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Fluorescent dyes specific for quadruplex DNA

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ABSTRACT

Fluorescent dyes which are specific for duplex DNA have found a wide range of applications from staining gels to visualization of chromosomes. Porphyrin dyes have been found which are highly fluorescent in the presence of quadruplex but not duplex DNA. These dyes may offer a route to the specific detection of quadruplex DNA under biologically important conditions. There are three types of DNA quadruplex structures, and these may play important roles in telomere, centromere, triplet repeat, integration sites and other DNAs, and this first set of porphyrin dyes show some selectivity between the quadruplex types.

INTRODUCTION

Most of the molecules which intercalate between the base pairs of duplex DNA, like ethidium and proflavin, have about the same surface area as a base pair. Thus, we were led to considering molecules with approximately the same surface area as a quartet of nucleic acid bases as selective binders to quadruplex DNAs. In addition, aptamer DNAs that have sequences compatible with quadruplex structure formation have been found which selectively bind to porphyrins (1,2). These considerations suggested examining the binding of porphyrins to quadruplex DNA.

DNA quadruplexes formed of four parallel strands of *anti* dG residues have four equivalent grooves (3–5). The quadruplexes formed with *anti-syn-anti-syn* alternation of the dG within each quartet have two narrow and two wide grooves and have edge loops (6–8) as shown in Figure 1. The quadruplexes formed with *anti-anti-syn-syn* alternation within each quartet have one wide, one narrow and two medium width grooves, and have crossover loops (6,9,10) as shown in Figure 1.

The selective detection of quadruplex DNA in the presence of complex mixtures containing many structural forms of DNA, such as single and double stranded DNA, is not possible by any current technique. Circular dichroism (CD) can discriminate between single stranded DNA and quadruplex structures quite readily (11–16). The deconvolution of a CD spectrum of a mixture of single stranded, duplex and quadruplex DNAs has not been carried out. Raman spectroscopy has been used to observe bands which are signatures for quadruplex DNAs (17–21). The Raman spectra of some samples containing a mixture of single stranded and quadruplex forms have been deconvoluted (22), though this may not be possible for a multi-component system. Specific detection of quadruplex DNA could be of considerable use in studies on telomere (23,24), fragile X (25–28) and other triplet repeat DNAs (28,29) and with DNAs which partially adopt quadruplex structures (23).

A large number of porphyrins are available for study; the two of central interest here are referred to as T4 and NMM, and their structures are illustrated in Figure 2 along with the structures of ethidium and the TS4 porphyrin. Prior studies have shown that some porphyrins do bind to duplex DNA (30,31) and that the binding is between the bases on one strand of the duplex DNA rather than intercalating between the bases on both strands (32). Porphyrins have been used to damage DNA in photodynamic therapy (31,33,34). A report that appeared while this work was in progress indicated that T4 binds to a parallel stranded quadruplex (35) in agreement with the prior results on aptamer DNAs (1,2), and another showed that T4 inhibits telomerase by interacting with quadruplex DNA (36).

MATERIALS AND METHODS

The DNA samples referred to as 12mer: d(GGGGTTTTGGGG), 14mer: d(GGTTGGTTGGTTGG), 16mer: d(GGTTGGTTTTG-GTTGG), 17mer: d(GGTUTGGTGTGGUTTGG), 20mer: d(GGTUTUGGUTUTGGUUTTGG) and 23mer: d(GGGGTT-GGGGTGTGGGGTTGGGG) were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). The $d(GGC)_3$, $d(GGC)_4$ and $d(GGC)_5$ samples were obtained from DNAgency (Malvern, PA). The 15mer, GGTTGGTGTGGTTGG, was obtained from Gilead Sciences (Foster City, CA). The DNAs were HPLC purified. To remove traces of salt the DNAs were ethanol precipitated three times.

The molar extinction coefficients used for the samples are: $d(GGC)_3$: 82 090, $d(GGC)_4$: 110 520, $d(GGC)_5$: 138 150, 12mer: 110 000, 14mer: 130 150, 15mer: 143 303, 16mer: 147 600, 17mer: 137 070, 20mer: 197 530, 23mer: 226 530. The extinction coefficient for calf thymus DNA is 6600 per nucleotide. All of the fluorescence and UV experiments were carried out with the samples in 20 mM HEPES, 140 mM NaCl, 5 mM KCl at pH 7.0. The 15mer, no potassium sample, was in the same buffer but without KCl. N-methyl mesoporphyrin IX (NMM), meso-tetra (N-methyl-4-pyridyl) porphine tetra tosylate (T4) and meso-tetra (4-sulfonatophenyl) porphine dihydrochloride (TS4) were obtained from Porphyrin Products (Logan, UT). Ethidium was obtained from Sigma and the purity of each dye checked by NMR. Excitation and emission wavelengths (in nm) of 484 and 595 for ethidium, 399 and 614 for NMM, 429 and 655 for T4 and 410 and

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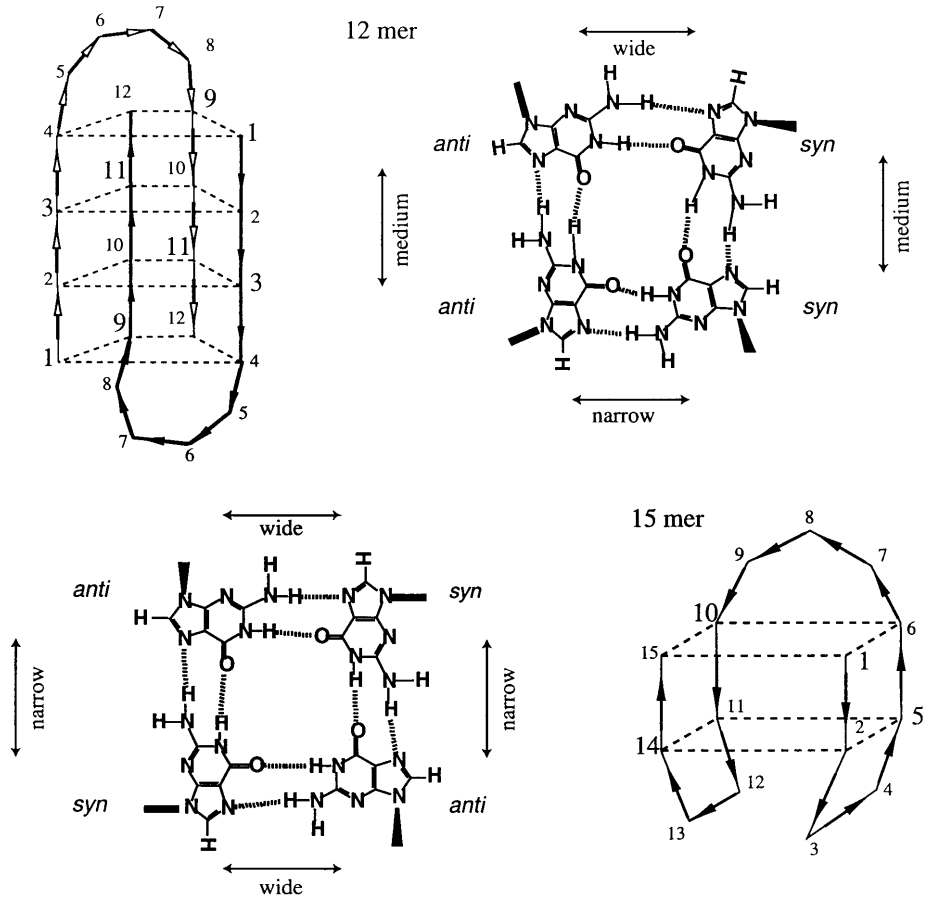

Figure 1. Schematic depictions of the chair type structure DNA quadruplex structure formed by the 15mer d(GGTTGGTGTGGTTGG) and the basket type structure formed by the 12mer d(GGGGTTTTGGGG). The positions of the *syn* G residues are indicated by the larger type. Also shown are depictions of the groove widths and base pairing schemes associated with these two types of structures.

646 for TS4 were used. All of the fluorescence experiments were carried out using a Hitachi F-2000.

The fluorescence titrations started with 600 µl of a 10^{-5} M dye solution to which DNA was added. The DNA concentration was increased from 10^{-7} to 10^{-3} M per nucleotide in a three series. In the first series the DNA concentration was increased from 10^{-7} to 10^{-6} M in steps of 2.5×10^{-7} M. In the second series the DNA concentration was increased from 2.5×10^{-6} to 2.5×10^{-5} M in steps of 2.5×10^{-6} M and in the last series the DNA concentration was increased from 5×10^{-5} to 10^{-3} M in steps of 2.5×10^{-5} M. The total sample volume increase during a titration was <3.3%. To minimize self absorption a cell of path length 10 mm was used.

After each addition of DNA the samples were annealed by heating to 80°C, followed by slow cooling to room temperature. Fluorescence measurements were repeated three times for each sample and the intensities averaged and corrected by running a blank before each series of experiments.

Error bars were determined by examining each of a set of five test samples, each prepared seven times and the fluorescence measured five times for each. A least-squares fit of the standard deviation against the average fluorescence intensity provided regression coefficients which were used to calculate the errors as a function of fluorescence intensity.

The absorption spectra were obtained with a 50:1 DNA nucleotide to drug ratio with the concentration of NMM at 1×10^{-5} M and that of T4 at 5×10^{-6} M. The samples were in the same buffer as used for the fluorescence experiments and were annealed before recording the absorption spectra.

RESULTS AND DISCUSSION

Two quadruplex DNAs of known structure were used for the initial binding studies. The 15mer, d(GGTTGGTGTGGTTGG), forms an intramolecular edge, or chair, type structure, as shown in Figure 1 (7,8). Dimers of the 12mer, d(GGGGTTTTGGGG), form a quadruplex of the crossover, or basket, form as shown in Figure 1 (37). The binding of ethidium to these two types of quadruplex DNA was examined and compared to that of duplex DNA. The results in Figure 3 show the familiar fluorescent enhancement in the presence of duplex calf thymus DNA; little or no fluorescence enhancement is observed in the presence of the 15mer, and modest enhancement is observed in the presence of the 12mer quadruplex DNA. Similar results were obtained with other dyes, like proflavin, which intercalate between the base pairs of duplex DNA, indicating that the usual duplex DNA intercalators appear not to bind well to quadruplex DNAs. Prior results indicate that parallel stranded quadruplex DNA has fewer

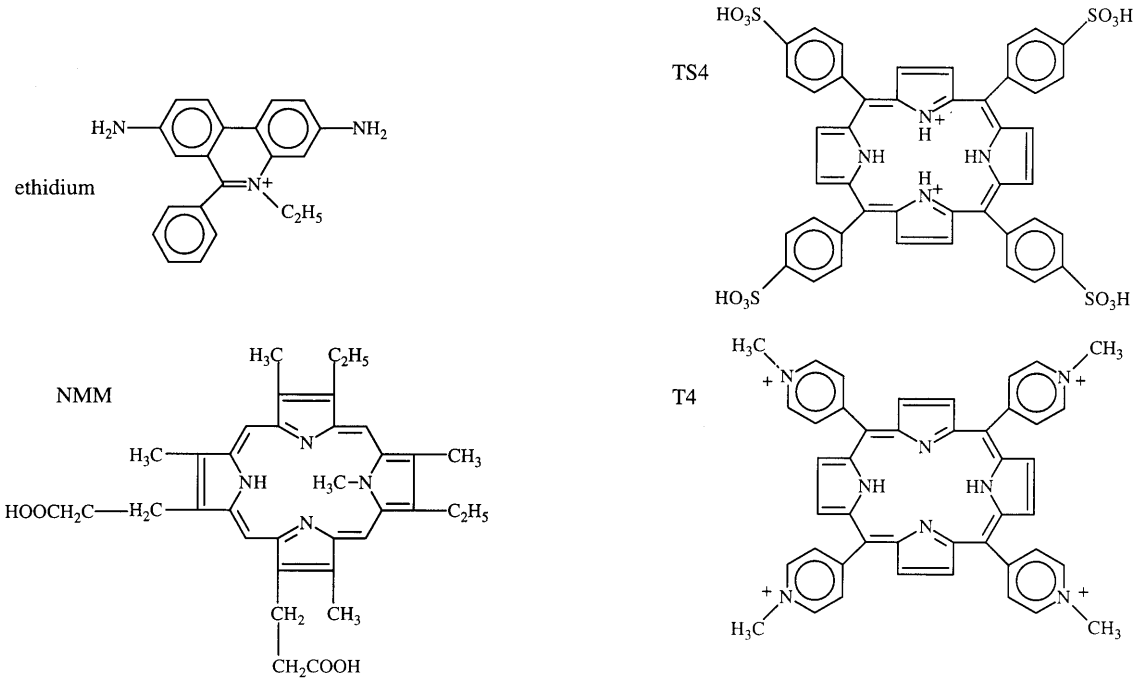

Figure 2. The structures of ethidum, T4, NMM and TS4 are shown.

binding sites per nucleotide for ethidium and that these binding sites are weaker than is the case for duplex DNA (38,39).

The binding of NMM to the 12mer and 15mer quadruplex DNAs was examined and compared to that of duplex DNA. The results in Figure 3 show that there is essentially no fluorescent enhancement in the presence of duplex calf thymus DNA, and significant fluorescence enhancement in the presence of the 12mer and 15mer quadruplex DNAs. The 16 and 17mers form edge type structures and also significantly enhance the fluorescence as does the 20mer which forms a basket structure. The 23mer and 14mer predominately form parallel structures and also significantly enhance the fluorescence. The structures of the 14, 16, 17, 20 and 23mers in the presence and absence of potassium have been recently determined (V.Marathias and P.H.Bolton, submitted). The 15mer, in the absence of potassium, does not form a quadruplex structure (7,8), nor does it significantly enhance the fluorescence. These results show that all three types of quadruplex DNAs significantly enhance the fluorescence of NMM while duplex DNA does not.

The binding of T4 to the 12mer and 15mer quadruplex DNAs was examined and compared to that of duplex DNA. The results in Figure 3 show that there is modest fluorescent enhancement of T4 in the presence of duplex calf thymus DNA, and significant fluorescence enhancement in the presence of the 15mer quadruplex DNA, but not the 12mer quadruplex DNA. The 16 and 17mers form edge type structures and significantly enhance the fluorescence. The 20mer forms a basket structure and also significantly enhances the fluorescence. The 23mer and 14mer predominately form parallel structures and do not significantly enhance the fluorescence. The 15mer, in the absence of potassium, does not form a quadruplex structure (7,8), nor does it significantly enhance the fluorescence. These results indicate that edge and crossover type quadruplex DNAs can significantly enhance the fluorescence of T4 while parallel quadruplex DNAs do not. Duplex DNA enhances the fluorescence more than the crossover and parallel quadruplex DNAs.

The triplet repeat DNAs, $d(GGC)_n$, associated with the fragile X syndrome, with n = 3, 4, 5 were examined since these have been shown to form a variety of structures (25–27). The solution structure of a quadruplex DNA containing sequence elements of the GGC fragile X repeat has been shown to be of the crossover type (26). The high level of fluorescence enhancement of ethidium observed in the presence of the $d(GGC)_n$ samples, ~70% of that observed for duplex calf thymus DNA, is consistent with these DNAs adopting, at least partially, duplex structures. The results with T4 indicate that significant amounts of edge or crossover quadruplex structures are not present, so the enhancement of the NMM fluorescence is consistent with a small percentage of parallel type quadruplex structures being present.

The absorption spectrum of T4 and NMM in the presence and absence of the DNAs was obtained and the spectra are shown in Figure 4. These results show that the absorption spectrum of NMM is not changed by the presence of calf thymus DNA while the wavelength of maximal absorption shifts ~20 nm to longer wavelengths in the presence of either the 12mer or 15mer DNAs. The absorption maximum of T4 shifts to longer wavelengths in the presence of calf thymus, 15mer and 12mer DNAs. The absorption results suggest that NMM may not be binding to duplex DNA. T4 exhibits similar absorption changes in the presence of the 12mer and 15mer while NMM does not. The fluorescence of NMM is enhanced more by the 12mer than by the 15mer.

The absorption changes are indicative of binding but the fluorescence enhancement of the T4 and NMM induced by binding to the quadruplex DNAs is not due to simple solvent exclusion as is the case for some dyes which bind to duplex DNA. For example, the fluorescence of T4 and NMM is about the same

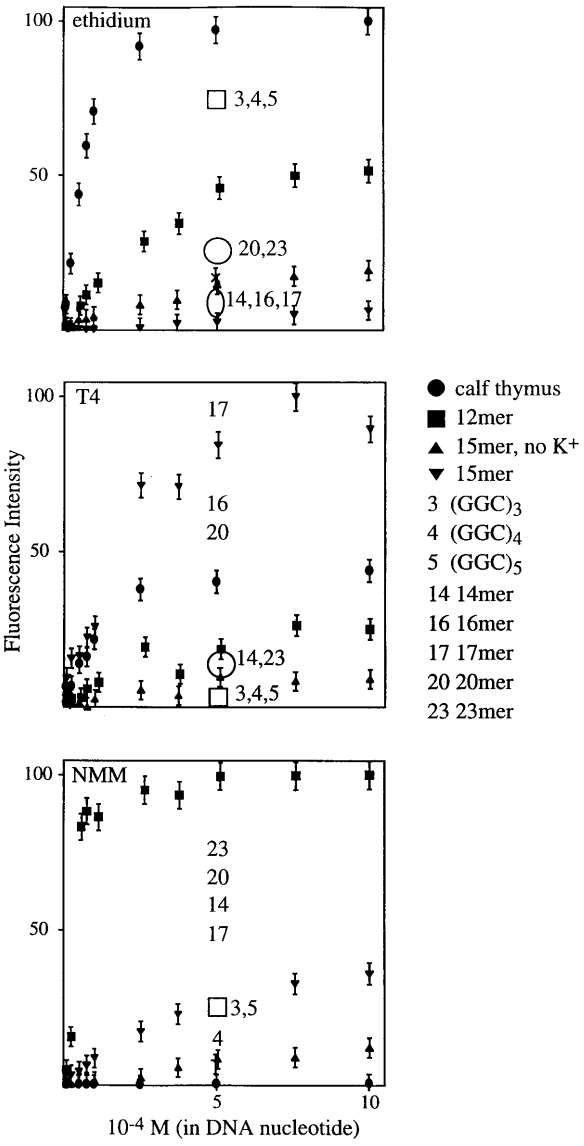

Figure 3. The data obtained on the fluorescence of the DNA samples with ethidium (top), T4 (middle) and NMM (bottom) are shown (32). The results as a function of concentration of calf thymus DNA (●), the 12mer dimer which forms a crossover type structure (■), the 15mer which forms an edge type structure in the presence (▼) but not in the absence of potassium (▲). Single data points for the $d(GGC)_n$ samples with n = 3, 4, 5 are shown. Also shown are single data points for the 14mer: d(GGTTGGTTGGTTGG), 16mer: d(GGTTGGTTTTGGTTGG), 17mer: d(GGTUTGGTGTGGUTTGG), 20mer: d(GGTUTUGGUTUTGGUUTTGG) and 23mer: d(GGGGTTGGGG-TGTGGGGTTGGGG).

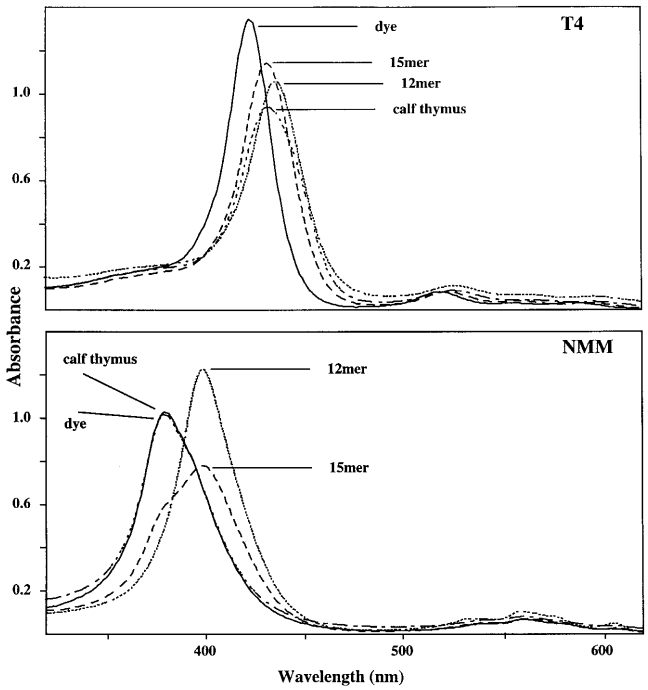

Figure 4. The absorption spectra of the dyes in the absence and in the presence of a 50:1 nucleotide to drug ratio are shown for the 12mer, 15mer and calf thymus DNA. The concentration of NMM is 1×10^{-5} M and that of T4 at 5×10^{-6} M.

in methanol as in aqueous solution, whereas that of ethidium is much stronger in methanol. This indicates that there are other interactions than water exclusion involved in the fluorescence enhancement.

The binding of TS4 to the quadruplex DNAs was also examined. This dye is highly fluorescent in the absence of DNA and no significant binding to any of the quadruplex DNAs was found. Neither NMM nor T4 is highly fluorescent in the free state. The relative maximal fluorescence of T4, ethidium and NMM under the conditions used here is ~1:3:11.

These results show that fluorescent porphyrin probes can be used to monitor the presence of quadruplex DNA in the presence of other structural forms of DNA. While these porphyrin dyes do not show absolute preference for quadruplex DNAs, their selectivity for quadruplex DNAs is quite high. Thus, the combination of the results from T4, NMM and ethidium can allow determination of the presence and approximate percentage of duplex and quadruplex DNA present in a mixture. A set of more selective dyes would allow determination of the percentage of each quadruplex type.

These, and other, porphyrin dyes will also allow the monitoring of the rates of formation of quadruplex structures induced by proteins and other factors. For example, the fluorescence of T4 is enhanced ~10-fold more by the 15mer in the presence of potassium than in the absence. The 15mer only adopts the chair type structure in the presence of potassium (7,8), and the fluorescence of T4 will allow monitoring the rate and presence of this conformation change under a wide range of conditions not otherwise accessible.

Telomere binding proteins have been shown to promote quadruplex formation (40,41). The monitoring of the displacement of the dyes from quadruplex DNA may allow investigation of the formation of protein–quadruplex DNA complexes. These dyes could also find a use in fluorescent microscopy in detecting the sites of quadruplex DNA in chromosomes and other systems in addition to their use as a counterstain (42). The use of combinatorial chemistry and high throughput screening, in combination with monitoring displacement of T4 and NMM from quadruplex DNAs, may allow finding molecules of even higher selectivity and other desirable qualities to specifically target telomere, centromere, triplet repeat, integration sites and other DNAs of known therapeutic interest (43–45). The structures of the complexes of the porphyrins with the quadruplex DNAs are being determined to further guide these applications.

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