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PULSE FLUOROMETRY OF CYCLO-(GLYCYL-L-TRYPTOPHYL)

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The fluorescence of cyclo-(glycyl-L-tryptophyl) in trimethyl phosphate has been studied in a temperature range varying from room temperature to −85°C.

At room temperature, the fluorescence decay is the sum of two exponentials, the relative amplitude of which depends on the emission wavelength. This can be explained by the presence of the two following emitting molecular states: on one hand the unfolded state, the fluorescence decay time and the emission spectrum of which are close to these of skatole; on the other hand the folded state which has a shorter decay time and a blue-shifted spectrum.

By lowering the temperature, the fluorescence spectrum shifts to the blue, while the skatole spectrum shifts to the red. This behavior corresponds to an increase of the folded conformation concentration in agreement with the NMR results. Furthermore the rate of exchange between the folded and the unfolded conformations decreases. Accordingly the wavelength dependence of the fluorescence decay lessens. There are two possible values of the conformational angle χ_2 differing by 180°, which correspond to the folded state; due to the indole asymmetry, the interactions between the indole and diketopiperazine rings differ in these conformers. Consequently the fluorescence decay remains biexponential even at −85°C.

1. Introduction‡

Tyrosine and tryptophan fluorescence in proteins can be partially quenched by the peptide group CONH. Some of the characteristics of this quenching have been reported [1–5]. Studies on the fluorescence of model compounds (amides, esters, peptides) have shown that this quenching occurs intramolecularly (and not by an inductive effect of the carbonyl group) and depends on the presence of a polar solvent that is able to solvate the carbonyl group by hydrogen bonding. The quenching efficiency also seems to be related to the relative orientation and the distance between the peptide groups and the chromophore.

In order to supply additional informations on the characteristics of this quenching, we have undertaken the investigation of the fluorescence decay of tryptophan diketopiperazines. This study led to the confirmation that the mechanism of quenching by the peptide group is due to a mesomeric effect rather than to an inductive effect, and that water plays a specific part in it [6].

In this previous work, we have shown that the emission spectra of the cyclopeptide in water and dimethylsulfoxide (DMSO) at room temperature result from the superposition of two component spectra which have different fluorescence maxima and are characterized each by a different value of the radiationless deactivation constant. These spectra were respectively attributed to the folded and unfolded forms, the existence of which had been reported by NMR studies [7–9]. However the ratio of the concentration of molecules in the folded form to the concentration of molecules in the unfolded form given by fluorescence decay measurements differs from that given by NMR. To explain this discrepancy, we assumed that an exchange reaction between the two forms occurs during the lifetime of the excited state.

However, in our kinetic interpretation, we did not take into account the existence of several conformations characteristic of the folded or unfolded form.

* With the technical assistance of J.C. Auchet.

‡ *Abbreviations used are*:

CGlyTrp	– cyclic diketopiperazine of glycine and tryptophan
DKP	– diketopiperazine
DMSO	– dimethylsulfoxide
TMP	– trimethylphosphate
PM	– photomultiplicator
NMR	– nuclear magnetic resonance
CPL	– circular polarization of luminescence.

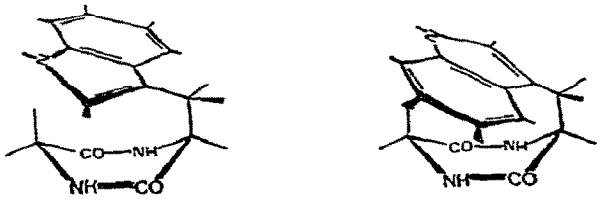

Fig. 1. The two folded conformations of the tryptophyl diketopiperazines. These two conformations are reached for two values of χ_2 differing by 180°C.

reached by rotation of the $C_\alpha - C_\beta$ and $C_\beta - C_\gamma$ bonds (described by the values of the angles χ_1 and χ_2, respectively). In particular, there are two possible values of χ_1, in the unfolded state, which differ by about 120°C (U_I and U_{II} forms in ref. [8]).

In the case of tryptophan, the examination of molecular models shows that two folded conformations can be distinguished because of the indole ring dissymmetry (fig. 1). If the degree of quenching of indole fluorescence by the peptide group depends on the relative orientation of the chromophore and quenching group, it is possible to anticipate a difference between the quantum yields and lifetimes of these two folded conformers. This difference does not seem to appear at room temperature since the decay is biexponential and one of the decay times, close to that of skatole in DMSO, must be attributed to the unfolded form.

In order to examine more carefully this phenomenon it appeared of interest to measure the fluorescence decay of these peptides at low temperature. Trimethylphosphate was chosen as solvent for this purpose. As will be shown, the fluorescence decay of CGlyTrp at room temperature in TMP was found to be analogous to that in DMSO.

With decreasing temperature, the concentration of molecules in the folded form increases, as showed by NMR; furthermore, the solvent viscosity increases and the molecular motions are slowed down.

One expects the overall fluorescence spectrum to shift towards shorter wavelengths since, according to our explanation, the folded form emission is blue shifted with respect to that of the unfolded form; at the same time the fluorescence decay should become monoexponential. Among these two predictions, the former only was found to be fulfilled in the case of CGlyTrp. These results are discussed in the present paper.

2. Material and methods

The diketopiperazine CGlyTrp (a gift from Dr. B. Donzel) was synthesized by a method described elsewhere [9] and was chromatographically pure. Skatole (3-Me-indole) was sublimated. Trimethylphosphate (Uvasol from Merck) showed strong absorption and non-negligible fluorescence in the UV; it was purified by treatment on active charcoal followed by distillation under vacuum. All solutions were prepared with freshly distilled solvent. The concentrations of solutions were about 10^{-4} M.

It has been shown in preliminary experiments that oxygen quenching of the fluorescence of indole was effective in TMP. In order to remove dissolved O_2, we submitted the solutions to a minimum of three freeze-pump-thaw cycles in vacuum at a pressure of 10^{-5} torr. After deaeration, the 1 × 1 cm quartz cell was sealed under vacuum.

A Cary 14 recording spectrometer and Jobin–Yvon spectrofluorimeter (modified in our laboratory) were used for measurements of absorption and fluorescence, respectively. Fluorescence spectra were corrected for the dispersion of the monochromator and the wavelength dependence of the PM response.

For the low temperature measurements, we used a small cryostat from Meric. The cell containing the sample is held on a copper support inside of which there is a flow of liquid nitrogen. The temperature needed can be chosen by regulating the nitrogen outflow and is measured on a semiconductor thermometer. The cell and its support are placed in a tight cylindrical tube where a 10^{-3} torr vacuum is established. The cylinder is supplied with three quartz windows which allow excitation of the sample and measurement of the fluorescence emission at 90°C from the exciting beam. This cryostat can be fitted in the spectrofluorimeter and the decay apparatus.

Fluorescence decay-time measurements by the single photoelectron method were made using a previously described apparatus [10]. Experimental conditions and the method of decay analysis were identical to those described in the preceding paper [6]. The apparatus response function $g(t)$ was obtained by using *p*-terphenyl in cyclohexane (decay-time: 0.96 ns) as reference solution [11].

3. Fluorescence of the peptide CGlyTrp and of skatole in TMP at room temperature

The fluorescence spectra of CGlyTrp and skatole were measured in TMP at room temperature: the fluorescence maxima are at 334 nm and 340 nm, respectively. So, according to what happens in DMSO and water, the fluorescence spectrum of the dipeptide is shifted towards lower wavelengths, with respect to that of skatole. It has been shown in DMSO and water [6] that the emission spectrum of the folded form of DKP shows a shift of 10 nm towards lower wavelengths compared with that of the unfolded form, the spectrum of which is close to that of skatole. In the same way, the shift of the overall spectrum of the dipeptide in TMP may be reasonably attributed to the existence of the folded form.

The shift of both spectra of CGlyTrp and skatole in TMP compared to the spectra in DMSO may be attributed to a difference in solvent polarity.

It was shown in ref. [6] that the fluorescence decay of the DKP of tryptophan in DMSO and H_2O at room temperature can be fitted to a sum of two exponential functions:

$$F(\lambda, t) = C_\alpha(\lambda) e^{-t/\tau_\alpha} + C_\beta(\lambda) e^{-t/\tau_\beta}, \qquad (1)$$

where $C_\alpha(\lambda) + C_\beta(\lambda) = 1$. τ_α and τ_β are independent of the emission wavelength λ, while the coefficients C_α and C_β change along the emission spectrum.

As it appears from the results in table 1, this behaviour is again found in TMP at room temperature. The values of the two time constants τ_α and τ_β are close to those found in DMSO, and the value of the decay time of skatole is only 0.2 ns higher in TMP (7.7 ns) than in DMSO. In addition, the contribution of the exponential function with the shortest time constant decreased with increasing emission wavelength. The likeness of the results in TMP and DMSO suggests

Table 1
Fluorescence decay analysis of CGlyTrp in TMP at 20°C: experimental values of the time constants τ_α and τ_β, and dependence of the coefficients $C_\alpha(\lambda)$ and $C_\beta(\lambda)$ on the emission wavelength λ

τ_α	2,1 nsec				
τ_β	6,8 nsec				
λ nm	320	330	340	350	360
$C_\alpha(\lambda)$	0,34	0,24	0,18	0,135	0,06
$C_\beta(\lambda)$	0,66	0,76	0,82	0,865	0,94

that the kinetic scheme involving an equilibrium between the folded and unfolded forms of the dipeptide is still valid TMP. From the experimental parameters τ_α, τ_β, C_α and C_β, it is possible to calculate two apparent spectra $F_\alpha(\lambda)$ and $F_\beta(\lambda)$ corresponding to the two exponential of eq. (1), and an apparent equilibrium constant C:

$$C = \left(\int F_\alpha(\lambda)\, d\lambda \Big/ \int F_\beta(\lambda)\, d\lambda\right)(\tau_\beta/\tau_\alpha).$$

Unfortunately, the decay measurements could not be performed at a wavelength lower than 320 nm, owing to the use of a reference compound for the determination of $G(t)$: it was not possible to find a reference with a short decay time, a sufficiently high quantum yield and which would have a fluorescence maximum around 300 nm. Hence the short wavelength region of the spectra $F_\alpha(\lambda)$ and $F_\beta(\lambda)$ could not be investigated. By extrapolation of the spectra it was possible, however, to estimate the ratio of the areas of the spectra, and to determine an approximate value of C. We found a value of 0.26. The NMR studies [7,8] have shown that the folded form is favoured in the ground state in a number of solvents, and this may be true for TMP. On the contrary, the value of C suggests that in the excited state it is the unfolded form that is favoured, the concentration of the two forms changing during the lifetime of the excited state: a similar behaviour was observed in DMSO.

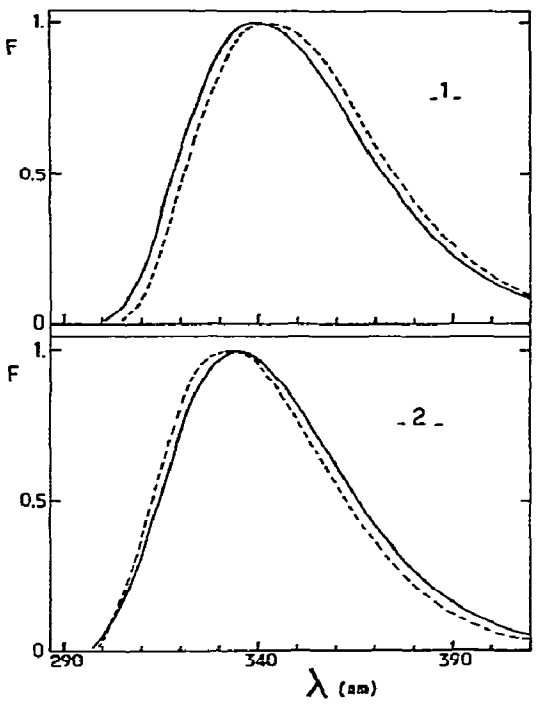

Fig. 2. Fluorescence spectra of skatole (1) and CGlyTrp (2) at 20°C (——) and −85° (– –) in TMP. Excitation was at 280 nm.

4. Study of the fluorescence spectra of skatole and CGlyTrp as a function of temperature

When the temperature is lowered, the fluorescence spectrum of skatole shifts gradually towards longer wavelengths, down to the freezing point (fig. 2); the shift is of 5 nm when the temperature falls from 20°C to −85°C. Thus the blue shift of the spectrum which generally occurs in the temperature range where the lifetime of the chromophore and the relaxation time of solvent molecules take similar value [12], is not observed in TMP.

In contrast to that of skatole, the fluorescence spectrum of the cyclopeptide undergoes a blue shift and a slight deformation in the emission maximum region. The blue shift is equal to 3 nm when the temperature goes from 20°C to −85°C. In fact one can assume that this shift results from the superposition of two phenomena: a red shift as in the case of skatole, and a larger shift towards lower wavelengths. Then the difference between the maximum wavelength of skatole and CGlyTrp increases from 6 nm at 20°C to 13 nm at −85°C. This behavior can be explained by the increase of the folded form concentration when the temperature is lowered and it is in agreement with the predictions made in the introductory section.

5. Fluorescence decay skatole and CGlyTrp at low temperature

As can be seen in table 2, the fluorescence decay of skatole can be fitted to a single exponential, over the whole explored temperature range. For each temperature, the time constant was found to be independent of the emission wavelength. This is consistent with the fluorescence spectra measurements which have shown (see preceding section) that during the lifetime of the excited state of indole, the solvent relaxation does not markedly interfere with the emission of light; otherwise the decay would not be monoexponential and independent of the emission wavelength [13].

We have also measured the fluorescence decay of CGlyTrp at three different temperatures (below 20°C) and at various emission wavelengths (figs. 3 and 4). As was done at room temperature we tried to analyse the decay curve as a sum of two exponential functions. The results of the analysis are shown in table 3. It is obvious from the data in table 3, that, contrary to what happens at room temperature, the two time constants τ_α and τ_β are no longer independent from the emission wavelength λ: they both increase with λ, for the three temperatures studied. For a given wavelength the average time constant $\langle\tau\rangle$ defined by

$$\langle\tau\rangle = C_\alpha\tau_\alpha + C_\beta\tau_\beta,$$

increases with temperature. When temperature falls from 20°C to −23°C the two time constants increase, this tendency being more pronounced for the short

Table 2
Fluorescence decay analysis of skatole in TMP at various temperatures T. τ is the time constant of the simple exponential. Excitation was at 280 nm

T °C	20°	−23°	−34°	−44°	−64°	−85°
τ ns±c	7.7	8,15	8,25	8,4	8,7	9

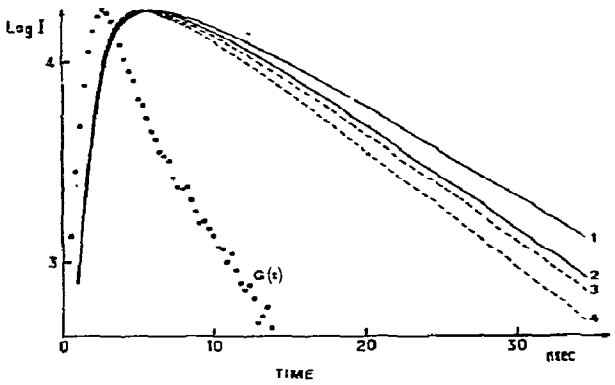

Fig. 3. Fluorescence decay of skatole (——) and CGlyTrp (– –) at 20°C (curve 2 and 4) and −85°C (1 and 3) in TMP, for an emission wavelength of 340 nm. Excitation was at 280 nm. $G(t)$ is the apparatus response function.

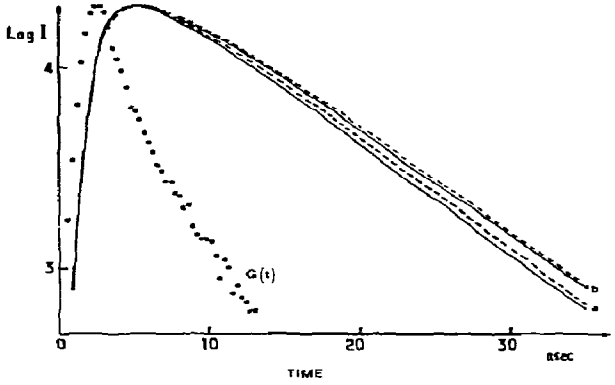

Fig. 4. Fluorescence decay of CGlyTrp at −23°C (a) and −85°C (b) in TMP, for two emission wavelengths: 320 nm (——) and 350 nm (– –). Excitation was at 280 nm. $G(t)$ is the apparatus response function

time constant. On the contrary, the value of this time constant is lower at −85°C than at −23°C or −43°C, its coefficient falling to a value of 0.1 practically independent of the emission wavelength.

Thus it appears that the kinetic scheme proposed to analyse the fluorescence decay of the peptide at room temperature is no longer valid at low temperature in TMP. In order to explain the observed behaviour at low temperature, we propose the following interpretation:

From the NMR results, it seems a reasonable assumption that at −85°C, the cyclopeptide in its ground state is almost entirely in the folded form. At this temperature, the medium is highly viscous and relaxation of molecular conformation is expected to be much slower than the deactivation of the excited state. The contribution of the folded form to the fluorescence emission must then be preponderant.

Table 3
Two components fluorescence decay analysis of CGlyTrp in TMP at various temperatures and emission wavelengths (λ). ⟨τ⟩ is the average time constant defined in the text

λ nm	T = −23°C				T = −43°C				T = −85°C			
	τ_α nsec	τ_β nsec	C_α	⟨τ⟩ nsec	τ_α nsec	τ_β nsec	C_α	⟨τ⟩ nsec	τ_α nsec	τ_β nsec	C_α	⟨τ⟩ nsec
320	3,6	7,3	0,38	5,9	3,2	7,1	0,24	6,2	—	—	—	—
330	4,2	7,6	0,36	6,4	3,5	7,4	0,23	6,5	2,9	7,2	0,14	6,6
340	4,5	7,8	0,33	6,7	4,3	7,8	0,30	6,7	3,4	7,5	0,11	7,05
350	4,7	7,8	0,29	6,9	4,4	7,8	0,24	7,0	3,7	7,7	0,10	7,3

Now the contribution of the exponential with the shortest time constant is only 10 percent of the overall decay. Moreover the longest time constant is about 1.5 ns, shorter than the decay time of skatole at the same temperature; it is then likely that this time constant cannot be attributed to an unfolded form.

We showed in the introductory section (fig. 1) that two folded conformations can be distinguished. As follows from the indole dissymmetry, the interaction between the indole chromophore and the peptide ring may not be similar in these two conformations; this consideration leads us to the assumption that the radiationless deactivation probability of the indole chromophore may be different in the two conformers, and may be characterized by two different fluorescence decay times, both of them being still smaller than the decay time of skatole. Moreover, if the interaction energy between the aromatic and dipeptide rings has not the same value in the two conformations, these may be differently populated. So the existence in the fluorescence decay of a weak contribution of an exponential with a short time constant and a strong contribution of an exponential with a long time constant can be explained and conciliated with NMR studies.

However, this new hypothesis does not account for the increase of both constants τ_α and τ_β with increasing emission wavelength. So it appeared to us that the decomposition of the decay in two exponentials was an approximation. In addition to the contribution of the two folded conformations to the overall emission,

there may exist a contribution of the unfolded form, increasing with the emission wavelength. Practically a resolution of the decay into more than two components would have been difficult and rather hazardous in this case, and we did not try it.

For temperatures higher than −85°C, one may expect an increase of the contribution of the unfolded form: accordingly, the analysis of the decays at −23°C and −43°C as a sum of two exponentials, led to the appearance of an increase of the longest time constant, as compared to its values at −85°C. The red shift of the fluorescence spectrum with increasing temperature reinforces this hypothesis, as was shown in the preceding section. On the other hand the gap between the radiationless deactivation probabilities of the two folded conformations may be assumed to diminish with increasing temperature, the biexponential decay at room temperature taking its true origin from the difference between the radiationless deactivation probabilities of the folded and unfolded forms.

Recently, Schlessinger et al. [14] have measured the circular dichroism (CD) and the circular polarization of luminescence (CPL) of several aromatic diketopiperazines.

In very viscous solvents, the CPL was similar to the CD while in dioxane, dimethylsulfoxide and glycerol, the CPL was zero. The authors' conclusion is that, in these solvents, the excited diketopiperazines unfold in a time very short compared to the fluorescence decay time.

As already mentioned in the introduction, there are two possible values of χ_1 in the unfolded state. In one of the corresponding conformations, an interaction may occur between the aromatic ring and one carbonyl belonging to the peptide ring, as an inspection of CPK molecular models shows. The fluorescence might be quenched in this conformation.

If we admit that emission proceeds from the unfolded conformations, one should have to assume that the exchange between the two conformations occurs in a time the order of magnitude of which is equal to or longer than the nanosecond. Otherwise the fluorescence decay would be monoexponential, which is in contradiction with our experiments. Furthermore it seems unlikely to have at the same time a very rapid unfolding and a relatively slow change of the angle χ_1 in the unfolded state.

On the other hand in the nonviscous solvents dioxane and DMSO the brownian rotation may be sufficient to explain the negligible CPL. However this explanation cannot be valid in glycerol. Further investigation with this solvent would be interesting.

Acknowledgement

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