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ORIGINAL PAPER

Nucleobase-Specific Enhancement of Cy3 Fluorescence

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Abstract We report on the role of dye–nucleobase interactions on the photophysical properties of the indocarbocyanine Cy3. The fluorescence efficiency and lifetime of Cy3 increase in the presence of all four nucleoside monophosphates. This behavior correlates with an increase in the activation energy for photoisomerization and a ~4 nm red shift in the fluorescence spectrum. Changes are more dramatic for the purines (dAMP, dGMP) than the pyrimidines (dCMP, dTMP), and for the nonsulfonated cyanine ($DiIC_2(3)$) than the sulfonated dye (Cy3–SE). These results are consistent with a model in which Cy3–nucleoside π–π interactions decrease the efficiency of photoisomerization, increasing the efficiency of fluorescence.

Keywords Cy dyes · Cy3 · Photoisomerization · Nucleobases · Nucleosides · DNA

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Introduction

Fluorescence techniques are important tools for investigating the structure and dynamics of nucleic acids [1–5]. In particular, fluorescence resonance energy transfer (FRET) measurements have played an important role in the study of the structure and function of nucleic acids for several decades, and the popularity of the technique continues to increase thanks to the latest advances in instrumentation and the availability of new and improved fluorescent dyes [6, 7].

The so-called "Cy dyes" are among the most widely used fluorescent reagents for covalently labeling proteins and nucleic acids. In particular, Cy3 (Fig. 1) is one of the most popular fluorophores used in biophysical applications due to its remarkable stability against photobleaching, compatibility with commonly available laser lines and detectors, and commercial availability. The term "Cy dye" was introduced by A. Waggoner and collaborators in 1989, and it refers to a series of symmetric indocarbocyanine dyes (Fig. 1) [8, 9]. It is important to stress that terms such as Cy3 or Cy5 are not well-defined in the chemical sense, and have been used in the literature to designate different molecules. For example, Cy dyes are commercialized as phosphoramidites, *N*-hydroxysuccinimidyl esters, maleimides, and hydrazides, all of which contain different groups attached to the imine nitrogens, and may or may not contain negatively charged sulfonate groups in positions 5 and 5′ of the aromatic rings. In particular, the phosphoramidites are commercialized by Glen Research (Sterling, VA), and do not contain sulfonates or any other substituent in the aromatic rings. Phosphoramidites are used in DNA and RNA solid state synthesis to incorporate the dye at the 3′ or 5′ terminus of the oligonucleotide. In contrast, oligonucleotides labeled at an internal position are pro-

duced post-synthesis by reaction of an amino-modified oligonucleotide with the Cy-dye *N*-hydroxysuccinimidyl ester [10]. The latter is commercialized by GE Healthcare (Piscataway NJ), and contains sulfonates at positions 5 and 5′ of the aromatic rings to improve water solubility and reduce dye aggregation.

In previous work, we investigated the photophysical properties of Cy3 linked covalently to DNA [11]. We observed that the fluorescence efficiency and lifetimes of Cy3 on DNA depend strongly on its specific location, and differ greatly from the values measured for the free dye in solution. The photophysical properties of the dye were interpreted in terms of the potential energy surface shown in Fig. 2 [12, 13]. Following light absorption, the dyes isomerize from the first excited singlet state (^{1}N) to a ground state non-fluorescent photoisomer (P). Isomerization occurs via a partially twisted intermediate excited state, which deactivates rapidly to the ground-state surface to yield the ground state photoisomer (P), or return back to the thermodynamically stable ground state (N). Once formed, the photoisomer undergoes a thermal back-isomerization reaction to yield the thermodynamically stable isomer (P→N). Interestingly, we observed dramatic differences in the properties of Cy3 attached covalently to the 5′ end of single-stranded or double-stranded DNA. The properties of Cy3 attached to DNA internally were also noticeably different from the properties of the 5′-labeled samples. We interpreted these differences as arising from variabilities in Cy3–DNA interactions in the different samples. In this model, Cy3–DNA interactions increase the energetic barrier for photoisomerization, increasing the fluorescence lifetime and quantum yield.

There have been other reports that document changes in the fluorescence properties of Cy3 upon covalent binding to biopolymers. Gruber et al. reported an anomalous fluorescence enhancement by twofold to threefold of Cy3 upon covalent linking to IgG and non-covalent binding to avidin [14]. Oiwa et al. measured the fluorescence lifetime and steady-state anisotropy of Cy3-labelled ATP and ADP analogs, and their complexes with Myosin Subfragment-1, observing an unexpected difference depending on whether the modification was at the 2′ or 3′ positions of the ribose moiety [15].

In order to gain a deeper understanding of the nature of the interactions between Cy3 and DNA, in this work we investigate the fluorescence properties of Cy3–SE and $DiIC_2(3)$ in the presence of DNA nucleoside monophosphates (see Fig. 1). We observe a marked increase in the fluorescence efficiency and lifetime of Cy3, which is more dramatic in the presence of purines (dGMP, dAMP) than pyrimidines (dTMP, dCMP). Furthermore, we observe that Cy3–dNMP interactions are more pronounced in the case of the non-sulfonated Cy3 molecule, as evidenced by a larger enhancement of the fluorescence emission and a larger spectral shift. These results suggest that π–π interactions between Cy3 and the DNA bases play an important role in determining the spectral and photophysical properties of the dye.

Materials and methods

Sample preparation The following DNA nucleoside monophosphates (dNMPs) were purchased from Sigma-Aldrich (St Louis, MO): 2′deoxyadenosine 5′-monophosphate (dAMP), 2′deoxycytidine 5′-monophosphate sodium salt (dCMP), 2′deoxyguanosine 5′-monophosphate sodium salt hydrate (dGMP), and thymidine 5′-monophosphate disodium salt hydrate (dTMP). All solutions were prepared in 10 mM Tris buffer at pH=7.2. The Cy3 *N*-hydroxysuccinimidyl ester (Cy3–SE) was purchased from GE Healthcare (Piscataway NJ). The nonsulfonated Cy3 dye, 1,1′-Diethyl-3,3,3′,3′-tetramethylindocarbocyanine iodide ($DiIC_2(3)$), was purchased from Sigma-Aldrich (St. Louis, MO). Both dyes were dissolved in 10 mM Tris buffer. The absorbance of the Cy3 solutions at 515 nm was ~0.05 for steady state experiments and ~0.2 for lifetime experiments.

Steady-state fluorescence Fluorescence spectra were measured using a QuantaMaster-4/2005SE Spectrofluorometer (PTI, NJ). Temperature was measured inside the cuvette with a calibrated thermocouple and controlled using a water circulation system. The samples were excited at 515 nm. Fluorescence quantum yields as a function of dNMP concentration were measured relative to the corresponding value for the free Cy3 dye. Fluorescence quantum yields as a function of temperature were determined using the value for the free dye at 25 °C as a reference.

Time resolved fluorescence Time-resolved fluorescence intensity decays were measured using time-correlated single-photon counting (TCSPC) at room temperature. The samples were excited using a Tsunami (Spectra-Physics, CA) Titanium Sapphire (Ti:S) laser. The laser has a pulse duration of 130 fs and is operated at 80 MHz. An excitation wavelength of 460 nm at 4 MHz was obtained by doubling the frequency using a model 3980 frequency doubler (Spectra-Physics, CA). Attenuating the excitation beam ensured that a single photon triggered the start of the count. The sample was excited with vertically polarized light while the emission polarizer was kept at the magic angle with respect to the excitation polarizer. Fluorescence emissions were detected using a Gemini-180 double-grating monochromater (Jobin-Yvon, NJ) and a R3809U-50 microchannel plate photomultiplier tube (Hamamatsu, Japan). An SPC-830 single-photon counting card (Becker-Hickl,

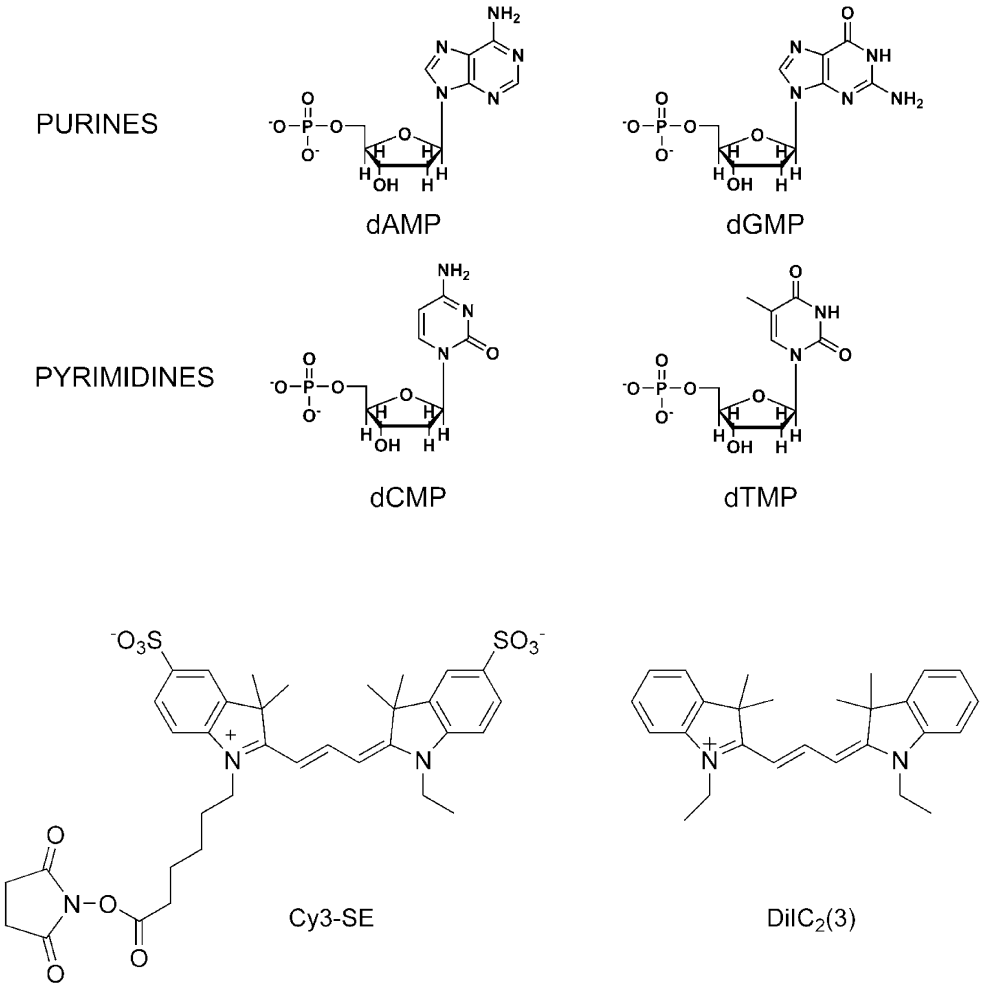


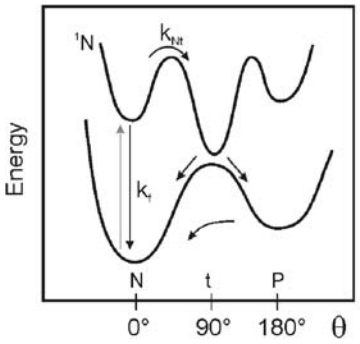
Fig. 1 Chemical structures of the nucleoside monophosphates and Cy3 dyes used in this work (see "Materials and methods")

Germany) was used for data acquisition. The instrument response function (IRF) was measured using a 2% Ludox scattering solution (Sigma Aldrich, MO). The measured FWHM of the IRF was typically about 40 ps. Intensity decays were obtained from the measured decays by iterative deconvolution assuming that the intensity decay function can be represented by a sum of exponentials. The goodness of the fit was evaluated from the χ^2 value and the randomness of the residuals.

Results

The absorption spectrum of Cy3 is not affected by the addition of any of the dNMPs. In contrast, the position of the fluorescence band of Cy3 shows a small but measurable red-shift (Fig. 3). The shift is more pronounced for the non-sulfonated compound in the presence of purines. The shift in emission wavelength is accompanied by an increase in fluorescence efficiency. For instance the fluorescence intensity of a 1 μM solution of $DiIC_2(3)$ increases by a factor of approximately two in the presence of 100 mM dGMP or dAMP (Figs. 3 and 4). Figure 4 shows the relative increase in fluorescence efficiency of Cy3–SE and $DiIC_2(3)$ in the presence of increasing concentrations of dNMPs. The fluorescence efficiency of both cyanines increases in the presence of all four nucleosides, but it is markedly higher in the presence of the purines than the pyrimidines. All changes are more pronounced for $DiIC_2(3)$ than Cy3–SE.

The same trend is observed in the time-resolved fluorescence intensity decays. The fluorescence decays of the free Cy3–SE and $DiIC_2(3)$ dyes are monoexponential, with lifetimes of 180 and 80 ps respectively. At least two exponential terms are needed to fit the decays in the presence of dNMPs. Consistent with the observed increase in fluorescence efficiency, the decays of Cy3 show longer lifetimes in the presence of the purines than in the presence of the pyrimidines (Fig. 5), and changes are more significant in the case of $DiIC_2(3)$ than Cy3–SE. Interestingly, the lifetimes of both Cy3 samples in the presence of 100 mM dGTP increase with increasing emission wavelength, while no such dependence was observed for the free



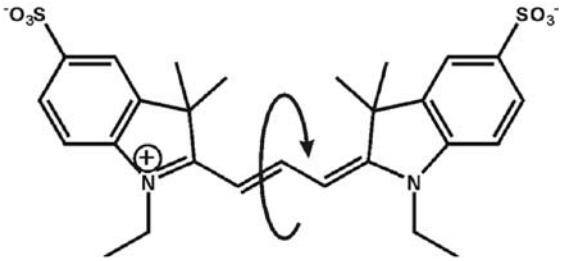


Fig. 2 Potential energy surface for cyanine photoisomerization. The angle θ represents the rotation coordinate, N the ground state of the normal form (*trans* isomer), ^1N the first excited singlet state of the normal form, t the twisted state, and P the ground state of the photoisomer

dyes (supplementary material, Fig. 1). The lifetimes and relative amplitudes of all fits can be found in the supplementary material.

Fluorescence quantum yields vary largely with the temperature of the solution, as expected for a molecule in which the main deactivation path is an activated process. The activation energy for photoisomerization (^{1}N→t process, Fig. 2) can be obtained from Eq. 1 [11, 16]:

$$\ln\left[\phi_f^{-1}(T) - \phi_{\mathrm{f,max}}^{-1}\right] = \ln A - E_a/\mathrm{RT} \qquad (1)$$

where ϕ_f is the temperature-dependent fluorescence quantum yield of Cy3, $\phi_{f,max}$ is the maximum fluorescence efficiency that would be measured in the absence of photoisomerization, A is a constant that depends on the local microscopic friction and the radiative lifetime of fluorescence, and E_a represents the activation energy for isomerization (^{1}N→t) [11, 16]. The value of $\phi_{f,max}$ was taken as 0.85 from previous work [11]. Results show that the activation barrier for photoisomerization increases in the presence of all four dNMPs, but it is larger in the presence of the purines than the pyrimidines. As an example, the results for Cy3–SE in the presence of 100 mM dNMPs are presented in Fig. 6. Activation energies were calculated from the slopes of the plots of Fig. 6 according to Eq. 1 as 29.4±0.6 kJ/mol for dGMP, 25.1±0.4 kJ/mol for dAMP, 23.1±0.9 kJ/mol for dCMP, and 21.6±0.2 kJ/mol for dTMP. The activation energy for Cy3–SE in the absence of nucleosides was measured as 19±1 kJ/mol in previous work [11].

Discussion

We have observed a nucleobase-dependent enhancement of the fluorescence efficiency of Cy3. The fluorescence efficiency and lifetime of the dye increase by addition of any of the four nucleobases, but changes are more dramatic in the case of the purines, dGMP and dAMP. The same is true for the observed spectral shifts in the emission spectrum of the dye. It is interesting to note that this increase in fluorescence efficiency and lifetime is opposite to the behavior observed with several fluorescent dyes such as fluorescein [17], coumarins [18], rhodamines and oxazines [19], which are quenched by G-residues via photoinduced electron transfer. Quenching by A-residues has been also observed, but to a lower extent due to the higher oxidation potential of dAMP compared to dGMP [20]. Cy3 does not have a favorable reduction potential, and it is not quenched by any of the nucleobases [20].

We interpret the increase in fluorescence efficiency as a consequence of interactions between the dye and the nucleoside monophosphates, which reduce the efficiency of *trans→cis* isomerization from the first excited state. This is supported by the measured increase in the activation energy for photoisomerization in the presence of all four dNMPs. Photoisomerization is the most efficient non-radiative deactivation path for these compounds [12, 16], so interactions that increase the activation barrier for isomerization will result in an increase of the fluorescent efficiency (Fig. 2).

In previous work, we observed a similar behavior for Cy3 attached covalently to the 5′ terminus of single

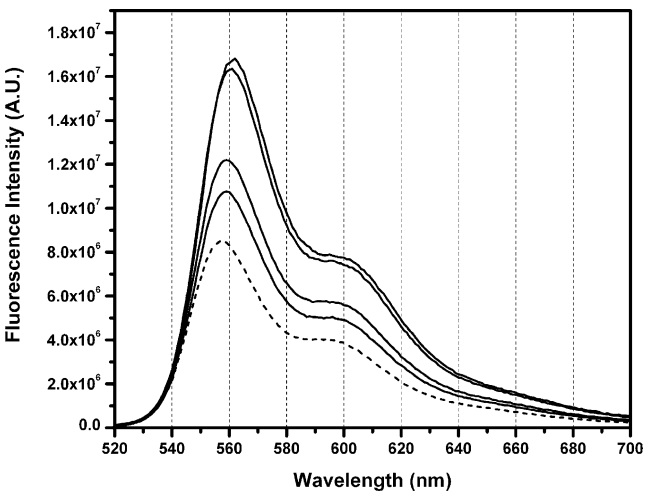


Fig. 3 Fluorescence spectra of diIC$_2$(3) (λ_{max}=557 nm, *dashed line*) and diIC$_2$(3) in the presence of 100 mM dNMPs (*full lines*). In order of increasing intensity: dCMP (λ_{max}=559 nm), dTMP (λ_{max}=559 nm), dAMP (λ_{max}=561 nm) and dGMP (λ_{max}=562 nm). Fluorescence intensities were corrected for differences in the absorbances of the solutions

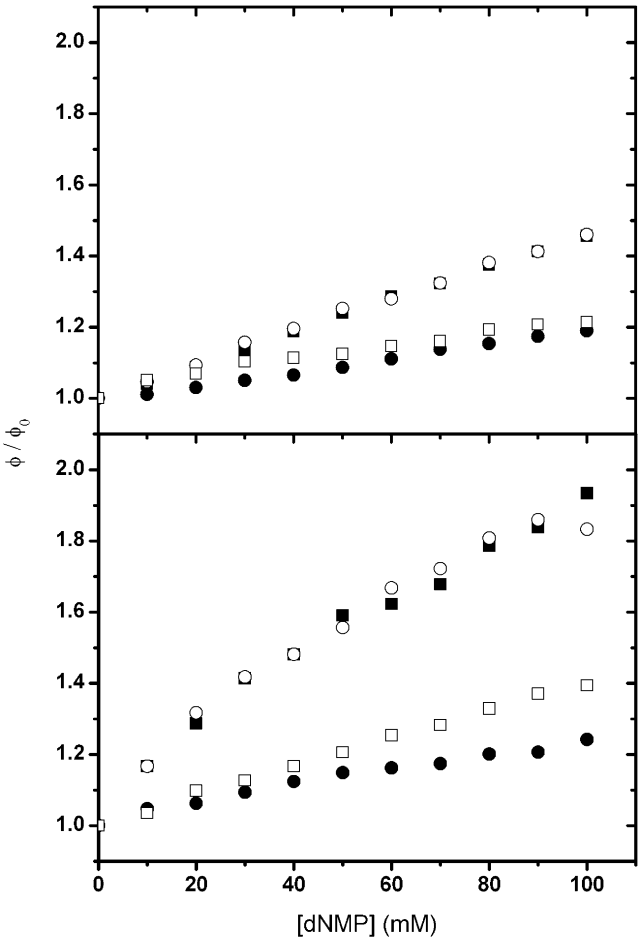


Fig. 4 Relative fluorescence quantum yields of Cy3–SE (*top*) and $diIC_2(3)$ (*bottom*) as a function of increasing concentrations of dCMP (*filled circles*), dTMP (*open squares*), dAMP (*filled squares*), and dGMP (*open circles*). Values are relative to the quantum yield of the dye in the absence of dNMPs (ϕ_0)

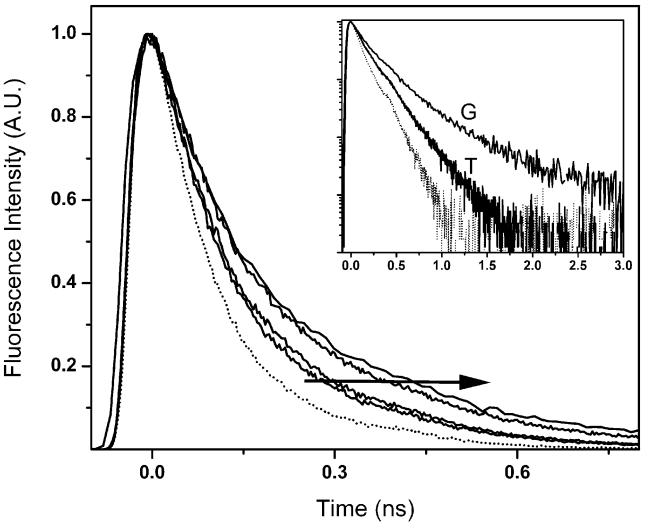


Fig. 5 Fluorescence intensity decay of $diIC_2(3)$ (*dashed line*) and $diIC_2(3)$ in the presence of 80 mM dNMPs (*full lines*). From *left* to *right*: dCMP, dTMP, dAMP, and dGMP. *Inset* $diIC_2(3)$ (*dashed line*), and $diIC_2(3)$ in the presence of 80 mM dTMP and dGMP (*full lines*) in an extended timescale. Intensities are plotted in logarithmic scale to emphasize the existence of multiple lifetimes in the presence of dNMPs

stranded DNA [11]. In this case, we observed that the increase in fluorescence quantum yield and lifetime was accompanied by a reduction in photoisomerization efficiency and a 4 nm red-shift in the emission spectrum. We observe a similar spectral shift for Cy3 in the presence of dNMPs. The spectral shift is more pronounced for the nonsulfonated dye in the presence of purines, which also present the longest fluorescence lifetime, greater increase in fluorescence quantum efficiency, and higher activation energy for photoisomerization.

The differences between Cy3–SE and DiIC2(3) suggest that the sulfonates present in Cy3–SE weaken the interactions that cause the more dramatic behavior observed for DiIC2(3). This is consistent with the remarkable differences we have observed in previous work between Cy3 covalently attached to single stranded DNA at the 5′ terminus or at an internal modified base [11]. Surprisingly, we measured a longer lifetime of fluorescence, higher fluorescence efficiency and lower photoisomerization yield for the dye attached to the 5′ terminus. Due to the different synthetic approaches, the dye lacks the sulfonate groups in this case, but has them in the internally-labeled oligonucleotide. It is likely that the lack of sulfonates in the 5′-labeled sample allow for stronger interactions between the fluorophore and the DNA bases, decreasing the ability of the dye to photoisomerize. The observed differences between the

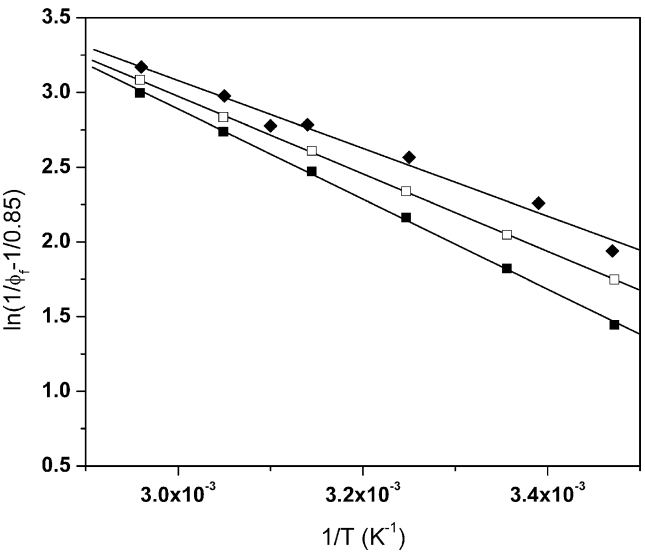


Fig. 6 Plot according to Eq. 1 for free Cy3–SE (*filled diamonds*) and Cy3–SE in the presence of 100 mM dAMP (*filled squares*) and dTMP (*open squares*). The activation energies for isomerization, calculated from the slopes of these plots, were: 19±1 kJ/mol (free Cy3, ref. [11]), 25.1±0.4 kJ/mol (dAMP) and 21.6±0.2 kJ/mol (dTMP). The data for dGMP and dTMP are not shown for clarity (see text)

purines and pyrimidines suggest that π–π stacking interactions between the carbocyanine and the nucleobases might be responsible for the measured enhancement in the fluorescence efficiency of the dye. Other types of interactions such as electrostatic interactions between the dye and the monophosphates are not ruled out, but these interactions alone cannot explain the marked differences between the purines and pyrimidines. The fact that spectral shifts were observed in the fluorescence spectrum, but not in the absorption, suggests that interactions occur in the excited state. This is significant because the conformation of Cy3 on DNA has been studied by NMR spectroscopy [21], and the results of these studies are often cited in the context of fluorescence experiments. It is possible that the interactions between the dye and the nucleobases in the ground state are different from the interactions in the excited state, which determine the fluorescence properties of the dye on DNA. Our results suggest that these interactions are dynamic in the nanosecond timescale, as evidenced by the dependence of the intensity decay on emission wavelength, and the fact that the lifetimes increase smoothly as the concentration of dNMPs is increased.

In summary, we have shown a nucleobase-dependent increase of the fluorescence efficiency of the dye Cy3 in the presence of nucleoside monophosphates. The increase in fluorescence efficiency and fluorescence lifetimes correlates with an increase in the activation energy for photoisomerization and a ~4 nm red shift in the fluorescence spectrum. These effects are more dramatic for the purines (A, G) than the pyrimidines (C, T), and for the nonsulfonated cyanine than the sulfonated dye. These results are consistent with a model in which Cy3–nucleoside π–π interactions decrease the efficiency of photoisomerization, thus increasing the efficiency of fluorescence. Other types of interactions, such as electrostatic interactions with the charged phosphates, are also likely to play a role, particularly when Cy3 is covalently attached to DNA.

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