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DNA Melting in the Presence of Fluorescent Intercalating Oxazole Yellow Dyes Measured with a Gel-Based Assay

Abstract: *We measured the effect of the intercalating oxazole yellow DNA dye quinolinium,4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(trimethylammonio)propyl]-,diiodide (YO-PRO) and its homodimer (YOYO) on the melting of self-complementary DNA duplexes using a gel-based assay. The assay, which requires a self-complementary DNA sequence, is independent of the optical properties of the molecules in solution. The melting temperature of the DNA is observed to increase in direct proportion to the number of occupied intercalation sites on the DNA, irrespective of whether the dye molecules are in monomer or dimer form. The increase is ~ 2.5°C for each intercalation site occupied in the presence of 38 m*M *[Na^+], for dye/duplex ratios in which less than 1/5 of the available intercalation sites are occupied.* © 2002 Wiley Periodicals, Inc. Biopolymers 65: 40–44, 2002

Keywords: *DNA melting; fluorescent intercalating oxazole yellow dyes; hairpin; gel-based assay*

INTRODUCTION

Major advances in single molecule research have been made with fluorescently labeled DNA.[1–10] The cyanine nucleic acid dyes YO-PRO and YOYO have a number of features especially desirable for such studies including high affinity for nucleic acids, low affinity for other biopolymers, low fluorescence when not bound to nucleic acids and large fluorescence enhancement upon binding to nucleic acids. However, the effect of these dyes on DNA melting has apparently gone unmeasured, perhaps because these dyes have absorption characteristics that complicate conventional uv measurements of DNA melting.

In this article, we report measurement of the effect of YO-PRO and YOYO on the melting temperature of duplex DNA with a new method, developed in the laboratory of G. Zocchi (UCLA). This method monitors the melting of double stranded "duplex" DNA by trapping melted (single-stranded) DNA in a well-defined "hairpin" secondary structure and measuring the proportion of duplex and hairpin populations by gel electrophoresis.

The method was designed to be complementary to uv absorption measurements in that it reports the number of melted duplexes, whereas uv absorption indicates the number of melted base pairs.[11] Besides this fundamental distinction, there are two major differences between the gel-based method used here and

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conventional uv absorption measurements for measuring DNA melting. First, the gel-based method is limited to self-complimentary DNA sequences. Second, the gel-based method is insensitive to the optical properties of DNA and other molecules in solution. We take advantage of this feature to measure the effect of optically active compounds on DNA melting.

We observe the duplex melting temperature increase by $\sim 2°C$ for every intercalation site occupied by YOYO or YO-PRO on our 30 base-pair (bp) duplexes. As an aside, we note that the presence of 2% β-mercaptoethanol (βME), alone or with dye, lowers the melting temperature of our duplex DNA by 3°C. βME is an essential component of enzymatic oxygen scavenging systems that are regularly used in conjunction with YOYO and YO-PRO to prolong the lifetime of fluorophores for fluorescence microscopy.[12] At typical concentrations, uv absorption by βME ($\sim$ 2%) overwhelms that of duplex DNA ($\sim$ 10 μM) making conventional melting studies impossible.

METHODS

A 30-bp high performance liquid chromatography (HPLC) purified DNA oligonucleotide with the sequence

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5′-GGG CTG AGT ATC CTG CAG GAT
                        ACT CAG CCC-3′
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was purchased from Operon Technologies (http://www.operon.com). This sequence was designed to have a perfect hairpin conformation, a melting temperature $\sim$ 70°C (in 34 mM [Na^+]), and no other stable secondary structure below the melting temperature.

The DNA was resuspended to a concentration of 200 pM/μL in concentrated phosphate buffered saline (10 $\times$ PBS: 1.5M NaCl, pH 7.4 at 25°C) with 1 mM EDTA, and incubated at 99°C for 20 min in a water bath (6 L capacity) to disrupt all base-pairing interactions. The water bath was then turned off and the DNA left incubating as it cooled slowly back to room temperature ($>$ 12 h). Due to the slow cooling rate and the high oligomer concentration, the DNA was annealed to $>$ 95% double-stranded duplex form (as determined by gel electrophoresis, described below). This concentrated, annealed DNA stock solution was stored frozen (−20°C).

Figure 1 summarizes the gel-based assay. Annealed, duplex DNA was diluted 1:40 in 1 $\times$ TBE (85 mM Tris, 85 mM boric acid, 2 mM EDTA at pH 8.0), making the final solution 0.25 $\times$ PBS (38 mM NaCl). Other ingredients were added during dilution as noted. The diluted samples were partitioned into 200 μL thin-walled tubes containing 10.5 μL each. Samples were incubated for 15 min (except as noted) by submersion in a water bath (Lauda RM6-S). Temperature in the bath was controlled to ±0.25°C. After incubation, samples were rapidly quenched to $<$ 5°C by transfer to an ice bath. The relative amount of duplex and hairpin conformations in the quenched samples was determined by electrophoresis under 1 $\times$ TBE through a 2.7% high-purity agarose gel (Promega—LMP, preparative grade) containing 0.5 μg/mL ethidium bromide (EtBr), taking care to cool the gel apparatus. Typical run conditions were 100 V and/or 150 mA. Bands were visualized based on EtBr fluorescence and captured on Polaroid 667 film using a Polaroid DS-34 camera with yellow filter (Tiffen 15). Exposure settings (*e.g.*, f = 4.5, 0.25 s) were such that bands of maximum intensity were below saturation in the print. Prints were digitally scanned (Epson 1640SU) at 600 dpi and band intensities were analyzed using NIH Image gel analysis macros (http://rsb.info.nih.gov/nih-image/).

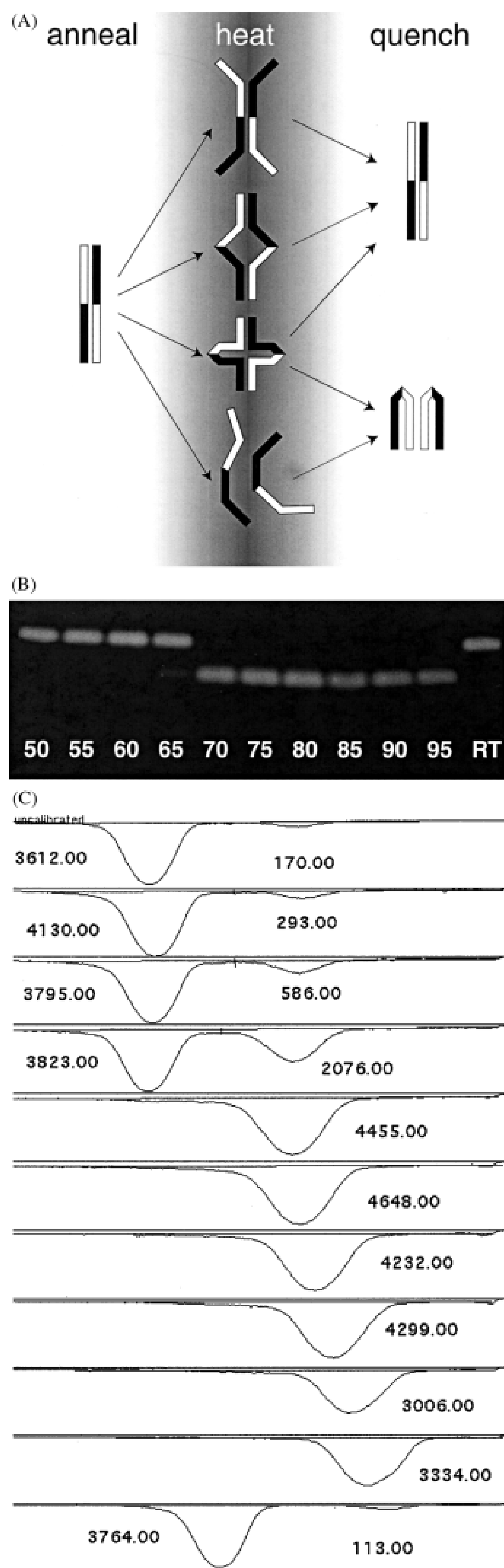
The %-hairpin DNA was calculated from the ratio of the lower band intensity to the total intensity in both bands. Error due to human variation in selecting the region of interest that represents each band was ±3%. The melting temperature, T_m, was defined as the temperature at which 50% of the DNA is in the hairpin conformation after quenching. To determine T_m, data was best fit with the extreme (minimum) value cumulative distribution function[13]:

$$f = 1 - f_o e^{-e^{[(T-T_o)/\Delta T]}}$$

where the parameters of location, T_o and scale, ΔT, both contribute to the melting temperature, $T_m = T_o + \Delta T \ln[\ln(2)]$ and the parameter f_o adjusts the offset to account for any hairpins present before the assay.

The nonlinear response of the film is expected to exaggerate the steepness of the melting curves, but not affect T_m. This was verified empirically by comparing exposure settings. The main source of error was a systematic offset that comes from assuming the amount of EtBr bound per base pair of DNA is independent of the hairpin vs duplex nature of the DNA. In fact, since 2 ± 1 of the 15 base pairs in a hairpin are unstacked in the loop, there may be a 10–20% loss of signal in the hairpin band. The associated undercounting of hairpins results in a T_m that is systematically higher than the true T_m by about 2°C.

Figure 2A shows the melting of our 30-bp self-complementary duplex DNA in 1 $\times$ TBE as a function of temperature, measured by the gel-based assay. Different symbols are used to represent each of four different incubation times: 3, 6, 15, and 30 min. Position along the vertical axis represents the %-hairpin DNA in the quenched solution. Figure 2B is a plot of T_m as a function of incubation time. The apparent melting temperature decreases exponentially as incubation time increases, approaching a steady state value, T_m = 65.9°C, with a time constant of 6 min. All subsequent measurements were therefore made on samples incubated for 15 min.



RESULTS AND DISCUSSION

We looked at the effect of fluorescent intercalating dyes YOYO and YO-PRO on DNA melting in the presence of 0.25 × PBS using a gel-based assay (Figure 1). We checked that measurements were made under steady-state, dilute conditions. We found it necessary to incubate samples for at least 15 min at a target temperature to achieve a steady-state reading of the relative proportion of melted duplexes (Figure 2). Increasing the DNA concentration by a factor of 6 had no effect on the duplex melting temperature. Decreasing the salt concentration by a factor of 6 (to 0.04 × PBS) lowered T_m by 2.5°C, as expected.[14,15]

Figure 3 shows the melting curves for different concentrations of YOYO and YO-PRO dye. Figure 4 is a plot of T_m as a function of the average number of dye molecules per DNA duplex. The relation between T_m and dye/duplex ratio is essentially linear, with a slope of 4.8°C/(dye/duplex) for YOYO, and approximately half as much, 2.4°C/(dye/duplex), for YO-PRO. These dyes bind double-stranded DNA by intercalating between the base pairs.[16] Their effect on T_m is the result of three physical effects.[17] First, the positively charged dye molecules screen the negatively charged phosphates in the DNA backbone. Second, the presence of the dye's aromatic rings enhances stacking interactions. Third, by locally stretching/unwinding the double helix, intercalated dye molecules reduce the charge density per unit length along the backbone.

The YOYO molecule is a dimer of the YO-PRO molecule. Therefore, a dye/duplex ratio of 1 for

FIGURE 1 Illustration of the gel-based assay used for measuring the melting temperature of self-complementary duplex DNA.[11] (A) Schematic of the procedure. Self-complementary DNA is annealed at high concentrations into the duplex form, then diluted and incubated at the query temperature for 15 min and rapidly quenched to < 5°C. Upon quenching, single-stranded oligomers close to form hairpin loops (half the length of the duplex) while partially melted duplexes reanneal. The quenched solution is analyzed by gel electrophoresis and the proportion of fully melted duplexes is quantified by comparing intensities of the two bands on the gel. (B) Example of gel data for melting of duplex DNA in 1 × TBE. DNA was visualized using EtBr. Lanes represent query temperatures ranging from 50 to 95°C in 5°C increments from left to right. The rightmost lane is a control, left at room temperature, indicating the low level of hairpin DNA in the stock solution. (C) Scan of integrated intensity along gel lanes shown in B. Left to right corresponds with top to bottom on the gel. Relative amounts of duplex and hairpin DNA were measured from integrated intensities under the peaks as shown.

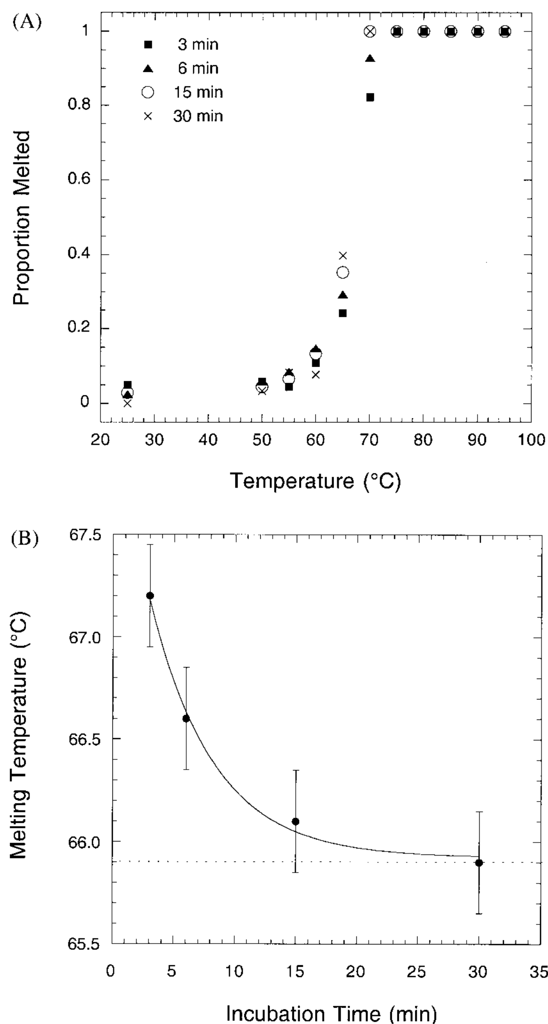


FIGURE 2 (A) Proportion of melted DNA as a function of temperature for self-complementary duplex DNA in 1 × TBE with no dye. Data for four different incubation times are shown. (B) Melting temperature, T_m, as a function of incubation time, extracted from the data shown in A. The exponential decrease in T_m asymptotically approaches T_m = 66°C with a time constant of 6 min.

YOYO is equivalent to a dye/duplex ratio of 2 for YO-PRO in terms of the number of intercalation sites occupied. The observed effect of both YO-YO and YO-PRO on DNA melting is roughly linear in the proportion of occupied intercalation sites per duplex for less than 1/5 sites occupied.

Occupation levels greater than 1/5 were not tested because they took the melting temperature beyond the boiling point of water. We note that it might be possible to test the effect of greater occupation levels in the presence of denaturants. For example, we found that in the presence of 13*M* urea, the melting temperature of duplex DNA with 2/5 sites occupied was lowered to between 25 and 50°C (data not shown).

CONCLUSIONS

We have measured the effect of the intercalating DNA dyes YO-YO and YO-PRO on the melting of self-complementary DNA duplexes using a gel-based assay. These dyes are widely used in physical studies of DNA by fluorescence microscopy but, to our knowledge, their effect on DNA duplex stability has not been quantified before. The melting temperature of the duplex DNA increases in the presence of these intercalating dyes, as is the case for other intercalating

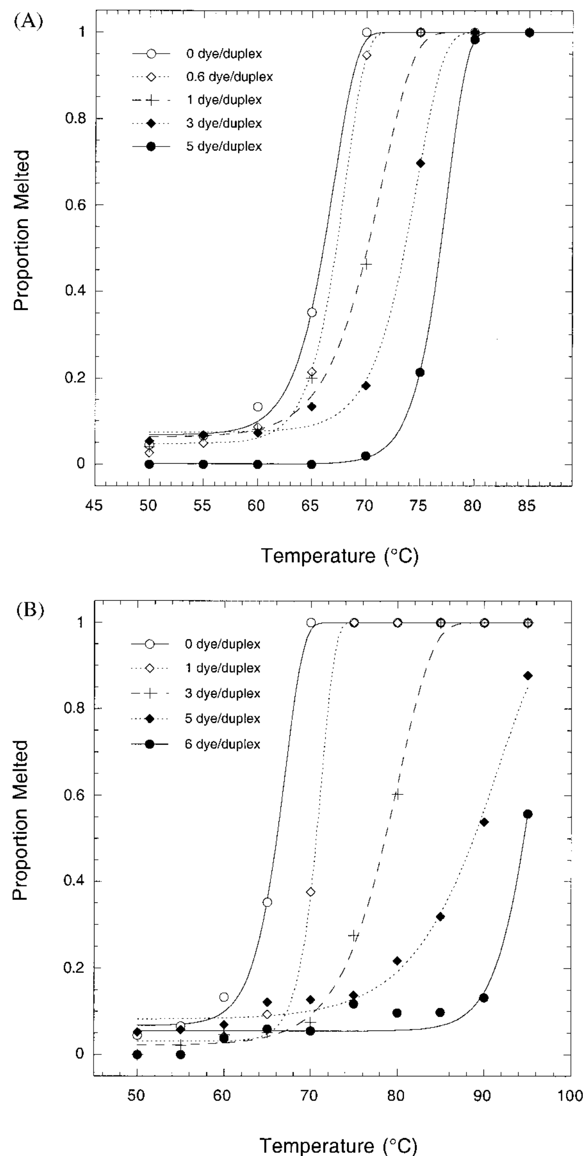


FIGURE 3 Effect of (A) YO-PRO and (B) YOYO on DNA melting. The proportion melted increases with temperature for self-complementary DNA duplexes in 1 × TBE with varying amounts of YO-PRO and YOYO. Curves shift to higher temperatures as the number of dye molecules per DNA duplex increases.

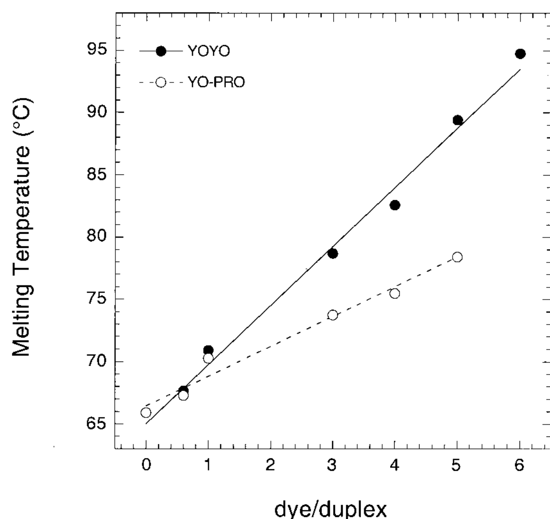


FIGURE 4 Melting temperature, T_m, as a function of the number of dye molecules per duplex for both YOYO and YO-PRO, extracted from data shown in Figure 3. The linear rise in T_m is 4.8°C/(dye/duplex) for the dimer YOYO, which is nearly twice that for the monomer YO-PRO, 2.4°C/(dye/duplex). This shows that for low occupation levels, duplex stability is a simple function of the proportion of occupied intercalation sites.

compounds.[18] The increase is directly proportional to the number of occupied intercalation sites on the DNA for dye concentrations that lead to less than 1/5 occupation of intercalation sites and appears insensitive to whether the dye molecules are in monomer or dimer form.

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