The non-fluorescence of 4-fluorotryptophan

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The derivative 4-fluorotryptophan was confirmed to have negligible fluorescence at 25 °C and 285 nm (tryptophan/4-fluorotryptophan quantum-yield ratio greater than 100:1). However, photolysis experiments on tryptophan and 4-fluorotryptophan, in which loss of starting material was measured by reverse-phase h.p.l.c., demonstrated that 4-fluorotryptophan was significantly more photochemically active than the parent tryptophan, with the 4-fluorotryptophan photolysis quantum yield being 7 times larger than that of tryptophan at 25 °C and 285 nm. In addition, at 77 K and 275 nm 4-fluorotryptophan displayed strong fluorescence and phosphorescence, with emission quantum yields comparable with those of tryptophan at 77 K and 275 nm.

INTRODUCTION

Tryptophan is found in almost all proteins, and its fluorescence dominates the emission of most native proteins (Lakowicz, 1983). An analogue of tryptophan, 4-fluorotryptophan, was recently shown to have no fluorescence at room temperature (Bronskill & Wong, 1988), and these authors suggested that substitution of 4-fluorotryptophan for tryptophan in proteins would result in the suppression of tryptophan fluorescence. Therefore fluorescence from an individual tryptophan residue of particular interest (e.g. located at the active site of an enzyme) could be seen by substituting the other tryptophan residue(s) with 4-fluorotryptophan residue(s). Another suggested use (Bronskill & Wong, 1988) of the 4-fluoro analogue was in the elimination of all of the tryptophan fluorescence, allowing tyrosine and phenylalanine fluorescence to be seen (Konev, 1967; Lakowicz, 1983).

A possible complication in proteins with 4-fluorotryptophan substituting for tryptophan would result if the 4-fluoro analogue were more photochemically active than tryptophan itself. One might expect this to be the case, since energy not present as fluorescence must ultimately appear in some other form. Thus photoproduct contamination could negate the spectroscopic advantages of substitution with 4-fluorotryptophan. It was therefore of interest to us to investigate the photo-reactivity of 4-fluorotryptophan and compare it with that of tryptophan. The emission properties of 4-fluorotryptophan at 77 K were also investigated in the course of our work.

MATERIALS AND METHODS

Tryptophan and 4-fluorotryptophan were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and were used as received without further purification. For room-temperature fluorescence and photolysis experiments tryptophan and 4-fluorotryptophan were prepared as 0.1 mM solutions in pH 6.5 h.p.l.c.-quality water from Fisher Scientific Co. (Atlanta, GA, U.S.A.). For the low-temperature (77 K) fluorescence and phosphorescence

experiments tryptophan and 4-fluorotryptophan were prepared at 0.1 mM in a 1:1 (v/v) solution of pH 6.5 h.p.l.c.-quality water and certified-grade ethylene glycol, both from Fisher Scientific Co.

The apparatus used for fluorescence, phosphorescence and u.v. irradiation was as follows. The excitation system consisted of a Hanovia 100 W xenon arc lamp, a quartz focusing lens, a 10 cm water filter, a Bausch and Lomb high-intensity grating monochromator and a second quartz lens. The sample was mounted in a dark box that allowed x, y and z sample-position changes to be made. The emitted light was detected at 90° to the excitation beam after passing through a quartz lens and a SPEX 1680 double monochromator. This emitted light was detected by an RCA 1P-28 photomultiplier tube and was amplified. Further signal processing was achieved with an IBM PS/2 model 30 computer with an Omega DAS-16F interface board and LAB TECH NOTEBOOK (Wilmington, MA, U.S.A.) software.

For fluorescence and phosphorescence experiments the excitation monochromator slits were set at 1.7 mm entrance and 1.0 mm exit, producing a bandpass of approx. 3 nm (full width, half maximum). The emission monochromator slits were set at 0.5 mm, entrance and exit. For photochemical experiments the slits of the excitation monochromator were set at 5 mm entrance and 3 mm exit, producing a bandpass of approx. 10 nm (full width, half maximum).

Photolysis of 1.0 ml of 0.1 mM-tryptophan or -4-fluorotryptophan solutions was carried out at a wavelength of 285 nm and a temperature of 25 °C. We irradiated at 285 nm because both tryptophan and 4-fluorotryptophan display strong absorption at this wavelength (Bronskill & Wong, 1988). Photolysis periods of 0, 10, 15, 20 and 40 min were used. For the 25 °C experiments 1.0 cm × 1.0 cm quartz cells were used for both fluorescence and photolysis. During photolysis a magnetic stir bar was used to mix the solution continuously. By use of reverse-phase h.p.l.c. the concentrations of the tryptophan and 4-fluorotryptophan solutions were monitored as described below. The data collected from these measurements led to the evaluation of photolysis rate constants. In separate experiments tryptophan was

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photolysed at 295 nm, to facilitate comparison of the present 285 nm data with previous tryptophan photolysis studies (Borkman *et al.*, 1986; Tallmadge *et al.*, 1989).

A chopper was used to eliminate scattered light and fluorescence in the phosphorescence spectra. Round 0.8 cm-diameter quartz tubes were used for both the fluorescence and phosphorescence spectra at 77 K. Sample position was optimized by varying *x*, *y* and *z* coordinates so as to collect the maximum emission intensity.

U.v.-absorption spectra were recorded with a Cary 14 spectrophotometer. By using absorption data for tryptophan and 4-fluorotryptophan at 285 nm (which agreed well with those of Bronskill & Wong, 1988) the difference in absorption was determined. This was used to calculate the relative fluorescence and photolysis quantum yields reported.

Tryptophan and 4-fluorotryptophan concentrations were measured after each photolysis period by using reverse-phase h.p.l.c. analysis. The chromatograph used was a Hewlett-Packard 1090 liquid chromatograph. The column was a VYDAC 218TP54 reverse-phase column (25 cm × 4.8 mm) run at a temperature of 35 °C. The samples were eluted with a linear gradient from 10% to 20% (v/v) methanol in water over a 10 min time period. The flow rate was set at 1 ml/min and the sample size injected was 20 μ l. The eluate was monitored by the Hewlett-Packard 1090 diode-array absorption detector. The wavelengths monitored were 254 and 295 nm.

RESULTS AND DISCUSSION

Our absorption spectra of tryptophan and 4-fluorotryptophan were consistent with those of Bronskill & Wong (1988). The absorption spectrum of 4-fluorotryptophan was shifted to the blue about 15–20 nm compared with that of tryptophan.

At room temperature (25%) tryptophan exhibited strong fluorescence whereas 4-fluorotryptophan had negligible fluorescence, with a tryptophan/4-fluorotryptophan quantum-yield ratio greater than 100:1. Typical room-temperature fluorescence spectra of tryptophan and 4-fluorotryptophan are shown in Fig. 1.

After 285 nm irradiation of both 0.1 mM-tryptophan and 0.1 mM-4-fluorotryptophan at 25 °C, reverse-phase h.p.l.c. was performed. The chromatograms, monitored

Fig. 1. Relative fluorescence spectra in aqueous solution of 0.1 mM-tryptophan (—) and 0.1 mM-4-fluorotryptophan (---) at 25 °C and 285 nm excitation

Fig. 2. Reverse-phase h.p.l.c. chromatograms of photolysed solutions of tryptophan and 4-fluorotryptophan at 25 °C and 285 nm excitation

Absorption of the eluent was monitored at 295 nm (upper trace, 20% full scale) and 254 nm (lower trace, 10% full scale). (a) 0.1 mM-Tryptophan before irradiation; (b) 0.1 mM-tryptophan after 20 min irradiation; (c) 0.1 mM-4-fluorotryptophan before irradiation; (d) 0.1 mM-4-fluorotryptophan after 20 min irradiation.

at both 295 nm and 254 nm, showed that 4-fluorotryptophan was more photoreactive at 25 °C and 285 nm than was tryptophan (Fig. 2). Fig. 2(a) shows the reverse-phase h.p.l.c. chromatogram of tryptophan before irradiation and Fig. 2(b) shows the 20 min-irradiated sample. Loss of starting material as well as photoproduct formation are both in evidence. Photoproducts of tryptophan that have been identified previously include *N*-formylkynurenine, kynurenine and tryptamine (Borkman *et al.*, 1986). Figs. 2(c) and 2(d) are the reverse-phase h.p.l.c. chromatograms of 4-fluorotryptophan before and after 20 min irradiation respectively. The rate of loss of 4-fluorotryptophan is seen to be several times greater than that of tryptophan. In addition, large amounts of photoproducts were formed that, on the basis of their retention times, appear to differ from the products of tryptophan photolysis, but no product identification was attempted for 4-fluorotryptophan.

Tryptophan was also photolysed at 25 °C and 295 nm to compare with previous work (Borkman *et al.*, 1986; Tallmadge *et al.*, 1989). No significant differences in the reverse-phase h.p.l.c. chromatogram for tryptophan photolysis at 295 nm compared with that at 285 nm were seen. Such a comparison was not done for 4-fluorotryptophan because its absorption is very weak at 295 nm.

The rates of photolysis were determined by monitoring the loss of original sample (tryptophan or 4-fluorotryptophan) by reverse-phase h.p.l.c. The concentrations before ($[A]$) and after ($[A_0]$) irradiation times of 0, 5, 10, 15, 20 and 40 min are plotted as $\log([A]/[A_0])$ versus time in Fig. 3. After correcting for the difference in absorbance

Fig. 3. First-order kinetics for photolysis of 0.1 mM-tryptophan (—) and 0.1 mM-4-fluorotryptophan (---) solutions

Samples were photolysed at a wavelength of 285 nm $\pm$ 5 nm at 25 °C. The data plotted are from the 254 nm absorption signal of the reverse-phase h.p.l.c. chromatograms.

Fig. 4. Total emission spectra of 0.5 mM-tryptophan (—) and 0.5 mM-4-fluorotryptophan (---) in water/ethylene glycol solution at 77 K and 275 nm excitation

for 4-fluorotryptophan compared with tryptophan at 285 nm, we found the tryptophan/4-fluorotryptophan relative photolysis quantum-yield ratio to be 1:7 at 25 °C and 285 nm.

At low temperature (77 K) 4-fluorotryptophan displayed strong fluorescence and phosphorescence. The low-temperature total emission spectra of tryptophan and 4-fluorotryptophan are shown in Fig. 4. An excitation wavelength of 275 nm was chosen for the low-temperature emission spectra (instead of 285 nm) to avoid overlap between the fluorescence and the scattered excitation peak. The emission quantum yields for tryptophan and 4-fluorotryptophan at 77 K are seen to be of comparable magnitude. The phosphorescence spectra of

Fig. 5. Relative phosphorescence spectra of 0.1 mM-tryptophan (—) and 0.1 mM-4-fluorotryptophan (---) in water/ethylene glycol solution at 77 K and 285 nm excitation

tryptophan and 4-fluorotryptophan are shown in Fig. 5 on an expanded scale.

We have shown that 4-fluorotryptophan has a significantly larger photolysis quantum yield than has tryptophan at 25 °C. Since there is a complete lack of fluorescence in 4-fluorotryptophan at room temperature, the data suggest that there may be a fast reaction pathway from the excited state of 4-fluorotryptophan, causing it to be more photolabile than tryptophan and resulting in fluorescence quenching. In any case it is noteworthy that 4-fluorotryptophan is more photoreactive than tryptophan at room temperature, and that substitution of 4-fluorotryptophan into proteins should be made only after recognition of this fact.

In addition, we have found that, although 4-fluorotryptophan fluorescence is absent at 25 °C, it becomes comparable in intensity with that of tryptophan at 77 K. Similarly, both compounds display strong phosphorescence at 77 K. Again, these facts should be taken into account when spectroscopic experiments on 4-fluorotryptophan-substituted proteins are contemplated.

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REFERENCES

- Borkman, R. F., Hibbard, L. B. & Dillon, J. (1986) *Photochem. Photobiol.* **43**, 13–19
- Bronskill, P. M. & Wong, J. T. (1988) *Biochem. J.* **249**, 305–308
- Konev, S. V. (1967) *Fluorescence and Phosphorescence of Proteins and Nucleic Acids*, Plenum Press, New York
- Lakowicz, J. (1983) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York
- Tallmadge, D. H., Huebner, J. S. & Borkman, R. F. (1989) *Photochem. Photobiol.* **49**, 381–386