propane or Freon-12 (CF_2Cl_2) to give a final ratio of approximately 1:0.5:4. For the generation of 1,1-difluoroethyl radical from 1,1-difluoroethane, bistrifluoromethyl peroxide was used, since di-*tert*-butyl peroxide under the same conditions did not afford sufficiently high concentrations of radicals for esr measurements. For the generation of 2-fluoropropyl radical from the photolytic abstraction of hydrogen from 2-fluoropropane, spectra of radicals with good signal to noise ratios could only be observed at temperatures higher than −80°.

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Study of the Conformation in the Excited State of Two Tryptophanyl Diketopiperazines[1]

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Contribution from the Centre de Biophysique Moleculaire, Orleans Cedex, France. *Received September 18, 1973*

Abstract: In this work, we studied two aromatic diketopiperazines in solution, by the method of "time resolved spectroscopy." It can be shown that, in DMSO, the folded form of both *cyclo*(glycyl-tryptophyl) and *cyclo*(alanyl-tryptophyl) is much less stable in the excited state than in the ground state. We are able to determine the rate parameters and the equilibrium constant characteristic of these excited-state interactions. The height of the potential barrier which restrains the exchange between the two conformers is about 6 kcal/mol. In addition to this, it is shown that the folded form of *cyclo*(glycyl-tryptophyl) is much more stable in aqueous solution than in DMSO. The nature of the forces responsible for this behavior is discussed.

The diketopiperazines are single rigid molecules, with restricted rotational freedom, which offer a model system for the study of the structure of natural peptides and proteins.

It has been shown by nmr[2] spectroscopy[3,4] that the preferential conformation in solution of the diketopiperazines containing an aromatic side chain (tyrosyl, phenylalanyl, tryptophanyl residues) is a folded one, in which the aromatic side chain faces the diketopiperazine ring. The two open forms, in which the aromatic side chain lies away from the dipeptide ring, are unfavored at room temperature. Studies in water, DMSO, and TFA suggest that this behavior is not dependent on the nature of the solvent. However, data are lacking for tryptophanyl cyclopeptide in water.

The existence of an optical activity in the lowest energy absorption band also supports this interaction between the aromatic chromophore and the dipeptide ring.[5a] These authors have found that the ellipticity is the same for the peptide *cyclo*(Gly-Trp), in water and DMSO.

The fluorescence of aromatic diketopiperazines is strongly quenched in water; in contrast, the quantum yields of the cyclodipeptides in DMSO and dioxane are very similar to the quantum yields of $AcTrpNH_2$ and $AcTyrNH_2$.[5b] These findings suggest that the interactions of the diketopiperazine rings are very different when the chromophore is in its ground state and when it is in its excited state. The quenching of fluorescence of phenylalanyl, tyrosyl, and tryptophyl residues by the peptide bond has been studied many times.[6] It is generally attributed to the interaction between the carbonyl and the aromatic ring. It may be interpreted in terms of intramolecular charge transfer from the excited aromatic ring to the carbonyl.[7,8]

In the present work, we study the peptides *cyclo*(Gly-Trp) and *cyclo*(Ala-Trp) in solution, using an aspect of the method of time resolved spectroscopy.[9-11] The fluorescence decay is studied as a function of the emission wavelength; the method allows one to detect the existence of various fluorescent species and to analyze the kinetics of interaction of excited molecules.

Experimental Section

Materials. Acetyltryptophanamide was brought from Sigma Chemical Co. The diketopiperazines were synthesized by a method described elsewhere[3] and were analytically and chromatographically pure. Skatole[12] was sublimated. DMSO was purchased from Merck; it was kept on molecular sieves and used in a dehydrated atmosphere.

Absorption spectra were measured in a Cary Model 14 spectrophotometer. Emission spectra were measured in a quartz cell (1 × 1 cm) with a Jobin Yvon spectrofluorimeter, modified in our

(1) Paper written with the technical assistance of J. C. Auchet.

(2) Abbreviations used are: $AcTrpNH_2$, acetyltryptophanamide; $AcTyrNH_2$, acetyltyrosinamide; *cyclo*(Gly-Trp), cyclic diketopiperazine of glycine and tryptophan; *cyclo*(Ala-Trp), cyclic diketopiperazine of alanine and tryptophan; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; nmr, nuclear magnetic resonance; CD, circular dichroism.

(3) B. Donzel, Thèse, Zürich, 1971.

(4) (a) K. D. Kopple and D. M. Marr, *J. Amer. Chem. Soc.*, **89**, 6193 (1967); (b) K. D. Kopple and M. Ohnishi, *ibid.*, **91**, 962 (1969).

(5) (a) H. Edelhoch, R. E. Lippoldt, and M. Wilchek, *J. Biol. Chem.*, **243**, 4799 (1968); (b) H. Edelhoch, R. S. Bernstein, and M. Wilchek, *ibid.*, **243**, 5985 (1968).

(6) I. Weinryb and R. F. Steiner in "Excited States of Proteins and Nucleic Acids," Macmillan, New York, N. Y., 1972, p 277.

(7) J. Tournon, E. Kuntz, and M. Ashraf el Bayoumi, *Photochem. Photobiol.*, **16**, 425 (1972).

(8) J. Tournon and M. Ashraf el Bayoumi, *J. Chem. Phys.*, **56**, 5128 (1972).

(9) N. G. Bakhshoiev, Y. T. Mazurenko, and I. V. Peterskaya, *Opt. Spectrosk.*, **21**, 307 (1966).

(10) W. R. Ware, in "Creation and Detection of the Excited States," A. A. Lamola, Ed., Marcel Dekker, New York, N. Y., 1971, p 123.

(11) Ph. Wahl and J. C. Auchet, *Biochim. Biophys. Acta*, **285**, 99 (1972).

(12) Skatole = 3-methylindole.

laboratory. Relative fluorescence quantum yields were determined by the method of Parker–Rees[13] using AcTrpNH$_2$ as a reference. The concentrations were identical for all the samples (OD_{280} = 0.46). The fluorescence lifetimes were determined by the single photoelectron counting method, which has been described earlier.[11,14] We used a free running flash lamp operating in deuterium. The wavelength of the exciting light is selected by a 500-mm Bausch and Lomb monochromator. The fluorescence beam at 90° of the exciting beam goes through a "high intensity" Bausch and Lomb monochromator. The wavelength band width is 10 nm for the exciting beam and 5.4 nm for the fluorescence beam. The wavelength of the exciting light was 280 nm. The concentrations of the samples were about 10^{-4} M. The measurements was made in 1 × 1 cm quartz cells maintained at a constant temperature.

The fluorescence observed is a convolution product of the form

$$i(t) = \int_0^t g(T)I(t - T)dT$$

where $g(t)$ is the apparatus response function and $I(t)$ the decay corresponding to an infinitely short excitation. In the case of a τ exponential decay

$$I(t) = C_1e^{-t/\tau_1} + C_2e^{-t/\tau_2}$$

The "true" decays $I(t)$ were determined using the modulating function method.[15] We have checked the fit of the recalculated convolution function $i^0(t)$ to the experimental curve $i(t)$ using a method proposed by Knight and Selinger.[16] These authors have shown that the best computed curve is obtained when the weighted mean variance of the fit is equal to the weighted mean variance of the Poisson distribution corresponding to the counts in the n channels describing the experimental curve. In that case, one obtains

$$R = \frac{1}{n}\sum_{i=1}^{n}\frac{1}{i(t)}[i(t) - i^0(t)]^2 = 1$$

In this treatment the statistical error introduced by $g(t)$ is neglected which is certainly an inaccurate assumption. We found that the best fits were obtained when R was around 2.

It has been found in this laboratory[17] that the response function of a single photon pulse fluorometer depends on the wavelength of the exciting and of the emission light. We have shown that the time response function can be calculated from the decay curve of a reference compound of known time constant, measured at the exciting and the emission wavelength chosen for the sample under study. In this work, we used the *p*-terphenyl and the 2,5-diphenyloxazole in cyclohexane as reference compounds, which have respective time constants of 0.96 and 1.36 nsec.

Principle of the Method of Decay Analysis

In this section, we intend to describe the method we have used in order to analyze our fluorescence decay measurements.

We suppose that the diketopiperazine can adopt two different conformations A_1 and A_2 in equilibrium in the ground state. The ratio of concentrations of the two conformations is given by

$$K = [A_1]/[A_2] \quad (1)$$

where K is the equilibrium constant.

The properties of molecules are not the same in their excited state and in their ground state; most of the time the concentrations $[A_1]$ and $[A_2]$ will not be the equilibrium concentrations in the excited state. Moreover, the lifetime of the excited states is very short, around 10^{-9} sec; as a result, the equilibrium will not be reached during the lifetime of the excited state. A similar situation can be found in the case of excimers, exciplexes, and quenching of fluorescence.[18-21]

In our case, the kinetic scheme is

$$\begin{array}{ccc} A_1^* & \underset{k'}{\overset{k}{\rightleftharpoons}} & A_2^* \\ \uparrow\downarrow k_2 & & k_1\uparrow\downarrow \\ A_1 & \underset{K}{\rightleftharpoons} & A_2 \end{array} \quad (2)$$

The vertical arrows represent the activation by light absorption, the deactivation by emission of a photon, and the radiationless deactivation.

The rate constants for deactivation are given by

$$\begin{aligned} k_1 &= k_{F_1} + k_{i_1} \\ k_2 &= k_{F_2} + k_{i_2} \end{aligned} \quad (3)$$

k_{F_1} and k_{F_2} correspond to radiative deactivation; k_{i_1} and k_{i_2} are the rate constants of radiationless deactivation. k and k' are the rate constants of exchange between the two species in the excited state.

The equilibrium constant in the excited state may be expressed as

$$K_e = k'/k \quad (4)$$

The fluorescence intensity of each component is given by

$$\begin{aligned} I_1 &= k_{F_1}[A_1^*] \\ I_2 &= k_{F_2}[A_2^*] \end{aligned} \quad (5)$$

where $[A_1^*]$ and $[A_2^*]$ are the concentrations of the species in the excited state. They fulfill a two differential equation system similar to the system which appears in the exciplexes or quenching of fluorescence theories. The fluorescence decays, corresponding to infinitely short excitation, are given by

$$\begin{aligned} I_1(t) &\approx a_1e^{-t/\tau\alpha} + b_1e^{-t/\tau} \\ I_2(t) &\approx a_2e^{-t/\tau\alpha} + b_2e^{-t/\tau\beta} \end{aligned} \quad (6)$$

with

$$\begin{aligned} a_1 &= X_1[(k_1 + k)K - k' - K/\tau_\beta] \\ b_1 &= X_1[K/\tau_\alpha - (k_1 + k)K + k'] \\ a_2 &= X_2[k_2 + k' - 1/\tau_\beta - kK] \\ b_2 &= X_2[1/\tau_\alpha - k_2 - k' + kK] \end{aligned} \quad (7)$$

$$X_1 = \frac{\epsilon_1 k_{F_1} I_0[A]}{(1 + K)\left(\frac{1}{\tau_\alpha} - \frac{1}{\tau_\beta}\right)}$$

$$X_2 = \frac{\epsilon_2 k_{F_2} I_0[A]}{(1 + K)\left(\frac{1}{\tau_\alpha} - \frac{1}{\tau_\beta}\right)}$$

where I_0 is the intensity of the exciting light, $[A] = [A_1] + [A_2]$, and ϵ_1 and ϵ_2 are the molar absorptivities of A_1 and A_2 at the excitation wavelength.

(13) C. A. Parker and W. T. Rees, *Analyst (London)*, **85**, 587 (1960).
(14) Ph. Wahl, *Biochim. Biophys. Acta*, **175**, 55 (1969).
(15) B. Valeur and J. Noirez, *J. Chim. Phys. Physicochim. Biol.*, **70**, 500 (1973).
(16) A. E. W. Knight and B. K. Selinger, *Spectrochim. Acta, Part A*, **27**, 1223 (1971).
(17) Ph. Wahl, J. C. Auchet, and B. Donzel, *Rev. Sci. Instrum.*, in press.
(18) J. B. Birks, D. J. Dyson, and J. M. Munro, *Proc. Roy. Soc., Ser. A*, **275**, 575 (1963).
(19) W. M. Vaughan and G. Weber, *Biochemistry*, **9**, 464 (1970).
(20) Ph. Wahl and J. C. Auchet, *C. R. Acad. Sci., Ser. B*, **274**, 1334 (1972).
(21) M. Fayet and Ph. Wahl, *Biochim. Biophys. Acta*, **229**, 102 (1971).

τ_α and τ_β are given by

$$\frac{1}{\tau_\alpha} + \frac{1}{\tau_\beta} = k_1 + k_2 + k + k'$$
$$\frac{1}{\tau_\alpha\tau_\beta} = k_1k_2 + k_1k' + k_2k \tag{8}$$

We shall further assume that

$$\epsilon_1 = \epsilon_2 = \epsilon$$
$$k_{F_1} = k_{F_2} = k_F \tag{9}$$

As we are dealing with very weak interactions of the chromophore, which have little influence on the absorption properties, we may assume that the emission rate constant k_F remains unchanged (Strickler and Berg relation[22]).

The total fluorescence intensity is then given by

$$I(t) = I_1(t) + I_2(t) = \frac{k_F\epsilon I_0[A]}{(1/\tau_\alpha) - (1/\tau_\beta)}\left[\left(M - \frac{1}{\tau_\beta}\right)e^{-t/\tau_\alpha} + \left(\frac{1}{\tau_\alpha} - M\right)e^{-t/\tau_\beta}\right]$$

with

$$M = \frac{k_1K + k_2}{1 + K} \tag{10}$$

The quantum yield may be expressed as

$$Q = \frac{\int I(t)\mathrm{d}t}{\epsilon I_0[A]} = Q_\alpha + Q_\beta$$

where Q_α and Q_β are the quantum yields associated with the decay times τ_α and τ_β.

An apparent equilibrium constant may be introduced

$$C = \frac{Q_\alpha}{Q_\beta}\frac{\tau_\beta}{\tau_\alpha} = \frac{M - (1/\tau_\beta)}{(1/\tau_\alpha) - M} \tag{11}$$

Q may be rewritten as

$$Q = k_F[\tau_\alpha + \tau_\beta - M\tau_\alpha\tau_\beta] \tag{12}$$

Experimental Determination of the Rate Constants

We assume that the emission spectra of the two species overlap each other. The decay of a part of the total emission restricted to a bandwidth $\mathrm{d}\lambda$ around the emission wavelength λ is

$$F(\lambda,t)\mathrm{d}\lambda = C_1(\lambda)\mathrm{d}\lambda[a_1e^{-t/\tau_\alpha} + b_1e^{-t/\tau_\beta}] + C_2(\lambda)\mathrm{d}\lambda[a_2e^{-t/\tau_\alpha} + b_2e^{-t/\tau_\beta}] \tag{13}$$

with

$$\int C_1(\lambda)\mathrm{d}\lambda = \int C_2(\lambda)\mathrm{d}\lambda = 1$$

which may be written

$$F(\lambda,t) = h(\lambda)[C_\alpha(\lambda)e^{-t/\tau_\alpha} + C_\beta(\lambda)e^{-t/\tau_\beta}] \tag{14}$$

with

$$C_1(\lambda)a_1 + C_2(\lambda)a_2 = h(\lambda)C_\alpha(\lambda)$$
$$C_1(\lambda)b_1 + C_2(\lambda)b_2 = h(\lambda)C_\beta(\lambda) \tag{15}$$

Setting

$$C_\alpha(\lambda) + C_\beta(\lambda) = 1$$

(22) S. J. Strickler and R. A. Berg, *J. Chem. Phys.*, 37, 814 (1962).

$h(\lambda)$ is determined by the condition

$$\int F(\lambda,t)\mathrm{d}t = F(\lambda)$$

where $F(\lambda)$ is the intensity of the emission spectrum of the solution at wavelength λ, under a continuous excitation. Hence we have

$$h(\lambda) = \frac{F(\lambda)}{C_\alpha(\lambda)\tau_\alpha + C_\beta(\lambda)\tau_\beta} \tag{16}$$

$h(\lambda)$, $C_\alpha(\lambda)$, $C_\beta(\lambda)$, τ_α, and τ_β can be experimentally measured. Moreover one may write

$$F(\lambda) = F_\alpha(\lambda) + F_\beta(\lambda)$$

with

$$F_\alpha(\lambda) = \frac{C_\alpha(\lambda)\tau_\alpha}{C_\alpha(\lambda)\tau_\alpha + C_\beta(\lambda)\tau_\beta}\cdot F(\lambda) \tag{17}$$
$$F_\beta(\lambda) = \frac{C_\beta(\lambda)\tau_\beta}{C_\alpha(\lambda)\tau_\alpha + C_\beta(\lambda)\tau_\beta}\cdot F(\lambda)$$

and finally

$$Q_\alpha/Q_\beta = S_\alpha/S_\beta \tag{18}$$

where S_α and S_β are the areas under $F_\alpha(\lambda)$ and $F_\beta(\lambda)$ given by

$$S_\alpha = \int F_\alpha(\lambda)\mathrm{d}\lambda$$
$$S_\beta = \int F_\beta(\lambda)\mathrm{d}\lambda$$

According to relations 10, 11, and 18 C and M become

$$C = \frac{\tau_\beta}{\tau_\alpha}\frac{S_\alpha}{S_\beta} \tag{19}$$

$$M = \frac{(1/\tau_\beta) + (1/\tau_\alpha)C}{1 + C} \tag{20}$$

$1/\tau_\alpha$, $1/\tau_\beta$, and M can be determined from fluorescence decays and emission spectra measurements.

If K is known from independent measurements in the ground state, the determination of the four rate constants of eq 2 requires further information about the excited state; we shall also assume that one of the deactivation rate constants is known. The other deactivation rate parameter will be given by relation 10.

From eq 8 one can obtain

$$k = \frac{[(1/\tau_\alpha) - k_1][k_1 - (1/\tau_\beta)]}{k_1 - k_2}$$
$$k' = \frac{[(1/\tau_\alpha) - k_2][(1/\tau_\beta) - k_2]}{k_1 - k_2} \tag{21}$$

Introducing the following expressions does not restrict the generality of our treatment: $k_2 < k_1$ and $\tau_\alpha < \tau_\beta$. Since we have necessarily

$$k \geq 0$$
$$k' \geq 0$$

relation 20 should lead to

$$k_2 \leq 1/\tau_\beta < k_1 \leq 1/\tau_\alpha \tag{22}$$

The two equalities become true for $k = 0$ and $k' = 0$, respectively.

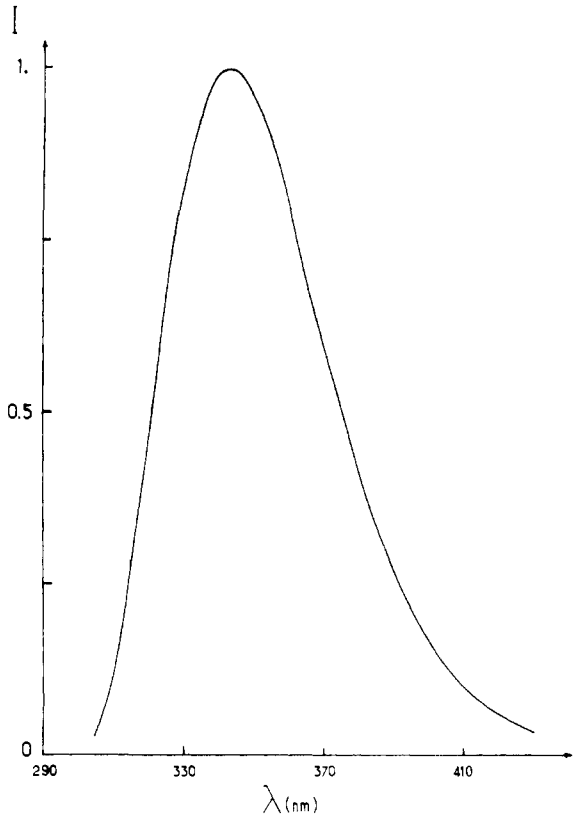

Figure 1. Fluorescence spectrum of $AcTrpNH_2$ in DMSO at 18°. Excitation was at 280 nm.

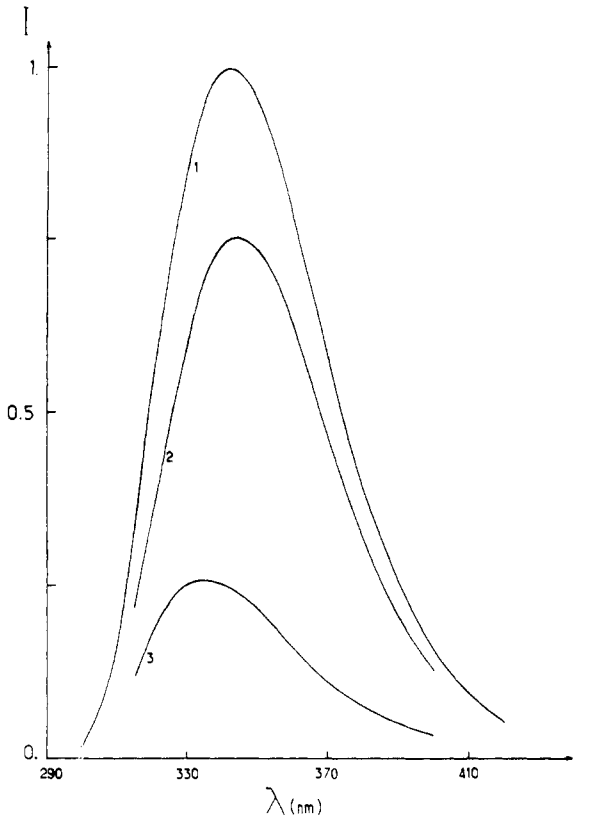

Figure 2. Fluorescence spectrum of *cyclo*(Gly-Trp) in DMSO at 18° (curve 1); decomposition into two components corresponding to the unfolded form ($F_2(\lambda)$, curve 2) and to the folded one ($F_1(\lambda)$, curve 3). Excitation was at 280 nm.

Determination of the Spectra of the Fluorescent Species

The emission intensity at wavelength λ of the species A_1 is given by

$$F_1(\lambda) = C_1(\lambda)\int I_1(t)dt = C_1(\lambda)[a_1\tau_\alpha + b_1\tau_\beta]$$

$C_1(\lambda)$ and $C_2(\lambda)$ can be obtained by solving eq 15. $F_1(\lambda)$ becomes

$$F_1(\lambda) = \frac{a_1\tau_\alpha + b_1\tau_\beta}{a_1b_2 - b_1a_2}\left[\frac{b_2}{\tau_\alpha}F_\alpha(\lambda) - \frac{a_2}{\tau_\beta}F_\beta(\lambda)\right] \quad (23)$$

$F_2(\lambda)$ will be determined from a similar equation, or, more simply, from

$$F_2(\lambda) = F(\lambda) - F_1(\lambda)$$

a_1, a_2, b_1, and b_2 can be calculated according to eq 7 in which the rate constants will be replaced by their value, previously determined.

Special Cases. (a) If k and k' are much smaller than k_1 and k_2, the equilibrium in the ground state is not perturbed. We shall have

$$1/\tau_\alpha = k_1 = 1/\tau_1$$
$$1/\tau_\beta = k_2 = 1/\tau_2$$

The decay time at the wavelength λ is given by

$$F(\lambda,t) = h(\lambda)[C_1(\lambda)e^{-t/\tau_1} + C_2(\lambda)e^{-t/\tau_2}]$$

The spectrum of the components 1 is given by

$$F_1(\lambda) = F_\alpha(\lambda) = \frac{C_1(\lambda)\tau_1}{C_1(\lambda)\tau_1 + C_2(\lambda)\tau_2}F(\lambda) \quad (24)$$

The equilibrium constant in the ground state is

$$C = K = \frac{\tau_2}{\tau_1}\frac{S_1}{S_2} \quad (25)$$

with

$$S_1 = \int F_1(\lambda)d\lambda$$
$$S_2 = \int F_2(\lambda)d\lambda$$

(b) On the contrary if k and k' are much greater than k_1 and k_2, the equilibrium in the excited state will be reached much before the emission of fluorescence occurs. One can show that the decay is reduced to a single exponential.[18,23] We have

$$I_1(t) \simeq \frac{K_e}{1 + K_e}e^{-t/\tau}$$
$$I_2(t) \simeq \frac{1}{1 + K_e}e^{-t/\tau} \quad (26)$$

with

$$\frac{1}{\tau} = \frac{k_2 + K_ek_1}{1 + K_e} \quad (27)$$

τ is independent of the emission wavelength.

Results and Discussion

***cyclo*(Gly-Trp) and *cyclo*(Ala-Trp) in DMSO.** Fluorescence spectra of $AcTrpNH_2$, *cyclo*(Gly-Trp) and *cyclo*(Ala-Trp) are shown in Figures 1–3. The fluores-

(23) J. Klein, R. Voltz, and G. Laustriat, *J. Chim. Phys. Physicochim. Biol.*, 67, 704 (1970).

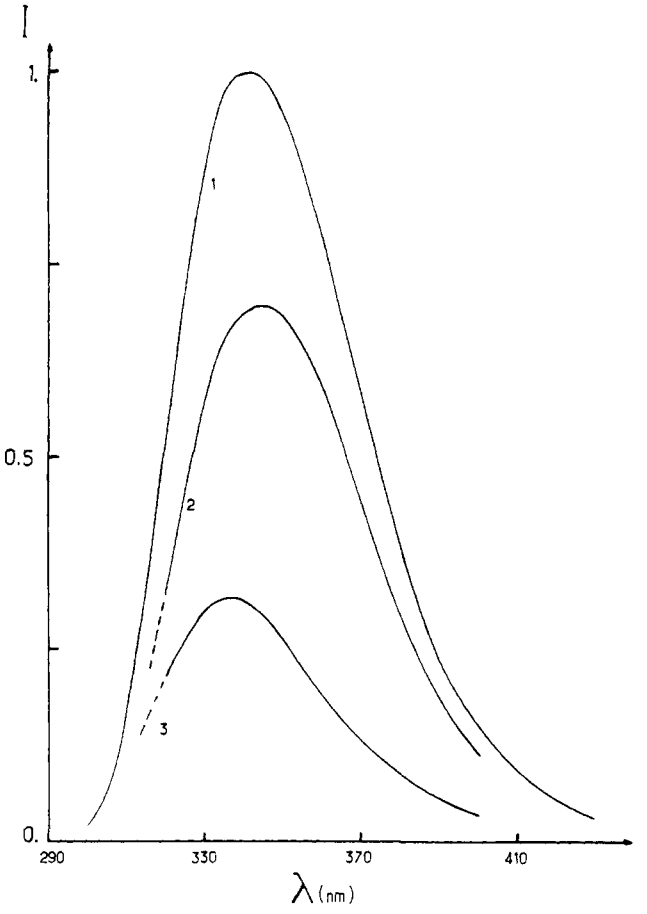

Figure 3. Fluorescence spectrum of *cyclo*(Ala-Trp) in DMSO at 18° (curve I); decomposition into two components corresponding to the unfolded form ($F_2(\lambda)$, curve 2) and to the folded one ($F_1(\lambda)$, curve 3). Excitation was at 280 nm.

Table I. Experimental Decay Times of *cyclo*(Gly-Trp) in DMSO and Water, and of *cyclo*(Ala-Trp) in DMSO[a]

	τ_α, nsec	τ_β, nsec	C	$M \times 10^{-9}$, sec^{-1}
cyclo(Gly-Trp) in DMSO	1.9	7	0.19	0.204
cyclo(Ala-Trp) in DMSO	2.7	7.3	0.26	0.185
cyclo(Gly-Trp) in water	0.3	3	14.5	3.14

[a] The values of the corresponding coefficients $C_\alpha(\lambda)$ and $C_\beta(\lambda)$ are given in Tables II, V, and III, respectively. C and M are calculated according to eq 19 and 20 (see text).

cence decays of these compounds together with that of skatole were measured at various emission wavelengths at 18°.

The decays of skatole and $AcTrpNH_2$ (Figure 4) are single exponential functions of time constants 7.5 and 7.3 nsec. They were found to be independent of the emission wavelength.

On the other hand, the decays of the diketopiperazines depend on the emission wavelength (Figures 4 and 5). The decay curves cannot be fitted to single exponential functions.

These results were analyzed using the modulating function method. The decays can be decomposed into two exponential decays the time constants τ_α and τ_β

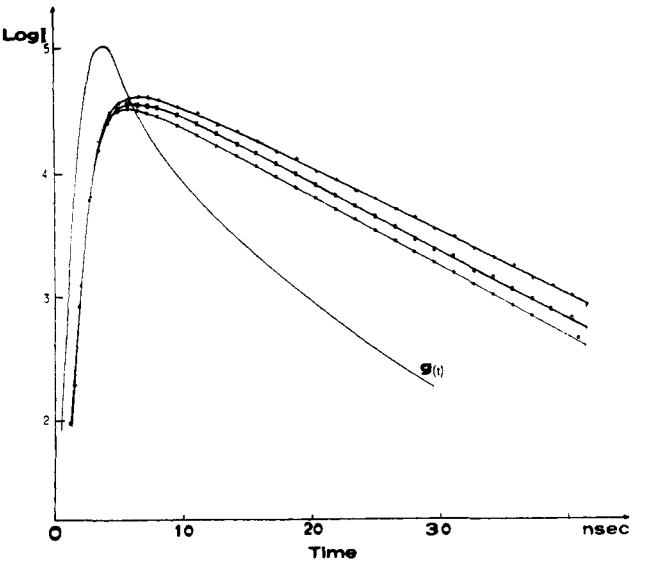

Figure 4. Fluorescence decays of $AcTrpNH_2$ in DMSO (+) emission wavelength 350 nm, and of *cyclo*(Gly-Trp) in DMSO at two emission wavelengths: 320 nm (●) and 360 nm (■). Excitation was at 280 nm, temperature = 18°.

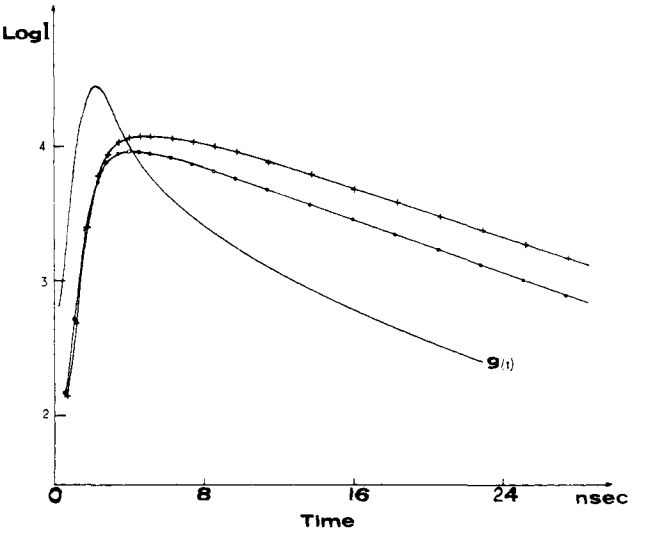

Figure 5. Fluorescence decays of *cyclo*(Ala-Trp) in DMSO at two emission wavelengths: 320 nm (●) and 380 nm (+). Excitation was at 280 nm, temperature = 18°.

of which are independent of the emission wavelength, within experimental error.

The best values for τ_α and τ_β are given in Table I. The coefficients $C_\alpha(\lambda)$ and $C_\beta(\lambda)$ are given in Tables II and III. $F_\alpha(\lambda)$ and $F_\beta(\lambda)$ can be calculated from (17) and the values of C and M can be derived from formulas 19 and 20. They are given also in Table I.

Hence the decay of the diketopiperazine is a two exponential decay; this is consistent with the presence of two fluorescent species, which we identify with the folded conformer and the average open conformer.

The rates of change of the conformation should not be too high, since in this case the decay would be a single exponential, according to relation 26.

It can be noticed that the time constant τ_β is close to the time constants of $AcTrpNH_2$ and skatole. It might be thought that this constant is characteristic of the unfolded conformation, in which the indole ring is

Table II. Fluorescence Decay of *cyclo*(Gly-Trp) in DMSO; Experimental Values of $C_\alpha(\lambda)$ and $C_\beta(\lambda)$ at Various Wavelengths, Temperature = 18°

λ, nm	315	320	325	330	335	340	350	360	380
$C_\alpha(\lambda)$	0.42	0.38	0.33	0.295	0.23	0.19	0.11	0.03	~0
$C_\beta(\lambda)$	0.58	0.62	0.67	0.705	0.77	0.81	0.89	0.97	~1

Table III. Fluorescence Decay of *cyclo*(Ala-Trp) in DMSO; Experimental Values of $C_\alpha(\lambda)$ and $C_\beta(\lambda)$ at Various Wavelengths, Temperature = 18°

λ, nm	320	330	340	345	350	355	360	365	370	380
$C_\alpha(\lambda)$	0.44	0.31	0.25	0.21	0.15	0.09	0.06	0.03	~0	~0
$C_\beta(\lambda)$	0.56	0.69	0.75	0.79	0.85	0.91	0.94	0.97	~1	~1

Table IV. Properties of the Excited State of *cyclo*(Gly-Trp) and *cyclo*(Ala-Trp) in DMSO[a]

	k_1, 10^8 sec^{-1}	$\tau_1 = 1/k_1$, nsec	Q_C/Q_A	k, 10^8 sec^{-1}	k', 10^8 sec^{-1}	K_e	ΔH_e, kcal/mol	ΔH^*, kcal/mol
cyclo(Gly-Trp)	0.239	4.2	0.92[b] 0.85[c]	2.7	0.23	0.085	−0.93	6.2
cyclo(Ala-Trp)	0.206	4.8	0.92[b] 0.87[c]	1.6				6.5

[a] τ_1 is the decay time of the isolated folded conformation. [b] Ratio Q_C/Q_A measured by the method of Parker and Rees.[13] [c] Q_C/Q_A calculated from decay measurements.

surrounded by solvent molecules. If this assumption was true, the equilibrium constants K and C should be identical (relation 25). However, the values given by nmr spectroscopy (K = 1.9 and 2.3 for *cyclo*(Gly-Trp) and *cyclo*(Ala-Trp), respectively[3,4]) are much greater than our values of C. This implies that the excited conformers are not in the equilibrium state. We must apply the general scheme described in the preceding section.

We assume that k_2 is equal to the reciprocal of the lifetime of $AcTrpNH_2$: $k_2 = 0.137 \times 10^9$ sec^{-1}.

Using the method previously described, one finds for k_1, k, k', and K_e the values given in Table IV. In the case of *cyclo*(Ala-Trp), the reciprocal of the decay time τ_β having the same value as k_2, one finds a zero value for k' and K_e (eq 21); it means that k' is too small to be experimentally determined.

The spectra $F_1(\lambda)$ and $F_2(\lambda)$ can be obtained from (23). They have been drawn in Figures 2 and 3. Their maxima are at 335 and 344 nm, respectively. They are the same for the two cyclopeptides studied.

These computations are based on the assumption that k_F is the same for two conformers. If this is true, k_F should also be the same for $AcTrpNH_2$. From (12) we obtain, if τ_A is the lifetime of $AcTrpNH_2$

$$\frac{Q_C}{Q_A} = \frac{k_F(\tau_\alpha + \tau_\beta - M\tau_\alpha\tau_\beta)}{k_F\tau_A} = \frac{\tau_\alpha + \tau_\beta - M\tau_\alpha\tau_\beta}{\tau_A}$$

where Q_C and Q_A are the quantum yields of the diketopiperazine and of $AcTrpNH_2$. The value of Q_C/Q_A calculated and directly measured by the method of Parker and Rees is given in Table IV.

The agreement between these two values is regarded as satisfactory. This finding reinforces the hypothesis of the identity of k_F for the two conformers.

In contrast to our finding in the excited state, the most favored conformation in the ground state is the folded one.[24] The enthalpy of interaction is −3.5 kcal/mol.[4b] The entropy change favors the unfolded form, and has a value of −8.1 eu.[4b] An important part of the entropy can be ascribed to internal rotations around C_α–C_β and C_β–Ar bonds. These rotations are blocked in the folded configuration and almost free in the open one. Making the assumption that the entropy change is the same in the excited state and in the ground state, the enthalpy change in the excited state will be obtained from the classical formula

$$\Delta H_e = -RT \ln K_e + T\Delta S$$

which provides the values given in Table IV.

This value is small. Hence the equilibrium in the excited state mainly depends on the entropy enhancement on going from the folded form to the unfolded one.

In other respects, the value of the rate constant of unfolding allows one to determine the activation energy. According to the absolute rate theory of Eyring, the rate constant of unfolding of the molecule is given by

$$k = \frac{2KT}{h} e^{-\Delta H^*/RT}$$

where K is the Boltzmann constant, h, the Planck constant, ΔH^*, the height of the potential barrier, that is the activation energy, and R the gas constant. The factor 2 is introduced because there are two possible rotations around the C_α–C_β bond which allow the opening of the folded form.

Taking the value of k determined above, we obtain the values of ΔH^* (Table IV).

cyclo(Gly-Trp) in Water. The fluorescence decays of skatole, $AcTrpNH_2$, and *cyclo*(Gly-Trp) were measured in water at a temperature of 6°.

The decays of skatole and $AcTrpNH_2$ (Figure 6) are single exponential functions without any dependence upon the emission wavelength. The time constants are 12.9 nsec for skatole and 3.8 nsec for $AcTrpNH_2$.

One can see that, in contrast to DMSO, the introduction of a linear peptide bond strongly quenches the

(24) In the case of *cyclo*(Gly-Trp).

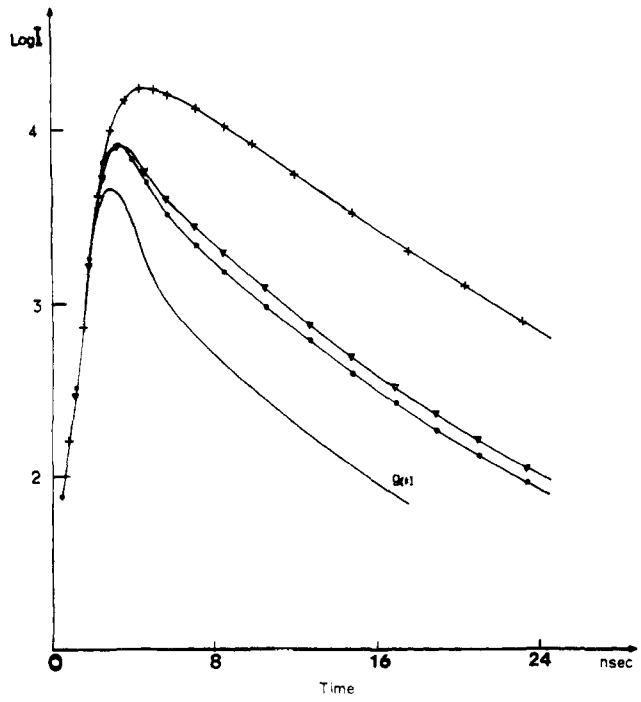

Figure 6. Fluorescence decays of $AcTrpNH_2$ in water (+) emission wavelength 360 nm and of *cyclo*(Gly-Trp) in water at two emission wavelengths: 320 nm (●) and 380 nm (▼). Excitation was at 280 nm, temperature = 6°.

Table V. Fluorescence Decay of *cyclo*(Gly-Trp) in Water; Experimental Values of $C_\alpha(\lambda)$ and $C_\beta(\lambda)$ at Various Wavelengths, Temperature = 6°

λ, nm	320	330	340	350	360	380
$C_\alpha(\lambda)$	0.964	0.945	0.94	0.934	0.931	0.911
$C_\beta(\lambda)$	0.036	0.055	0.06	0.066	0.069	0.089

fluorescence in water. This result is well known in accordance with many previous studies.[6]

The fluorescence decay of *cyclo*(Gly-Trp) is much more rapid than that of $AcTrpNH_2$. It depends on the emission wavelength as shown in Figure 6.

The analysis by the modulating functions method has shown that these decays can be fitted to a sum of two exponential functions; the values of τ_α and τ_β are given in Table I. The coefficients $C_\alpha(\lambda)$ and $C_\beta(\lambda)$ are given in Table V. The spectrum $F(\lambda)$ is drawn in Figure 7. Applying eq 19 and 20, one obtains the values of C and M given in Table I.

In this case we assume that k_2 is equal to the reciprocal of the lifetime of skatole, since the lifetime of $AcTrpNH_2$ is greatly diminished by the interaction of the peptide bond with the aromatic ring (which does not exist in DMSO): $k_2 = 0.0775 \times 10^9\ \text{sec}^{-1}$. For the aqueous solution of *cyclo*(Gly-Trp), the value of K has not been determined by nmr, due to the low solubility of the product. Hence we were not able to calculate the true rate constants and the true equilibrium constant in the excited state, K_e.

However, replacing in eq 10 M and k_2 by their value, k_1 can be expressed as a function of K, and then

$$\frac{1}{\tau_\alpha} - k_1 = 0.193\left(1 - \frac{15.9}{K}\right) \times 10^9\ \text{sec}^{-1}$$

From relation 22 the first term of this equality must be

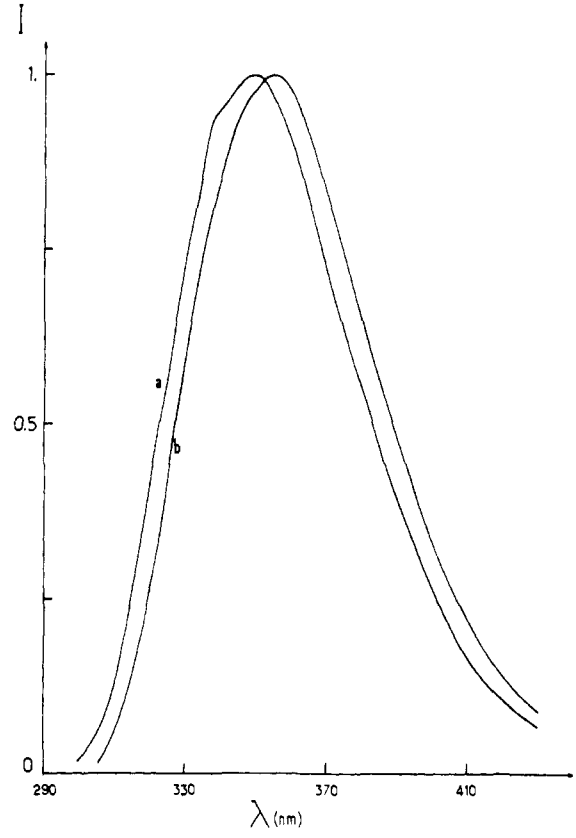

Figure 7. Fluorescence spectra of *cyclo*(Gly-Trp) (a) and $AcTrpNH_2$ (b) in water at 6°. Excitation was at 280 nm. Each spectrum has been normalized to 1 at the maximum.

positive or equal to zero, which implies for the second term

$$K \geq 15.9$$

Using formulas 21 and 4, it is not difficult to show that k_1, k, k', and K_e have a monotonous variation in the range of variation of K, so the following table can be set

$$15.9 \leq K < \infty$$

$$3.14 \times 10^9\ \text{sec}^{-1} < k_1 \leq 3.33 \times 10^9\ \text{sec}^{-1}$$

$$0 \leq k < 1.77 \times 10^8\ \text{sec}^{-1}$$

$$2.55 \times 10^8\ \text{sec}^{-1} \leq k' < 2.71 \times 10^8\ \text{sec}^{-1}$$

$$\infty > K_e \geq 1.53$$

As for *cyclo*(Gly-Trp) in DMSO, the times of exchange between the folded and unfolded forms are in the order of nsec and might even be greater for the unfolding of the molecule (characterized by the parameter k).

The value of k' may be considered to be already determined, since its variation with K does not exceed experimental imprecision. So one can take

$$k' = 2.6 \times 10^8\ \text{sec}^{-1}$$

In contrast, the value of k depends on K. One always has: $k < k'$, which is the opposite of what happens in DMSO. Hence K_e is always greater than 1. The folded form is favored in the excited state as in the ground state. It can be shown that for $K = 17.3$ one

has $K = K_e$; if $K > 17.3\ K_e$ is always smaller than K. The folded form again appears to be less stable in the excited state than it is in the ground state.

A more precise conclusion will have to await more accurate determinations of K in the ground state.

Let us reconsider the behavior of $AcTrpNH_2$. The rotation about the various nonrigid bonds may be assumed to be very fast, so that the concentrations of the excited conformers are in equilibrium, at the time of emission. In this case, the time constant is given by (26). Taking for k_1 and k_2 the value used for *cyclo*-(Gly-Trp), this formula leads to $K_e = 0.06$.

The excited indole ring would spend 5% of its time in the neighborhood of the peptide bound, and 95% of its time in contact with the water molecules.

Conclusion

The time resolved spectroscopy measurements give a number of precise details about the interactions in the aromatic diketopiperazines.

For the diketopiperazines in DMSO, according to the quantum yield measurements,[5b] the interaction is found to be weaker in the excited state than in the ground state (studied by nmr). Our results show that the concentrations of the conformer cannot reach their equilibrium value during the lifetime of the excited state. The rotation barrier which restricts the rate of exchange is about 6.4 kcal/mol. The equilibrium constant in the excited state is 0.08 for *cyclo*(Gly-Trp), while the nmr gives a value of 1.9 in the ground state. In the excited state, the energy of interaction of the indole ring with the diketopiperazine ring is not very different from the energy of interaction of indole with DMSO.

The behavior of *cyclo*(Gly-Trp) is very different in water. In this case a complete investigation is not possible, because the value of K has not been measured in the ground state. However, our results indicate that $K \geq 16$. Hence, the interaction of the indole ring in the ground state with the diketopiperazine is much stronger than in DMSO. (It must be noticed that the measurements in water and DMSO were not performed at the same temperature; however, the variation of temperature is too small to account satisfactorily for the change in equilibrium constant K.)

An increased stability of the folded conformer of the tryptophan side chain in water soluble cyclodipeptides has been observed,[3] but these result disagree with the nmr experiments made by Kopple and Ohnishi[4b] and Kopple and Marr[4a] on the diketopiperazine of tyrosine and alanine. These authors have found similar values of K in solvents as different as DMSO, dimethylformamide, trifluoroacetic acid, and water. Moreover, our results are not in accordance with those of Edelhoch, *et al.*,[5a] based on measurements of the CD of *cyclo*(Gly-Trp) in DMSO and water. Let us point out that these authors have not determined the values of K.

Our results also suggest that the folded form of *cyclo*(Gly-Trp) in water although favored in the excited state would be destabilized as compared to the ground state.

Kopple and Marr[4a] have discussed the nature of the forces acting in the conformational equilibrium of the aromatic diketopiperazines. They have emphasized the role of dipole–dipole induced interaction between dipole moments of the peptide bonds and the polarizable aromatic ring. If these forces play an actual part, DMSO, because of the great polarity of its molecules, should tend to favor the unfolded form, rather than water, the molecules of which have a smaller dipolar moment. Moreover, in water, hydrophobic interactions might increase the stability of the folded form. Our results are in good agreement with these assumptions. Surprisingly the case is different for the cyclopeptide of tyrosine and phenylalanine, as it follows from nmr studies. It must be noticed, however, that the nmr measurements are performed at a very high concentration of peptide (0.2 M). The intermolecular interaction might not be negligible in this range of concentration.