TYROSINATE FLUORESCENCE MAXIMA AT 345 nm IN PROTEINS LACKING TRYPTOPHAN AT pH 7

A. G. SZABO, K. R. LYNN, D. T. KRAJCARSKI and D. M. RAYNER
Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A OR6, Canada

Received 11 July 1978

1. Introduction

Advantage is often taken of the different fluorescence properties of two aromatic amino acids, tyrosine and tryptophan, to study the conformations, environmental properties and interactions of proteins containing these amino acids [1,2]. In this work we report the novel fluorescence properties of two peptides, isolated from the Indian Cobra *Naja naja* which do not contain any tryptophan yet whose fluorescence spectra include a maximum at 345 nm.

2. Materials and methods

Corrected fluorescence spectra were measured on a Perkin Elmer MPF-44A spectrofluorimeter equipped with a microprocessor corrected spectral attachment unit. The solutions of the peptides in either 0.01 M cacodylate or 0.01 M phosphate buffers, at pH 7, had an absorbance <0.05 at the excitation wavelength as measured on a Cary 118C spectrophotometer.

The two cytotoxins designated herein as CTA and CTB were isolated from *Naja naja* venom purchased from Ross Allen Venom Laboratory, Silver Springs, FL, according to procedures outlined in [3]. The two peptides were recycled on the ion-exchange column used in the original separation, desalted and lyophilized. Their amino acid compositions agreed with those reported for CM-XI and CM-XII, respectively [4]. Paper electrophoresis showed the fractions to be homogeneous. N-terminal sequences were determined by the methods in [5]. The first 24 residues of each peptide, CTA and CTB, agreed with those in [6,7]. The absence of tryptophan was verified by analysis with *p*-toluenesulphonic acid [8] and the procedure in [9].

The *S*-carboxymethyl derivatives of the two peptides were prepared by first reducing the cysteine bonds in mercaptoethanol (100 μmol) and 8 M urea under nitrogen for 4 h. Recrystallized iodoacetic acid (500 μmol) in 1 M sodium hydroxide was added and maintained at pH 8.0 during the addition with 4 M sodium hydroxide. After 12 h the reaction mixture was adjusted to pH 5 and the *S*-carboxymethyl derivatives were obtained in 70% yields by chromatography on a Sephadex G-15 column, elution with 1% acetic acid/water and subsequent lyophilization. Gel electrophoresis showed these derivatives to be homogeneous. Amino acid analysis of acid hydrolysates demonstrated the complete absence of cystine residues and the formation of 8 *S*-carboxymethylated cysteine residues/molecule.

3. Results

The corrected fluorescence spectra of buffered (pH 7) solutions of CTA and CTB are presented in fig.1. With excitation at 275 nm the fluorescence spectrum of CTA is characterized by a broad band with a maximum at 344 nm and a small shoulder at ~305 nm while that of CTB has a maximum at 304 nm with a marked shoulder between 335 nm and 350 nm. With excitation changed to 290 nm the fluorescence spectrum of CTA shows a slight shift in its maximum to 345 nm accompanied by a loss of the small shoulder at ~305 nm, while that of CTB shows a marked change with its maximum now at 346 nm.

Issued as NRCC no. 16950

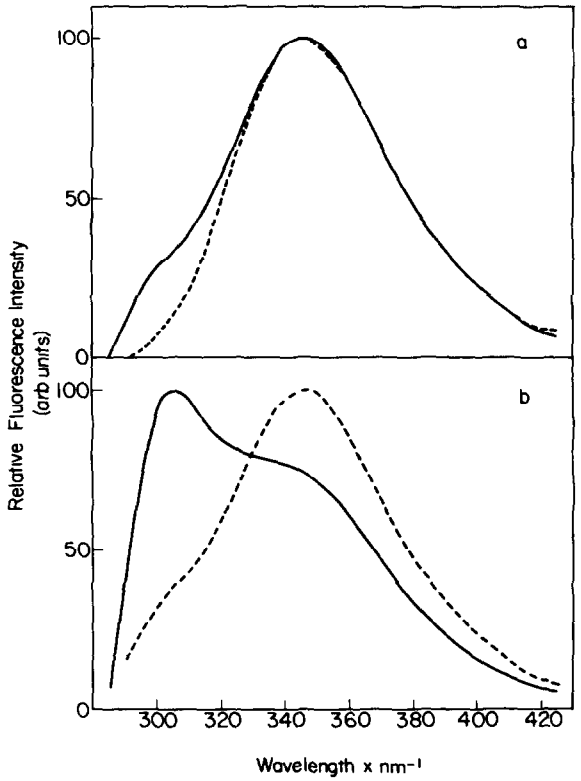

Fig.1. Corrected fluorescence spectra of cytotoxins with normalized peak heights; λ_{ex} 275 nm (——); λ_{ex} 290 nm (- - -); 0.01 M cacodylate buffer, pH 7: (a) CTA; (b) CTB.

These fluorescence spectral properties would normally be considered [1] as typical of the fluorescence properties of proteins containing tyrosine and tryptophan with a change in the excitation wavelength to 290 nm resulting in selective excitation of the tryptophan residues. The previously determined sequences [6,7] of CTA and CTB were shown to contain three tyrosine residues each and no tryptophan whatsoever. The observation of fluorescence maxima at 345 nm in proteins containing only tyrosine has not been reported previously. Because of the implications of these results the additional tests reported in section 2 were performed to confirm the absence of tryptophan in both peptides.

The fluorescence spectra of the *S*-carboxymethyl derivatives of the two peptides are shown in fig.2. In the case of the CTB derivative the fluorescence spectrum (λ_{ex} = 275 nm) has a clear maximum at

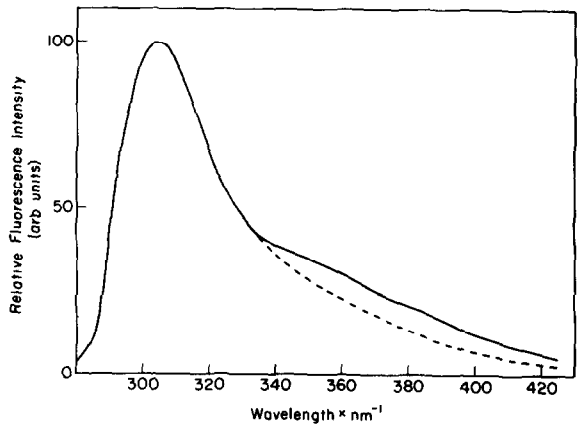

Fig.2. Corrected fluorescence spectra of the carboxymethyl derivatives of CTA (- - -); and CTB (——); normalized peak heights; λ_{ex} 275 nm; 0.01 M cacodylate buffer, pH 7.

305 nm, typical of 'normal' tyrosine fluorescence. For the CTA derivative the fluorescence spectrum also has a strong maximum at 305 nm but a weak shoulder persists at longer wavelengths.

4. Origin and significance

In our work we conclusively demonstrated that neither peptide contained tryptophan, whose emission properties may have otherwise explained our observations. The fluorescence spectrum of tyrosinate, the conjugate phenolate base of tyrosine, was presented [10]. We reported that the excited state p*K* of the phenol group of tyrosine was 4.2, c.f. 10.3 in the ground state. The fluorescence maximum of tyrosinate at 345 nm and the band shape (fig.3) is similar to that of CTA at either excitation wavelength and to that of CTB when excited at 290 nm. The broad shoulder in the fluorescence spectrum of CTB when excited at 275 nm occurs in the same wavelength range as tyrosinate emission. This spectral similarity with tyrosinate; the low excited state p*K* of tyrosine and the presence of tyrosine as the only fluorescent amino acid in both peptides requires the conclusion that the observed emission at 345 nm in the peptides, at pH 7, be assigned to tyrosinate emission. It is highly unlikely that at pH 7 there is any tyrosinate present in the ground state. Therefore we suggest that

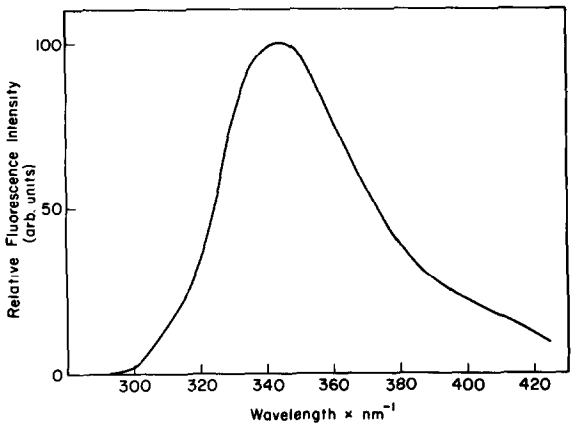

Fig.3. Corrected fluorescence spectrum of tyrosinate, pH 13, peak height normalized to 100; λ_{ex} 275 nm.

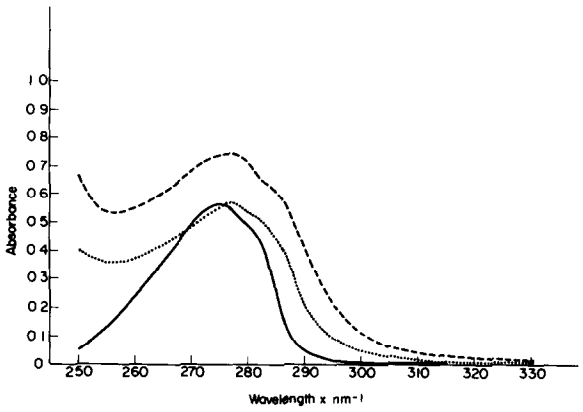

Fig.4. Absorption spectra of CTA (- - -); CTB (· · ·); and tyrosine (——). All solutions were 1.3×10^{-4} M tyrosine.

the tyrosinate emission in the peptides originates from an intramolecular excited state proton transfer from the phenolic hydroxyl of one or more of the tyrosine residues of the peptides to a protein acceptor. The carboxylate group of the amino acids glutamic acid and aspartic acid would be suitable proton acceptors.

The change in fluorescence spectra with excitation wavelength indicates the presence of at least two types of tyrosine residues in the peptides. The absorption spectra (fig.4) of both peptides show that the A_{290} is greater than that of tyrosine at the same wavelength. This spectral perturbation of the tyrosine residues may be due to hydrogen bonded complexes with glutamic and/or aspartic acid residues in the peptides. Such a complex would favour a rapid proton transfer such as we suggest in the excited state. It is interesting to note that in the case of CTA there is an additional glutamic acid residue at position 52 adjacent to tyrosine 51. This may account for the fluorescence spectral differences between CTA and CTB with excitation at 275 nm.

The fluorescence spectra of the *S*-carboxymethyl derivatives provide important confirmation of our assignment of the 345 nm emission to tyrosinate in the intact peptides. The fluorescence maximum at 305 nm in the spectra of these derivatives is characteristic of tyrosine emission found in several proteins. These spectra verify the absence of tryptophan in CTA and CTB. Furthermore the loss of fluorescence at 345 nm indicates that in the intact peptides specific conformations and amino acid interactions are required in order for the excited state proton transfer to occur.

The interaction of tyrosine with the carboxylic amino acids leading to fluorescence quenching of the tyrosine in proteins has been reported earlier [2]. However, tyrosinate fluorescence in protein originating from this interaction has not previously been reported.

Our results have obvious important implications in the fluorescence properties of proteins, especially those which contain both tyrosine and tryptophan residues. Often conformational and/or structural perturbations in proteins are monitored by changes in the fluorescence intensity in the 330–350 nm spectral region. These are usually interpreted as being due to changes in the tryptophan environment or in the tyrosine–tryptophan relationship leading to changes in energy transfer between these amino acids. Our finding indicates that these interpretations will require more careful consideration.

In summary, we have demonstrated that tyrosinate emission with a maximum at 345 nm can be observed at pH 7 in the small cytotoxin peptides CTA and CTB. Studies are in progress to further characterize this emission in these peptides and its implications in biochemical fluorescence studies.

References

[1] Longworth, J. W. (1971) in: Excited States of Proteins and Nucleic Acids (Steiner, R. F. and Weinryb, I. eds) pp. 319–484, M. Dekker, New York.
[2] Cowgill, R. W. (1976) in: Biochemical Fluorescence Concepts (Chen, R. F. and Edelhoch, H. eds) vol. 2, pp. 441–486, M. Dekker, New York.
[3] Larsen, P. R. and Wolff, J. (1968) J. Biol. Chem. 243, 1283–1289.
[4] Takechi, M., Sasaki, T. and Hayashi, K. (1971) Naturwisch. 58, 323–324.
[5] Seligy, V., Roy, C., Dove, M. and Yaguchi, M. (1976) Biochem. Biophys. Res. Commun. 71, 196–202.
[6] Hayashi, K., Takechi, M. and Sasaki, T. (1971) Biochem. Biophys. Res. Commun. 45, 1357–1362.
[7] Takechi, M., Hayashi, K. and Sasaki, T. (1972) Mol. Pharmacol. 8, 446–451.
[8] Liu, T. Y. and Chang, Y. H. (1971) J. Biol. Chem. 246, 2842–2848.
[9] Spies, J. R. and Chambers, D. C. (1949) Anal. Chem. 21, 1249–1266.
[10] Rayner, D. M., Krajcarski, D. T. and Szabo, A. G. (1978) Can. J. Chem. 56, 1238–1245.