Vol. 114, No. 1, 1983 **BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS**
July 18, 1983 Pages 268-274

FLUORESCENCE STUDY ON THE CONFORMATION OF A CYCLIC ENKEPHALIN ANALOG IN AQUEOUS SOLUTION

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Received April 25, 1983

Conformational parameters of the potent cyclic enkephalin analogs [D-Cys^2,D-Cys^5]enkephalinamide and [D-Cys^2,Trp^4,D-Cys^5]enkephalinamide were determined by steady-state fluorescence measurements. The very low tyrosine fluorescence quantum yield observed with [D-Cys^2,D-Cys^5]enkephalinamide reflects a close intramolecular interaction between the phenol ring of Tyr^1 and the disulfide bridge. Evaluation of resonance energy transfer between Tyr^1 (donor) and Trp^4 (acceptor) of [D-Cys^2,Trp^4,D-Cys^5]enkephalinamide on the basis of Förster's equation resulted in an average intramolecular distance of 9.7 ± 0.2 Å between the two aromatic rings. This mean distance is nearly identical with the average Tyr^1 - Trp^4 distance (9.5 ± 0.3 Å) previously determined with the linear analog [D-Ala^2,Trp^4,Met^5]enkephalin.

The conformation of the opioid peptides Met- and Leu-enkephalin (Tyr-Gly-Gly-Phe-Met(or Leu)) in various states has been the subject of numerous investigations. While no consensus has been reached with regard to the solution conformation(s) of the enkephalins, increasing evidence for the existence of a conformational equilibrium in aqueous solution has been obtained in recent years (cf. ref. 1). Furthermore, the notion of conformational heterogeneity and flexibility of these peptides is quite compatible with the lack of specificity observed in their interaction with various opiate receptor subclasses. Recently, highly potent cyclic enkephalin analogs with either μ- or δ-receptor selectivity have been synthesized (2-6). Obviously, the number of conformational possibilities is greatly reduced in these semi-rigid cyclic analogs. Therefore, it is of interest to study their solution conformation to obtain insight into the receptor-bound conformation, since these compounds are unlikely to undergo drastic conformational changes during the process of binding to the receptor.

For the purpose of a conformational study by fluorescence techniques the 4-tryptophan analog of a potent cystine-containing enkephalin analog,

0006-291X/83 $1.50

[D-Cys^2,D-Cys^5]enkephalinamide (H-Tyr-D-Cys-Gly-Phe-D-Cys-NH_2) (4), was synthesized. Like its parent compound the resulting cyclic analog, [D-Cys^2,Trp^4,D-Cys^5]enkephalinamide (H-Tyr-D-Cys-Gly-Trp-D-Cys-NH_2) (Fig. 1), shows about 300-times higher potency than [Leu^5]enkephalin in the guinea pig ileum assay and is about as potent as the latter peptide in the mouse vas deferens assay (7). The tryptophan-containing cyclic analog permits the determination of the average intramolecular distance between the phenol ring of Tyr^1 (donor) and the indole moiety of Trp^4 (acceptor) by evaluation of singlet-singlet energy transfer between donor and acceptor on the basis of the Förster equation (8). In order to assess the effect of the conformational restriction on the separation between the two aromatic rings in positions 1 and 4, it is of interest to compare the average Tyr^1-Trp^4 intramolecular distance resulting for the cyclic peptide with the corresponding distance previously determined with the open-chain analog [D-Ala^2,Trp^4,Met^5]enkephalin (9). Finally, determination of the tyrosine and tryptophan fluorescence quantum yields of the cyclic analogs [D-Cys^2,D-Cys^5]enkephalinamide and [D-Cys^2,Trp^4,D-Cys^5]enkephalinamide and their interpretation in relation to the quantum yields obtained for the corresponding open-chain analogs can be expected to provide some insight into the side-chain orientation in positions 1 and 4.

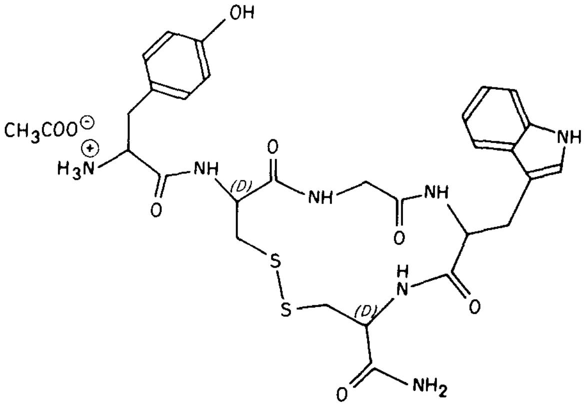

Figure 1. Structural formula of the cyclic enkephalin analog H-Tyr-D-Cys-Gly-Trp-D-Cys-NH_2.

MATERIALS AND METHODS

The preparation and pharmacological characterization of [D-Cys^2,Trp^4,D-Cys^5] enkephalinamide will be described elsewhere (7). The syntheses and biological activities of [D-Cys^2,D-Cys^5]enkephalinamide and of [D-Ala^2,Met^5]enkephalin and [D-Ala^2,Trp^4,Met^5]enkephalin have been reported (4,9).

The extinction coefficients of the disulfide chromophore in the wavelength region from 260 to 330 nm were determined by subtracting the absorption spectrum of two phenylalanine residues from that of the analog [Phe^1,D-Cys^2,D-Cys^5]enkephalinamide (7). The contribution of the disulfide moiety to the absorption spectrum was taken into account in all fluorescence determinations with the cyclic analogs. For fluorescence studies 3 x 10^{-5}M solutions of peptides were made up in tridistilled H_2O and fluorescence spectra were recorded on a Hitachi-Perkin Elmer fluorometer MPF-2L. Fluorescence quantum yields were measured by comparison of the emission spectra of the peptides with those of L-tyrosine and L-tryptophan (9), whose quantum yields in H_2O were taken as 0.14 and 0.13, respectively (10).

Tyr-Trp distances, r, were determined based on Förster's equation (8) using the relation

$$r = (E^{-1} - 1)^{1/6} R_o \qquad (1),$$

whereby the transfer efficiency, E, was calculated from the measured donor (tyrosine) quantum yields in the presence (ϕ_D) and absence (ϕ_D^o) of transfer ($E = 1-\phi_D/\phi_D^o$). ϕ_D was obtained as described (11), and ϕ_D^o was determined with the corresponding analogs containing phenylalanine in place of tryptophan in position 4. The Förster critical distance, R_o (cm), is determined experimentally according to eq. 2

$$R_o = [(8.79 \times 10^{-25})(\kappa^2/n^4)\phi_D^o\, J_{AD}]^{1/6} \qquad (2),$$

where κ^2 = dipole-dipole orientation factor, n = refractive index and $J_{AD} = \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$ = spectral overlap integral between the molar decadic absorption coefficient of the acceptor, ε_A, and the spectral distribution of the donor fluorescence normalized to unity, F_D, modified by the wavelength factor λ^4. A value of 2/3 was used for κ^2 on the basis of arguments presented elsewhere (9) and a value of $J_{AD} = 4.8 \times 10^{-16}\ M^{-1}cm^6$ was adopted from the literature (12).

RESULTS AND DISCUSSION

The tyrosine fluorescence quantum yield determined with [D-Cys^2,D-Cys^5]-enkephalinamide ($\phi^{Tyr} = 0.009$) is nearly 2.5 times lower than that of the linear analog [D-Ala^2,Met^5]enkephalin (Table 1) and, in fact, represents the lowest quantum yield in absence of energy transfer which has been reported for a tyrosyl peptide to date (cf. ref. 13). The comparison with the linear peptide indicates that the drastic quenching of the tyrosine fluorescence observed with the cyclic analog is caused by the adjacent disulfide group. The mechanism by which disulfide moieties quench tyrosine or tryptophan fluorescence has not yet been clearly established; however, studies with model compounds (14) showed that close proximity (< 6 Å) between the aromatic ring of the

Table 1. Tyrosine and Tryptophan Fluorescence Quantum Yields, ϕ, of Enkephalin Analogs[a]

Analog	ϕ^{Tyr}	ϕ^{Trp}
H-Tyr-D-Cys-Gly-Phe-D-Cys-NH$_2$	0.009 ± 0	–
H-Tyr-D-Ala-Gly-Phe-Met-OH[b]	0.022 ± 0.001	–
H-Tyr-D-Cys-Gly-Trp-D-Cys-NH$_2$	0.006 ± 0.001	0.036 ± 0.001
H-Tyr-D-Ala-Gly-Trp-Met-OH[b]	0.008 ± 0.001	0.059 ± 0.001

[a] Mean of three determinations ± S.E.M. [b] Results taken from ref. 9.

fluorescent residue and the disulfide group, permitting a sulfur-¶ bond interaction, is required. Therefore, we can exclude extended conformations of the tyrosyl side-chain pointing away from the disulfide bridge which could result in an intramolecular distance between the centers of the phenol ring and the S-S moiety of up to 9 Å. In fact,it is quite possible that the phenol ring and the disulfide group form an energetically favorable intramolecular complex. The heat of formation for this type of complex has recently been shown to be of the order of 1 kcal/mol (15,16) at an optimal distance of about 3.5 Å between the plane of the aromatic ring and one of the sulfur atoms. Based on these findings and arguments a close interaction between the tyrosyl side-chain and the disulfide bridge in [D-Cys^2,D-Cys^5]enkephalinamide is proposed.

The tryptophan fluorescence quantum yield of the cyclic analog [D-Cys^2,-Trp^4,D-Cys^5]enkephalinamide is reduced by 40% as compared to that of the corresponding linear analog [D-Ala^2,Trp^4,Met^5]enkephalin (Table 1). Even though this reduction is not as drastic as in the case of the tyrosyl residue of [D-Cys^2,D-Cys^5]enkephalinamide, some quenching of the tryptophan fluorescence by the disulfide group in the cyclic peptide is indicated by this result. The emission maximum at 350 nm observed with [D-Cys^2,Trp^4,D-Cys^5]enkephalinamide

Table 2. Förster Parameters and Average Intramolecular Tyr^1-Trp^4 Distances, r, of Enkephalin Analogs[a]

Analog	ϕ_D^0	E	R_0 [Å]	r [Å]
H-Tyr-D-Cys-Gly-Trp-D-Cys-NH_2	0.009	0.37 (0.35-0.40)	8.9	9.7 ± 0.2
H-Tyr-D-Ala-Gly-Trp-Met-OH[b]	0.022	0.62 ± 0.05	10.3	9.5 ± 0.3

[a] Values shown are the mean of three determinations ± S.E.M. Numbers in parentheses represent upper and lower limit. [b] Data taken from ref. 9.

indicates that the tryptophyl residue is located in a predominantly aqueous environment, as is the case with [D-Ala^2,Trp^4,Met^5]-enkephalin.

The efficiency of energy transfer between Tyr^1 and Trp^4 in [D-Cys^2,-Trp^4,D-Cys^5]enkephalinamide is considerably lower (E = 0.37) than that observed between the corresponding residues in [D-Ala^2,Trp^4,Met^5]enkephalin (E = 0.62) (Table 2). However the R_0-value of the cystine-containing analog is also smaller because of the low tyrosine fluorescence quantum yield and, therefore, a similar average intramolecular distance (9.7 ± 0.2 Å) between the phenol ring of Tyr^1 and the indole moiety of Trp^4 results for the cyclic analog as for the linear peptide (9.5 ± 0.3 Å).

A theoretical conformational analysis performed with [D-Ala^2,Trp^4,Met^5]enkephalin indicated the existence of an equilibrium of conformers with Tyr^1-Trp^4 intramolecular distances ranging from 5 to 15 Å (17). The average transfer efficiency computed for this ensemble of conformers was found to be in good agreement with the experimentally determined value. The predominant Tyr^1-Trp^4 distance of 9 Å resulting from this analysis is comparable to the intramolecular distance between the two aromatic rings contained in the potent morphine derivative 7α-(1-(R)-hydroxy-1-methyl-3-phenylpropyl)-6,14-endo-ethenotetrahydrooripavine (PEO). This observation is noteworthy in view of the proposal that the aromatic side-chain in position 4 of enkephalin might play the same role as the phenylethyl substitutent on carbon-19 of PEO in the interaction with opiate receptors (18). It would be of considerable interest to perform a theoretical conformational analysis with the cyclic analog and to compare the resulting distance distribution with that of the linear peptide. Heterogeneity in the overall conformation of the cyclic peptide is of course reduced because of the introduced conformational constraint. Nevertheless, inspection of a CPK-model of [D-Cys^2,Trp^4,D-Cys^5]enkephalinamide reveals that depending on the orientations of the exocyclic tyrosyl residue and the tryptophyl side-chain intramolecular distances between the two aromatic rings ranging from 6 to 16 Å can be obtained. However, the energetically favorable interaction between the phenol ring and the disulfide group mentioned above might reduce the conformational heterogeneity of the tyrosyl side-chain and thereby narrow the range of intramolecular Tyr^1-Trp^4 distances.

It has been proposed (19) that the binding sites for the aromatic rings in positions 1 and 4 of enkephalin may lie closer together in μ-receptors than in δ-receptors. In the present study no difference in the average phenol-indole intramolecular distance is observed between the δ-receptor-selective linear analog (7) and the cyclic analog which shows a slight preference for μ-receptors (4). However, it is possible that the predominant conformer(s) of the cyclic analog might show a shorter distance between the two aromatic rings. Of course, it has also to be kept in mind that the side-chain topography might change in the process of binding to the receptor.

ACKNOWLEDGMENT

This work was supported by operating grants from the Medical Research Council of Canada (MT-5655) and the Quebec Heart Foundation.

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