Table II: Chemical Shifts and NH Temperature Dependency for CCK_8 and NS CCK_8 in Me_2SO-d_6

	CCK_8[a]				NS CCK_8[a]			
	NH[b]	CHα	$CH_2\beta$	others	NH[b]	CHα	$CH_2\beta$	others
Asp^{26}		3.75	2.53–2.47			3.70	2.52–2.47	
Tyr^{27}	8.61 (−5.1)	4.37	3.00–2.70	7.17–7.00	8.45 (−7.6)	4.30	2.89–2.67	6.97–6.57, OH = 9.12
Met^{28}	8.22 (−4.2)	4.24	1.81–1.78	γ = 2.32–2.30, SCH_3 = 1.95	8.13 (−4.8)	4.25	1.86–1.80	γ = 2.36–2.32, SCH_3 = 1.94[c]
Gly^{29}	7.94 (−2.3)	3.71, 3.52			7.92 (−2.0)	3.70, 3.51		
Trp^{30}	8.22 (−3.8)	4.43	3.06–2.93	6.87 (t), 6.98 (t), 7.16 (s), 7.25 (d), 7.52 (d), NH = 10.74	8.17 (−7.6)	4.44	3.06–2.97	6.88 (t), 6.98 (t), 7.16 (s), 7.24 (d), 7.52 (d), NH = 10.74
Met^{31}	8.28 (−5.2)	4.20	1.78–1.75	γ = 2.30–2.27, SCH_3 = 1.95	8.28 (−8.8)	4.21	1.76–1.71	γ = 2.30–2.24, SCH_3 = 1.95[c]
Asp^{32}	7.97 (−2.8)	4.40	2.53–2.42		8.00 (−4.0)	4.41	2.56–2.41	
Phe^{33}	7.79 (−3.7)	4.27	3.00–2.77	~7.16	7.81 (−4.8)	4.28	3.00–2.78	~7.14

[a] Chemical shifts are given in ppm from Me_4Si used as internal reference. [b] NH temperature dependencies (ppm/°C × 10^3) are given in parentheses. [c] These values may be reversed.

small, but significant, difference observed in the Trp $^3J_{\alpha-\beta}$ between CCK_7 and CCK_8 shows an increase of tg^- population in the latter (Table IS, supplementary material). Using other Karplus-type relations for $^3J_{\alpha-\beta}$ (ϕ) in the literature (Demarco et al., 1978; Cung et Marraud, 1982), the computed percentages of the three rotamers are slightly different, but the most populated conformation remains the same.

^1H NMR Studies in Me_2SO-d_6. This study was performed on CCK_8 and NS CCK_8, and the results obtained were compared to those corresponding to CCK_7 and NS CCK_7 under the same conditions. The assignment of these spectra is not as easy as in aqueous solutions since a strong overlap of the α-protons is observed especially for the octapeptides. In order to obtain complete information about chemical shifts, a 2D correlation spectroscopy experiment (DQF COSY) was done, as shown on Figure 4 for CCK_8.

Nevertheless, some ambiguities remained for the assignment of Met^{28} and Met^{31} signals, respectively. NOE experiments, leading to a sequential assignment of peptide residues, allowed the discrimination between the α-protons of these two amino acids. For the complete attribution of Met side-chain protons, a combination of NOE and spin-decoupling experiments was performed. The difference spectra, resulting from two experiments, in which the γ-Met protons are presaturated, with the selective irradiation of γ-Met protons during acquisition for the first one and with off-resonance irradiation for the other one, allowed us to obtain the chemical shift and the coupling constants of the corresponding β-protons. The assignment of the CCK_8 spectrum in Me_2SO-d_6 is identical with that described by Koizuka et al. (1984).

The determination of the chemical shifts and coupling constants of aspartyl β-protons, whose signals were under the residual Me_2SO peak, was possible by using the association of an inversion–recovery sequence for Me_2SO peak suppression and difference spectrum between selective irradiation of Asp α-protons and off-resonance irradiation. Finally, in order to verify the absence of peptide aggregation in Me_2SO-d_6, a comparison of the CCK_8 spectrum at 5×10^{-3} and at 5×10^{-4} M was made. No variations in the chemical shifts nor the line widths were observed (not shown).

(A) Comparison between NS CCK_8 and CCK_8. As reported in Table II, a great similarity was observed in the proton chemical shifts of CCK_8 and NS CCK_8 since the single significant difference was a deshielding of the tyrosine signals in CCK_8. This effects is probably related to the electroattracting properties of the sulfate group. However, the solvent accessibility of the amide protons in both peptides was slightly different. In the unsulfated peptide, the Gly NH (-2.0×10^{-3} ppm/°C) and Asp^{32} NH (-4.0×10^{-3} ppm/°C) appeared protected from the solvent, by contrast with the other amides, which showed temperature coefficients between -5×10^{-3} and

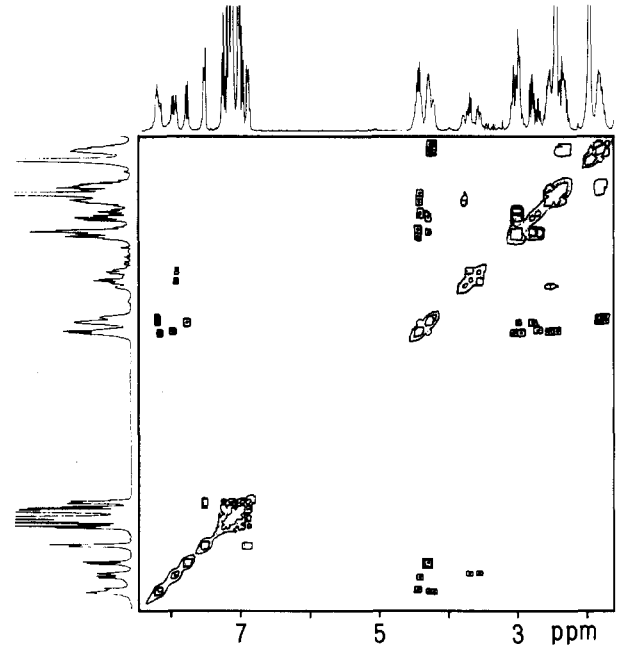

FIGURE 4: 2D correlation spectrum for CCK_8 in Me_2SO-d_6 solution ($c = 5 \times 10^{-3}$ M).

-8×10^{-3} ppm/°C. In CCK_8, Gly and Asp^{32} NH's elicited weak temperature coefficients (-2.3×10^{-3} and -2.0×10^{-3} ppm/°C, respectively), but Phe and Trp NH's with slopes around -3×10^{-3} ppm/°C may be also considered as buried protons. Furthermore, for the other CCK_8 amide protons the greatest value of the temperature coefficient was only -4.2×10^{-3} ppm/°C.

The coupling constants $^3J_{NH-\alpha}$ (Table IIS, supplementary material) were not significantly modified from one peptide to the other and were all in the range 7–8.5 Hz. The determination of the side-chain orientation from $^3J_{\alpha-\beta}$ coupling constants indicated, as in water, a significant preference for the tg^- conformer.

(B) Comparison between CCK_7 and CCK_8. The chemical shifts of α and side-chain protons in the heptapeptide (Table II) were almost identical with the corresponding protons in CCK_8 except for the signal of Tyr, which occurs as the N-terminal amino acid in CCK_7 and as an internal residue in CCK_8 (Table III). Contrastingly, at the level of the amide protons, large differences were shown between both peptides since, in Me_2SO-d_6 as in aqueous medium, a shielding of Gly NH (0.35 and 0.34 ppm, respectively) and a deshielding of Met^{31} NH (−0.22 and −0.32 ppm, respectively) were observed for CCK_8 related to CCK_7. Furthermore, the upfield shift of Gly NH in CCK_8 was accompanied by a decrease in its NH temperature coefficient, whereas no significant change was

Table III: Chemical Shifts, NH Temperature Dependency, and Coupling Constants for CCK$_7$ in Me_2SO-d_6 (Neutral Medium)

	chemical shifts[a]				coupling constants[b]		
	NH[c]	Hα	$CH_2\beta$	others	$J_{NH\alpha}$	$J_{\alpha-\beta}$	conformers[d]
Tyr^{27}		3.41	2.88–2.54	7.15–7.01		4.50–9.00	g^+g^- (25%) tg^- (58%) tg^+ (17%)
Met^{28}	8.50 (−16)	4.26	1.83–1.74	$\gamma \sim 2.28$, S–CH_3 = 1.92[e]		4.50–9.0	g^+g^- (25%) tg^- (58%) tg^+ (17%)
Gly^{29}	8.29 (−6.6)	3.69, 3.57			5.5		
Try^{30}	8.03 (−4.4)	4.44	3.09–2.91	6.88 (t), 6.97 (d), 7.24 (d), 7.49 (d), 7.15 (s), NH = 10.80	7.5	4.50–9.50	g^+g^- (20%) tg^- (63%) tg^+ (17%)
Met^{31}	8.06 (−5.2)	4.15	1.83–1.74	$\gamma \sim 2.28$, S–CH_3 = 1.93[e]	7.5	4.50–9.50	g^+g^- (20%) tg^- (63%) tg^+ (17%)
Asp^{32}	7.94 (−3.4)	4.32	2.37–2.30		7	7.00–7.00	g^+g^- (20%) tg^- (40%) tg^+ (40%)
Phe^{33}	7.88 (−5.0)	4.23	3.01–2.76	~7.15	7	4.50–9.50	g^+g^- (20%) tg^- (63%) tg^+ (17%)

[a] Chemical shifts are given in ppm (±0.005) from Me_4Si used as internal reference. [b] Coupling constants are given in Hz (±0.25). [c] NH temperature dependencies (ppm/°C × 10^3) are given in parentheses. [d] Rotamer populations are calculated from Pachler. [e] These values may be reversed.

observed for the other amides.

(C) Spectra of NS CCK$_8$ and NS CCK$_7$ in Acidic Conditions. The comparison of the NMR parameters for NS CCK$_8$ in acidic (Table IIIS) and neutral conditions (Table III) showed an important deshielding of Asp^{26} protons at low pH, accounting for change in the ionization state of the peptide. Strikingly, the same phenomenon did not appear for Asp^{32} signals, which were slightly more shielded in acidic than in neutral medium. It can be noted that very weak variations in the chemical shifts were observed for the other residues. The most important features were the degeneracy of the Gly Hα signals and the various NH temperature coefficients, which corresponded to solvent-exposed protons. These two latter results were also observed for NS CCK$_7$ in acidic conditions (Table IIIS).

Fluorescence Studies

NS CCK$_7$ and NS CCK$_8$. Information on the conformations of the unsulfated forms of CCK$_7$ and CCK$_8$ can be deduced from the analysis of fluorescence quantum yields and of the energy transfer between the chromophores. Fluorescence parameters determined for both peptides being very similar, results concerning these compounds will be presented simultaneously.

(A) Fluorescence Spectra and Quantum Yields. For each peptide the total emission quantum yield was determined for excitation at 280 nm where both Tyr and Trp residues absorb and 295 nm where Trp is selectively excited. The spectral parameters of the emission spectra excited at 295 nm (λ_{max} = 350 nm, $\Delta\lambda$ = 63 nm) indicate that the Trp residues of these peptides are exposed to the solvent. This result is corroborated by external quenching experiments, which have shown a large accessibility of the emitting residue to acrylamide.

When excited at 280 nm, the peptide emission is composite with only a small contribution from Tyr (Figure 5). The respective contributions of tyrosine and tryptophan to the total quantum yield (ϕ = 0.073) may be evaluated after decomposition of the 280-nm excited emission spectrum into its two components. If one assumes, as in previous studies (Fournié-Zaluski et al., 1985), that the fluorescence emitted at 380 nm originates from Trp only, the difference between the 280- and 295-nm excited fluorescence spectra normalized

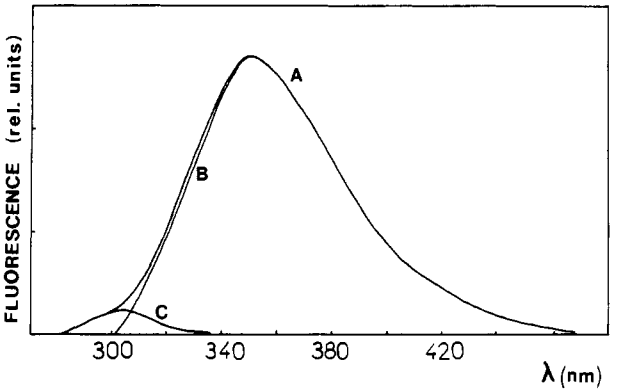

FIGURE 5: Fluorescence spectra of NS CCK$_7$ or NS CCK$_8$ obtained upon excitation at 280 (curve A) and 295 nm (curve B), normalized at 380 nm, and difference between spectra A and B (curve C).

at 380 nm should give the emission spectrum of tyrosine.

For CCK$_7$ and CCK$_8$, the fraction yields are

$$\phi_p^{280}(\text{Trp}) = \phi_p^{280}(S_B/S_A) = 0.070$$

$$\phi_p^{280}(\text{Tyr}) = \phi_p^{280}(S_c/S_A) = 0.003$$

where S denotes the area under the curve indicated in subscript.

The quantum yield of each residue is corrected from its fractional absorption f:

$$\phi_{Trp} = \phi_p^{280}(\text{Trp})/f(\text{Trp}) \qquad \phi_{Tyr} = \phi_p^{280}(\text{Tyr})/f(\text{Tyr})$$

with $f_{Trp} = \epsilon(\text{Trp})/\epsilon(\text{Trp}) + \epsilon(\text{Tyr})$.

The peptides NS CCK$_7$ and NS CCK$_8$ are characterized by very weak tyrosine quantum yields (ϕ = 0.015). Further analysis of this quantum yield requires the determination of the Tyr → Trp transfer efficiency, which could account for the observed quenching.

(B) Energy-Transfer Measurement. The transfer efficiency E between Tyr and Trp in NS CCK$_7$ and NS CCK$_8$ was determined from the measurement of the acceptor quantum yield in the presence (λ_{exc} = 280 nm) or in the absence of the donor (λ_{exc} = 295 nm, where Tyr no longer absorbs). For both peptides, E was found equal to 0.10 ± 0.05 (Table IV). The distance between the donor and the acceptor was calculated

Table IV: Fluorescence Quantum Yields of Tyrosine and Average Intramolecular Distances between Tyrosine and Tryptophan Chromophores in Sulfated and Unsulfated Forms of CCK_7 and CCK_8

	pH	ϕ_{Tyr}	$E_{Tyr-Trp}$	R (Å)
NS CCK_7	7	0.015	0.10	15.0 ± 0.5
or NS CCK_8	4	0.025	0.50	10.0 ± 0.5
CCK_7 or CCK_8	7	0.035	0.60	15.0 ± 0.5

from eq 1 under the assumption of random donor–acceptor orientation ($\kappa^2 = {}^2/_3$) in both peptides. The choice for this κ^2 value, which implies a free rotation around the peptidic backbone for the phenol and indole rings, is supported by the fluorescence (external quencher studies) and the NMR results. Moreover, a value of $\kappa^2 = {}^2/_3$ is very appropriate in the case of phenol and indole rings, since indole is characterized by two linear transition moments and phenol by a planar or partially planar moment. These features minimize the case where κ^2 is very small.

Under these conditions, the R_0 value was calculated to be 10 Å from data of Eisinger et al. (1969) with $n = 1.33$ and assuming a small value of $\phi_D = 0.02$ (eq 2). The experimental results correspond to a distance of $R = 15 \pm 1$ Å (Table IV).

After determination of the transfer efficiency, the Tyr fluorescence quantum yield in absence of transfer process can be evaluated from the following relationship, which is deduced from the relation of Eisinger et al. (1969):

$$\phi_{Tyr} = \phi_p^{280}(Tyr)/f_{Tyr}(1 - E)$$

This Tyr quantum yield is very low for NS CCK_7 and NS CCK_8 ($\phi = 0.017$), which indicates that tyrosine fluorescence is strongly quenched. This quenching could be due to the interaction of Tyr with the aspartate residue at position 32 or other quenching groups, which supposes a particular folding of the molecule.

In order to test the effect of carboxylate protonation on the Tyr quantum yield, the peptide conformation was studied at pH 4. At this pH, Tyr fluorescence is enhanced ($\phi = 0.025$), and the average distance between the two chromophores is descreased ($R = 10$ Å).

CCK_7 and CCK_8. This study required the determination of the critical distance R_0 for the donor acceptor Tyr-(SO_3H)–Trp pair and the evaluation of the spectral overlap integral between the fluorescence spectrum of the donor Tyr(SO_3H) and the absorption spectrum of the acceptor Trp. The computed value of R_0 was 16 Å.

The experimental determination of intramolecular distance between this donor–acceptor pair was complicated by the fact that both the molar extinction coefficient of Tyr(SO_3H) is low ($\epsilon_{max} = 210$ M^{-1} cm^{-1}) and its absorption spectrum is blue-shifted with respect to that of tyrosine itself.

In order to determine the transfer efficiency from the donor fluorescence quenching, we have compared the donor fluorescence intensity of CCK_7 and CCK_8 with the fluorescence of a mixture of peptidic fragments containing either the donor or the acceptor groups. Therefore, a mixture of Tyr-(SO_3H)-Met-Gly and Gly-Trp-Met-Asp-Phe-NH_2 was used for CCK_7 and a mixture of Asp-Tyr(SO_3H)-Met-Gly and Gly-Trp-Met-Asp-Phe-NH_2 for CCK_8.

The fluorescence intensity at 288 nm [maximum of the fluorescence spectrum of Tyr(SO_3H)] was decreased for CCK_7 and CCK_8 solutions as compared to solutions of identical absorbance containing the mixture of peptidic fragments, where no energy transfer can occur. Assuming that the quenching is only due to the transfer process, the efficiency E was 0.60 ± 0.05 (Table IV) for the two peptides, which leads to a distance between the Tyr(SO_3H) and Trp chromophores of 15 ± 0.5 Å.

Conformational Calculations. In order to mimic the behavior of the sulfated and unsulfated CCK_8 in water at neutral pH, the N-terminal nitrogen is considered as positively charged (NH_3^+) and the side chains of Asp^{26} and Asp^{32} as negatively charged (COO^-). The sulfate moiety of the Tyr^{27} residue in CCK_8 is taken as SO_3^-, and its electronic charges are calculated from the quantum mechanical MINDO method applied to the C_6H_5–SO_3^- model compound. The detailed discussion of the samples of conformations obtained will be published elsewhere (B. Maigret et al., unpublished results), and only comparison with experimental data are given here.

(A) The most relevant features coming from the Metropolis sampling procedure concern average properties. We have obtained, during the calculations, data that can be easily compared to experimental results obtained by NMR and fluorescence-transfer measurements. With the usual Bystrov–Karplus relation, the coupling constants $^3J_{NH-H\alpha}$ may be calculated from the ϕ angles determined for each residue from the conformational sampling. The comparison with the experimental values for CCK_8 and NS CCK_8 is reported in Table IVS. A relatively good accordance is observed between computed and experimental data for the unsulfated peptide, while larger differences are obtained for CCK_8. A comparison may be also carried out between the chromophore mean distance calculated from fluorescence-transfer measurement and from the samples of conformations (Table IVS). A Tyr–Trp distance of 15 Å, analogous to that obtained by fluorescence experiments, is calculated for CCK_8 and NS CCK_8. Curiously, the mean value is not influenced by the presence or the absence of the sulfate group. On the contrary, the calculated Tyr–Phe distance is significantly smaller in CCK_8 (11 Å) than in NS CCK_8 (13 Å).

Another parameter that may be significant, as far as the statistical representation of the molecules is concerted, is the N–C end to end distance. It appears that the simulated samples mainly contain two families of conformers, characterized by average distances of 8–9 and 15–16 Å, respectively. The most stable conformers are found in the latter group.

We can also calculate the mean accessibility of each residue to the solvent and the ability to form hydrogen-bonded structures. Regarding accessibility, one of the most relevant results indicates that the glycine residue is completely buried, especially its NH group, and that the Trp side chain is largely accessible.

(B) The most stable conformers that can be found in the samples of conformations resulting from the present Metropolis calculations on CCK_8 are given in Table VS and schematized in Figure 6. All of them present for Asp and Tyr residues the ϕ and ψ dihedral angle values localized in the β-region of the Ramachandran energy map and various backbone foldings.

Indeed, several hydrogen-bonded structures appear at the present level of calculation, especially those associated with β-turns in both the N- and C-terminal parts of the molecules: CO of Tyr^{27} bonded to NH of Trp^{30} (conformers 2 and 3) and CO of Gly bonded to NH of Asp^{32} (conformer 1). γ-Turns can also be found centered around the Gly residue (conformer 1), the Met^{28} residue (conformer 5), the Tyr^{27} residue (conformer 3) and Phe^{33} (conformer 3).

The most favored conformation (conformer 2) presents a β-sheet structure at the N-terminus followed by a small distorted helical moiety at the C-terminus ($\beta\beta$turn$\beta\beta\alpha\alpha$). The conformation proposed from experimental studies for CCK_7 (Durieux et al., 1983), and stabilized by a γ-turn followed by

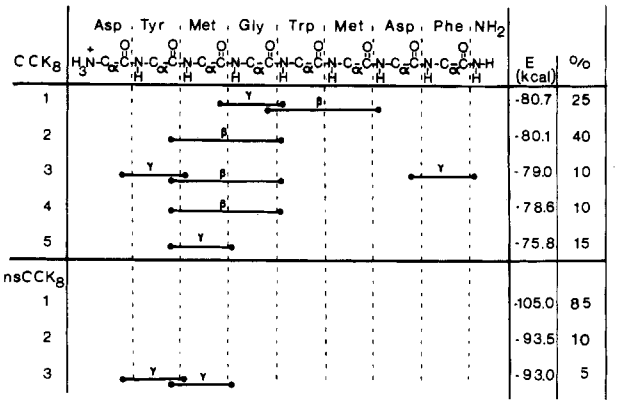

FIGURE 6: Schematic representation of the various turns observed in the most stable conformations of CCK_8 and NS CCK_8 obtained by Metropolis calculations.

a β-turn in the Met^{31}-Asp^{32} sequence is found as the second most probable conformer in these calculations (conformer 1). Most of the conformational constraints described here imply that the residues Met^{28}-Gly^{29}-Trp^{30} appear to be crucial for the flexibility of the molecule. The SO_3^- group is often included in interresidue interactions with the N atom of the Trp side chain (conformer 1), with the NH_2 C-terminal (conformer 2), or with the NH_3^+ (conformer 3).

For the unsulfated derivatives (Table VS, Figure 6), less organized structures are observed. The only H-bonded model found contains γ-turns involving Tyr^{27} and Met^{28} residues in a conformation (conformer 3) that is destabilized by 12 kcal/mol as compared to the most stable one.

Discussion

In this study we used ^1H NMR spectroscopy and fluorescence-transfer measurements as complementary methods to determine the conformational behavior in solution of CCK_7 and CCK_8 in their sulfated and unsulfated forms. Both experimental methods demonstrate that in physiological pH conditions CCK_7 and CCK_8 exist preferentially in folded conformations in both aqueous solution and more lipophilic medium. Indeed, in water the titration of CCK_7 and CCK_8 functional groups induced chemical shift variations on the protons of almost all amino acids (Figure 3). These results, previously reported for CCK_7 and NS CCK_7 (Durieux et al., 1983), reflect through-space effects that are indicative of backbone folding (Bundi & Wuthrich, 1977), a finding corroborated by the ionization constants of the β-Asp^{32} carboxylate group in these peptides. Indeed, as compared to the pK_a (3.65) of the β-carboxylate in aspartic acid, the increased values for Asp^{32} carboxylate groups in CCK_7 ($pK_a \sim 3.85$) and especially in CCK_8 ($pK_a \sim 4.50$) are indicative of their involvement in a hydrophobic surrounding. This feature is reminiscent of the pK_a increase of Asp from 3.65 in CCK_4 to 4.10 in CCK_6 associated with an increased folding tendency for the longer compound (Fourniê-Zaluski et al., 1985a). Interestingly, the pK_a (3.70) of Asp^{26} in CCK_8 shows that this N-terminal amino acid is solvent-exposed. The occurrence of folded conformations in the studied peptides is reinforced by the weak temperature dependency observed for some amide protons both in aqueous medium (pH 6) and in Me_2SO-d_6. Indeed, small amide coefficients ($<-3 \times 10^{-3}$ ppm/°C) are often considered as an indication that the NH proton is involved in hydrogen bonding or is at least buried from the solvent. As a general feature, looking at this parameter as a reflection of the folding tendency of a peptide, it is interesting to note that CCK_8 is more constrained than CCK_7 and that the latter seems to be more structured in water than in Me_2SO-d_6. Indeed for CCK_8 (Tables I and II), three amide groups (Asp^{32}, Gly, and Phe) present weak slopes in the two solvent conditions. Contrastingly, for CCK_7 only Asp^{32} NH shows a small temperature coefficient in Me_2SO-d_6 (Table III), whereas in aqueous medium, Asp^{32}, Trp, and Phe NH's may be considered as buried protons (Table I).

The implication of Asp^{32} NH in a H-bonded structure was previously observed in CCK_5 and CCK_6 (Fourniê-Zaluski et al., 1985a), suggesting that the presence of a β-turn including the four residues Gly-Trp-Met-Asp with Trp and Met at the corners of the chain reversal is likely to correspond to a common conformation characteristic in CCK peptides.

Furthermore, a second folding tendency on the C-terminal part of CCK_7 and CCK_8 seems to occur around the Phe residue, as suggested by the relatively solvent-buried position of the Phe NH. This indicates as proposed for the small analogues CCK_4, CCK_5, and CCK_6 (Fourniê-Zaluski et al., 1985a) the occurrence of a possible γ-turn including the three last amino acids Met-Asp-Phe.

The most significant difference between the conformational behavior of CCK_7 and CCK_8 is related to the flexibility of the N-terminal part of these peptides. In the octapeptide, the low-temperature coefficient of Gly NH (Tables I and II) is indicative of an additional folding that does not appear in CCK_7. A β turn including the four residues Asp-Tyr-(SO_3H)-Met-Gly or a γ-turn including Tyr(SO_3H)-Met-Gly may account for this feature. In addition, the Trp NH behaves also as a buried proton in Me_2SO-d_6 solution of CCK_8, suggesting that the N-terminal part of the peptide exists in equilibrium between various folded structures. In contrast, the N-terminal sequence of CCK_7 appears to be more flexible, although the intermediate value of the Trp-NH temperature coefficient (Table III) could indicate the existence of a γ-turn including the sequence Met-Gly-Trp.

Another interesting result, obtained from NMR data, concerns the difference between the sulfated and the unsulfated peptides. In both the heptapeptide and the octapeptide, the presence of the sulfated group increases the folding tendency. However, strikingly, the sulfate-induced constraint does not implicate the same part of the sequence in CCK_7 and CCK_8, since no organization of the N-terminus occurred in the unsulfated form of CCK_7 (Durieux et al., 1983), while for NS CCK_8 (Table II) an unfolding of the C-terminal sequence is clearly observed.

The analysis of the fluorescence parameters confirm the CCK folding tendency proposed from NMR data for the four peptides in physiological conditions. Indeed, at pH 7 the quantum yield of tyrosine is very low suggesting the occurrence of a quenching process with the tyrosine residue hydrogen bonded to a carbonyl group of the peptide backbone or to the carboxylate side chain of Asp^{32}. Whatever the origin of this feature, the proximity between the tyrosine and the quenching group supposes the occurrence of folded conformations. This result is reinforced by the large mean distance (R = 15 Å) obtained between the chromophores Tyr and Trp by fluorescence-transfer measurement. This value is consistent with a folding of the backbone pushing the two aromatic chromophores far from each other (Figure 7).

When the experiment is performed at pH 4, an increase in the quantum yield of Tyr and a concomitant decrease in the donor–acceptor distance (R = 10 Å) are observed. This may be interpreted as an enhancement in the amount of extended structures by a lowering of the pH. This assumption is consistent with the fact that addition of 6 M guanidinium chloride,

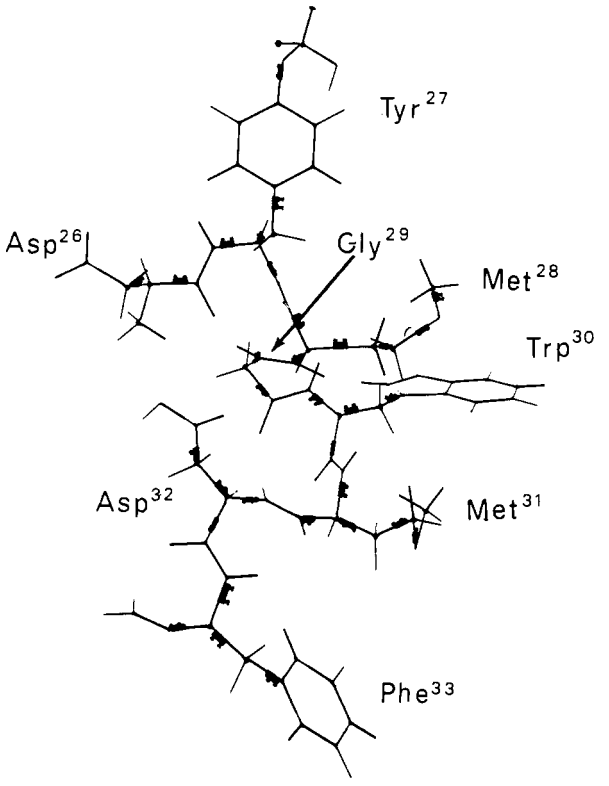

FIGURE 7: Dreiding model of the preferential folded conformation of CCK_8 obtained from ^1H NMR and fluorescence data.

which induces a denaturation of the molecules, is accompanied by an increase of the tyrosine quantum yield and a modification of the distance between phenol and indole rings (R = 10 Å), identical with that obtained through a pH decrease. Taken together, these results suggest that an ionic interaction between the free ammonium group and the Asp^{32} carboxylate is partially responsible for the folding tendency of CCK peptides. Likewise, the distance of 10 Å, determined from the Tyr → Trp transfer measurement in Boc-NS-CCK_7 (Schiller et al., 1978), is consistent with an extended conformation, which is probably due to the protection of the amino group cancelling the possibility of an ionic interaction in this peptide.

Similar conclusions can be deduced from the NMR study of NS CCK_7 and NS CCK_8. In Me_2SO-d_6, the protonated peptides show large temperature coefficients for all the NH groups, which could be related to extended structures (Table IIIS). The same feature was also known in aqueous medium for NS CCK_7. Indeed, at pH 2, all NH's have temperature coefficients higher than -5.0×10^{-3} ppm/°C, while buried NH's were observed in neutral conditions (-3.6×10^{-3} ppm/°C for Asp NH and -4.5×10^{-3} ppm/°C for Phe and Trp NH's).

Finally, it is important to note that, even though all the experimental data seem to indicate the occurrence of constraints in the backbone of the studied peptides in neutral conditions, it is clear by looking at several parameters like $^3J_{NH-\alpha}$ coupling constants, which are all in the same range, that an equilibrium between various folded conformations exists in solution.

Therefore, the experimental data have to be compared to those obtained by Metropolis calculations for CCK_8 and NS CCK_8. In the case of CCK_8, an important degree of spatial organization in the N-terminal moiety of the peptide was shown by the two methods: both β- and γ-turns including Trp and Gly NH are proposed as stabilized structures by Metropolis calculations, whereas the NMR data are consistent with an equilibrium between these folded conformations. However, the folding tendency of the C-terminal part of the peptide, observed by NMR spectroscopy, is not so clearly shown by calculations. Only one stabilized structure (conformer 2) contains a β-turn at the level of the Gly-Trp-Met-Asp sequence. In all the other structures, these residues are not in a random-coil situation but organized in such a way that no dominant conformational type appears, apart from an equilibrium between α and β structures (Table VS). This fact may be related to the absence, in the stable conformations computed for molecules in vacuo, of the ionic interactions between Asp^{26} NH_3^+ and Asp^{32} COO^-, which seem to play an important role in the stabilization of the folded structures in solution. This assumption is corroborated by some characteristics of the calculated conformations that are not observed experimentally. Thus, in the three most stable calculated conformations, the sulfate group interacts strongly with the NH of the indole ring or the $CONH_2$ group of Phe. In aqueous solution, or in Me_2SO, the sulfate group is most probably solvated and cannot participate in such interresidue interactions. The overestimation of this effect in the calculations may explain the differences observed between the experimental and calculated coupling constants $^3J_{NH-H\alpha}$ in the studied peptides.

CONCLUSIONS

All the data reported in this study indicate a close analogy between the conformational behavior of CCK_7 and CCK_8. In neutral conditions, a folding of both peptide backbones was observed in lipophilic solvent and, more interestingly, in aqueous medium. The preferential conformation adopted by the C-terminal part of CCK_7 and CCK_8 in both solvents was identical with that encountered in smaller fragments. Taking into account that all the endogenous CCK-related peptides found in the brain contain the C-terminal sequence Trp-Met-Asp-Phe-NH_2, which is the minimal fragment exhibiting a high affinity for brain receptors, it might be suggested that this common conformational behavior is related to a structural characteristic of CCK agonists. Furthermore, the β- or γ-turns, occurring in the N-terminal part of CCK_7 and CCK_8, suggest that the flexible glycine residue may play a role of a hinge between the folded N- and C-terminal parts of these peptides. The importance of this amino acid in both the conformational and biological properties of CCK analogues was recently demonstrated by the complete loss of potency following its replacement by the sterically constrained Aib residue in CCK_8 (Fournié-Zaluski et al., 1985b). In acidic medium, protonated CCK_7 and CCK_8 exist preferentially as extended structures. This result seems to indicate that an ionic interaction involving the positively charged amino group of Asp^{26} plays a role in the folding tendency of these peptides. However, the Asp^{26} ammonium group is not essential, since its protection by various agents does not affect the biological activity.

Nevertheless, the conformational change observed by protonation indicates that extended and folded structures are energetically closely related. Therefore, according to the results of calculations that emphasize the stability of folded forms devoid of internal ionic interactions, one can assume that, in the lipidic surrounding of the receptor or during the receptor binding process, CCK_8 or biologically active related peptides might adopt folded conformations similar to those described in solution for free CCK_7 and CCK_8. This hypothesis is now being tested by conformational studies of CCK peptides in phospholipids and through the synthesis of cyclic peptides mimicking the various tendencies evidenced in this work.

ACKNOWLEDGMENTS

We are grateful to Dr. M. Delepierre for assistance with the NMR experiments and to E. Fellion for the peptide synthesis. We thank Dr. A. Beaumont for stylistic revisions and A. Bouju for typing the manuscript. Calculations were performed in the Centre de Calcul Vectoriel pour la Recherche (CCVR), Ecole Polytechnique, 91128 Palaiseau Cedex, France.

SUPPLEMENTARY MATERIAL AVAILABLE

Five tables showing experimental data of CCK_7, NS CCK_7, CCK_8, and NS CCK_8 (5 pages). Ordering information is given on any current masthead page.

Registry No. CCK octapeptide, 25126-32-3; CCK heptapeptide, 20988-64-1; nonsulfated CCK octapeptide, 25679-24-7; nonsulfated CCK heptapeptide, 47910-79-2.

REFERENCES

Bodanszky, M., Natarajan, S., Hahne, W., & Gardner, J. D. (1977) *J. Med. Chem. 20*, 1047–1050.

Bundi, A., & Wuthrich, K. (1977) *FEBS Lett. 77*, 11–14.

Chen, R. F. (1967) *Anal. Lett. 1*, 35–42.

Crawley, J. N. (1985) *Ann. N.Y. Acad. Sci. 448*, 283–292.

Cung, M. T., & Marraud, M. (1982) *Biopolymers 21*, 953–967.

Demarco, A., Llinas, M., & Wuthrich, K. (1978) *Biopolymers 17*, 617–636.

Dockray, G. J. (1976) *Nature (London) 264*, 568–570.

Dodd, J., & Kelly, J. S. (1981) *Brain Res. 205*, 337–350.

Durieux, C., Belleney, J., Lallemand, J. Y., Roques, B. P., & Fournié-Zaluski, M. C. (1983) *Biochem. Biophys. Res. Commun. 114*, 705–712.

Elsinger, J., Ferrer, B., & Lamola, A. A. (1969) *Biochemistry 8*, 3908–3915.

Förster, T. (1948) *Ann. Phys. (Leipzig) 2*, 55–75.

Fournié-Zaluski, M. C., Gacel, G., Maigret, B., Prêmilat, S., & Roques, B. P. (1981) *Mol. Pharmacol. 20*, 484–491.

Fournié-Zaluski, M. C., Durieux, C., Lux, B., Belleney, J., Pham, P., Gérard, D., & Roques, B. P. (1985a) *Biopolymers 24*, 1663–1681.

Fournié-Zaluski, M. C., Belleney, J., Durieux, C., Gacel, G., Roques, B. P., Bégué, D., Menant, I., Lux, B., & Gérard, D. (1985b) *Ann. N.Y. Acad. Sci. 448*, 598–600.

Gardner, J. D., Coulon, T. P., Klaeveman, H. L., Adanes, T. D., & Ondetti, M. A. (1975) *J. Clin. Invest. 56*, 366–375.

Gaudreau, P., Quirion, R., St. Pierre, S., & Pert, C. B. (1983) *Eur. J. Pharmacol. 87*, 173–174.

Guyon-Gruaz, A., Demonte, J. P., Fournié-Zaluski, M. C., Englert, A., & Roques, B. P. (1981) *Biochemistry 20*, 6677–6683.

Hays, S. E., Beinfeld, M. C., Jensen, R. T., Goodwin, F. K., & Paul, S. M. (1980) *Neuropeptides 1*, 53–62.

Hore, P. J. (1983) *J. Magn. Reson. 55*, 283–300.

Innis, R. B., & Snyder, S. H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6917–6921.

Ivy, A. C., & Oldberg, E. (1928) *Am. J. Physiol. 86*, 599–613.

Jeener, J. (1971) *Ampère International Summer School II*, Basko Pelje, Yugoslavia.

Koizuka, I., Watari, H., Yanaihara, N., Nishina, Y., Shiga, K., & Nagayawa, K. (1984) *Biomedical Res. 5*(Suppl.), 161–168.

Maigret, B., Prêmilat, S., Fournié-Zaluski, M. C., & Roques, B. P. (1981) *Biochem. Biophys. Res. Commun. 99*, 267–274.

Marion, D., Garbay-Jaureguiberry, C., & Roques, B. P. (1983) *J. Magn. Reson. 53*, 199–212.

Mutt, V., & Jorpes, J. E. (1971) *Biochem. J. 125*, 57–58.

Ondetti, M. A., Rubin, B., Engel, S. L., Pluscec, J., & Sheeham, J. T. (1970) *Am. J. Dig. Dis. 15*, 149–156.

Pachler, K. G. R. (1964) *Spectrochim. Acta 20*, 581–587.

Piantini, U., Sorensen, O. W., & Ernst, R. R. (1982) *J. Am. Chem. Soc. 104*, 6800–6801.

Pinget, M., Strauss, E., & Yalow, R. S. (1979) *Life Sci. 25*, 339–342.

Prêmilat, S., & Maigret, B. (1977) *J. Chem. Phys. 66*, 3418–3425.

Rehfeld, J. F. (1978) *J. Biol. Chem. 253*, 4022–4030.

Roques, B. P., Garbay-Jaureguiberry, C., Oberlin, R., Anteunis, M., & Lala, A. K. (1976) *Nature (London) 262*, 778–779.

Sakomoto, C., Goldfine, I. D., & Williams, J. A. (1984a) *Biochem. Biophys. Res. Commun. 118*, 623–628.

Sakomoto, C., Williams, J. A., & Goldfine, I. D. (1984b) *Biochem. Biophys. Res. Commun. 124*, 497–502.

Schiller, P. W., Natarajan, S., & Bodanszky, M. (1978) *Int. J. Pept. Protein Res. 12*, 139–142.

Steigerwalt, R. W., & Williams, J. A. (1984) *Regul. Pept. 8*, 51–59.

Vanderhaeghen, J. J., Signeau, J. C., & Gepts, W. (1975) *Nature (London) 257*, 604–605.

Zajac, J. M., & Roques, B. P. (1985) *J. Neurochem. 44*, 1605–1614.

Zhou, Z. Z., Eng. J., Pan, Y. C. E., Chang, M., Hulmes, J. D., Raufman, J. P., & Yalow, R. S. (1985) *Peptides (N.Y.) 6*, 337–341.