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Two new fluorophore pairs, tyrosine–4′-aminophenylalanine and tyrosine–4′-dimethylaminophenylalanine, in the determination of molecular dimensions in peptides

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Peptides and polypeptides containing tyrosine and 4′-aminophenylalanine or tyrosine and 4′-dimethylaminophenylalanine were studied by electronic absorption and fluorescence spectroscopy. Positions of the band maxima, pK_a values of the aromatic ammonium group and distances between the two fluorophores in seven different compounds are compared.

1. Introduction

It has previously been shown that 4′-aminophenylalanine (APhe) and 4′-dimethylaminophenylalanine (DMAPhe) can be used in structural, fluorescence investigations of peptides [1,2]. The aim of the present work is to compare the results of fluorimetric and spectrophotometric studies of several biologically active peptides and polypeptides containing tyrosine (Tyr) and APhe or DMAPhe fluorophores.

The Tyr-APhe pair was used for determination of the molecular dimensions of oligopeptides. The APhe residue was introduced into the peptide chain in place of the naturally occurring phenylalanine. In this manner, we investigated two enkephalin analogs and a hexapeptide fragment of proline-rich polypeptide (PRP). The latter polypeptide has been found in sheep colostrum [3] and possesses immunoregulatory activity.

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The Tyr-DMAPhe pair was used in studying the spatial structure of a trypsin inhibitor isolated from squash seeds (CMTI-I), which is a polypeptide containing 29 amino acid residues. For the labelling of CMTI-I with DMAPhe, the azalactone of *N*-acetyl-DMAPhe was used. The reaction conditions were determined previously, using lysozyme as a standard protein [2].

2. Experimental

The syntheses of the oligopeptides studied have been described elsewhere (for references and abbreviations see table 1).

CMTI-I was obtained according to the method described in ref. 4. For CMTI-G, a sample of CMTI-I was subjected to guanidylation with *o*-methylisourea according to the method of Kimmel [5]. Lysozyme was purchased from C. Both (F.R.G.). The syntheses of the azalactones of *N*-acetyl-APhe and *N*-acetyl-DMAPhe have been described by Siemion et al. [2].

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2.1. Modification of CMTI-I, CMTI-G and lysozyme with the azalactone of N-acetyl-DMAPhe

To a stirred solution of 1 mg of protein or polypeptide in 0.3 ml phosphate buffer (0.66 M, pH 7.4), a 5% solution of the azalactone in dioxane was added. A 200-fold molar excess of azalactone was used. The turbid solution was stirred for 1–2 h until it became transparent. The reaction mixture was applied to a Sephadex G-15 column (1–70 cm) equilibrated with 0.1 M ammonium carbonate (pH 8.4). The eluent was checked for the presence of the modified material and DMAPhe by means of the absorbance at 220 and 250 nm, respectively. Fractions containing labelled protein or inhibitor were freeze dried and the product rechromatographed under the same conditions. The amount of marker in the samples was determined spectrophotometrically (220 and 250 nm). Unmodified material and *N*-acetyl-DMAPhe were used as standards. The results were processed using the formula published in the paper by Jaffe and Orchin [6].

Homogeneity of the samples was controlled by gel electrophoresis. The amino acid composition of CMTI-I and CMTI-G was analyzed using an AAA 881 amino acid analyzer (Microtechna, Czechoslovakia). Protein samples were hydrolyzed in 6 N HCl at 100 °C for 24 h. The content of basic amino acids in CMTI-I was: His, 1.00; Arg, 1.85; Lys, 2.27; homo-Arg, 0.00; in CMTI-G, the respective values were: His, 1.00; Arg, 1.76; Lys, 0.25; homo-Arg, 1.63.

2.2. Spectrophotometric and spectrofluorimetric measurements

Absorption spectra were recorded with a Specord ultraviolet-visible spectrophotometer (C. Zeiss, G.D.R.) and fluorescence spectra using a Perkin-Elmer (U.S.A.) model 204 spectrofluorimeter. Details of the experimental procedures are given elsewhere [1,2,7]. Measurements on fluorescence polarization were made with a Perkin-Elmer (U.S.A.) MPF 44 apparatus equipped with a polarization accessory produced by Hitachi (Japan).

The efficiency of intramolecular energy transfer from Tyr (donor) to APhe or DMAPhe (acceptor) in the peptides investigated was determined via two independent approaches; (i) from the increase in acceptor fluorescence and (ii) from the decrease in donor emission intensity due to energy transfer.

In method i, the formula given by Efremov et al. [8] was employed. The equation of Schiller [9] was used in method ii. The fluorescence quantum yield of donor in the absence of energy transfer, needed for method ii and for the determination of R_0 (see below), was estimated by acidification of the solution to a pH of at least 3.2, which gives rise to bleaching the APhe and DMAPhe [7]. The distance (r) between the centers of the aromatic rings of Tyr and APhe (DMAPhe) in the investigated compounds was calculated from eq. 1 (see ref. 9):

$$r = (E^{-1} - 1)^{1/6} R_0 \qquad (1)$$

where E is the efficiency of energy transfer and R_0 Förster's critical distance characteristic for a given donor-acceptor pair. The R_0 value was determined to be 1.06 nm for the Tyr-APhe pair and 1.08 nm for the Tyr-DMAPhe pair [1,2].

The pK_a values of the aromatic ammonium group of APhe and DMAPhe were determined graphically from the pH dependence of the extinction coefficient at 236 or 250 nm. The band maxima of APhe and DMAPhe in peptides containing Tyr were determined from the maximal deflection in the spectrophotometric titration curves.

3. Results and discussion

The substances studied are summarized in table 1. The positions of the absorption and fluorescence band maxima and pK_a values of APhe and DMAPhe in the peptides investigated are given in table 2.

The absorption spectra of APhe and DMAPhe in the near-ultraviolet region resemble those of aniline and dimethylaminobenzene; in DMAPhe the lowest lying absorption band is poorly resolved and, in some cases, its maximum position could not be determined.

Table 1

Description of the compounds studied

Compound	Formula	Abbreviation	Ref.
(1) (APhe^4,Leu^5)enkephalin	H-Tyr-Gly-Gly-APhe-Leu-OH	AEnk	17
(2) Cyclic enkephalin analog	H-Tyr-D-Lys-Gly-APhe-Leu	c-AEnk	18
(3) Hexapeptide fragment of proline-rich polypeptide (PRP)	H-Tyr-Val-Pro-Leu-APhe-Pro-OH	A-PRP	19
(4) Ethyl ester of APhe-glycylglycine	Ac-APhe-Gly-Gly-OEt	APGGOEt	2
(5) Ethyl ester of DMAPhe-glycylglycine	Ac-DMAPhe-Gly-Gly-OEt	DMAPGGOEt	2
(6) Trypsin inhibitor from squash seeds modified with DMAPhe		CMTI-I-DMAPhe	
(7) Guanidylated trypsin inhibitor from squash seeds modified with DMAPhe		CMTI-G-DMAPhe	
(8) Lysozyme modified with DMAPhe		lysozyme-DMAPhe	

One can see from table 2 that the microenvironment (i.e., the local electron charge density and local dielectric constant) can influence the pK_a of the aromatic ammonium group considerably, especially in proteins (CMTI-I and lysozyme). The same phenomenon may be responsible for the variation in positions of the absorption and fluorescence bands in the peptides studied. Differences in fluorescence quantum yield suggest that the peptide bonds and the protonated carboxyl group [7] quench the fluorescence of APhe and DMAPhe through an intramolecular interaction.

Table 3

Mean values of energy-transfer efficiency (E) and distance (r) between centers of aromatic rings in peptides and polypeptides containing the Tyr-APhe or Tyr-DMAPhe fluorophore pair

E_i and r_i, calculated from increase in acceptor fluorescence; E_{ii} and r_{ii}, calculated from decrease in donor fluorescence. AU values given have an accuracy of ± 0.01.

Compound	E_i	E_{ii}	r_i (nm)	r_{ii} (nm)
AEnk	0.53	0.48	1.04	1.08
c-AEnk	0.24	0.32	1.28	1.20
A-PRP	0.32		1.19	
CMTI-I-DMAPhe	0.196	0.26	1.35	1.29

Table 2

Absorption and fluorescence band maxima and pK_a values of the aromatic ammonium group in APhe and DMAPhe, in different molecular environments (for abbreviations see table 1)

Compound	Absorption band maximum (nm)		Fluorescence maximum (nm)	Fluorescence quantum yield	pK_a
	L_a	L_b			
APhe, unsubstituted	236.0	284.3	345	0.086 ± 0.001	4.72 ± 0.01
DMAPhe, unsubstituted	248.0	285	365	0.220 ± 0.001	5.40 ± 0.01
APGGOEt	237.6	286.7	348	0.067 ± 0.001	4.25 ± 0.01
DMAPGGOEt	250.5	285	360	0.125 ± 0.001	5.09 ± 0.01
AEnk	237		345	0.04 ± 0.01	4.90 ± 0.01
c-AEnk	241		350	0.01 ± 0.01	
A-PRP	239		352	0.03 ± 0.01	4.80 ± 0.01
Lysozyme-DMAPhe	249				4.00 ± 0.01
CMTI-I-DMAPhe	250		362		5.50 ± 0.01

The efficiencies of intramolecular, radiationless energy transfer and mean distances between the aromatic rings of Tyr and APhe (DMAPhe) in the peptides investigated are listed in table 3.

It can be seen from these results that the agreement between the two methods of determination of energy-transfer efficiency described in section 2 is reasonable. Method i (using the increase in acceptor fluorescence) is considered to be theoretically more exact than method ii (employing the decrease in donor emission) [12]. The latter approach, however, is simpler and therefore less prone to experimental error. One of the difficulties encountered here is the need for precise determination of the intrinsic fluorescence quantum yield of the donor, in a system where both donor and acceptor are present. Our procedure, consisting of the acidification of the solution to pH 3.2 which bleaches APhe and DMAPhe as acceptors of energy, is valid only under the condition that lowering the pH does not influence the donor (Tyr) fluorescence intensity. However, it is well known [10] that the fluorescence of Tyr is strongly quenched by a protonated carboxyl group at pH < 3. This effect depends on the actual pK_a of the quenching carboxyl group positioned near Tyr in the molecule under study – a value which is usually not known with sufficient precision. Therefore in most cases, the value of r obtained via method ii is less reliable than that calculated according to method i. In the case of c-AEnk (see table 1) however, which has no free carboxyl group, method ii is more reliable.

3.1. Intramolecular distances (r) in oligopeptides

The distance between Tyr and APhe in AEnk ($r = 1.08$ nm) that we have determined is in agreement with the results obtained for different enkephalin analogs by others [11,12]. This value of r suggests that a distinct proportion of folded conformational forms appears in the conformational equilibrium of AEnk in water.

In organic solvents of moderate polarity, e.g., acetonitrile, lower value of r for AEnk (0.85 nm) has been reported [13].

Comparison of the r values for AEnk and its analog c-AEnk ($r = 1.2$ nm) shows that cyclization considerably increases the mean distance between the aromatic rings.

In A-PRP hexapeptide, Tyr and APhe are separated by three amino acid residues, instead of two as in AEnk and c-AEnk. Therefore, one can anticipate a greater interfluorophore distance in A-PRP than in AEnk, which is indeed the case ($r = 1.19$ nm).

The mean conformation in a DMSO solution of the hexapeptide fragment of PRP (Tyr-Val-Pro-Leu-Phe-Pro) has been studied by ^{1}H- and ^{13}C-NMR spectroscopy (I.Z. Siemion et al., in preparation). The results of this study are not inconsistent with our spectrofluorimetric data.

The fluorescence polarization of A-PRP was also measured. The results (table 4) support the conclusion that the fluorophore groups in A-PRP in aqueous solution rotate rapidly relative to the fluorimetric time scale.

It can be concluded from the results described above that APhe may be useful in the investigation of the mean conformation of peptides in solution.

Table 4

Fluorescence polarization of A-PRP

Excitation wavelength (nm)	Emission wavelength (nm)	Degree of polarization	
		0.1 M phosphate buffer (pH 7.4)	50% polyvinyl alcohol (pH 7.4)
275	300	0.075 ± 0.001	0.12 ± 0.001
250	350	0.067 ± 0.001	–

3.2. Molecular dimensions in polypeptides (CMTI-I)

The synthesis of natural peptide analogs containing the APhe residue is a relatively simple task. Hydrogenation of a peptide containing 4′-nitrophenylalanine transforms this group into an APhe residue. We were interested by the consideration of using APhe as a fluorescent label for proteins. For this purpose, we investigated the reaction of the azolactone of *N*-acetyl-APhe with proteins, followed by hydrogenation of nitro groups to amino groups. The results were unsatisfactory, however, due to failure of the hydrogenation reaction. Therefore, we used the azolactone of

N-acetyl-DMAPhe which is quite stable and easy to obtain. The labelling reaction was investigated using lysozyme. This approach was also employed in studying the molecular dimensions of CMTI-I (trypsin inhibitor from squash seeds).

The azolactone of *N*-acetyl-DMAPhe reacts principally with the amino groups in proteins [2]. In the trypsin inhibitor CMTI-I, only the α-amino group of Arg^1 and ϵ-amino groups of Lys^{11} and Lys^{12} can be modified. To obtain a derivative specifically labelled only at Arg^1, the guanidylated inhibitor CMTI-G was used. As the guanidine groups (i.e., homoarginine residues) do not react with azolactones under the conditions used, substitution of CMTI-G can occur only at the N-terminus of the polypeptide. CMTI-I possesses only one Tyr residue (Tyr^{27}). Therefore, the labelled inhibitor CMTI-G-DMAPhe represents a 1:1 donor-acceptor system. Spectrophotometric determination of the substitution yield showed that, on average, one DMAPhe residue was introduced per molecule of CMTI-G. Amino acid analysis of CMTI-G (see section 2) and electrophoresis of CMTI-G-DMAPhe (fig. 1) show that labelled CMTI-G was not completely homogeneous. These small amounts, of impurities did not (see below) appear to affect the results of the measurements.

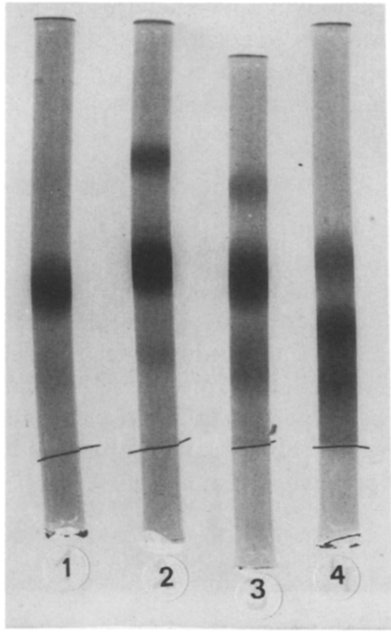

Fig. 1. Polyacrylamide gel (pH 8.6) electrophoresis of: (1) CMTI-I, (2) CMTI-G, (3) CMTI-G-DMAPhe, (4) CMTI-I-DMAPhe.

The reaction of CMTI-I with the azolactone of *N*-acetyl-DMAPhe gave a product containing, on average, two residues of fluorescent marker per inhibitor molecule. This material was also electrophoretically inhomogeneous (fig. 1). The amino groups of Lys^{11} and Lys^{12} could be substituted in this case, in addition to the N-terminal amino group of Arg^1. The possible presence of a small amount of material with both ϵ-amino groups substituted could not be excluded. The isolation of individual fractions from this mixture was not possible because of the very small amount of product obtained. Based on the estimation of possible contributions by variously substituted CMTI-I-DMAPhe molecules, we considered the r value calculated from energy-transfer determinations as the mean distance between Tyr^{27} and the sequence Lys^{11}-Lys^{12}.

Accurate determination of R_0, the critical Förster's distance, is of primary importance for the correct evaluation of the distance between the fluorophore pair. In order to determine R_0, it is necessary to know the orientation factor of donor and acceptor transition moments (κ). In the case of Tyr-APhe and Tyr-DMAPhe pairs in the compounds studied, a value of $\kappa^2 = 2/3$ was used [1,2], which is characteristic of freely rotating fluorophores. The assumption of free rotation of fluorophores was confirmed for A-PRP by fluorescence polarization measurements. For a polypeptide like CMTI-I, the possibility of restricted rotation of some amino acid side groups must be taken into account. Therefore, experimental verification of the assumed value of $\kappa^2 = 2/3$ was needed. For this purpose, the fluorescence polarization of CMTI-G-DMAPhe was measured (table 5).

By exciting the sample at 250 nm and analyzing the emitted light at 365 nm the polarized fluorescence of the DMAPhe residue was measured. The fluorescence polarization of the Tyr residue was determined by excitation at 275 nm and detection at 300 nm. From the results of these experiments (table 5), the error in determining the distance

Table 5

Fluorescence polarization of CMTI-G-DMAPhe in 0.1 M phosphate buffer (pH 7.4)

Excitation wavelength (nm)	Emission wavelength (nm)	Degree of polarization
250	365	0.0399
295	365	0.0345
275	300	0.2368
275	365	0.0250

between fluorophores due to inaccuracy in the κ value was estimated to be ± 0.15 nm, using the procedure of Haas et al. [14].

Average values for the interfluorophore distances in CMTI-I-DMAPhe are given in table 3. More detailed results for CMTI-I-DMAPhe and CMTI-G-DMAPhe are presented in table 6. The experimental values of the energy-transfer efficiency (E) were corrected following the procedure of Epe et al, [15]. One can observe from table 6 that intramolecular energy transfer between DMAPhe bound to Arg^1 and Tyr^{27} in CMTI-G-DMAPhe is negligible. In only one out of three measurements was a small effect detected corresponding to a distance r between the fluorophores of approx. 1.9 nm. However, the E value was within experiment error. The range of applicability of measurements of energy transfer between Tyr and DMAPhe for distance determination was estimated to be 1.7 nm (see table 6). This leads to the conclusion that the distance between DMAPhe anchored at the Arg^1 and Tyr^{27} residues is at least 1.7 nm. This weak interaction between the two fluorophores allows us to ignore its effect on the results concerning CMTI-I-DMAPhe, where lysine residues as well as Arg^1, are substituted. The total amount of energy transfer of CMTI-I-DMAPhe can be attributed to the interaction between Tyr^{27} and DMAPhe bound to the Lys^{11}-Lys^{12} sequence. Thus, based on our results, it is possible to assess the distance between Tyr^{27} and DMAPhe bound to the Lys^{11}-Lys^{12} fragment to be approx. 1.35 nm. According to a model of the CMTI-I molecule proposed by Siemion et al. [16], it can be assumed that Arg^1 and the Lys^{11}-Lys^{12} fragment are situated on two opposite edges of the CMTI-I molecule, on two opposite sides of Tyr^{27}. Taking into account our results, it follows that the length of the long axis of the CMTI-I molecule should be at least 3.05 nm.

Table 6

Energy-transfer efficiency (E) and intramolecular distance (r) between fluorophores in CMTI-I-DMAPhe and CMTI-G-DMAPhe

Sample	Marker/peptide concentration ratio	E_i [a]	E_{ii} [b]	r (nm) calculated from E_i
(1) CMTI-G-DMAPhe	1.4:1.5	0.033 [c]	0.110	1.9
(2) CMTI-G-DMAPhe	1.4:1.8	0.000	0.000	
(3) CMTI-G-DMAPhe	1.5:1.5	0.000		
(4) CMTI-I-DMAPhe	1.2:0.6	0.150		1.41 ± 0.16 [d]
(5) CMTI-I-DMAPhe	2.8:1.6	0.227		1.32 ± 0.16 [d]
(6) CMTI-I-DMAPhe [e]	3.4:1.8	0.211	0.26	1.34 ± 0.16 [d]

[a] Determined from the increase in acceptor fluorescence.

[b] Determined from the decrease in donor fluorescence.

[c] Within the range of experimental error, which was estimated to be 0.06, taking into account the reproducibility of a particular measuring series. For $E = 0.06$ one obtains (eq. 1) $r = 1.7$ nm. Therefore, the latter value represents the maximum distance for which the method operates accurately.

[d] Error estimate includes the errors resulting from measurements of E (see [c]) and that due to inaccuracy in the orientation factor of the transition moments of fluorophores (κ) (see ref. 14).

[e] Sample modified at higher pH (pH 7.9).

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