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Yuichi Ohya[a]; Kentaro Yabuki[a]; Tatsuro Ouchi[a]
[a] Department of Applied Chemistry, Faculty of Engineering and High Technology Research Center, Kansai University, Suita, Osaka, Japan

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Sequence Dependence of Fluorescent Quenching of Chromophores Covalently Bonded to Oligo-DNAs before and after Duplex Formation

YUICHI OHYA*, KENTARO YABUKI and TATSURO OUCHI

Department of Applied Chemistry, Faculty of Engineering and High Technology Research Center, Kansai University, Suita, Osaka 564-8680, Japan

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The labeling of oligo- and polynucleotides with fluorescent probes is an important technique for the analysis of DNAs and RNAs. The effect of duplex formation with complementary oligo-DNA on the quenching behavior of two fluorescent chromophores (eosin, Eo and tetramethylrhodamine, TMR) attached to the 5′-terminal of various 10mer oligo-DNAs was investigated and the dependence of the quenching on DNA base sequence is discussed. We found that guanine residues played a major role in the quenching of the fluorescence of the chromophores. Guanine residues on the complementary DNA near the chromophores, in particular, had a significant influence on the quenching.

Keywords: Fluorescent probe; Quenching; DNA; Duplex formation

INTRODUCTION

Polynucleotide (DNA or RNA) hybridization assays have been an essential component of biological research for over a decade and are becoming more and more important for the clinical detection of genetic disorders and single nucleotide polymorphisms (SNPs). Many kinds of DNA detection systems have been proposed [1–4]. The labeling of oligo- and polynucleotides with fluorescent probes is a classical but still very important technique for analysis of DNAs and RNAs. The spectral change of the probes and fluorescent resonance energy transfer (FRET) between probes covalently attached to DNAs and RNAs have been investigated for detection of duplex or triplex formations of DNA [5,6], for structural analyses of DNA and RNA [7–9], for diagnostic detection of specific genes [10,11] and for other purposes [12–14].

DNA duplexes contain a rich π-electron system comprising four bases stacked upon each other. A number of researchers have investigated electron transfer behaviors through DNA duplexes or conductivity of DNA duplexes through the use of various fluorescent probes [15–18]. Recently, we reported the construction of a sequential chromophore array on a DNA assembly system, and multi-step energy transfer behavior, using some fluorescent chromophores covalently bonded to various oligo-DNAs [19,20]. In those studies, however, we sometimes observed quenching of the fluorescence of the chromophores (decrease in quantum yield) due to covalent bonding with oligo-DNA as well as through duplex formation of fluorescent-probe-modified oligo-DNA with complementary oligo-DNA. In order to apply the fluorescence labeling technique to DNA analysis and other fields, it is quite important to reveal the photochemical influence of DNA itself on the chromophores. The quenching behavior of some chromophores, especially fluorescein (FL) and pyrene (Py), when covalently bonded to oligo-DNAs, on duplex formation with complementary oligo-DNAs, has been previously reported [5,21–23]. For example, Cardullo *et al.* reported that the fluorescence of FL attached to oligo-DNA was quenched on association with a complementary oligo-DNA, and quenching of the fluorescence did not depend on the length of the oligo-DNA [5].

*Corresponding author. Tel.: +81-6-6368-0818. Fax: +81-6-6339-4026. E-mail: yohya@ipcku.kansai-u.ac.jp

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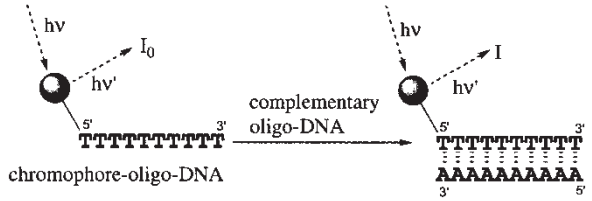

FIGURE 1 Schematic diagram of the experiment.

The effect of DNA sequence on the spectra and the intensity of covalently bonded fluorescent chromophores has not, however, been investigated in detail. In the present study, we attached fluorescent chromophores to oligo-DNAs of various sequences and investigated the dependence of fluorescence intensity on sequence for the chromophore-oligo-DNA conjugates before and after duplex formation with complementary oligo-DNA. We focused, in particular, on the effect of guanine residues on fluorescence quenching because there have been reports that guanine residues have great influence on electron transfer through DNA duplexes due to a hole transport mechanism [24–27]. As mentioned above, in a previous study, we reported the fluorescence resonance energy transfer behavior of a sequential chromophore array on a DNA assembly [19]. We used tetramethylrhodamine (TMR) and eosin (Eo) as chromophores in that system, and experienced quenching of these chromophores by DNAs. So, in this study, to investigate the sequence effect of DNAs on the quenching behavior of TMR and Eo, we attached these two fluorescent chromophores to the 5′-terminal of 10mer oligo-DNAs containing guanine residues at various sites, and observed the fluorescence intensity before and after duplex formation with the complementary oligo-DNA (Fig. 1).

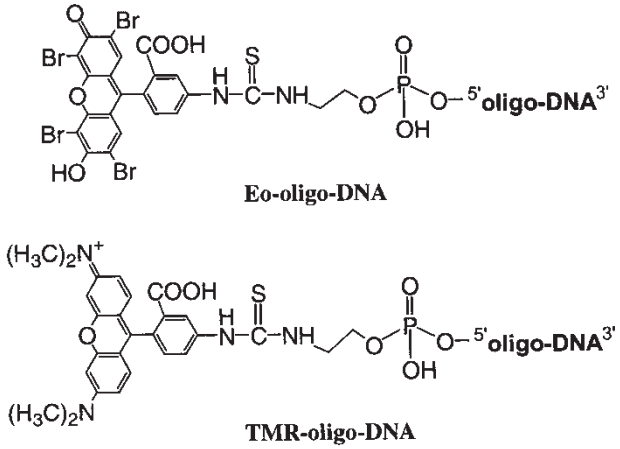

FIGURE 2 Chemical structures of chromophore-oligo-DNA conjugates.

RESULTS AND DISCUSSION

The chemical structures of the chromophore-oligo-DNA conjugates prepared are shown in Fig. 2, and their sequences and their abbreviations are summarized in Table I. These sequences were carefully chosen in order to investigate the effect of the guanine residues. The sequences T_{10}, GT_9, G_2T_8 and G_4T_6 were used to determine the effect of differing number of guanine residues. The sequences G_2T_8, GCT_8, CGT_8 and C_2T_8 were used to determine the effect of guanine residues located on the strand to which the chromophore was covalently bonded compared with those on the complementary (not covalently bonded) strand. The T_4GCT_4 sequence was provided to confirm the effect of distance of a GC pair from the terminal to which the chromophore was attached.

All chromophore-oligo-DNA conjugates with the complementary oligo-DNAs exhibited a melting point T_m in the range 30–45°C. Lowering of T_m due to bonding of chromophores was within 5°C of T_m of non-modified oligo-DNAs. No rise in T_m was observed. Specific interaction of the chromophore with a DNA duplex, such as intercalation, was not detected by this T_m measurement nor by measurement of fluorescence spectra using free chromophore and unmodified DNA duplex. The fluorescence intensity of the conjugates was investigated before and after hybridization with the complementary oligo-DNA in 0.05 M Tris–HCl 0.5 M NaCl buffer (pH 7.5) at 10°C, a temperature significantly lower than the T_m. The excitation and emission wavelengths were 525 and 547 nm for Eo-oligo-DNA conjugates and 554 and 578 nm for TMR-oligo-DNA conjugates, respectively.

Figure 3 shows a typical example of change in fluorescence spectrum for TMR-C_2T_8 on titration by the complementary oligo-DNA (A_8G_2). A change in fluorescence intensity was observed but there was no observed change in spectral shape or shift in wavelength. Figure 4 shows the relative fluorescence intensity of TMR-C_2T_8 at 578 nm mixed with A_8G_2, at various ratios. The fluorescence intensity of TMR bonded to C_2T_8 was decreased by duplex formation with A_8G_2. The quenching of fluorescence of TMR was almost saturated when the A_8G_2:TMR-C_2T_8 ratio was 1:1. When a non-complementary oligo-DNA was used instead of A_8G_2, no significant change in fluorescence intensity was observed. Almost all other chromophore-oligo-DNA conjugates studied showed this pattern of fluorescence with complementary and non-complementary oligo-DNAs. Figures 5 and 6 show the relative fluorescence intensities of Eo-oligo-DNA conjugates and TMR-oligo-DNA conjugates before and after mixing with complementary oligo-DNAs. The fluorescence intensity was expressed as a value relative to fluorescence

TABLE I Sequences and the abbreviations of chromphore-oligo-DNA conjugates and the complementary oligo-DNAs

Sequence	Chromphore		Complementary oligo-DNA
	Eosin	Tetramethyl-rhodamine	
5′TTTTTTTTTT3′	Eo-T10	TMR-T_{10}	A_{10}
5′GTTTTTTTTT3′	Eo-GT_9	TMR-GT_9	A_9C
5′GGTTTTTTTT3′	Eo-G_2T_8	TMR-G_2T_8	A_8C_2
5′GGGGTTTTTT3′	Eo-G_4T_6	TMR-G_4T_6	A_6C_4
5′CCTTTTTTTT3′	Eo-C_2T_8	TMR-C_2T_8	A_8G_2
5′GCTTTTTTTT3′	Eo-GCT_8	TMR-GCT_8	A_8GC
5′CGTTTTTTTT3′	Eo-CGT_8	TMR-CGT_8	A_8CG
5′TTTTGCTTTT3′	Eo-T_4GCT_4	TMR-T_4GCT_4	A_4GCA_4

of free Eo or TMR. The mixing ratio of complementary oligo-DNA:chromophore-oligo-DNA conjugate was 2:1. White bars show the data before mixing with complementary oligo-DNA, that is, the data for the chromophore-oligo-DNAs in the single strand (ss) state. The fluorescence intensities of chromophores covalently bonded to oligo-DNA containing guanine residue(s) were weaker than that of the chromophores bonded to T_{10}. Eo-GT_9, Eo-GCT_8 and Eo-CGT_8, containing one guanine residue, showed similar but weaker fluorescence intensity than Eo-T_{10}. TMR-GT_9, TMR-GCT_8 and TMR-CGT_8 containing one guanine residue also showed similar but somewhat weaker fluorescence intensity than TMR-T_{10}. The fluorescence intensities of Eo-G_4T_6 and TMR-G_4T_6, containing four guanine residues, were minimum among Eo-oligo-DNA conjugates and TMR-oligo-DNA conjugates, respectively. Eo-C_2T_8 and TMR-C_2T_8, containing no guanine residues, showed similar fluorescence intensity to Eo-T_{10} and TMR-T_{10}, respectively. These results indicated that fluorescence of Eo and TMR was quenched by guanine residue(s) covalently bonded near the chromophores and the quenching efficiency depended on the number of guanine residues.

Half-tone bars in Figs. 5 and 6 show the data after mixing with two equivalents of complementary oligo-DNA, that is, the data for the oligo-DNA/chromophore-oligo-DNA double strand (ds) state. Table II summarizes the quenching efficiency (Q%) of the duplex formation calculated by the following equation:

$$Q(\%) = (1 - I/I_0) \times 100$$

where I_0 is fluorescence intensity without complementary oligo-DNA and I is fluorescence intensity with complementary oligo-DNA. Although Eo-T_{10} and TMR-T_{10} did not show significant change in fluorescence intensity, the fluorescence intensities of Eo-C_2T_8, Eo-GCT_8, Eo-CGT_8, TMR-C_2T_8, TMR-GCT_8 and TMR-CGT_8 were significantly lower in the ds

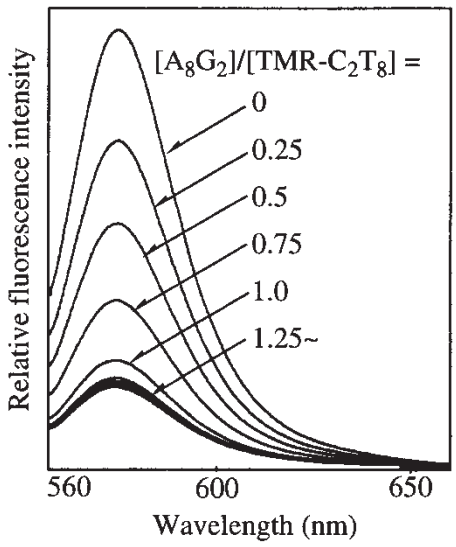

FIGURE 3 Fluorescence spectra for titration of TMR-C_2T_8 by the complementary oligo-DNA (A_8G_2) in 0.05 M Tris–HCl 0.5 M NaCl buffer (pH 7.5) at 10°C. [TMR–C_2T_8] = 5.0×10^{-7} mol dm^{-3}, [A_8G_2]/[TMR–C_2T_8] = 0–2.0. Excitation wavelength: 554 nm.

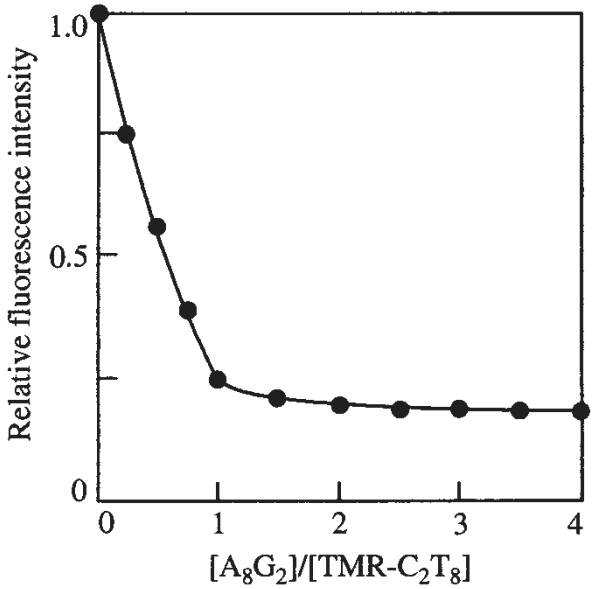

FIGURE 4 Plots of relative fluorescence intensity at 578 nm vs. ratio of TMR-C_2T_8 to the complementary oligo-DNA (A_8G_2) in 0.05 M Tris–HCl 0.5 M NaCl buffer (pH 7.5) at 10°C. [TMR–C_2T_8] = 5.0×10^{-7} mol dm^{-3}, [A_8G_2]/[TMR–C_2T_8] = 0–2.0. Excitation wavelength: 554 nm.

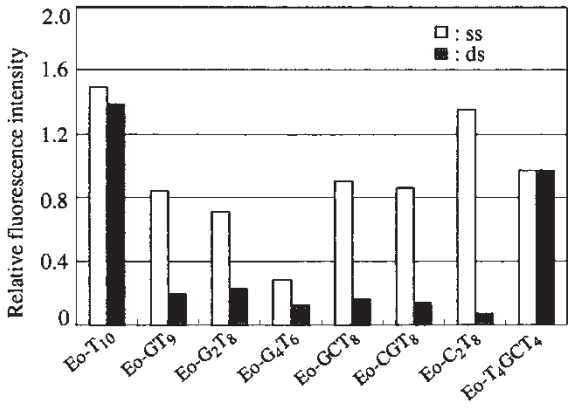

FIGURE 5 Sequence dependence of fluorescence intensity of Eo-oligo-DNA before and after hybridization with the complementary oligo-DNAs. The fluorescence intensity was expressed as a value relative to fluorescence of free Eo. ss: single strand before hybridization, ds: double strand after hybridization. Excitation wavelength: 525 nm, emission wavelength: 547 nm.

state than in the ss state. In these cases, the complementary oligo-DNAs for these conjugates contained guanine residues near their 3′-terminus. *Q* (%) values observed for Eo-C_2T_8, TMR-GCT_8 and Eo-CGT_8 were larger than those of Eo-T_{10}, Eo-GT_9, Eo-G_2T_8 and Eo-G_4T_6. Similarly, *Q* (%) values observed for TMR-C_2T_8, TMR-GCT_8 and TMR-CGT_8 were also larger than those of TMR-T_{10}, TMR-GT_9, TMR-G_2T_8 and TMR-G_4T_6. Interestingly, in Eo-oligo-DNA conjugates and TMR-oligo-DNA conjugates, Eo-C_2T_8 and TMR-C_2T_8, respectively, showed minimum fluorescence intensity in both the ss and ds states, even lower than for Eo-G_4T_6 and TMR-G_4T_6, which have four guanine residues. These results suggest that a guanine residue on the complementary oligo-DNA near the chromophores has a greater influence on the quenching of the chromophores

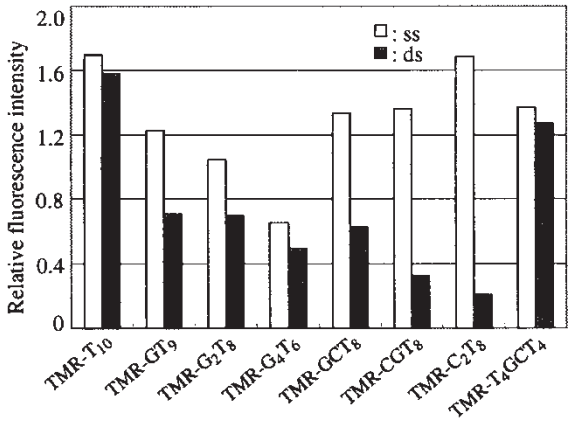

FIGURE 6 Sequence dependence of fluorescence intensity of TMR-oligo-DNA before and after hybridization with the complementary oligo-DNAs. The fluorescence intensity was expressed as a value relative to fluorescence of free TMR. ss: single strand before hybridization, ds: double strand after hybridization. Excitation wavelength: 554 nm, emission wavelength: 578 nm.

TABLE II Sequence dependence of quenching by duplex formation for chromophore-oligo-DNA conjugates

	Q(%)†	
Sequence*	Eo	TMR
T_{10}	7.4	7.0
GT_9	77.5	42.8
G_2T_8	67.9	34.1
G_4T_6	56.2	23.8
GCT_8	82.8	53.5
CGT_8	84.2	76.1
C_2T_8	95.0	88.0
T_4GCT_4	−0.4	6.7

*Sequenced of oligo-DNA covalently attached to chromophores. †Quenching (%) = $(1 - I/I_0) \times 100$, where: I_0, fluorescence intensity without complementary oligo-DNA; *I*, fluorescence intensity with complementary oligo-DNA.

than a guanine residue on the covalently linked strand. This is probably because accessibility of the covalently linked chromophore against the end of complementary strand is easier than against the covalently linked strand (Fig. 7), and the π−π stacking of the chromophore with guanine at the end of complementary strand may occur. In contrast, Eo-T_4GCT_4 and TMR-T_4GCT_4, whose complementary oligo-DNAs have guanine at five residues distance from their 3′-terminus, did not show significant quenching when mixed with the complementary oligo-DNAs, and were similar in this regard to Eo-T_{10} and TMR-T_{10}. These results suggest that five residues distance is enough to avoid quenching by a guanine residue on the complementary strand.

CONCLUSION

Sequence dependence of DNA on the fluorescence quenching of the Eo and TMR attached to 5′-teminal of 10mer DNAs before and after duplex formation with the complementary oligo-DNA was investigated. All results obtained suggest that guanine residues, especially on the complementary strand, play a major role in the quenching of Eo and TMR. It has been reported that guanine could be an electron donor and plays major role in electron transfer through the DNA duplex [24–27]. While it is not

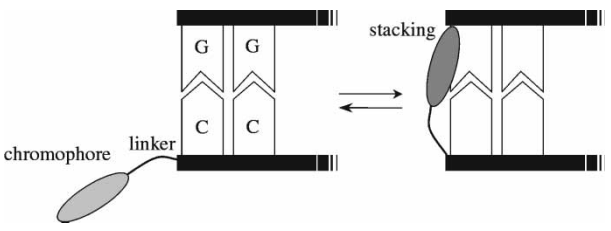

FIGURE 7 Speculative illustration for the interaction of covalently linked chromophore with the complementary strand.

clear that the quenching phenomena observed in these experiments are attributable to an electron transfer reaction between chromophores and guanine residues, these fundamental findings are quite informative in various fields for analysis of DNA by fluorescent probe labeling.

MATERIALS AND METHODS

Materials

Eosin isothiocyanate (EITC) and tetramethylrhodamine isothiocyanate (TRITC) were purchased from Molecular Probes, Inc. (USA). 5′-End free fully protected oligo-DNAs on solid support (control pore glass, CPG) and non-modified oligo-DNAs were obtained from Toagosei Co., Ltd. (Japan) or Hokkaido System Science, Co. (Japan). Aminoethanol, ethyl trifluoroacetate, 1*H*-tetrazole/acetonitrile solution and anhydrous acetonitrile were purchased from Wako Pure Chemical Industries Ltd. (Japan). 2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite was purchased from Aldrich Chemical Co. Inc. (USA). Water was purified using a reverse-osmotic membrane. *N*,*N*-Dimethylformamide and other organic solvents were purified by usual distillation methods. Other materials were of commercial grade and used without further purification. 5′-Aminoethyl-oligoDNA prepared by the methods reported previously using 5′-end free fully protected oligo-DNAs on solid support, 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, aminoethanol and ethyl trifluoroacetate [19]. Oligo-DNAs covalently bonded with the chromophores via C2 linker at 5′-terminal, chromophore-oligo-DNA conjugates, were synthesized by using 5′-amiaminoethyl-oligoDNAs obtained and EITC or TRITC according to the same methods reported previously [19,20].

Spectroscopic Studies

The UV-VIS absorption spectrum was recorded on UV-2500PC (Shimadzu, Japan) spectrophotometers. Concentration of unmodified oligo-DNA was calculated from the UV absorbance at 260 nm (A260), and extinction coefficients were determined using the nearest–neighbor approximation. Concentration of each chromophore-oligo-DNA conjugate was determined using UV-VIS absorbance at λ_{max} of Eo or TMR (525 or 554 nm) for Eo-oligo-DNA conjugate or TMR-oligo-DNA conjugate, respectively. The concentration of conjugate in solution was confirmed by the fact that their hypochromicity with complementary unmodified oligo-DNAs was maximal at 1/1 mole ratio. Hypochromisity measurement was performed by monitoring A260 of the solution containing the conjugate and complementary oligo-DNA in various ratios. Melting curves were recorded by starting at a temperature sufficiently above T_m and reducing temperature at a rate of 10°C/h to a temperature sufficiently below T_m. Absorbance values were continuously recorded at intervals of 5°C. Fluorescence measurements were performed on a F4010 (Hitachi, Japan) spectrophotometer using a 1×1 cm quartz cell. Excitation wavelengths used were 525 and 554 nm for Eo and TMR, respectively. Fluorescence spectra for the mixture of chromophore-oligo-DNA conjugate and complementary matrix oligo-DNA were obtained by varying the mixing ratios. The following procedure shows a typical example for titration of the complex of Eo-T_{10} by A_{10}. "Solution A" containing Eo-T_{10} (5.0×10^{-7} mol dm^{-3}) and "Solution B" containing Eo-T_{10} (5.0×10^{-7} mol dm^{-3}) and A_{10} (20.0×10^{-7} mol dm^{-3}) were prepared. The fluorescence spectra of "Solution A" were measured under titration by "Solution B". The final ratio of Eo-T_{10} to A_{10} was 4.0. All fluorescence experiments were performed in 50 mM Tris–HCl buffer, pH 7.5, containing 500 mM NaCl at 10°C.

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