Fluorescence probing of nucleic acids: I. singly and doubly labeled dithymidine phosphate: fluorescence and energy transfer studies

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ABSTRACT

Dithymidine phosphate labeled at its 5' end with a naphthalene-carbamate has been prepared, DTpT. It is shown to exist in several conformations which are characterized by chromatography and absorption and fluorescence measurements. Models for three possible conformers where the dye is solvated, stacked on the first thymine or intercalated between the two thymines are given with their absorption and fluorescence spectra. The doubly labeled molecule, DTpTA, where D and A form a donor-acceptor energy transfer pair has also been prepared. The energy transfer rate has been measured from the donor fluorescence lifetime decay.

INTRODUCTION

Fluorescence probing has proved to be a powerful technique to study conformations of biomolecules and to investigate interactions involving biopolymers. Although many studies have been reported of fluorescence probing of protein conformations, only few studies have dealt with fluorescence probing of nucleic acids conformations. The difficulty in probing nucleic acids lies in their structure; there is no unique site for interaction of a fluorescent dye with nucleic acids in contrast with a protein where a probe often interacts with the active site. In addition aromatic amino-acids, particularly tryptophan, can act as intrinsic probes of proteins. In nucleic acids, however, the bases exhibit only very weak fluorescence. One approach to tackle the problem is to prepare fluorescent base analogues. Our approach is to label nucleic acids with dyes at specific sites, for example the 3' and 5' positions of the end riboses of the chain. Such singly labeled nucleic acids can be used to study proteins-nucleic acids interactions. One may also prepare a doubly labeled nucleic acid and use the efficiency of energy transfer to study conformational changes due to its interaction with another nucleic acid chain or a protein. Obviously this technique is limited to short labeled DNA chains since energy transfer can hardly be measured beyond 50 Å.

In this paper we shall discuss the preparation and spectroscopic
properties of a dithymidine monophosphate which is labeled at its 5'end with a naphthalene derivative and at its 3'end with an anthracene derivative, forming a donor-acceptor pair (DTpTA). The energy transfer rate in this doubly labeled nucleic acid is obtained from fluorescence lifetime measurements. Implications of these results for the determination of the chain conformations are discussed.

I - CHEMICAL SYNTHESIS

The method used to synthesize the doubly labeled dithymidine phosphate DTpTA is applicable to longer chains; it consists of three steps:

a) Preparation of the 5' labeled monomer (DT)

Preparation of 5' naphthalene-2-carbamate thymidine-3'OH (5'NC-Thy-3'OH) compound II in Fig. 1 was described in an earlier paper. It is prepared by the reaction of 2-naphthalene isocyanate on thymidine protected at its 3' position.

Fig. 1: Compounds referred to in the text.

b) Condensation (DTpT)

An excess of the 5' labeled nucleotide is condensed with 5'-monophosphate-thymidine to give the dimer. The condensation is done by the procedure used for oligothymidilic acids in dry pyridine with dicyclohexyl-
carbodiimide as the condensing agent. To avoid condensation of non-labeled nucleotides we used a 3' acetyl protected phosphate thymidine. The condensation reaction is written as follows:

\[
5'\text{NC} - \text{Thy} - 3'\text{OH} + 5'\text{PO}_3\text{H} - \text{Thy} - 3'\text{Ace} \rightarrow 5'\text{NC} - \text{Thy} - \text{PO}_3\text{H} - \text{Thy} - 3'\text{Ace}
\]

The condensed product can be deacetylated easily without removing the 5' label in ammoniacal solution at pH 11.

Separation of the condensation products was done on a DEAE-cellulose column, ammonium acetate was used as eluent. The elution pattern is shown on Fig. 2a. Three main peaks are observed : A, C, D. Thin layer chromatography (TLC) (on silicagel sheets with eluent : 80% acetonitrile - 20% water) shows that product A is 5'-naphthalene carbamate-thymidine (R_f = 0.94) and that products C (R_f = 0.73) and D (R_f = 0.65) are new compounds. C is four times more abundant than D. The nature of products C and D was checked by enzymatic hydrolysis with snake venom phosphodiesterase, which gives back the two starting compounds, i.e. 5'-naphthalene-2 carbamate-thymidine-3'OH (compound II of Fig. 1) and 5'phosphate-thymidine-3'OH, identified by TLC. These two hydrolysis products were separated on DEAE-cellulose columns and their quantities determined by absorption spectroscopy.

Both compounds C and D were found to contain equal amounts of the two hydrolysis products. From this analysis two conclusions can be drawn:

- C and D are both condensation products of one 5'Naphthalene carbamate-thymidine and one 5'phosphate thymidine.
- In both compounds condensation is through a O-P-O bridge, since this is the basic requirement for phosphodiesterase action. These results can be taken as evidence that both products C and D correspond to compound III, Fig. 1, they will be called IIIC and IIID.

c) Labeling at the 3' end (DTPtA)

Labeling of the 3'OH end of compound IIIC was done by action of anthracene-2-isocyanate in dry pyridine at room temperature. Anthracene-2-isocyanate is not commercially available and is prepared by action of phosgene on 2-anthramine. Excess of anthracene isocyanate after completion of the reaction is removed by addition of methanol to give 2-anthracene-methyl carbamate. The product is separated on a DEAE-cellulose column and the elution diagram is shown on Fig. 2b. The first eluting product is shown by TLC to be anthracene-methyl carbamate. The second product is shown by comparison of its absorption and fluorescence spectra to those of IV (see next paragraph) to contain Anthracene carbamate, it is considered as product V, Fig. 1.
Fig. 2: Chromatography on DEAE cellulose column.

a) Elution diagram of compounds after condensation (I b). B produced in small amount does not contain naphthalene, it is not studied further.
b) Elution diagram after labeling the 3' end (I c).

In addition, 5'OH thymidine-3'anthracene carbamate (IV on Fig. 1) was prepared by direct action of 2-anthracene isocyanate on 5'-trityl-thymidine 3'OH and subsequent detritylation as described for 5'OH-thymidine-3'naphthalene. The product was analysed by NMR and shown to contain both anthracene and thymine. Compound IV was used as a reference for absorption and fluorescence studies.

II - OPTICAL MEASUREMENTS

Absorption spectra were run on a Beckman Acta III spectrometer, fluorescence spectra on a Jobin-Yvon spectrophotometer. Fluorescence spectra were not corrected for the wavelength dependent response of the instrument. Lifetime measurements were done with an instrument built on single photon counting techniques. The excitation light is produced at a repetitive rate of 60,000 per second by a spark in air; it has a width of 2.5 ns. Optical filters are used for excitation and emission lights: for the donor fluorescence a MTO 308 in excitation and a Kodak Wratten VOM 26 in emission, for the acceptor fluorescence a MTO 404 for excitation and a VOM 22
for emission. Excitation light was convoluted with different exponentials to fit the fluorescence decay curves. Details of the set up will be given in a later publication by M. Lebret and J.M. Le Pecq.

III - RESULTS AND DISCUSSION

a) Study of compound II

Absorption and fluorescence, spectra and fluorescence quantum yield of II in methanol have already been reported\textsuperscript{3}. From methanol to water while negligible change in absorption is observed, the maximum of the emission band shifts from 350 nm to 375 nm (Fig. 3). The fluorescence yields and lifetimes of II in methanol and water are shown on Table I. For comparison we have tabulated the fluorescence yields and lifetimes of the isomer of II, i.e. 5'OH-thymidine-3'a-naphthylcarbamate.

The data from Table I lead to the following observations:

1) Thymine drastically quenches the fluorescence of the dye when it is bound to the 5' position of thymidine but not when bound to the 3' position.

2) This quenching effect is much larger in water than in methanol.

Molecular models show that in thymidine dye-thymine stacking can occur when the dye is at the 5' position (Fig. 4a) but not at the 3' position.

Fig. 3: Absorption and fluorescence spectra of compound II in water at room temperature.
It has been shown that stacking can produce quenching of fluorescent molecules by enhancement of their intersystem crossing rate due to charge-transfer interaction. On the other hand, stacking in polynucleotides is stronger in water than in organic solvents such as methanol. These remarks suggest that fluorescence quenching in II is caused by a dye-thymine stacking interaction. Table I shows in addition that fluorescence lifetimes for compounds I and II are the same in a given solvent in spite of very different quantum yields. This striking result can be understood if we consider the following model:

1) The dye in II can be in two states, in the first the dye is not stacked with thymine (Fig. 4b) and has the same fluorescence yield as in I, in the second state the dye is stacked with thymine (Fig. 4a) and its fluorescence is completely quenched.

2) The dye cannot pass from one state to the other during the fluorescence lifetime.

These two states can be two conformations, or two sets of conformations of molecule II. Quantum yield values show that in water, fluorescent non stacked molecules represent only 5% of all molecules II.
b) Study of compounds C and D

Results of enzymatic analysis given above show that the two fractions C and D correspond to the same structure III of Fig. 1. Absorption spectra of C and D are closely related, the maximum of the first absorption band of C is at 268 nm, while that of D is at 260 nm.

The fluorescence spectrum of IIIC (fig.5) has its maximum at 375 nm like those of I and II. Its fluorescence quantum yield is still smaller than that of II, while its fluorescence lifetime is the same (Table 1). These results indicate along the model given in paragraph IIla that the conformations where the dye is stacked on the neighbouring thymine (Fig. 4a) is still more favoured for IIIC than it is for II, and that the molecules for which the dye is not stacked (Fig. 4b) represent in this case only 2% of all molecules present.

![Fig. 4 - a - molecules C in "stacked" conformation](image)

The fluorescence spectrum of compound IIID is considerably red-shifted compared to those of I, II and III C, its maximum being at around 435 nm (fig. 5). If as stated above III C and III D correspond to the same structure they should be conformational isomers (or conformers). These conformers are stable at room temperature in water and in the conditions of chromatography on cellulose. We looked for pathways from one conformer to the other, the conversion being monitored by the fluorescence spectra. The stability of conformers III C and III D seems quite high since heating to 90°C in water...
proved insufficient. It is well known that large concentrations of urea can destroy tertiary structure in nucleic acids. The presence of urea to concentrations up to 7 M is insufficient at room temperature but at 90°C it can induce transition from III C to III D (fig. 5) but not from III D to III C, indicating that conformer III D is more stable than conformer III C.

![Fluorescence spectra of conformers IIIC and IIID and transition from IIIC to IIID monitored by fluorescence spectra after heating in 7 M urea at 90°C.](image)

**Fig. 5**: Fluorescence spectra of conformers IIIC and IIID and transition from IIIC to IIID monitored by fluorescence spectra after heating in 7 M urea at 90°C

1 - Conformer IIIC
2 - After heating 20 min
3 - After heating 1 h 30
4 - After heating 5 h, nearly complete conversion to IIID

An isostilbic point appears at 340 nm

Observation of molecular models shows that the naphthalene dye is bound to thymidine with enough flexibility so that it can pass under the neighbouring thymine. This leads us to a tentative model for structure of conformer IIID where the naphthalene dye is intercalated between the two thymines (Fig. 4c). The intercalation could explain the fluorescence spectrum of IIID at 435 nm which is never observed with the isolated dye I, or the labeled monomer II. The hydrophobicity of the dye would make the intercalated conformer (Fig. 4c) more stable than the conformers in which the dye is solvated (Fig. 4b) or stacked with the neighbouring thymine (Fig. 4a). The dye cannot intercalate between the bases by a simple translation movement parallel to the bases but has to perform a translation plus a rotation which require that the two thymines separate by more than the thickness of the dye. This stretching of the
molecule in the transition state must be energetically disfavoured which explains the difficulty to pass from IIIC to IIID. During the formation of compound III the condensation of 5'-phosphate thymidine on compound II traps the dye in conformations where it is on one side or the other of thymine, which will correspond to conformers IIIC or IIID. This trapping would explain how conformer IIIC can be five times more abundant though less stable than conformer IIID.

c) Absorption and emission spectra of the energy acceptor in IV

![Absorption spectrum of compound IV in water at room temperature](image)

The first and second absorption bands of the acceptor IV in water are shown in Fig. 6. Excitation and emission spectra for fluorescence are shown in Fig. 7. The region of interest shows two excitation bands and an excitation minimum at 300 nm. The quantum yield of IV in water is 0.09; a solution of anthracene in cyclohexane was used as a reference. The fluorescence lifetime of IV was measured as 16 nsec.
d) **Energy transfer in the doubly labelled dimer V**

The emission spectrum of V in water excited at 308 nm, (fig. 8), shows both donor and acceptor emissions. The excitation wavelength was chosen such that direct excitation of the acceptor moiety is at a minimum while the donor is efficiently excited. Evidence of energy transfer can be derived from a comparison of excitation spectra of the acceptor fluorescence in the absence (compound IV) or in the presence (compound V) of the donor moiety. It can also be derived from comparison of the donor fluorescence yields in III C and V. However these methods are not direct and they require a number of corrections:

1) Direct excitation of the acceptor must be corrected for.
2) Corrections must be made for absorbancies at 308 nm so that the fraction of light that is absorbed by the donor moiety alone is calculated.
3) Changes in quantum yield of the donor by the presence of the acceptor moiety may be induced by factors other than energy transfer, for example the acceptor moiety may induce changes of conformation of III C. This effect occurs in compounds II and III C, the presence at the second thymine in III C decreasing the donor fluorescence yield (Table 1).

In contrast evaluation of energy-transfer from lifetime values is direct and does not require correction for absorption of the other moieties in the molecule. Moreover we have seen that the donor fluorescence lifetime remains constant in I, II, and III C and seems to be quite insensitive to stacking.
We have measured the donor fluorescence lifetime in V and compared it to that in III. A short lifetime component of 3 ns is observed which accounts for 99% of the emitted light (a second component of 16 ns is shown to be acceptor fluorescence passing through the emission filter). The drastic drop in the donor fluorescence lifetime is taken as evidence that a new deexcitation process of the donor fluorescence is operating in V due to the presence of the acceptor dye. This deexcitation process can be energy-transfer to the acceptor or can be induced by conformation changes in V due to the presence of the acceptor on the molecule. However this second hypothesis can be ruled out on the following grounds:

1) In I, II and IIIC donor fluorescence lifetime is the same in spite of the presence of the dithymidine phosphate and further addition of the acceptor separated from the donor by two thymines is very unlikely to cause any serious perturbations on the latter.

2) The acceptor fluorescence excited at 400 nm has the same lifetime in molecules IV and V (Table 1), which shows that the presence of the donor dye at one end of the dithymidine phosphate does not affect the acceptor fluorescence lifetime at the other end.

In conclusion, we consider the measured decrease in the donor fluorescence lifetime as a direct evidence for energy transfer to the acceptor in compound V. We call $k_D$ the fluorescence rates of the donor in II and IIIC and $k_T$ the energy transfer rate in V. From lifetime measurements of compounds IIIC and V (Table 1):

![Emission spectrum of V excited at 300 nm in water, at room temperature.](image)
Forster's formula for the donor–acceptor energy transfer rate constant $k_T$ can be written as a function of $R$:

$$k_T = k_D \left( \frac{R^6}{R_0^6} \right)^{1/6}$$

with

$$R_0^6 = 0.87 \times 10^{-24}$$

where $R_0$ is the donor–acceptor distance at which $k_T$ equals the donor fluorescence rate constant $k_D$. $Q_F$ is the fluorescence quantum yield of the donor, i.e., the dye in non-stacked conformations, i.e., $Q_F = 1$.

$$I = \int_{\nu_1}^{\nu_2} \frac{f_D(\nu) \epsilon_A(\nu)}{\nu^4} d\nu$$

is the overlap integral of donor fluorescence and acceptor absorption, where $-\nu$ is the wave number in cm$^{-1}$ and $f_D(\nu)$ is the spectral distribution of donor fluorescence normalized such that

$$\int_{\nu_1}^{\nu_2} f_D(\nu) d\nu = 1, \quad \epsilon_A(\nu)$$

is the acceptor decadic extinction coefficient.

$n$ is the refractive index of the solvent, in this case water. $K$ is the orientation factor. $K = \cos \phi_{DA} - 3 \cos \theta_D \cos \theta_A$, where $\phi_{DA}$ is the angle between the transition moment vectors of both molecules, $\theta_D$ and $\theta_A$ are the angles between vector DA and D and A transition moment vectors.

If donor and acceptor chromophores are free to rotate at a rate faster than the transfer rate, the average value of $K^2$ is $2/3$, in this case the calculated value for $R_0$ using (2) is $32 \text{ Å}$. From the donor fluorescence lifetime measurement we find $R = 24 \text{ Å}$ from (1). This value is consistent with the structure given for pTPy and molecular models.

However, there is no evidence that may support the assumption that the two dyes are free to rotate on the chain. On the contrary, this work has shown that naphthalene carbamate dyes bound in 5' position fluoresce and can act as donors in only a fraction of all molecules $V$ present in solution. These correspond to conformers where the dye and the thymine are not stacked. These conformers are separated from stacked conformers by an energy barrier that cannot be overcome at room temperature during the donor fluorescence lifetime of 18 ns. Hence the possible directions that donor transition...
moments can assume on the chain may be limited. On the 3'-end of molecule V the anthracene carbamate acceptor dye is seriously limited in its rotation by the steric hindrance of the ribose ring to which it is directly bound.

In conclusion, this work on TpT labelling shows that oligo-nucleotides can be labelled with fluorescent dyes specifically at both ends. This specificity gives to such systems a potential use to study their conformational changes due to interaction with proteins and nucleic acids. The relatively long lifetime value for the fluorescent donor and the accuracy in fluorescence lifetime measurements allow determination of energy transfer rates for longer oligo-nucleotides containing up to 10 residues.

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