Conformational Studies of Dansylated Enkephalins by Fluorescence Transfer Measurements, Proton Nuclear Magnetic Resonance Spectroscopy, and Theoretical Calculations†

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ABSTRACT: Dansylated on their C-terminal part, enkephalins retain all the biological properties of their parent compounds and would be useful fluorescent probes to study the enkephalin–receptor recognition process or interactions between these peptides and their degrading enzymes. Such explorations require in a first step a conformational analysis of these compounds in both aqueous and less polar solvents. This was performed by using three comparative methods: fluorescence energy transfer using the dansyl group (Dns) as an acceptor, ^{1}H NMR, and theoretical conformational analysis. The fluorescent enkephalins, Tyr^1-Gly^2-Gly^3-Phe^4-Met^5-NH-$(CH_2)_2$-NH-Dns (**1**), its D-Ala^2 derivative **2**, and a tetrapeptide analogue, **3**, lacking the Met^5 residue exhibit a large energy transfer from Tyr to Dns with a strong additional quenching of the tyrosine fluorescence in H_2O. Therefore, the distance r_1 between the two luminophores was computed from acceptor fluorescence. The folding tendency of the three compounds evidenced by short Tyr–Dns distances (r_1 = 12.4–14.9 Å) increases from H_2O to trifluoroethanol (TFE) with in the case of **2** a stacking interaction between Tyr and Dns in TFE. These features are supported in both solvents by selective shielding of the proton chemical shifts in **1** and **2** as compared to their precursors without the Dns ring. All these results agree with intramolecular distances computed for a statistical model of **1** in H_2O and a flexible β-turn model for **1** in TFE and **2** in both solvents. In all the solvents used, dansylated enkephalins remain very flexible and exhibit computed average distances between Tyr^1 and Phe^4 (10–11 Å) near those determined in biologically active Trp^4-Met^5-enkephalin analogues and in the highly potent morphine derivative 7α-[(*R*)-1-hydroxy-1-methyl-3-phenylpropyl]-6,14-*endo*-ethenotetrahydrooripavine [Schiller, P. W., & St. Hilaire, J. (1980) *J. Med. Chem. 23*, 290–294]. These features associated with a folding tendency of these fluorescent peptides allow their binding to μ receptors probably through a trans conformational process. The significant δ selectivity of **1** and **2** is in accordance with the lengthening of the peptide sequence whereas the loss of activity of **3** confirms the crucial role attributed to the simultaneous presence of a D^2-amino acid and a fifth residue for the biological activity [Fournié-Zaluski, M. C., Gacel, G., Maigret, B., Premilat, S., & Roques, B. P. (1981) *Mol. Pharmacol.* (in press)].

The addition of a dansyl group (Dns) on the C-terminal part of enkephalins provides fluorescent peptides such as Tyr-Gly-Gly-Phe-Met-NH-$(CH_2)_2$-NH-Dns (Met-E-C_2-Dns) (**1**) and Tyr-D-Ala-Gly-Phe-Met-NH-$(CH_2)_2$-NH-Dns (D-Ala^2-Met-E-C_2-Dns) (**2**) which retain all the biological properties of their parent compounds (Fournié-Zaluski et al., 1978). The peptide **1** has been used for fluorometric measurements of enkephalin-degrading aminopeptidase activity (Guyon et al., 1979) whereas visualization of opiate receptors in neuroblastoma cells was performed by using a rhodamine-labeled enkephalin (Hazum et al., 1979). Consequently, due to their high binding affinity to opiate receptors (Fournié-Zaluski et al., 1978) or their ability to recognize specific peptidases, fluorescent enkephalins could represent useful probes for the study of peptide–receptor or peptide–enzyme interactions by the various methods, already used in the case of cholinergic (Waksman et al., 1976; Heidmann & Changeux, 1979) or adrenergic receptors (Atlas & Levitski, 1977). Such experiments could possible in the near future since several groups (Simonds et al., 1980; Bidlack & Abood, 1980) have been able to solubilize opiate receptors in a state that reversibly binds opioid ligands whereas Schnebli et al. (1979) have purified an aminopeptidase involved in the specific cleavage of the Tyr–Gly bond of enkephalins.

Nevertheless, binding experiments with fluorescent peptides require first a study of their conformational properties in both aqueous and more hydrophobic solutions. The main question which arises with respect to the state of the enkephalin in solution is whether this peptide exists under a single preferred conformation or under an equilibrium population of conformers. In dimethyl-d_6 sulfoxide (Me_2SO-d_6) solution, arguments in favor both of a unique conformation (β turn) (Roques et al., 1976; Jones et al., 1976; Stimson et al., 1979) and of an equilibrium situation (Bleich et al., 1976; Spirtes et al., 1978; Higashijima et al., 1979) have been presented. It should be emphasized that in H_2O a unique conformation, similar to the one proposed for enkephalin in Me_2SO-d_6, has not been detected for these small peptides (Jones et al., 1977). In order to examine whether the experimentally determined properties agree better with one of the two possible situations, we have studied the conformational states of **1** and **2** and of a shorter and less active analogue, Tyr-Gly-Gly-Phe-NH-$(CH_2)_2$-NH-Dns (**3**), in Me_2SO, trifluoroethanol (TFE), and H_2O solutions by three comparative methods: fluorescence energy transfer measurements, ^{1}H NMR experiments, and theoretical conformational analysis. Indeed, in these fluorescent compounds, the dansyl group has potentially a large degree of freedom. Therefore, the dansyl residue behaves as a very useful conformational probe for fluorescence and ^{1}H NMR experiments, taking into account its fluorescence property and its strong magnetic anisotropy. The experimental results were compared with those derived from either a statistical model (1) or a

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flexible β_I-turn 2 → 5 model (2). In all the solvents used, dansylated enkephalins remain very flexible and exhibit computed average distances between Tyr[1] and Phe[4] (10–11 Å) near those determined in biologically active Trp[4]-Met[5]-enkephalin analogues and in the highly potent morphine derivative 7α-[(*R*)-1-hydroxy-1-methyl-3-phenylpropyl]-6,14-*endo*-ethenotetrahydrooripavine (PEO) (Schiller & St. Hilaire, 1980). Therefore, despite the large steric hindrance of the dansyl ring, the persistence of a large degree of freedom for this C-terminal fluorescent moiety and the folding tendency of these dansylated peptides allow their interaction with both opiate receptors and degrading aminopeptidases.

Materials and Methods

(1) Chemical Methods. Met-enkephalin (Met-E) is from Bachem (Switzerland); Met-enkephalinamide, D-Ala[2]-Met-enkephalinamide, and dansylated enkephalins were prepared as described (Fournié-Zaluski et al., 1977) and purified by chromatography on Bio-Gel P-2 (3 × 100 cm) using a solution of 0.1 M acetic acid as solvent. The fractions containing the pure products in their acetate forms were pooled and lyophilized. The purity was checked by thin-layer chromatography on silica gel glass plates with 4:1:1 BuOH:AcOH:H_2O as an eluent. The spots were detected by both UV fluorescence and ninhydrin spray. For UV and fluorescence measurements, peptide solutions (1.5×10^{-5} M) were prepared from stock solutions in 2% ethanol (Merck-Uvasol) diluted with the desired solvent [phosphate buffer, pH 7, and trifluoroethanol (Merck-Uvasol)]. Solutions for ^{1}H NMR spectroscopy were prepared by dissolution of the lyophilized enkephalins in pure Me_2SO-d_6 (Merck) or in 5% Me_2SO-d_6 final concentration of Me_2SO-d_6 diluted with D_2O (100% deuterated) or TFE-d_3 (Merck)].

UV spectra were recorded on an Unicam SP 8-100 spectrophotometer and fluorescence spectra on a Perkin-Elmer MPF 44A spectrofluorometer equipped with a $DCSU_2$ corrected spectra unit and fitted with a thermostated cell holder. UV and fluorescence measurements were performed at 25 ± 1 °C through temperature regulation by an external circulating water bath.

NMR studies were performed at 25 ± 1 °C on a Bruker WH 270-MHz spectrometer operating in the Fourier-transform (FT) mode and equipped with a BST 100/700 temperature controller. Chemical shifts (in parts per million ± 0.01 ppm) were measured from hexamethyldisiloxane (HMDS) as an internal reference. Coupling constants are given in hertz (±0.2 Hz). Assignments were performed by homodecoupling experiments and temperature variation studies as described (Roques et al., 1976). Analysis of the backbone conformation and determination of rotamer populations were performed according to Pachler (1964). The concentrations of the peptide solutions are 10^{-3} M in Me_2SO-d_6 and 10^{-4} M in both TFE-d_3 and D_2O.

(2) Energy-Transfer Measurements. As predicted by Förster's theory (Förster, 1948), the dependence of the efficiency, E, of singlet energy transfer between two luminophores on their relative distance, r_1, is

$$E = R_0^6/(R_0^6 + r_1^6) \quad (1)$$

R_0 is the Förster critical distance for which the energy transfer rate is equal to the rate of all other deactivation processes of the donor and

$$R_0 = 9.79 \times 10^3 (Q'_{D_0} \kappa^2 n^{-4} J_{AD})^{1/6} \text{ Å} \quad (2)$$

with

$$J_{AD} = \int_0^\infty \frac{I_D(\bar{\nu})}{\int_0^\infty I_D(\bar{\nu})\, d\bar{\nu}} \epsilon_A(\bar{\nu}) \bar{\nu}^{-4}\, d\bar{\nu} \text{ cm}^6 \text{ M}^{-1} \quad (3)$$

where J_{AD} = the overlap integral, Q'_{D_0} = the donor fluorescence quantum yield in the absence of transfer, n = the refractive index of the solvent, κ^2 = the orientation factor of the two fluorophores ($\kappa^2 = {}^2/_3$ for a random orientation), $I_D(\bar{\nu})$ = the fluorescence intensity of the donor at $\bar{\nu}$, and $\epsilon_A(\bar{\nu})$ = the molar absorption coefficient of the acceptor at $\bar{\nu}$.

The energy transfer efficiency, E, allowing the determination of the distance r_1 through eq 1 has been obtained from the relative increase of acceptor fluorescence (Einsinger, 1969).

Q'_{D_0} (Tyr quantum yield obtained in Met-E) and Q_D (Tyr quantum yield in the presence of transfer occurring in dansylated enkephalins) were computed in H_2O or TFE by comparison with the quantum yield ($Q_{Tyr} = 0.14$) of a standard aqueous solution of L-Tyr (Schiller et al., 1977) by using

$$Q_X/Q_{Tyr} = (A_{Tyr}/A_X)[IF_X/(IF_{Tyr})](n^2/n_{H_2O}^2) \quad (4)$$

X corresponds to Met-E or dansylated enkephalins, IF_X and IF_{Tyr} are the areas of the fluorescence spectra, and A_X and A_{Tyr} are the absorbances of tyrosine in X and in L-Tyr, respectively. n and n_{H_2O} are the refractive indices of the solvent used and of water. Their square values are introduced in eq 4 to correct for the effect of the refractive index of fluorescence intensity. A value of $Q'_{D_0} = 0.030$, identical with that reported by Schiller et al. (1977), was obtained for Met-E in H_2O whereas a value of $Q'_{D_0} = 0.056$ was measured in TFE. These values were introduced in the relation 2 by using a value of $\kappa^2 = {}^2/_3$. This is justified by both the occurrence of two transition moments for the dansyl fluorophore (Haas et al., 1978) and the existence of a distribution of the orientations of two chromophores as evidenced by NMR and the theoretical calculations (see below). Therefore, $\kappa^2 n^{-4} = 0.200$ and 0.241 for phosphate buffer and for TFE solutions, respectively. Q'_{D_0}, the Tyr quantum yield in the absence of transfer for the dansylated compounds, was computed from the transfer efficiency E and Q_D by using

$$E = 1 - Q/Q'_{D_0} \quad (5)$$

On the other hand, according to Einsinger (1969), the transfer efficiency E was determined from the relative increase of acceptor fluorescence. For such a purpose, normalized absorption and fluorescence spectra of the dansylated compounds were compared to those of Met-E which contain only the donor moiety and Gly-Gly-Phe-Met-C_2-Dns (**4**) as a pure acceptor standard. The fractions of light absorbed by the tyrosine (f_{Tyr}) and dansyl (f_{Dns}) chromophores in the dansylated peptides are given by $f_{Dns}(\lambda) = \epsilon_{Dns}(\lambda)/[\epsilon_{Dns}(\lambda) + \epsilon_{Tyr}(\lambda)]$ and $f_{Tyr}(\lambda) = 1 - f_{Dns}(\lambda)$ where ϵ_{Dns} and ϵ_{Tyr} are the molar absorption coefficients of Gly-Gly-Phe-Met-E-C_2-Dns (acceptor) and Met-E (donor), respectively. The transfer efficiency from the relative increase of acceptor fluorescence, E, was obtained from the ratio $Q/Q_{Dns} = f_{Dns}(\lambda) + Ef_{Tyr}(\lambda)$ where Q and Q_{Dns} are respectively the quantum yields of the dansyl group at the same wavelength, λ, in a dansylated peptide and in **4**. E values were accurately determined from the fit between experimental data and computed theoretical curves Q/Q_{Dns} as a function of λ for different values of E (Figure 1).

Finally, the distance r_1 between the two luminophores was computed from eq 1 by using the values of E determined by the precedent method.

(3) Theoretical Calculations. The average values of the properties of interest are computed from a representative

Table I: Fluorescence Quantum Yields and Intramolecular Tyr-Dns Distances in Dansylated Enkephalins[a]

compd	solvent	E^c	Q_D	Q'_{D_0}	R_0 (Å)	r_1 (Å)[d]
1	H_2O[b]	0.50 ± 0.02	0.0084	0.0168	14.6	14.5 ± 0.4
	TFE	0.84 ± 0.03	0.0078	0.049	17.7	13.4 ± 0.2
2	H_2O	0.37 ± 0.03	0.0066	0.0105	12.5	13.7 ± 0.2
	TFE	0.77 ± 0.07	0.0087	0.038	16.9	13.9 ± 0.1
3	H_2O	0.37 ± 0.03	0.0048	0.00762	12.8	14.0 ± 0.3
	TFE	0.75 ± 0.05	0.0050	0.020	15.2	12.7 ± 0.3

[a] Peptide concentration = 6×10^{-6} M. [b] Phosphate buffer (0.1 M) at pH 7.0. [c] Mean of five determinations ± SEM. [d] Computed from E_A on the assumption of fixed separation of luminophores.

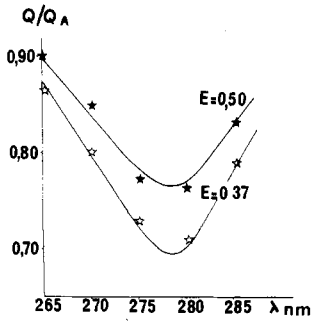

FIGURE 1: Computed theoretical curves Q/Q_{Dns} as a function of λ for different values of E and experimental values obtained for Met-E-C_2-Dns (**1**) (☆) and D-Ala^2-Met-E-C_2-Dns (**2**) (☆) in phosphate buffer, pH 7.0.

sample of conformations obtained from a Monte-Carlo method including long-range interactions as described previously (Premilat & Maigret, 1977; Leclerc et al., 1978). All the parameters for potential functions and geometries of amino acids are taken from Scheraga (1968) and used with a dielectric constant equal to 3.5, but a distinction is introduced between hydrophobic and hydrophilic residues (Leclerc et al., 1978). The geometrical parameters of the dansyl luminophore have been obtained from X-ray data on related compounds (O'Connel & Moslen, 1967); the charge distribution has been computed by an INDO quantum mechanical program. The potential functions for the residue -$(CH_2)_2$-NH-Dns have been optimized by comparison of experimental (Conrad & Brand, 1968) and computed transfer efficiencies in Trp-CO-$(CH_2)_5$-NH-Dns (J.-P. Demonte and A. Englert, unpublished experiments).

Two models have been considered: in model 1, the equilibrium population of molecular chains is being selected from all local low-energy states of the amino acids as described by Premilat & Maigret (1977); in model 2, the same method is used except for the torsion angles accessible to Gly^3 and Phe^4, ϕ_3, ψ_3, ϕ_4, ψ_4, which are restricted to the vicinity of a β_I turn (Venkatachalam, 1968). Model 2 corresponds to a flexible open β_I turn, stabilized by interaction with a water molecule, hydrogen bonded to CO of residue 2 and to NH of residue 5 separated by an appropriate distance. A reference for such a structure is a hydrated crystal of Tyr-Gly-Gly-Phe (Fournié-Zaluski et al., 1978). The energy of stabilization by the solvent, not represented explicitly, is assumed to be constant in the population of conformers, derived from the intramolecular energy surface. Both models are tentatively used for the discussion of experimental results whatever the solvent.

In the enkephalins considered here, the change of the distance between luminophores during the transfer is of the order of 1 Å, as $\Delta r = (D\tau_D^\circ)^{1/2}$, and the diffusion coefficient of chain ends relative to one another, D, and the lifetime of the donor luminophore, τ_D°, can be estimated to be respectively 10^{-7} cm^2 s^{-1} (Haas et al., 1978) and 1.8×10^{-9} s (Feitelson, 1969). In the case of a population of conformers, the experimentally

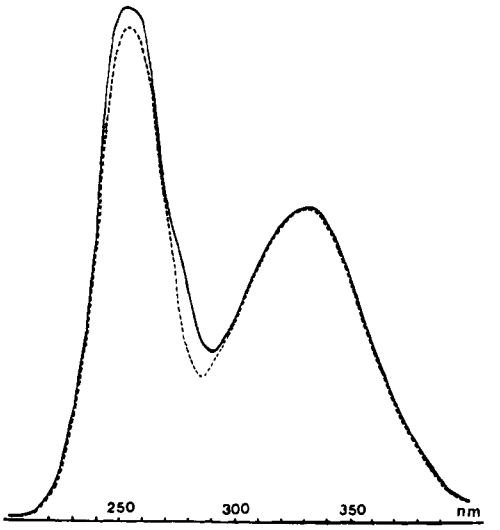

FIGURE 2: Fluorescence-corrected excitation spectrum of Gly-Gly-Phe-Met-C_2-Dns (**4**) (---) and Met-E-C_2-Dns (**1**) (—) in phosphate buffer (λ_{em} = 660 nm).

determined efficiency corresponds therefore to a static averaging regime.

The relations between the ^{1}H NMR coupling constants and the rotational angles are taken from Cung et al. (1974). The error on the computed averages of E, r_1, and J_{NH-CH_α} inherent to the statistical method used is estimated to be ±0.03, ±0.3, and ±0.5, respectively.

Results and Discussion

The well-known overlap between the tyrosine emission spectrum (λ_{max} = 303 nm) and the absorption spectrum of the dansyl group (λ_{max} = 330 nm) allowed energy transfer measurements in a variety of dansylated polypeptides. Such a transfer is clearly evidenced by a comparison of the corrected fluorescence excitation spectra (λ_{em} = 660 nm) of Met-E-C_2-Dns (**1**) and the peptide Gly-Gly-Phe-Met-C_2-Dns (**4**) which is devoid of the tyrosine residue (Figure 2).

Furthermore, the incorporation of the dansyl group at the end of a peptide sequence does not change its absorption properties as shown by the almost identical molar extinction coefficients measured at 330 nm for dansylethylamine and the dansylated peptides **1** and **4**. Likewise, the absorption spectrum of Met-E-C_2-Dns is almost identical with the addition of the absorption spectra of Met-E and dansylethylamine at 280 nm. The same features were already found in Trp^4-Met^5-E by Schiller et al. (1977).

The fluorescence properties of the dansylated enkephalins **1**, **2**, and **3** in phosphate buffer, pH 7, and TFE are reported in Table I. The value of Q'_{D_0} (fluorescence quantum yield of tyrosine in the absence of transfer) was computed from E and Q_D by using eq 5 for peptides **1**–**3**. These values are considerably lower than those of Q'_{D_0} (tyrosine quantum yield in the parent compound Met-E; Q'_{D_0} = 0.030 in H_2O and 0.056 in TFE). Such a quenching has already been observed by

Conrad & Brand (1968) and Schiller (1975), but the mechanism responsible for this phenomenon is not clear at this time. The distance r_1 has been calculated from relations 1 and 2 by using $J_{AD} = 3.29 \times 10^{-15}$ cm^6 M^{-1} and $\kappa^2 n^{-4} = 0.2$ in phosphate buffer and $J_{AD} = 2.94 \times 10^{-15}$ cm^6 M^{-1} and $\kappa^2 n^{-4} = 0.241$ in TFE. The distances r_1 in the three dansylated enkephalins are intermediate between a strongly folded form with a stacking of the dansyl ring upon the tyrosine one, $r_1 \sim 4.5$ Å, and a completely extended form, $r_1 \sim 24$ Å, as determined from molecular models. Moreover, the distance r_1 is slightly but significantly shorter in D-Ala^2-Met-E-C_2-Dns (**2**) than in **1** and **3**. This result could indicate a stronger folding tendency in **2** due to the replacement of Gly^2 by the more hydrophobic D-Ala^2 residue. This assumption seems to be reinforced by the blue shift (4 nm) of the fluorescence emission of the dansyl group in **2** ($\lambda_{max} = 550$ nm) as compared to those **1** and **3** ($\lambda_{max} = 554$ nm), indicating a more hydrophobic environment for the acceptor fluorophore (Schiller et al., 1978). All these features are in accordance with the results of NMR experiments and theoretical calculations (see below).

On the other hand, the lower value of the tyrosine quantum yield, Q'_{D_0}, in **3** is probably related to different backbone conformations in this dansylated tetrapeptide as compared to the pentapeptide ones. Indeed, the quenching of tyrosine fluorescence by peptide bonds is strictly related to the conformational state of the studied peptides (Edelhoch et al., 1968; Schiller & St. Hilaire, 1980). From this point of view, it is very interesting to remark that in solution Tyr-Gly-Gly-Phe, the parent compound of **3**, has the CO of Tyr^1, which could behave as the nearest quencher group, involved in a hydrogen bond with the NH of Phe ($1 \rightarrow 4$ β turn) (Fournié-Zaluski et al., 1978). Contrastingly, in Met-E and D-Ala^2-Met-E, which are the precursors of **1** and **2**, the CO groups of Tyr^1 remain free since the folding occurs around the Gly^3–Phe^4 bond ($2 \rightarrow 5$ β turn) (Roques et al., 1976). These results could indicate that a significant proportion of the stabilized conformers occurring in the parent compounds are still present in the fluorescent derivatives.

As expected, the fluorescence emission of the dansyl moiety is strongly enhanced with a concomitant large blue shift of its emission maximum ($\lambda_{max} = 536$ nm) when the dansylated peptides are dissolved in TFE. Moreover, it must be observed that, in this organic solvent, very similar transfer efficiencies are found from either the acceptor or the donor (not shown here) quantum yields. This finding suggests that the additional quenching of Tyr introduced by the presence of the dansyl ring is reduced in a less polar solvent.

One of the purposes of this work was to compare the results obtained from fluorescence transfer experiments with those deduced from NMR studies. Indeed, due to its strong diamagnetic anisotropy, the dansyl group can be used as a convenient conformational probe. Consequently, the NMR spectra of **1** and **2** were compared with those of their parent compounds Met-enkephalinamide (**5**) and D-Ala^2-Met-enkephalinamide (**6**) in three solvents, Me_2SO-d_6, D_2O, and TFE-d_3. The low solubility ($\sim 10^{-4}$ M) of the dansylated enkephalins in water and trifluoroethanol requires the use of the deuterated solvents D_2O and TFE-d_3 where the amide protons are exchanged. Therefore, in the first step, the NMR spectra were performed in Me_2SO-d_6 and the signals assigned by homodecoupling experiments. Aggregation between zwitterionic forms of small peptides could occur by intermolecular head-to-tail interactions at concentrations higher than 10^{-4} M (Higashijima et al., 1979). The stabilization of the formed dimers should involve electrostatic interactions between the positively charged ammonium group of one molecule and the negative carboxylate function of the other. However, the presence of such aggregates was established from the occurrence of very small variations in the chemical shifts and amide temperature dependencies as a function of the concentration of Met or Leu-enkephalins in Me_2SO-d_6 solutions. Recent studies performed on these peptides by relaxation measurements (Stimson et al., 1979) or on the highly folded tetrapeptide Tyr-Gly-Gly-Phe by ^{1}H NMR and ultracentrifugation experiments (M. C. Fournié-Zaluski S. Premilat, and B. P. Roques, unpublished experiments) do not confirm the proposed intermolecular associations. In any case, the dansylated enkephalins and their parent compounds which contain a C-terminal group and are consequently studied under their cationic forms cannot aggregate by head-to-tail interactions. This was clearly confirmed by the lack of fluorescence modifications in TFE and H_2O from 10^{-6} to 10^{-4} M and by the absence of changes in chemical shifts for more concentrated solutions (5×10^{-5}–5×10^{-2} M in Me_2SO-d_6) of **1**–**3**.

The first two interesting results of the NMR studies of these fluorescent enkephalins are the following: (i) In all compounds, the methylene groups of the chain linking the peptide moiety and the dansyl ring remain very sharp according to a large degree of freedom for this C-terminal group. Indeed, in a molecule as small as the 1,3-bis(9-adenyl)propane (Bolte et al., 1979), the restriction in the rotational motion around C–C bonds in the propyl bridging chain due to the occurrence of an intramolecular highly stabilized stacked form induces a large and selective broadening of the CH_2 signals. (ii) No severe steric hindrance occurs in the lateral chains of compounds **1**–**3**, as shown by the existence of three rotamer populations for Tyr^1, Phe^4, and Met^5.

The chemical shifts, coupling constants, and rotamer populations for the compounds **1**, **2**, **5**, and **6** in Me_2SO-d_6, D_2O, and TFE-d_3 are reported in Tables IS–IIIS (see paragraph at end of paper regarding supplementary material). The most interesting results of these studies are the following. In Me_2SO-d_6, no large changes in both chemical shifts and coupling constants, especially $^3J_{NH-H_\alpha}$, occur between the amidated peptides **5** and **6** and their dansylated derivatives **1** and **2**. This indicates that the preferential solvated structures or the average conformations of **5** and **6** were not significantly modified by the introduction of the dansyl ring. Consequently, this fluorophore remains far from the peptide backbone in a solvated-surrounding environment. Contrastingly, in D_2O, the dansyl residue exhibits a large tendency to fold on the peptide moiety as shown by the changes in the chemical shifts in **1** and **2** as compared to those for their parent compounds **5** and **6**. For instance, in **1** a strong shielding occurs on the Met^5 residue ($H_\alpha = 0.27$, $H_\beta = 0.20$, $H_\gamma = 0.10$, and $SCH_3 = 0.06$ ppm) whereas the H_α and H_β of Phe^4 are more weakly upfield shifted (0.08 ppm). The interaction between the dansyl group and the Met^5 side chain is also reflected by a slight increase of the tg^+ rotamer of Met^5 in **1** as compared to that in **5**.

In TFE, both the N-terminal part and the Met residue of **1** are affected by the Dns current shift since the H_α and H_β protons of Tyr are deshielded (~ -0.10 ppm) while H_α Met is upfield shifted (+0.10 ppm). So, even in this solvent which usually increases the intramolecular interactions, compound **1** exists under several conformations including those exhibiting a spatial proximity between the dansyl ring and the Met^5 or Tyr^1 residues. Furthermore, the presence of a weak deshielding effect on the Tyr moiety is in accordance with the shorter distance, r_1, found by fluorescence in TFE as compared to that in H_2O.

Table II: Average Properties Related to Nonradiative Energy Transfer from Tyr to Dns and Average Dimensions (Å) of Dansylated Enkephalins **1** and **2** Derived from Experimental Data and Computed Models 1 and 2

compd		$\langle E \rangle^a$ H_2O	$\langle E \rangle^a$ TFE	$\langle r_1 \rangle^b$	$\langle r_\alpha \rangle^c$	$\langle r_{Phe-Dns} \rangle^d$	$\langle r_{Dns-CH_3} \rangle^e$
1	model 1	0.48	0.66	15.3	9.0	11.5	10.6
	model 2	0.75	0.88	11.7	6.5	11.0	9.9
	exptl data	0.50	0.84	14.5[f]			
2	model 1	0.50	0.77	12.9	6.9	13.4	11.8
	model 2	0.50	0.79	12.6	5.7	13.2	10.8
	exptl data	0.37	0.77	13.7[f]			

[a] Transfer efficiency in a static averaging regime for $\kappa^2 = 2/3$. [b] Average distances between aromatic rings of Tyr and Dns. [c] Average distances between α carbons of Tyr and Met. [d] Average distances between Phe[4] and Dns rings. [e] Average distances between CH_3 of Met[5] and Dns ring. [f] Distance r_1 computed from E in H_2O on the assumption of fixed separation of luminophores.

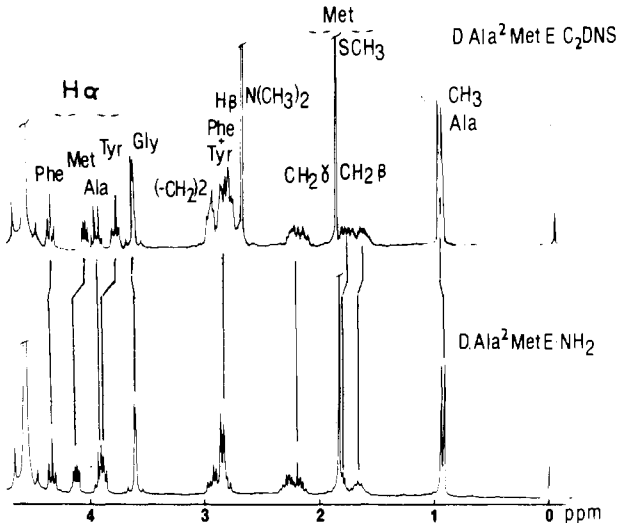

FIGURE 3: ^1H NMR spectra of D-Ala[2]-Met-E-C_2-Dns (**2**) and D-Ala-Met-enkephalinamide (**6**) in phosphate buffer, pH 7.

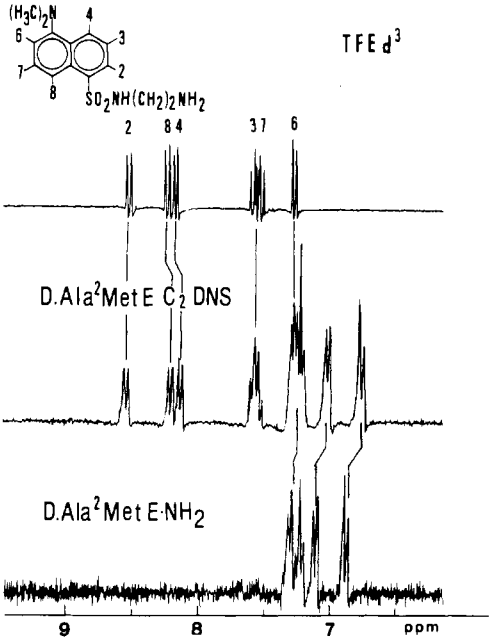

FIGURE 4: ^1H NMR spectra (aromatic part) of dansylethylamine, D-Ala[2]-Met-E-C_2-Dns (**2**), and D-Ala-enkephalinamide (**6**) in TFE-d_3.

The replacement of Gly[2] by the more hydrophobic D-Ala[2] residue leads to a stronger folding of the dansyl moiety on the peptide backbone. Indeed, the H_α and H_β protons of both the Tyr[1] and Met[5] residues are shielded in the aqueous solution of **2** (as compared to the parent amide **6**) (Figure 3) whereas only the Met[5] residue of **1** is affected in the same solvent. This interaction between the two fluorophores is in good accordance with the blue shift of the emission maximum of the dansyl fluorescence. Such preferential folded conformations with a proximity between the Tyr[1] and Dns moieties are even more populated in TFE. As shown in Figure 4 through a comparison of the spectra of **2**, **6**, and dansylethylamine (basic form), the influence of the aromatic ring of Dns leads to important shielding effects on the tyrosine protons. Moreover, it is very interesting to observe that in TFE only the protons belonging to these aromatic residues are significantly affected. This could indicate the existence of a preferential conformation with a shortening of the distance between the Tyr and Dns rings or a set of conformers including extended and highly folded forms (with stacking between Tyr and Dns groups). This latter assumption seems to be more adequately related to the fluorescence (Table II) and theoretical calculations (see below).

All the experimental results from fluorescence and NMR studies were compared with those obtained from computed conformations (Tables I, II, and III).

In the two models, the computed average value of κ^2 defined as in Haas et al. (1978) is $^2/_3$, and furthermore, in each family of conformers characterized by a given interluminophore separation r_1, κ^2 is close to the random value of $^2/_3$. In the statistical model 1, the distributions of distances, r_1, of the population of conformers are relatively wide, with 85% of the total having an interluminophore separation within ±5 Å from the average distance, $\langle r_1 \rangle$. Conformers with distances smaller than 5 Å are not found in the statistical sample. For Met-E-C_2-Dns (**1**), it appears that the statistical model 1 is in reasonable agreement with the experimental values of the transfer efficiencies in phosphate buffer. This result is in accordance with the presence of a more extended conformation of Met-enkephalin itself in aqueous medium. In TFE, the shortening of the distance between the two fluorophores determined by both fluorescence and NMR experiments is more adequately related to the "flexible β_I-turn" model 2 and is in accordance to a folding effect of this solvent.

In the case of D-Ala[2]-Met-E-C_2-Dns (**2**), the increased proximity between Tyr and Dns is clearly evidenced by the three methods. Thus, for this compound, the $^3J_{\alpha\text{-NH}}$ coupling

Table III: Experimental and Average Coupling Constants, J_{NH-CH_α} (in Hertz), for Dansylated Enkephalins 1 and **2** Derived from Computed Models 1 and 2

compd		X[2]	Gly[3]	Phe[4]	Met[5]
1[a]	model 1	5.3	5.1	9.1	7.5
	model 2	5.2	6.1	8.5	7.9
	exptl data[c]	5.5	6.0	8.0	8.0
2[b]	model 1	6.6	4.7, 6.6	9.0	8.3
	model 2	7.9	5.2, 6.8	8.1	9.2
	exptl data[c]	7.3	5.4, 6.0	7.8	7.8

[a] X = Gly. [b] X = D-Ala. [c] In Me_2SO-d_6.

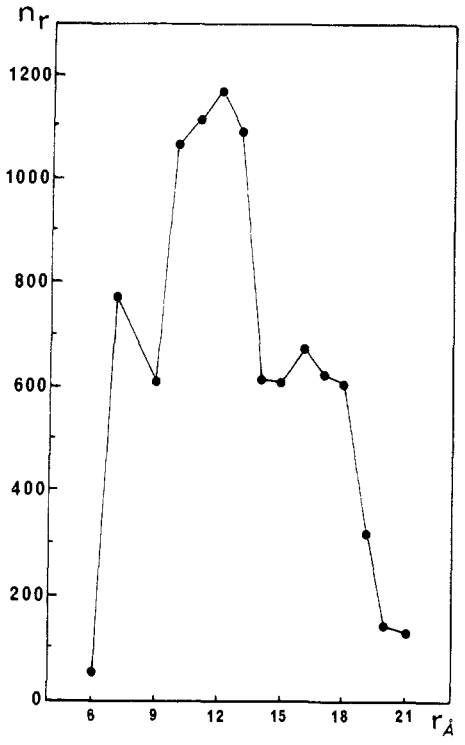

FIGURE 5: Distribution functions of distances ($r_Å$) between Tyr and dansyl fluorophores in compound **2**. n_r = number of conformers for a given r value.

constants computed for a flexible 2,5 β_I turn (model 2) are in good agreement with the experimental values in Me_2SO-d_6 (Table III). Such folding is probably retained in TFE. However, the enhanced shielding of the Tyr protons in TFE relative to D_2O is not reflected by the corresponding transfer efficiencies (Table I). Since the dansylated enkephalins are in rapid equilibrium between several conformations occurring at least at the level of the dansylethylamino chain, this discrepancy might be related to the differences in the strength of the effect measured in the NMR and fluorescence methods. For instance, the shielding effect is more strongly "distance dependent" than the transfer efficiency.

As already emphasized by Conrad & Brand (1968), the distance determined by energy transfer measurements corresponds to a nonlinear average of the different preferential conformers. This is clearly demonstrated in the present study by the 0.1-ppm shielding of both the tyrosine ortho and meta protons in **2**. This upfield shift is necessarily due to a proximity of the dansyl and the Tyr^1 residues, which corresponds to a shorter distance than the mean value (~12 Å) computed from fluorescence experiments. Indeed, the isoshielding curves calculated for the naphthalene ring (Bovey, 1969; Haigh & Mallion, 1971) show a lack of induced chemical shifts for protons located at distances superior to 8 Å from the dansyl ring center. Contrastingly, a shielding of about 0.5 ppm should be observed if the tyrosine ring is stacked at 4.5 Å (minimum distance computed from molecular models) upon the dansyl residue. Therefore, the experimental upfield shifts (~0.1 ppm) measured on ortho and para protons of tyrosine could be interpreted by the occurrence of about 20% of such a stacked conformation. Obviously, this is an oversimplification of the conformational equilibrium since several forms with different stacked geometries could lead to identical shielding effects. Nevertheless, NMR results clearly indicate that at least one conformer with a close proximity between tyrosine and dansyl rings exists although forms with distances smaller than 5 Å are not found in the computed statistical sample (Figure 5).

However, the statistical relevance of distribution functions by Monte-Carlo remains questionable. On the other hand, it is very difficult to calculate the lifetime of a well-stacked conformer, but it is probably longer than the lifetime of the donor luminophore. In such a case, the value of $\kappa^2 = {}^2/_3$ for this conformation may be inappropriate although the low polarization of the dansyl chromophore leads to values near $\kappa^2 = {}^2/_3$ for a large number of orientations. Nevertheless, only an accurate determination of the respective orientations of the two fluorophores and knowledge of the proportion of such a stacked form could permit determination of the right κ^2 value. This cannot be performed at this time for molecules so flexible.

Finally, comparison between all these results shows that r_1 values determined by energy transfer correspond to distances which are well correlated with the computed distributions of conformers (Figure 5). However, particular conformations which could be of special relevance for receptor recognition (as discussed below) are not visualized by fluorescence techniques. In contrast, although the great flexibility of peptide structures leads to a rapid equilibrium between several conformers of similar energy and then to a weighted average of all NMR parameters (chemical shifts, coupling constants, and relaxation times), the use of an appropriate probe, as the dansyl group in the present study, could allow characterization of the occurrence of conformers with specific geometry.

The strong opiate activity of **1** and **2** (Fournié-Zaluski et al., 1978) demonstrates that these compounds are able to adopt the required biologically active conformation at the receptor site. A large number of studies [see Gorin et al. (1978) for a review] have shown topological analogies between opioid peptides and opiate alkaloids. However, such a comparison runs against the occurrence of at least two kinds of binding sites for enkephalins in brain and in peripheral organs (Lord et al., 1977). Nevertheless, it appears that the tyramine moiety of the opioid peptides carries the pharmacological message whereas according to both its chemical content and its conformational behavior the remaining peptide sequence provides the specificity for one kind of receptor. So structure–activity studies (Roques et al., 1979) and theoretical calculations (Maigret et al., 1981) show that enkephalins with specific μ-agonist properties exhibit a high folding tendency. However, the μ-receptor activation probably involves a transconformational binding process of the opioid peptides since none of the folded conformations (1–4 or 2–5 β bends) found in the solid or solvated states are able to fit the μ-opiate pharmacophore (Fournié-Zaluski et al., 1981). This assumption has been recently strongly supported by the preferential μ-agonist potency of a cyclic enkephalin analogue (Di Maio & Schiller, 1981). This compound is characterized by (i) a cyclic and obviously folded conformation devoid of classical β structures and (ii) an intramolecular Tyr^1–Phe^4 distance around 10 Å. This spatial relationship between the two aromatic residues was already evidenced by fluorescence measurements in biologically active Trp^4 analogues of enkephalins and in the potent opiate PEO (Schiller & St. Hilaire, 1980).

Although the presence of an aromatic residue in the fourth position of enkephalins is not absolutely required for μ-receptor recognition, it is very interesting to observe that similar computed average distances between Tyr^1 and Phe^4 were also found in the highly potent fluorescent enkephalins **1** and **2**. So, according to the present study, the occurrence in solution of both a relevant Tyr^1–Phe^4 distance and a folding tendency for the dansylated peptides could explain their high biological potency and their binding property. On the other hand, comparative pharmacological assays on guinea pig ileum and

mouse vas deferens show that addition of the dansyl moiety enhances the δ selectivity as compared to Met-enkephalin addition (Fournié-Zaluski et al., 1978). This finding agrees with the shift to δ specificity occurring by the lengthening of the enkephalin sequence (Gacel et al., 1980). Finally, the use of three independent methods demonstrates the large degree of freedom of the additional dansyl ring in the fluorescent enkephalins allowing the recognition by these compounds of both μ and δ receptors as well as brain-degrading aminopeptidases. Therefore, the large decrease of activity for **3** (10% of the potency of **1**) can be related to the simultaneous absence of both a D residue in place of Gly^2 and a hydrophobic fifth amino acid (Fournié-Zaluski et al., 1981).

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Supplementary Material Available

Tables IS–IIIS containing ^1H NMR data for compounds **1**, **2**, **5**, and **6** (3 pages). Ordering information is given on any current masthead page.

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