FACTORS INFLUENCING THE PHOTOSENSITIZING PROPERTIES AND PHOTOLUMINESCENCE OF THIOFLAVIN T*

ROBERT B. CUNDALL, A. KEITH DAVIES, PETER G. MORRIS and JEFFREY WILLIAMS

Department of Biochemistry, University of Salford, Salford M5 4WT (Gt. Britain)

Summary

The fluorescence intensity of aqueous solutions of thioflavin T greatly increases when the dye is bound to ribonucleic acid and to single-stranded polynucleotides containing purine bases. Binding is due to both ionic and hydrophobic interactions. Thioflavin is an inefficient photosensitizer for the oxidation of histidine but the photosensitizing power of thioflavin increases considerably when the dye is bound to polynucleotides. These effects are interpreted in terms of a stabilization of the excited singlet and triplet states of the dye in the bound state.

1. Introduction

Bellin and Yankus [1] have demonstrated the photodynamic degradation of certain amino acids using several dyes as sensitizers. They found that binding of the dyes to the high molecular weight polymers polyvinyl pyrrolidone and deoxyribonucleic acid (DNA) caused a decrease in the photosensitized oxidation of histidine. They were unable to detect any photosensitized oxidation when thioflavin was used as the sensitizer even though this dye had been shown previously [2] to photosensitize the oxidation of *p*-toluenediamine.

Parker and Joyce [3] examined the photoluminescence properties of a number of dyes, and with thioflavin T they showed that there was an increase in the quantum yield of fluorescence from 0.0003 to 0.046 on binding to DNA. Scott and Willet [4] measured the critical electrolyte concentration of a number of dyes and showed little binding of thioflavin to polyanions such as hyaluronate and alginate but strong binding to ribonucleic acid (RNA).

We had noticed that, although an aqueous solution of thioflavin shows only extremely weak fluorescence, a brilliant yellow-green fluorescence is obtained when the dye solution is spotted onto filter paper and dried. Furthermore, a solution of thioflavin in a glucose glass at room temperature shows an intense fluorescence. A possible explanation of these effects is that thioflavin is extremely sensitive to factors affecting the rigidity of the molecule. We were interested to dis-

* Paper presented at the Xth International Conference on Photochemistry, Iraklion, Crete, Greece, September 6 - 12, 1981.

0047-2670/81/0000-0000/$02.50

cover how the binding of the dye to macromolecules, particularly polynucleotides, would affect the luminescence properties of thioflavin. In addition, in view of the work of Bellin and Yankus [1], we wanted to find out how the photosensitizing properties of the dye were affected by binding.

2. Experimental details

2.1. Materials

Thioflavin T was obtained from ICN Pharmaceuticals Inc., New York. The dye was purified by repeated crystallization from a 30vol.%toluene–70vol.%ethanol mixture with charcoal treatment.

Polynucleotides, poly-L-lysine, protamine sulphate and carrageenan were obtained from the Sigma Chemical Co. Sodium dodecyl sulphate (SDS) (specially pure) was obtained from BDH. Other chemicals were AnalaR grade. Water was doubly distilled.

2.2. Methods

Absorption spectra were measured on a Pye Unicam SP 1800 spectrophotometer. Fluorescence and phosphorescence spectra were measured with a corrected-grating spectrofluorimeter and a Hitachi Perkin–Elmer MPF-4 spectrofluorimeter. Laser flash photolysis was carried out with a frequency-doubled *Q*-switched ruby laser (JK Lasers Ltd.) giving up to 0.5 J per flash at 347 nm with a pulse width of 30 ns.

Oxygen absorption measurements were carried out in a Pyrex Clark-type oxygen electrode cell (Rank Brothers, Cambridge). The light source was a Hanovia 100 W medium pressure mercury lamp. The light was filtered through Pyrex glass (giving wavelengths greater than 300 nm) or through a combination of soda glass and Wood's glass filters (giving predominantly a wavelength of 365 nm).

3. Results and discussion

Firstly, the interaction of thioflavin with polynucleotides and SDS was studied by absorption spectrophotometry. For a 3.3×10^{-5} M solution of thioflavin (over a pH range of 5 - 9), the addition of RNA caused a bathochromic shift in the longest-wavelength absorption band of the dye together with an increase in absorbance. These spectral changes were greatly reduced by the presence of propan-2-ol. With native double-stranded calf thymus DNA (over the pH range 5.0 - 7.7), the longest-wavelength band of thioflavin showed only very minor shifts (about 2 nm). However, DNA solutions at pH 9, and DNA that had been heated in solution to separate the strands and cooled rapidly to prevent renaturation, produced marked shifts in the longest-wavelength absorption band of thioflavin. Polyadenylic acid (poly-A) produced shifts similar to those caused by RNA and denatured DNA. The most striking change in the absorption spectrum of thioflavin occurred in the presence of polyguanylic acid (poly-G) at pH

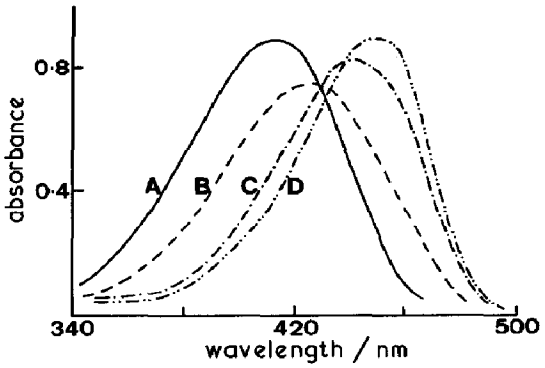


Fig. 1. The effect of poly-G on the longest-wavelength absorption band of thioflavin T for ratios of nucleotide to dye of 0 (spectrum A), 7 (spectrum B), 35 (spectrum C) and 250 (spectrum D).

7.7: a ratio of nucleotide to dye of 250 resulted in a 40 nm red shift in the longest-wavelength maximum to 452 nm (Fig. 1). The pyrimidine-based polynucleotides, *i.e.* polycytidylic acid (poly-C) and polyuridylic acid (poly-U), produced only very small red shifts (about 3 nm) in the longest-wavelength band of thioflavin.

The absorption spectrum of thioflavin was also affected by the anionic detergent SDS. Low concentrations of SDS caused a decrease in the absorbance at the longest-wavelength maximum; this was possibly due to induced dye aggregation. This effect was then reversed, a bathochromic shift in the absorption band occurring as the concentration of SDS was increased.

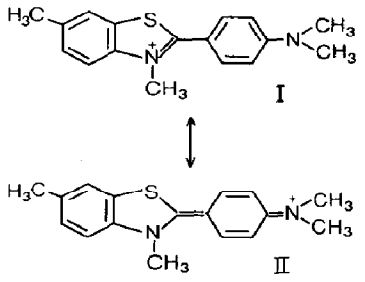
The shifts to longer wavelength, induced by polynucleotides at high ratios of phosphate to dye, depend on the particular polynucleotide: the shift with poly-G is considerably more than that with poly-A even though the two polyanions possess identical phosphate groups with similar spacing between the anionic sites. We attribute the spectral changes to hydrophobic interactions between the dye and the polynucleotide bases. Evidence for this is provided by the influence of propan-2-ol which tends to reverse the spectral changes; the presence of solvents of lower polarity is known to weaken hydrophobic interactions. It is significant that with SDS, which also provides the possibility of hydrophobic interactions, thioflavin shows spectral shifts similar to those observed with polynucleotides.

The interaction of thioflavin with polynucleotides appears to be specific to those containing purine bases since poly-C and poly-U have only very minor effects. Furthermore, the results with DNA show that there is a much stronger interaction with single-stranded polynucleotides than with the double-helical form. It is envisaged that the hydrophobic interactions are achieved by the insertion of thioflavin between the stacked bases in the single strands.

The next stage of the investigation concerned the influence of the thioflavin–polynucleotide interactions on the luminescence properties of the dye. In a 0.05 M tris–hydrochloric acid buffer solution of pH 7.7 in the presence of RNA, poly-A or poly-G, the fluorescence emission maximum of thioflavin was

about 490 nm. The quantum yields of fluorescence of thioflavin in the free state and bound to polynucleotides were determined by reference to a standard fluorescein solution. For thioflavin complexed to poly-G at a ratio of nucleotide to dye of 100, the quantum yield of fluorescence was 0.36 whereas that of unbound thioflavin was less than 0.001. Although RNA and poly-A considerably enhanced the fluorescence of thioflavin, the quantum yields were small (about 0.02 and 0.01 respectively). The excitation spectra for fluorescence of these thioflavin–polynucleotide complexes closely followed their absorption spectra.

It is useful to compare the fluorescence properties of thioflavin with those of the triphenylmethane dyes. In common with thioflavin, these dyes contain the dimethylaminophenyl group. Like thioflavin, they are only weakly fluorescent in fluid solution while being strongly fluorescent in rigid glasses. Auramine O, for example, does not fluoresce in solvents of low viscosity, but in glycerol at 0 °C or when bound to polymethacrylic acid the dye exhibits intense fluorescence [5]. It appears probable that intramolecular rotation of the dimethylaminophenyl group in the triphenylmethane dyes [5] and thioflavin is responsible for the dissipation of singlet and triplet state energy. It is reasonable to assume that the ground state of thioflavin is a resonance hybrid, two important canonical forms being the following:



The double-bond character of the link between the thiazole ring and the dimethylaminophenyl group would be expected to result in a planar ground state conformation. Electronic excitation of thioflavin by a $\pi \rightarrow \pi^*$ transition would be expected to reduce the double-bond character between the thiazole ring and the dimethylaminophenyl group. It is envisaged that as a result there will be a loss of molecular rigidity in the π,π^* excited singlet and triplet states owing to a reduction in the energy barrier for the rotation of the dimethylaminophenyl group. Rotation will provide a pathway for the dissipation of the electronic excitation energy. Procedures for restricting this motion, such as adsorbing the dye on filter paper, lodging it in a rigid glass or binding it to polynucleotides, lead to stabilization of the excited states of the dye and an increased probability of radiative processes.

An aqueous glass at 77 K formed from an acetate-buffered solution of thioflavin (initially at pH 5.0) showed intense prompt fluorescence (λ_{max} = 490 nm) together with phosphorescence. Low concentrations of RNA initially enhanced the phosphorescence, possibly by inducing aggregation of the dye owing to ionic binding along the phosphate–sugar backbone of the polynucleotide strand. Higher concentrations of RNA produced a marked reduction in the phos-

phorescence intensity. Frozen aqueous solutions also exhibited a weak long-lived (greater than 1 ms) emission (λ_{max} = 490 nm). The excitation spectrum of this emission showed a curious wavelength effect. It did not correspond to the absorption spectrum of thioflavin but had a maximum at 320 nm. The intensity of this long-lived emission increased with increasing amounts of RNA. It corresponded closely to the prompt fluorescence of thioflavin complexed to RNA and so had the appearance of delayed fluorescence. E-type delayed fluorescence was ruled out because of the low temperature. Furthermore, P-type delayed fluorescence was eliminated because the emission showed a linear dependence on the intensity of the exciting light. We conclude that the long-lived 490 nm emission is due to triplet–singlet energy transfer from a donor to thioflavin:

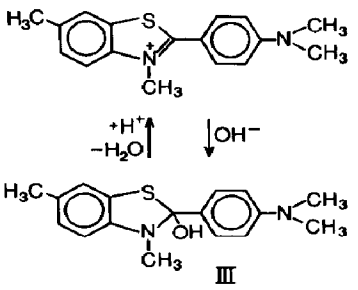
$$^3X^* + TF \rightarrow X + {}^1TF^*$$

$$^1TF^* \rightarrow TF + h\nu'$$

where TF represents thioflavin and X is the donor.

It was discovered that the intensity of the long-lived emission was greater for those solutions initially buffered at a higher pH. The same pH effect was observed in solutions of thioflavin containing SDS in place of RNA. Although the pH values cannot, of course, be applied at 77 K, it appears that hydroxide ion plays a role in this effect. For this reason, the influence of pH on thioflavin solutions was investigated. It was noticed that as the pH of an aqueous thioflavin solution was raised from 10 to 11 there was a reduction in the intensity of the long-wavelength absorption band and a corresponding growth at shorter wavelengths. If the pH was increased to 12, the solution became completely colourless and the colour was not recovered on lowering the pH to 7. On reducing the pH to 2 - 3 the colour was restored. These effects are shown in the scheme in Fig. 2. We believe that hydroxylated thioflavin (structure III), which is inevitably present in small quantities even in solutions of purified thioflavin, is responsible for triplet–singlet energy transfer to bound thioflavin, resulting in the long-lived emission. This conclusion is supported by the observation that hydroxylated thioflavin has an absorption band close to the excitation maximum for the long-lived 490 nm emission of thioflavin.

Laser flash photolysis revealed another delayed emission from thioflavin polynucleotide complexes in aqueous glasses at 77 K, this time in the microsecond



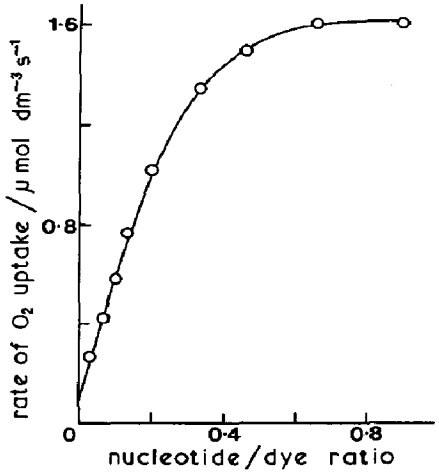
III

Fig. 2. Scheme showing the effect of pH on the structure of thioflavin.

time scale. The kinetics of decay of this 490 nm emission were complex, possibly because of the inhomogeneous nature of the cracked glasses. We believe that this emission is P-type delayed fluorescence caused by triplet–triplet annihilation between dye molecules situated between the stacked bases in the polynucleotide strands.

Laser flash photolysis experiments were also carried out on aqueous solutions of thioflavin at room temperature. The triplet state of unbound thioflavin was not detected, but when the dye was complexed to poly-A a transient was observed that had a strong absorption at a wavelength not less than 600 nm. The transient, which decayed by first-order kinetics and was quenched by oxygen with a second-order rate constant of $(7.3 \pm 0.8) \times 10^8$ mol^{-1} dm^3 s^{-1}, is attributed to the triplet state of thioflavin. The triplet was detected also in thioflavin solutions containing poly-G, although the intensity and lifetime were reduced compared with those for solutions containing poly-A. The triplet state was also readily observed when the dye was lodged in a glucose glass at room temperature.

The final stage of the investigation concerned an examination of the influence of polynucleotides on the photosensitizing properties of thioflavin. L-histidine was chosen as an oxidizable substrate at a concentration of 3.3×10^{-2} M. The solutions were prepared in 0.05 M tris–hydrochloric acid buffer of pH 7.7 and the dye concentration was 3.3×10^{-5} M. Solutions were irradiated in the oxygen electrode cell and the rate of oxygen absorption was calculated from the initial slope of chart recorder traces. In the free state, thioflavin was a very inefficient photosensitizer. The addition of poly-A or polyinosinic acid (poly-I) produced marked increases in the rate of oxygen absorption (a sixteenfold increase at a ratio of nucleotide to dye of 0.5 (Fig. 3)). Free adenine had no



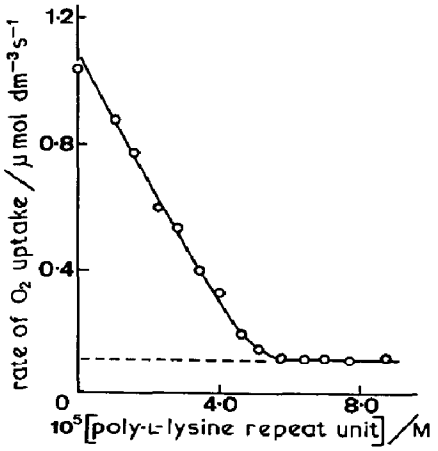


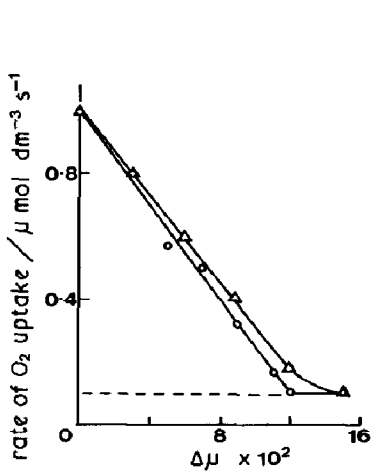
Fig. 3. The influence of poly-A on the thioflavin-sensitized photooxidation of histidine in air-saturated aqueous solution at pH 7.7.

Fig. 4. The influence of poly-L-lysine on the thioflavin-sensitized photooxidation of histidine in the presence of poly-A. The ratio of nucleotide to dye was 0.2. The reactions were carried out in air-saturated aqueous solution at pH 7.7.

effect on the rate. Poly-G also enhanced the photosensitizing efficiency of thioflavin but was less effective than poly-A and poly-I. The "rate enhancement effect" was only observed with polynucleotides having purine bases, *i.e.* pyrimidine-based polynucleotides such as poly-C and poly-U produced no enhancement. Indeed, with poly-C there was a reduction in rate. RNA exhibited a rate enhancement effect but double-stranded DNA did not. The rate of photosensitized oxidation in solutions containing poly-A, poly-I or poly-G was enhanced by deuterium oxide and decreased by azide ion in accordance with a type II singlet oxygen mechanism.

The rate enhancement effect of polynucleotides on the thioflavin-photosensitized oxidation of histidine was suppressed by polycations such as poly-L-lysine (Fig. 4) and protamine sulphate which form ionic complexes with polynucleotides. The addition of sodium sulphate or sodium chloride removed the enhancement effect with poly-A (Fig. 5) and RNA although with poly-G a small residual effect remained.

The rate of photosensitized oxidation of histidine was measured in the presence of complementary polynucleotides which hydrogen bond to form double-stranded helixes. With poly-A–poly-U mixtures no enhancement was observed until a ratio of poly-A to poly-U of unity was reached, after which the rate of photosensitized oxidation increased rapidly with increasing poly-A concentration. This demonstrates that, while single-stranded poly-A shows the rate enhancement effect, the poly-A–poly-U double helix does not. Similar effects were observed with the poly-I–poly-C system; no enhancement in the rate of oxygen absorption was observed until the ratio of poly-I to poly-C was close to unity.



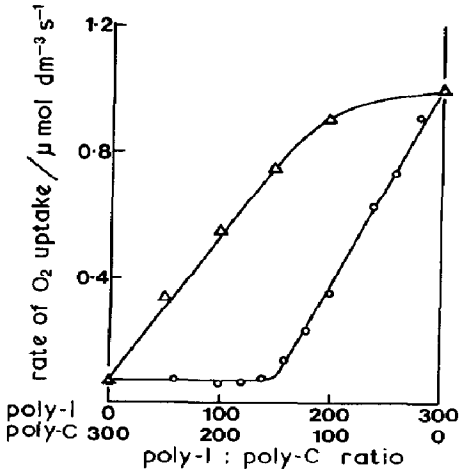


Fig. 5. The influence of ionic strength on the thioflavin-sensitized photooxidation of histidine (the ratio of nucleotide to dye was 0.2; the reactions were carried out in air-saturated solution at pH 7.7): ○, NaCl; △, Na_2SO_4: – – –, rate of photosensitized oxidation with unbound thioflavin.

Fig. 6. The effect of poly-I–poly-C mixtures on the rate of thioflavin-sensitized photooxidation of histidine: ○, rates for systems containing poly-I–poly-C mixtures; △, rates for systems containing poly-I only.

Beyond this equivalence point, the rate increased with increasing concentrations of poly-I (Fig. 6).

It is significant that the purine-based polynucleotides poly-G, poly-I and poly-A, which enhance the fluorescence of thioflavin in solution, also show the rate enhancement effect. The pyrimidine-based polynucleotides enhance neither the fluorescence of thioflavin nor the photosensitized oxidation of histidine.

The binding of thioflavin to poly-A, poly-I and poly-G stabilizes the excited states of the dye against intramolecular deactivation as is reflected in the fluorescence enhancement. This will increase the possibility of intersystem crossing to form the triplet state which is responsible for transferring energy to molecular oxygen.

The reduction in the rate of photosensitized oxidation of histidine, in the presence of polynucleotides, on the addition of polycations is expected if there is competition between the polycations and thioflavin to form ionic bonds with the phosphate groups on the polynucleotide. Further evidence that an ionic interaction between thioflavin and polynucleotides is essential for rate enhancement is provided by the observed reduction in the rate with increasing ionic strength. However, an electrostatic interaction between thioflavin and polynucleotides cannot solely be responsible for the rate enhancement effect, for otherwise the pyrimidine-based polynucleotides (poly-C and poly-U) would enhance the rate. Furthermore, polyanions such as carrageenan and polyvinyl sulphate have only a small influence on the rate. It is clear that there is an important interplay between hydrophobic and ionic interactions, both being required for the rate enhancement.

The interaction of thioflavin with polynucleotides is representative of a wider class of interaction of small molecules with biological macromolecules; such interactions are of interest in the study of many biochemical and pharmacological problems. For example, there are implications in this work for the understanding of phototoxic and photoallergic side effects of drugs. The present work shows that, in assessing a drug for potential phototoxicity by *in vitro* studies, careful account should be taken of the influence of the binding of the drug to macromolecules; this is highly probable in a cellular environment. From a simple examination of thioflavin in the unbound state we might conclude that, because it is a poor photosensitizer, it is unlikely to cause adverse light-induced effects in the living cell. The present work shows that the binding of thioflavin to RNA in the cell could greatly increase photosensitized damage to cell components, *e.g.* enzymes, cell membranes and nucleic acids.

References

1 J.S. Bellin and C.A. Yankus, *Arch. Biochem. Biophys., 123* (1968) 18.
2 G. Oster, J.S. Bellin, R.W. Kimball and M.E. Schrader, *J. Am. Chem. Soc., 81* (1959) 5095.
3 C.A. Parker and T.A. Joyce, *Photochem. Photobiol., 18* (1973) 467.
4 J.E. Scott and I.H. Willett, *Nature (London), 209* (1966) 985.
5 G. Oster and Y. Nishijima, *J. Am. Chem. Soc., 78* (1956) 1581.