Spectroscopic recognition of guanine dimeric hairpin quadruplexes by a carbocyanine dye

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ABSTRACT Isolated guanine quadruplex structures have been described at high resolution both in solution and in the solid state. The existence of this unusual DNA structure in vivo and its biological significance remain to be determined. We describe the binding of 3,3′-diethyloxadicarbocyanine to dimeric hairpin guanine quadruplexes. This interaction results in a set of unique spectrophotometric signatures, none of which arises from binding to single strands or Watson–Crick duplexes. These unique signatures include a new absorbance peak (λ̂ = 534 nm), an induced circular dichroism (λ = 534–626 nm), a quenching of the dye fluorescence upon excitation with visible light, and strong energy transfer from DNA. This last effect provides the basis for detecting hairpin quadruplex structures in the presence of excess amounts of nonquadruplex DNA structures, such as single strands and Watson–Crick duplexes. The mechanism of quadruplex recognition by this dye is discussed, along with the possibility of using this dye as a probe for hairpin quadruplex structures in vitro and in vivo.

Telomeres, the nucleoprotein complexes localized at the ends of eukaryotic chromosomes, are critical to chromosome stability and integrity (1). They contain repeats of short guanine tracts separated by several thymines and possibly adenines, with 12–16 bases forming a 3′ single-stranded overhang beyond the duplex region. The biological significance of quadruplex structures is of particular interest. They may facilitate chromosome association and alignment during meiosis (2). A nuclease from Saccharomyces cerevisiae capable of recognizing quadruplex structure and cutting the single-stranded region on the 5′ side has been identified (3). A protein that stabilizes the quadruplex form of the IgG switch region DNA has also been described (4). Yet evidence for their existence in vivo remains elusive, due in part to the lack of suitable probes for their detection.

Single-, double-, and quadruple-stranded forms of quadruplexes have been characterized in vitro (5). All are stabilized by Hoogsteen hydrogen bonding among four guanine bases arranged in a square planar configuration. The four-stranded linear quadruplexes consist of four parallel strands, with all deoxyguanosines in the anti conformation, while the one- and two-stranded, folded forms include antiparallel guanine sequences containing both syn and anti deoxyguanosine conformations. In general, quadruplex structures are stabilized by cations such as Na+ or K+ and, depending on sequence and cation, a particular oligonucleotide may exist as a mixture of several different quadruplex forms.

In contrast to the numerous studies on the formation and structural elucidation of various quadruplex DNA structures, little has been reported on the interaction of quadruplexes with ligands. Like some guanine- or xanthine-containing compounds, the folded quadruplex d(T2G4)4 and d(T2G4)4 were found to be capable of enhancing the fluorescence intensity of terbium(III) through the triplet energy transfer (6). Ethidium was reported to interact with the four-stranded linear quadruplex d(T4G4)4 as it does with duplex DNA in terms of spectroscopic properties and binding affinities (7). However, ethidium did not interact significantly with the unimolecular d(T2G4)4 folded quadruplex in the presence of Na+. In another study, Chen (8) has indicated that actinomycin D, ethidium, and chromomycin all bind poorly to the quadruplexes formed by d(T2G4)4 and d(T4G4)4 (8). Preferential cleavage of loop guanines in the unimolecular d(T2G4)4 quadruplex by bleomycin-Ni(III) was demonstrated, while no guanines were cleaved in the d(G4T2G4)4 quadruplex (9). However, it was not determined whether this preferential cleavage required the quadruplex structure.

In this paper, we present experimental results on the interaction between 3,3′-diethyloxadicarbocyanine (DODC), a carbocyanine dye (Fig. 1), and dimeric hairpin quadruplexes using UV absorbance, CD, fluorescence, and PAGE techniques. DODC was identified through a data base searching procedure (ref. 10; K. Burdick, Q.C., and I.D.K., unpublished data). We demonstrate that this ligand exhibits unique spectroscopic signatures when bound to hairpin quadruplex structures that are not observed in the presence of single-stranded, B-conformation double-stranded, or linear quadruplex DNA. This finding leads to the exciting possibility of using DODC as a probe for hairpin quadruplex structures in vitro and in vivo.

MATERIALS AND METHODS

Sample Preparation. DODC iodide was purchased from Kodak Eastman. Oligodeoxynucleotides were purchased from Genset, Inc., extensively dialyzed against 1 mM Tris-HCl (pH 7.5), and stored at −20°C in the same buffer. A stock solution of DODC was prepared in methanol. Stock solutions of Tris-HCl buffer, dye, salt (NaCl or LiCl), and oligodeoxynucleotide were mixed to give the desired dye concentrations. Residual methanol was <1% (vol/vol). Final buffer concentrations were 10 mM Tris-HCl and 150 mM NaCl (pH 7.5). The dye concentration was below 6 μM, while the concentrations of DNA samples ranged from 10 to 36 μM strand. DNA structures (Table 1) were verified from their CD spectra, melting curves, non-denaturing PAGE mobility, sedimentation equilibrium ultracentrifugation profile, and/or guanine imino proton NMR spectra: dimeric hairpin quadruplexes, [d(G1T2G4)]2, [d(G1T2G4)]2, [d(G3T2G3)]2, [d(G1T3CG4)]2, [d(G2CTG4)]2, and [d(G3TCT2G4)]2; linear four-stranded quadruplex, [d(TG3T4)]4; bimolecular Watson–Crick duplexes, [d(GCGAATTCCG)]2 and [d(CCGGAATTCGCC)]2; unimolecular hairpin Watson–Crick duplex, [d(CCGGT4CGCG)]2.

Absorbance and CD Measurements. Absorbance spectra were measured on a Varian Cary 3E UV-visible spectrophotometer in 1-cm cells, with a 2-nm band pass with temperature regulation. CD spectra were averages of 8–16 scans on a Jasco

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Abbreviation: DODC, 3,3′-diethyloxadicarbocyanine.
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Energy Transfer Studies. Energy transfer from DNA to DODC was determined from fluorescence excitation spectra of the dye, measured from 230 to 330 nm, in the presence of various oligonucleotides. The emitted fluorescence at 596 nm was observed at right angles to the incident light in a 10-mm cell. The inner filter effect was corrected with a factor of $10^{A/2}$, where $A$ is the total absorbance of the sample. The conventional methods to evaluate energy transfer efficiency require determination of the absorbance parameters of the DNA-bound dye (11, 12). However, the absorbance and the extinction coefficients of bound DODC were poorly determined in the range 230–280 nm, because ~99% of the absorbance in this range arises from the DNA in the complex.

The energy transfer efficiency, $\phi_{et}$, can be written as

$$\phi_{et} = \frac{F_s}{F_t} \left( \frac{\phi_t}{\phi_s} \right) \left( \frac{A_b}{A_d} \right);$$  

and free dye fluorescence intensities, respectively, $\phi_t$ is the quantum yield of the free dye, $\phi_s$ is the quantum yield of the bound dye in the absence of energy transfer, $A_s$ and $A_t$ are the absorbance of oligodeoxyxynucleotide and free dye, respectively, and $m$ is the fraction of oligodeoxyxynucleotide molecules that actually have dye molecules attached ($m = 1$ in the presence of excess dye).

All terms in Eq. 2 except $F_t$ and $A_t$ can be determined independently. Because $A_t$ constitutes ~99% of the total absorbance from the complex, we can neglect the absorbance contribution from the bound dye. If $(\phi_s/\phi_t)$ is small and the energy transfer efficiency is high, $F_t$ can also be estimated reliably according to

$$F_s = F_b - F_p = F_b - \left( \frac{\phi_t}{\phi_s} \right) \left( \frac{A_b}{A_t} \right) F_t,$$

where $A_b$ is the absorbance of the bound dye, $F_b$ is the fluorescence intensity of bound dye, and $F_t$ is the fluorescence intensity due to direct photon absorbance of the bound dye, even though $A_b$ is poorly determined. In the case of DODC, for example, $(F_b/F_t) > 7$ and $(\phi_t/\phi_s) = 0.52$. With the assumption that $(A_b/A_t) = 1$, even a relative error of 50% for $A_t$ will produce an uncertainty of only 4% or less for both $\phi_t$ and $F_t$.

**Gel Electrophoresis.** Nondenaturing PAGE was carried out in 10 mM Tris-HCl and 150 mM NaCl (pH 7.5) with 15% polyacrylamide. Gels were run at ~2 V/cm (~10 mA) for 48 hr at 4°C. DNA concentration ranged from 8–971 µM strands. After photography with UV shadowing, gels were stained in a solution containing ~30 µM DODC, ~1% methanol, 10 mM Tris (pH 7.5), and 150 mM NaCl for ~15 min at room temperature, rinsed briefly with distilled water, and then photographed under UV light at 254 nm.

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RESULTS

Absorbance Spectroscopy. In aqueous solution, the visible absorbance spectrum of DODC at low concentration (<10 μM) has a maximum at 576 nm with a small shoulder at 530–550 nm and a relative intensity ratio of \(A_{576}/A_{534} \approx 3\). Upon addition of \([d(G_4T_6G_4)]_2\), a new peak appears at 534 ± 1 nm along with an intensity decrease and small red shift for the 576-nm peak (Fig. 1). Similar spectral changes are also observed with other oligonucleotides that form bimolecular hairpin quadruplex structures under these conditions: \([d(G_4T_6G_4)]_2\), \([d(G_4T_6G_6)]_2\), \([d(G_4T_3C_3T)]_2\), \([d(G_4T_3C_3G)]_2\), or \([d(G_4T_3C_3T)]_2\) (data not shown). The binding to \([d(G_4T_6G_4)]_2\) appeared weaker than to \([d(G_4T_6G_4)]_2\), based on the DNA concentration required to induce the peak at 534 nm.

In contrast, we have found no other DNA structures that induced the 534-nm peak. Instead, they decreased the absorbance intensity at 534 nm in almost the same ratio as at 576 nm, without any shift in the maximum (Fig. 1). These other structures (summarized in Table 1) included the linear four-stranded quadruplex \([dT(G_4T)_4]_2\), the linear Watson–Crick duplexes \([d(CGCGAATTTCGC)]_2\) and \([d(CGCGAATTTCG)]_2\), the hairpin Watson–Crick duplex \([d(GCGT,GCG)]_2\), and the single-stranded forms of \([d(G_3A_3A_3T)]_2\) and \([d(G_3T_3A_3)]_2\) in the presence of LiCl, which is known not to promote any higher-order structures for these last two sequences (5). These results strongly suggest a different mode of dye binding to these forms of DNA, which we will refer to as the nonspecific binding mode.

The emergence of the 534-nm peak under the above conditions can be considered as a unique signature of the specific binding of the dye to the dimeric hairpin quadruplex structure. Binding curves derived from DNA-induced changes in absorbance are presented in Fig. 2. The apparent binding affinity of both specific and nonspecific binding modes is on the order of \(10^6\) M\(^{-1}\), with binding to the \([d(G_4T_6G_4)]_2\) quadruplex \(\approx 5\) times stronger than to the \([d(CGCGAATTTCG)]_2\) Watson–Crick duplex. It is, therefore, reasonable to expect that both bound forms coexist in the dye–\([d(G_4T_6G_4)]_2\) system.

Induced CD Spectroscopy. Generally, achiral dyes will show induced CD when tightly bound to a helical molecule. Fig. 3A shows the induced CD spectrum of DODC in the presence of \([d(G_4T_6G_4)]_2\). Except for a small positive bump at \(\approx 580\) nm, the spectrum is roughly conservative with a positive band at 535 nm and a negative band at 628 nm, suggesting contributions from the exciton effect. The same induced CD spectrum was also observed in the presence of \([d(G_4T_6G_4)]_2\) or \([d(G_4TCTG_4)]_2\). The hairpin quadruplexes \([d(G_4C_3T_3G_3)]_2\) and \([d(G_4T_3C_3G_3)]_2\) induced CD spectra, which differed both from each and from those induced by the other hairpin quadruplexes (Fig. 3B), suggesting that the thymine bases adjacent to the guanine quartet play important roles in determining the orientation and the position of the dye in the complex. Significantly, no induced CD signals were observed for the free dye or for the dye complexed with the linear quadruplex or with any of the nonquadruplex structures.

Surprisingly, the induced CD spectrum in the presence of the \([d(G_4T_6G_4)]_2\) hairpin quadruplex was completely different in position and relative sign from those induced by the \([d(G_4T_6G_4)]_2\) quadruplexes, as illustrated in Fig. 3A. The CD spectrum is blue-shifted with the positive band on the longer wavelength side. The separation between positive and negative peaks was much smaller than that observed in the case of \([d(G_4T_6G_4)]_2\) quadruplexes. A more obvious feature is the roughly conservative nature of the spectrum in the 500- to 660-nm range, with the zero midpoint right at \(534\) nm, indicating an exciton effect in this case. For all systems, the induced CD signal disappeared completely and reversibly at temperatures corresponding to the dissociation of the hairpin quadruplex.

Fluorescence Emission Spectroscopy. The fluorescence emission of the dye undergoes a dramatic red shift in intensity (~50%) along with an ~6-nm red shift in the presence of \([d(G_4T_6G_4)]_2\) (data not shown). The relative fluorescence quantum yield is 0.52 compared to that of free dye. None of the nonquadruplex structures, including the single-stranded form of \([d(G_4T_6G_4)]_2\) in the presence of Li\(^+\), significantly altered the fluorescence emission spectrum of the dye. As expected, \([d(G_4T_6G_4)]_2\) had a smaller red shift of only 3 nm and larger relative quantum yield of 0.85, presumably due to a weaker specific binding affinity. However, it was surprising that the fluorescence intensity in the presence of the \([d(TG_4T)_4]_2\) linear quadruplex was increased by 18%, implying the existence of yet another binding mode. The fluorescence quenching of the dye by the dimeric hairpin quadruplex is distinct from all other complexes, providing an additional unique signature of the dimeric hairpin quadruplex binding mode.

Fluorescence Collisional Quenching Studies. Stern–Volmer plots (Fig. 4) indicate that the dye bound to the Watson–Crick duplex or the linear quadruplex is completely accessible to the quencher, as their Stern–Volmer constants (\(K_{SV} = 23 \pm 1\) and \(21 \pm 1\), respectively) were almost the same as that of the free dye (\(K_{SV} = 24 \pm 1\)). On the other hand, the \(K_{SV}\) value for the dye bound to the \([d(G_4T_6G_4)]_2\) hairpin quadruplex was re-
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Ksv value with at by LM 8 7.5. protection case of produced that nonspecific dimeric more deeply involves outside excitation spectra almost identical 0.40 of On the other hand, dye With DNA. fluorescence quantum emission due to intensity free weaker and closely inside the DNA about one-fourth that of the free dye, demonstrating substantial protection from the quencher. As expected, the Ksv value for the dye bound to [d(G3T4G3)]2 was larger than that in the case of [d(G1T4G4)]2 but smaller than that in the case of free dye or the nonspecific binding modes, consistent with a weaker specific binding. These results suggest that the dimeric hairpin quadruplex binding mode differs from the nonspecific binding mode in that the former involves binding more deeply inside the DNA structure, while the latter most likely involves outside binding.

Energy Transfer from DNA Bases to Bound Dye. The excitation spectra of the free dye and dye–duplex complex look almost identical to their respective absorbance spectra (Fig. 5). On the other hand, in the presence of [d(G3T4G3)]2, the new large peak closely mirrors the DNA absorbance spectrum with the maximum intensity at ~260 nm (uncorrected excitation spectrum). Quantitative analysis of these data results in a value of 0.40 for φet, the energy transfer efficiency, for the complex with [d(G3T4G3)]2 and essentially 0 for φet in complexes with nonquadruplex DNA.

Gel Electrophoresis Visualized by Sensitized Fluorescence. With dye excitation in the UV, the signal loss from the lower fluorescence quantum yield, compared to excitation with visible light, is overwhelmed by the strongly enhanced fluorescence emission due to energy transfer. Fluorescence from dye bound to other DNA structures will be even lower than the background, due to the inner filter effect from DNA UV absorbance.

Fig. 6A shows a gel visualized by UV shadowing, where both the [d(CGGAATTCCG)]2 duplex and [d(G4T4G4)]2 hairpin quadruplex are visible as dark bands. The quadruplex migrated faster than the duplex having the same molecular weight because of its more compact structure. After staining by DODC, Fig. 6B shows that only the [d(G4T4G4)]2 quadruplex bands in the gel are distinguishable from the background as bright (pink) bands under 254 nm UV light. In contrast, the Watson–Crick duplex band actually looked slightly darker than the background. As expected, only the fluorescence signals from the dye bound to the quadruplex were detectable above the background. The concentration ratios of the duplex to the quadruplex in the gel ranged from 15 to 121 from left to right. This demonstrates the ability to detect a small amount of dimeric hairpin quadruplex in the presence of a large amount of duplex DNA. The concentration ratio was limited by the capacity of the gel and the limits of detection of the dye emission. But, in theory, this ratio can increase indefinitely because larger duplex concentrations will actually generate increasingly negative signals relative to the free dye background.

DISCUSSION

These results provide clear evidence that DODC has unique spectroscopic properties in the presence of dimeric hairpin quadruplexes, including (i) appearance of an absorbance peak at 534 nm, (ii) induction of a CD signal in the visible wavelength region, (iii) reduction in the intensity of fluorescence emission upon excitation with visible light, (iv) protection from collisional quenching, (v) energy transfer from the DNA bases. While the dye does bind to other forms of DNA, such as single strands, Watson–Crick duplexes, and linear, four-stranded guanine quadruplexes, none of the five features described above is observed. Furthermore, the hairpin quadruplex conformation can be detected even in the presence of large amounts of nonquadruplex DNA (Fig. 6).

The precise binding mechanism of the dye to the hairpin quadruplex remains uncertain. Spectral reversibility, along with the fact that the bound ligand can be completely extracted from its specific complex with 1-butanol, clearly indicate a noncovalent interaction. We can imagine four possible noncovalent binding modes: intercalation between quartets, binding in the grooves, interaction with thymine loops, and outside.

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**Figure 4**. Stern–Volmer plots for fluorescence quenching of 0.3 μM DODC by potassium ferrocyanide in 10 mM Tris/200 mM KCl, pH 7.5. DODC alone (●) and in the presence of 10 μM [d(CGCGATTCG)]2 (○), 16 μM [d(TG1T1)]2 (●), 16 μM [d(G1T4G4)]2 (□), and 8 μM [d(G3T4G3)]2 (○). λem = 534 nm; λem = 597 nm. All measurements were carried out at 25°C except for [d(G3T4G3)]2, which was performed at 15°C.

**Figure 5**. Fluorescence excitation spectra of 0.3 μM DODC alone (——) and in the presence of 8 μM [d(CGCGATTCG)]2 (—) and 8 μM [d(G3T4G3)]2 (⋯). λem = 597 nm. Same conditions as in Fig. 1.

**Figure 6**. PAGE of [d(CCGGAATTCCG)]2 and [d(G4T4G4)]2 in nondenaturing gels visualized by UV shadowing (A) and by sensitized fluorescence of DODC (λex = 254 nm) (B). DNA concentrations were as follows: [d(CCGGAATTCCG)]2, 971 μM (lane 1); [d(G4T4G4)]2, 63, 32, 12, and 8 μM strands (lanes 2–5, respectively). Same conditions as in Fig. 1.
binding. The possibility of outside binding can be easily eliminated according to the fluorescence collisional quenching data and the different CD spectra. Binding only to the loops is not supported by experimental results since \([d(G_4T_4G_4)\_2\], \([d(G_3T_4G_3)\_2\], \([d(G_4T_4G_4)\_2\], \([d(G_3T_4G_3)\_2\], \([d(G_4T_4G_4)\_2\] have similar loops but very different induced CD spectra. Furthermore, the dye does not bind specifically to the unique hairpin Watson–Crick duplex containing a T₄ loop.

Thus, groove binding and intercalation remain as the two possible modes of specific binding. Although energy transfer from DNA bases to the bound dye is commonly used as indirect evidence for intercalation, all that is needed is dye-base stacking for this effect to be observed (11–14). Several results argue against intercalation. The increase in melting point of the quadruplex in the presence of the dye is substantially smaller than what is usually seen for intercalation (data not shown). Also, it would be highly unusual for a ligand to intercalate into the hairpin quadruplex and not into the linear quadruplex or the Watson–Crick duplex. In fact, others have reported data suggesting that ethidium intercalates into a linear quadruplex but not into a folded quadruplex (7).

This leaves groove binding, which alone cannot easily explain the energy transfer results and the different induced CD spectra in complexes with \([d(G_4T_4G_4)\_2\], \([d(G_3T_4G_3)\_2\], \([d(G_4T_4G_4)\_2\], \([d(G_3T_4G_3)\_2\], \([d(G_4T_4G_4)\_2\]. A possible resolution may be based on binding of the dye in a groove of the quadruplex stem, with one end projecting into the thymine loop region and stacking between the loop thymines and the terminal guanine quartet. This model rationalizes both the energy transfer and the loop-sensitive CD results.

Additionally, groove binding provides an explanation for the apparently weaker, specific binding to \([d(G_4T_4G_4)\_2\] compared to \([d(G_4T_4G_4)\_2\]. In the latter, the deoxyguanosines display strictly alternating syn–anti conformations (15, 16) while in the former syn–syn as well as anti–anti sequences appear (17), with no anti–syn sequence. These differences should affect the groove structure. Similarly, structural differences between linear and hairpin quadruplexes may also account for the specific dye recognition of the latter and not of the former. Such differences include the parallel orientation of all four strands and the anti conformation for all deoxyguanosines in the former, compared to the antiparallel strand orientation and syn and anti conformations in the latter. Moreover, the linear quadruplex is characterized by four identical grooves (18), while the folded quadruplex has wide, medium, and narrow grooves (16). Definitive structure determination will require high-resolution methods such as x-ray crystal structure or NMR analysis.

In summary, DODC recognizes dimeric hairpin quadruplexes and the resulting complex possesses unique spectral properties not observed in other nucleic acid complexes. Thus, this dye possesses the qualities necessary for a probe of hairpin quadruplex structures both in vitro and, potentially, in vivo. Our results demonstrate that the presence of excess amounts of nonquadruplex DNA may actually enhance the ability to detect the quadruplex form. This probe may provide important information concerning the existence and possible biological significance of telomeres and other quadruplex-forming DNA sequences.

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