

Calculating Helix-to-Coil Transitions of Duplexes Formed by Phenazine-Conjugated Oligonucleotide, Using Fluorescence Melting Data

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A novel intercalating phenazine derivative (Pzn) was covalently linked to the 3'-end of decathymidylate via a ribose residue of the dye. A fluorescence technique was used to study double helix formation by this conjugate with poly(rA) in aqueous solutions of neutral pH, at the presence of 0.1 and 1 M sodium ions. Proportionality between thermally induced changes in the fluorescence intensity of the free conjugate and bound one was revealed, that made it possible to calculate the helix-to-coil transition from fluorescence melting data using a simple equation. The transition curves were found to be in well conformity with those constructed from absorption measurements. It was shown that the attachment of Pzn significantly enhanced the stability of poly(rA) · (dT)₁₀ duplex due to intercalation of the dye chromophore into the adenine strand. The temperature of half-dissociation was increased by 12°C, and the stabilizing increment of standard free energy was 3.2–3.6 kcal/mol at 37°C.

KEY WORDS: Dye fluorescence; phenazines; modified oligonucleotides; duplex stability.

INTRODUCTION

Covalent attachment of intercalating dyes to anti-sense oligonucleotides is being considered for stabilization of their molecular hybridization [1]. The accepted method for quantitative evaluation of oligonucleotide complex formations is based on analysis of their thermal helix-to-coil transitions. Measurements of thermally induced changes in UV nucleotide absorbance are widely used for obtaining melting curves. In the case of oligonucleotides modified with dyes, fluorescence techniques can be also applied owing to a sensitivity of the attached chromophore emission to hybridization.

In this paper we expound a method of construction of the helix-to-coil transitions from fluorescence melting data obtained as the temperature dependences of the fluorescence intensity of a dye-oligonucleotide conjugate. The double helix formed between decathymidylate modified with a novel phenazine derivative (Pzn) and poly(rA) was studied. The transition curves were found to be well consistent with those constructed from absorption measurements. It was revealed that Pzn attachment significantly enhanced the stability of poly(rA) · (dT)₁₀ duplex. Thermodynamic evaluation of the stabilizing effect has been made using a two-state model of transitions.

EXPERIMENTAL

Synthesis

Poly(rA) was obtained from Sigma Chemical Co. Decathymidylate ((dT)₁₀) was synthesized by normal H-

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phosphonate procedure [2]. Decathymidylate-phenazine conjugate ((dT)₁₀Pzn) was obtained by attachment of phenazole ribonucleoside to the 3'-terminus of (dT)₁₀ via the phosphodiester bond (Fig. 1), using the method of solid phase synthesis. A special silica support was prepared by the attachment of 5'-O-monomethoxytrityl derivative of imidazo[4, 5-d]phenazine N1-β-D-ribofuranoside [3] to aminopropyl silica Silochrom S-80 via succinate linker group, according to reported procedures [4]. H-Phosphonate oligonucleotide synthesis of (dT)₁₀ was performed on this support. The yield of the coupling reaction was about 98%. After synthesis, (dT)₁₀Pzn conjugate was removed from the support by treatment overnight with concentrated NH₃ at room temperature. The oligomer was isolated by preparative electrophoresis in 20% denaturing polyacrylamide gel.

The product band was easily visualized in the gel by its strong yellow fluorescence. After elution from the gel, the conjugate was desalted by gel-filtration on a PD-10 cartridge (Pharmacia). The efficiency of labelling was checked by comparison of the observed ratio of conjugate absorbance at short-wavelength band maximum (at 264 nm) to the maximal visible absorbance of the attached dye (at 384 nm) with the ratio of the sum absorbances at the same wavelengths of separate (dT)₁₀ and Pzn calculated using known extinction coefficients. The values of these ratios were coincided within 7%.

METHODS

All the experiments were performed in a 10 mM sodium cacodylate buffer, pH 7, containing 0.5 mM

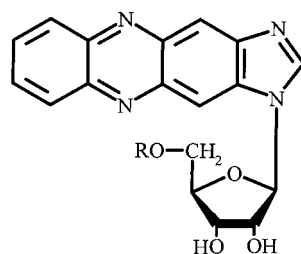
EDTA and 0.1 or 1 M NaCl. Oligonucleotide and polynucleotide concentrations were determined spectrophotometrically using reported extinction coefficients: 8600 M⁻¹cm⁻¹ at 266 nm for (dT)₁₀ (at 21–23°C) [5] and 10100 M⁻¹cm⁻¹ at 257 nm (at 20°C) for poly(rA). The concentration of (dT)₁₀Pzn conjugate was calculated from the absorbance of the dye moiety at 384 nm using extinction coefficient 22000 M⁻¹cm⁻¹. Duplex formations by (dT)₁₀Pzn or (dT)₁₀ with poly(rA) at 1:1 mole ratio of adenines to thymines were investigated. Oligomer strand concentration was 7 μM in all samples.

Light absorption was recorded on a M-40 spectrophotometer (VEB Carl Zeiss, Jena). Steady-state fluorescence measurements were performed by the method of photon counting on the instrument which is described earlier [6]. Melting curves were obtained by monitoring thermally induced change in the fluorescence intensity of the attached dye as well as in the complex absorbance at 260 nm. Fluorescence was excited at a wavelength of 450 nm and registered at the emission maximum of (dT)₁₀Pzn, which was found at 537 nm. The absorbance and fluorescence were measured in the same special quartz cuvette (volume 60 microliters, optical path length 0.2 cm). The cuvette was inserted into a copper holder, temperature of which was changed by means of a computer-operated Peltier element. The spectrophotometer and a spectrofluorimeter were attached to a personal computer for data collection. Melting curves were recorded at a heating rate of 0.5°C/min. They were reproduced in repeated experiments with maximal temperature shift of ±0.2°C.

RESULTS AND DISCUSSION

Absorption and fluorescence properties of the free Pzn were previously reported [6]. Pzn absorption spectrum in the visible spectral region shows one band with a maximum at 384 nm (extinction coefficient 22400 M⁻¹cm⁻¹) and a broad long-wave shoulder. On covalent attachment of Pzn to the end of (dT)₁₀ this absorption band changed weakly, with a red shift of ~0.6 nm and a hypochromism of about 5%. The absorption spectrum of (dT)₁₀Pzn conjugate is a superposition of absorption spectra decathymidylate and the phenazine derivative (Fig. 2). The maximum of its absorbance is occurred at 264 nm. The non-structured emission spectrum of the free Pzn has a maximum at 543 nm. After the conjugation it shifted to 537 nm and the fluorescence intensity decreased (by about 30% at 20°C) (Fig. 2).

Figure 3 demonstrates the temperature dependence of the relative fluorescence intensity for the free Pzn,



(I) R=H
Phenazole ribonucleoside

(II) R=5'-(dT)₉dT-O-P(=O)(O⁻)₂
3'-Modified oligonucleotide

Fig. 1. Molecular structures of phenazine derivative (I) and modified decathymidylate (II).

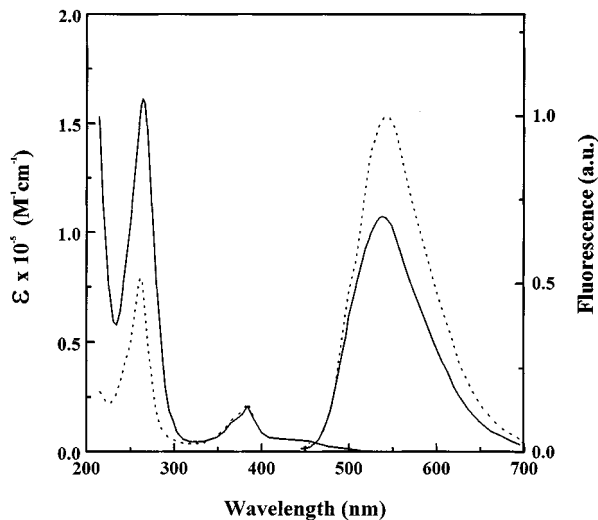


Fig. 2. Absorption (left) and fluorescence (right) spectra of the free Pzn (dotted lines) and $(dT)_{10}$ Pzn conjugate (solid lines) measured at 20°C in 10 mM sodium cacodylate buffer, pH 7, containing 0.5 mM EDTA and 1 M NaCl. Fluorescence was excited at a wavelength of 450 nm.

conjugate $(dT)_{10}$ Pzn and poly(rA) + $(dT)_{10}$ Pzn mixture. As can be seen, upon increasing the temperature from 5°C to 70°C, the emission of the conjugate decreased monotonically more than two-fold. In contrast, the weak

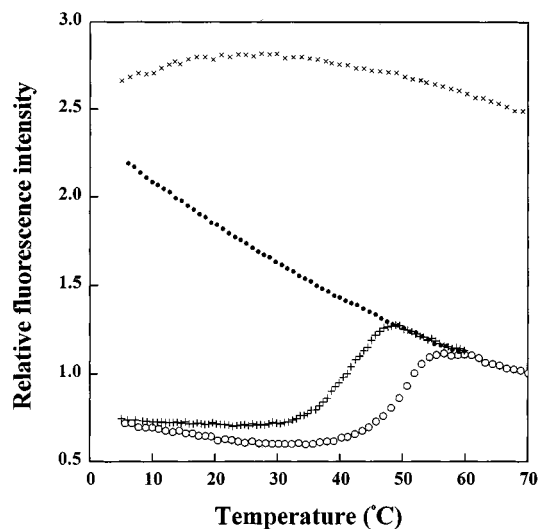


Fig. 3. Temperature dependence of the relative fluorescence intensity of Pzn (\times), $(dT)_{10}$ Pzn (\bullet) and equimolar mixture poly(rA)+ $(dT)_{10}$ Pzn measured in 10 mM sodium cacodylate buffer, pH 7, 0.5 mM EDTA in the presence of 0.1 M NaCl ($+$) and 1 M NaCl (\circ). Concentration of Pzn and $(dT)_{10}$ Pzn was 7 μ M each. The data points for the $(dT)_{10}$ Pzn and poly(rA)+ $(dT)_{10}$ Pzn samples were normalized using their own emission intensity values at 70°C. The data for Pzn are presented in the units used for the $(dT)_{10}$ Pzn.

temperature effect on the fluorescence of unconjugated Pzn is observed (up to 30°C the emission increased due to dissociation of dye dimers). Insignificant effect of NaCl on the both dependences has been revealed. Since upon the Pzn attachment its absorption spectrum changed insignificantly, but the fluorescence spectrum substantially shifted (by 6 nm) and the emission decreased on rising the temperature, the fluorescence quenching seems to be dynamic [7] occurred due to a collision between Pzn chromophore and thymine residue.

The data of fluorescence intensity for $(dT)_{10}$ Pzn and poly(rA)+ $(dT)_{10}$ Pzn mixture shown in Fig. 3 were normalized using their own values at 70°C (Fig. 4). Such procedure has improved the co-ordination of these data due to the elimination of influence of a certain unreproducibility of the exciting power or the concentration of fluorophores. The duplex formation in the mixture manifested itself in quenching the conjugate fluorescence at low temperatures. The quenching effect evidenced the insertion of the dye chromophore into the adenine strand, as it was observed on the interaction of the free Pzn with poly(rA) [6]. On heating, when the strands separation occurred, the emission intensity of sample (F) increased and, after the total complex dissociation, its temperature dependence became identical to that of the free $(dT)_{10}$ Pzn.

In order to construct melting transitions from fluorescence data, besides the temperature dependence of the fluorescence intensity for the free conjugate (F_f), we have to know that for the completely bound one (F_b). Because completeness of the duplex formation is not clear from

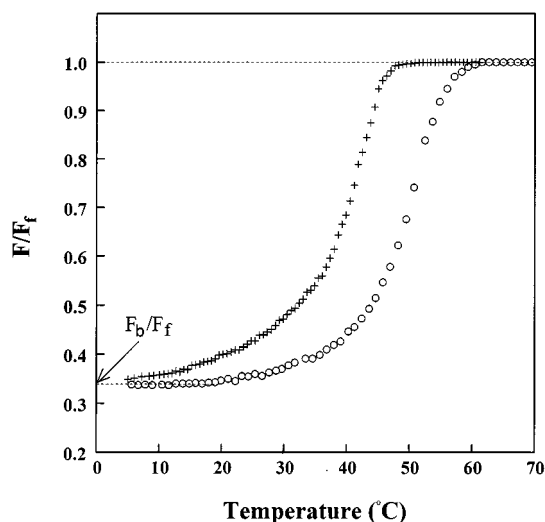


Fig. 4. Temperature dependence of the ratio between the fluorescence intensity of the poly(rA)+ $(dT)_{10}$ Pzn mixture and that of the free $(dT)_{10}$ Pzn obtained at 0.1 M ($+$) and 1 M NaCl (\circ). Other conditions as in Fig. 3.

the primary data, it was useful to present the fluorescence data as the temperature dependence of the ratio F/F_f (Fig. 4). As can be seen, at low temperatures, F/F_f for the sample with 1 M NaCl has reached a steady level which corresponds to the duplex form of the sample. Evidently, the ratio for the sample with 0.1 M NaCl tends to the same level. This finding suggests that there is a proportionality between thermally induced quenching effects for the free and bound $(dT)_{10}$ Pzn. Thus, the ratio F_b/F_f is the temperature-independent quantity that made it possible to calculate the fraction of bound conjugate using the simple equation

$$\Theta = (1 - F/F_f)/(1 - F_b/F_f) \quad (1)$$

By means of this calculation helix-to-coil transitions for $\text{poly}(\text{rA}) \cdot (\text{dT})_{10}$ Pzn duplex were constructed (Fig. 5). The melting profiles of this sample as well as its unmodified counterpart $\text{poly}(\text{dA}) \cdot (\text{dT})_{10}$ were also obtained from absorption melting curves according to the standard procedure [8] and presented in Fig. 5. As can be seen, transitions in the modified complex constructed from fluorescence and absorption measurements are in well con-

formity. They were found to be shifted to higher temperatures in comparison with transition curves of the unmodified complex that is an evidence of duplex-stabilizing action of the attached Pzn. The temperature of half-dissociation (T_m) was increased by $\sim 12^\circ\text{C}$ at the both ionic conditions, as compared to the unmodified counterpart. The effect is most likely caused by a stacking interaction of the dye chromophore with adenine bases of $\text{poly}(\text{rA})$.

In order to evaluate thermodynamic parameters of the duplex-to-coil transitions, the melting data were fitted to an equation which follows from the simple all-or-non model (solid lines in Fig. 5),

$$\ln\{\Theta/[1 - \Theta]^2 C\} = \Delta S/R - \Delta H/RT \quad (2)$$

where C is the total strand concentration of $(dT)_{10}$ or $(dT)_{10}$ Pzn and Θ is oligomer fraction in the duplex state. Taking into a consideration the possibility of triplex formation [9], this procedure was performed in such a way that achieved the best fits of the high-temperature portions of transition data. The obtained values of transition enthalpy and entropy as well as T_m data are collected in Table I.

As can be seen from Fig. 5, the two-state model equation well describes the melting data in all the temperature interval only for the unmodified duplex at 0.1 M NaCl. In the cases of other samples, a deviation of the experimental points from the theoretical curves in low-temperature stages is observed indicating the presence of triple-stranded complexes. Partial formation of the triplex structure in $\text{poly}(\text{rA}) + (\text{dT})_{10}$ system at a high ionic strength is confirmed by the previous observations [5, 10]. The attachment of Pzn enhanced the triplex formation at the both ionic conditions.

The values of transition enthalpy obtained for $\text{poly}(\text{rA}) \cdot (\text{dT})_{10}$ duplex, $-\Delta H = 7.8 - 8.4$ kcal/mole of base pair (Table I), are substantially greater than reported for dissociation of $\text{rA} \cdot \text{dT}$ oligomer-oligomer

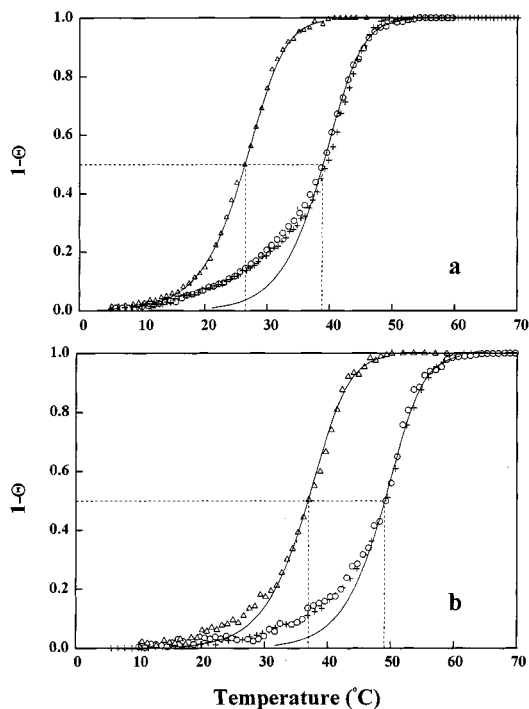


Fig. 5. Helix-to-coil transitions in the duplexes $\text{poly}(\text{rA}) \cdot (\text{dT})_{10}$ (Δ) and $\text{poly}(\text{rA}) \cdot (\text{dT})_{10}$ Pzn (\circ) at 0.1 M NaCl (a) and 1 M NaCl (b). Θ is the fraction of oligomer in the duplex state. The data points denoted by (+) were obtained from fluorescence measurements and the others from absorbance hyperchromicity at 260 nm. The dashed lines indicate the positions of the midpoint of transitions. The solid curves are the best fits to a two-state model eq. (2).

Table I. Thermodynamic Parameters for Duplex Formation by $(dT)_{10}$ or $(dT)_{10}$ Pzn and $\text{Poly}(\text{rA})^a$

Oligonucleotide	NaCl (M)	T_m ($^\circ\text{C}$) ^b	$-\Delta H$ (kcal/mol)	$-\Delta S$ (cal/mol·K)
$(dT)_{10}$	0.1	26.5	78	235
$(dT)_{10}$ Pzn	0.1	38.8	88	257
$(dT)_{10}$	1.0	36.9	84	246
$(dT)_{10}$ Pzn	1.0	49.0	96	273

^a Thermodynamic parameters were calculated per mole of the oligomer strand.

^b T_m values were determined as the temperature at half-dissociation.

complexes, ~ 5 kcal/mol [11]. Undoubtedly, the $-\Delta H$ increase is a result of the cooperative stacking interaction between $(dT)_{10}$ oligomers accompanying the duplex formation with poly(rA), the phenomenon which has been investigated earlier with poly(C) + oligo(I) system [12, 13]. Since the slope of melting transitions for the modified and unmodified duplexes is almost the same, one may presume that the attachment of Pzn did not substantially affected the intensity of this cooperative interaction.

As can be seen from Table I, the stabilizing effect of the Pzn attachment is characterized by increasing in the transition enthalpy by 10–12 kcal/mol. Calculated from the ΔH and ΔS data the increment of transition free energy at 37 °C is 3.2 and 3.6 kcal/mol at 0.1 M and 1 M NaCl, respectively. The stabilizing action of the studied phenazine derivative with a neutral chromophore on the duplex formation appears to be comparable with similar effects produced by other intercalating dyes, for instance an acridine derivative [14, 15] or a cationic phenazine derivative [16], when they have been attached to 3'-end of oligonucleotides via a linker. However, for antisense technology the oligonucleotide modification discussed has a certain advantage in comparison with widely used method of dye attachment via a polymethylene chain, since in this case the phenazole ribonucleoside incorporates into the oligonucleotide like an usual nucleoside

that predetermines the position of its chromophore in the target sequence.

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