Stabilizing and destabilizing effects of intercalators on DNA triplexes

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Abstract Oligonucleotide-directed triplex formation attracts much attention due to its potential usefulness in diagnostic and biotechnological applications (for review, see [1,2]). Among other aspects, the research embraces numerous studies probing the influence of intercalating ligands on triplex stability. The effect of the intercalator on triplex formation and stability is known to depend on nucleotide sequence, type of intercalator and solution conditions (for review, see [3]). The present work is aimed at determining the average number of intercalated ethidium bromide (EtBr) and acridine orange (AO) molecules leading to the most effective stabilization of triplexes. First, fluorescing complexes of intramolecular parallel (recombinant) triplex 5′-d(CATGCTAACT)-L-d(AGTTAGCATG)-L-d(CATGCTAACT)-3′ (parARB) and classical antiparallel 5′-$(dA)_{10}$-L-$(dT)_{10}$-L-$(dT)_{10}$-3′(antiATT) (L = -pO$(CH_2CH_2O)_3$p-) with EtBr and AO were characterized, binding constants were obtained and compared to those for homologous DNA duplexes. Then the total EtBr and AO concentrations corresponding to an average of one, two or three intercalated molecules per oligonucleotide were estimated. Thermal denaturation of parARB and antiATT complexes with an average of one, two or three bound molecules was carried out, thermodynamic parameters of the triplex-to-duplex and duplex-to-open-strand transitions were evaluated using a three-state model. The ability of EtBr and AO to stabilize or destabilize both parallel (recombinant) and classical antiparallel triplexes was found to depend strongly on the concentration of bound intercalator. The triplexes were shown to be stabilized by intercalation of the first and second EtBr or AO molecules, while binding of the third intercalator molecule to 10 nucleotide long triplex resulted in significant triplex destabilization.

Key words: Parallel triplex; Intercalator; Stabilization; Thermodynamics

1. Introduction

To use oligonucleotides for the recognition of native DNA through site-specific triplex formation one should understand the dependence of triplex stability on numerous factors, in particular, on the binding of intercalators. The data on binding of intercalators to conventional triplexes with two pyrimidine or two purine strands oriented antiparallel to each other are abundant; the stabilization effect of intercalators was shown to depend on their type and on the triplex sequence [4–13]. The stereochemical possibility of the novel type parallel (recombinant) triplexes or R-form DNA was theoretically predicted [14]; the triplex contains two identical strands in parallel orientation and in principle can be formed for any nucleotide sequence. Importantly, the R-form can be constructed in the absence of recombinases. Experimentally, the formation of the intramolecular R-form was observed in the specially designed oligonucleotide, 5′-d(CATGCTAACT)-L-d(AGTTAGCATG)-L-d(CATGCTAACT)-3′ (denoted parARB), and the conditions for its stabilization were determined [15]); here L denotes the linker, -pO$(CH_2CH_2O)_3$p-. Furthermore, the thermodynamics of triplex formation and its spectral characteristics, e.g. FTIR, were studied in the complex with propidium iodide [16,17]. Although the detailed structure of the parallel R-type triplex has not been verified by high resolution methods, such as X-ray or NMR, the general feasibility of the R-form [14] has been demonstrated recently when the parallel triplets GC:G and CG:C were observed crystallographically [18,19]. Notice, however, that the stability of the parARB triplex was shown to be relatively low [15]. Therefore, additional stabilization is required to achieve formation of an intermolecular parallel triplex that seems to be an encouraging goal significantly extending the repertoire of target sequences. One of the possible ways to increase the affinity of the third strands for their target duplexes is to use triplex-forming oligonucleotides (TFO) bearing a covalently linked intercalator either at the 5′- or at the 3′-end [20]. In this approach a gain in stability arises from intercalation of the linked intercalator in energetically favorable sites at the triplex/duplex junction [21]. Additional covalent attachment of intercalators between the bases to the sugar-phosphate backbone of TFO can potentially increase the affinity of the TFO-ligand conjugates for target duplexes provided that intercalation of the chosen dye in the triple helix is energetically advantageous and increases the triplex stability. Further application of this approach for recognition of the 14-bp DNA fragment comprising all four bases involved covalent tethering of different numbers of intercalators to the sugar-phosphate backbone not only at the TFO ends but also between the bases [22]. The importance of determining the optimal number of attached intercalators was demonstrated.

Nevertheless, the plentiful literature on binding conventional oligopurine and oligopyrimidine triplexes to intercalators added in the bulk of solution lacks data on the dependence of triplex stability on the concentration of bound intercalators. Earlier, the intercalator propidium iodide was shown to stabilize the parARB triplex. Besides, the effects of the first, second and following bound dye molecules were different [23]. Therefore, in the present work we studied how the triplex stability is affected by the binding of ethidium bromide (EtBr) and acridine orange (AO) [24–26]. More specifically, we analyzed the correlation between the stabilizing (or destabilizing) effect of the intercalators and the average ratio [bound dye/base triplets] for two triplexes: the parallel triplex parARB and the conventional antiparallel triplex 5′-$(dA)_{10}$-L-$(dT)_{10}$-L-$(dT)_{10}$-3′(antiATT).

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Abbreviations: EtBr, ethidium bromide; AO, acridine orange; TFO, triplex-forming oligonucleotides

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Table 1
Fluorescence characteristics of EtBr binding to double and triple helical oligonucleotides

Oligonucleotide	q/q_0[a]	τ (ns)	[b]K ($\times 10^{-5}$ M^{-1})	[b]n	[b]ω	[c]K ($\times 10^{-5}$ M^{-1})	[c]n
parARB	1.01 ± 0.05	25 ± 1	1.7 ± 0.1	2.4 ± 0.1		1.80 ± 0.05	2
dsARB	1.10 ± 0.05	26 ± 1	0.80 ± 0.05	3.2 ± 0.1		0.97 ± 0.02	3
antiATT	1.05 ± 0.05	25 ± 1	1.7 ± 0.1	3.5 ± 0.1			
antiA/T	1.04 ± 0.1	25 ± 1	0.17 ± 0.03	4.0 ± 0.2	7 ± 2		
Calf thymus DNA	1.0	25 ± 1					

[a]q is the quantum yield of fluorescence of the samples, q_0 the quantum yield of fluorescence of EtBr bound to DNA, 0.15 ± 0.01 [29,34].
[c]For the limited length of DNA [30].

2. Materials and methods

2.1. Oligonucleotides

Oligonucleotides able to form intramolecular triplexes 5′-d(CATGCTAACT)-L-d(AGTTAGCATG)-L-d(CATGCTAACT)-3′ (parARB) and 5′-$(dA)_{10}$-L-$(dT)_{10}$-L-$(dT)_{10}$-3′ (antiATT), as well as intramolecular double helices 5′-d(CATGCTAACT)-L-d(AGTTAGCATG)-3′ (dsARB) and 5′-$d(A)_{10}$-L-$d(T)_{10}$-3′ (antiA/T), where L = -$pO(CH_2CH_2O)_3p$-, have been synthesized by E.N. Timofeev (parARB, antiATT), B.K. Chernov (antiA/T) and BioTeZ GmbH (Germany) (dsARB). Oligonucleotides were purified by HPLC and desalted. Absence of intermolecular associates was tested by measuring rotational relaxation times of oligonucleotide-EtBr complexes [15,27].

2.2. Lifetimes of fluorescence

Lifetimes of fluorescence (τ) were measured with a phase fluorometer [28]. The lifetimes of EtBr fluorescence (τ_{EtBr}) were measured at excitation wavelengths of 365 nm and 546 nm and an emission wavelength above 600 nm. The data were averaged over two excitation wavelengths. The lifetime of AO fluorescence (τ_{AO}) was measured at excitation wavelength of 436 nm and an emission wavelength above 510 nm.

2.3. Quantum yields of fluorescence

Quantum yields of fluorescence (q) were calculated using absorption and fluorescence emission spectra of the dyes bound to oligonucleotides in relation to those bound to DNA [29].

2.4. Fluorescence emission spectra

Fluorescence emission spectra were registered with an Aminco SPF-1000 spectrofluorimeter in thermostatted cells at 3–4°C. Binding studies were carried out at constant nucleic acid concentrations by titration with EtBr or AO. The fluorescence intensity changes on binding increasing concentrations of EtBr were monitored at 615 nm with excitation at 530 nm, on binding AO at 520 nm with excitation at 420 nm. The fluorescence emission of fully bound EtBr and AO was obtained from parallel control titrations of solutions containing a large excess of the oligonucleotides or DNA, whereas emission of free dyes was measured from parallel control titration of buffer solution. Concentrations of the bound dye were calculated from equation:

$$C_2/C_0 = (I-I_1)/(I_2-I_1) \quad (1)$$

where I, I_1 and I_2 are the measured fluorescence intensities of the sample I, controls with free I_1 and fully bound I_2 dye, $C_o = C_1+C_2$ is the sum of free and bound dye concentrations. In order to take into account the finite length of the studied short DNA duplexes and triplexes we used the theoretical consideration of statistical mechanics for binding ligands with exclusion length n to a finite lattice [30]. For the special case of non-cooperative binding of large ligands to a homogeneous oligonucleotide lattice this theory is consistent with later works [31,32].

2.5. Thermal denaturation experiments

Thermal denaturation experiments were carried out with a Beckman 26 spectrophotometer at 259 nm in thermostatted cells at a constant heating rate of 0.2°C/min or with a Kontron Uvikon 820 spectrophotometer at intervals of 1°C. Samples and reference buffer solutions contained equal total concentrations of dyes. Analysis of helix-coil transitions was performed with a three-state model for successive monomolecular triplex-to-duplex and duplex-to-open-strand transitions. Satisfactory fits were obtained with the fit program *fit_helix_14* developed for two successive concerted reactions of the intramolecular DNA structure [33].

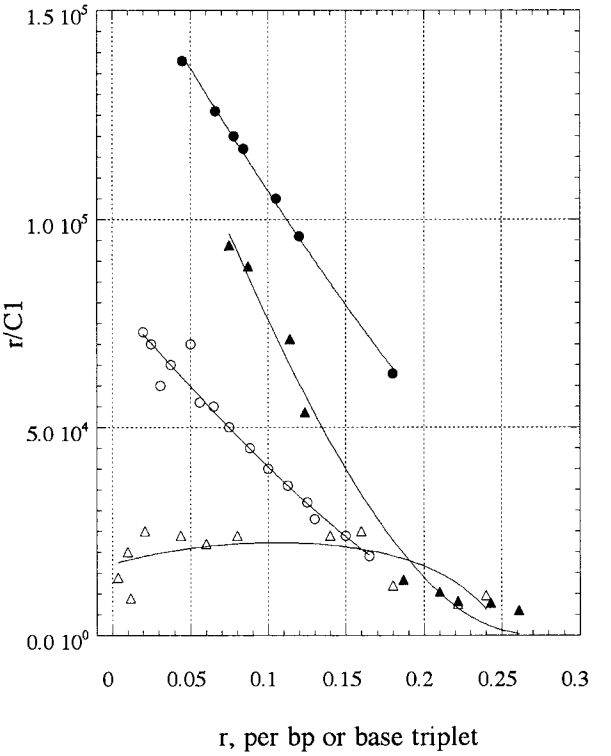

Fig. 1. Scatchard plots for the binding of ethidium bromide to intramolecular 10-bt triplexes parARB (●), antiATT (▲) and 10-bp duplexes dsARB (○), antiA/T (△). Solution conditions were 0.1 M NaCl, 0.01 M Na phosphate buffer, pH 7, temperature 3–4°C. Oligonucleotide concentration did not exceed 2×10^{-5} M(N); absorption extinction coefficients in water at 90°C per mol of nucleotides were taken to be: $\varepsilon_{260} = 9200$ M^{-1} cm^{-1} for parARB, $\varepsilon_{260} = 9400$ M^{-1} cm^{-1} for antiATT, $\varepsilon_{260} = 9300$ M^{-1} cm^{-1} for dsARB, $\varepsilon_{260} = 10200$ M^{-1} cm^{-1} for antiA/T [36]. Smooth curves are fits to the equations of McGhee and Von Hippel [31]

$$\frac{r}{C_1} = K(1-nr)\left(\frac{1-nr}{1-(n-1)r}\right)^{n-1} \quad (2)$$

where K is the binding constant for an isolated bound dye and n the maximal number of base pairs per bound dye. The binding curve of the cooperative type for binding antiA/T was fitted with equation

$$r/C_1 = K(1-nr)\cdot\left(\frac{(2\omega-1)(1-nr)+(r-R)}{2(\omega-1)(1-nr)}\right)^{n-1}\cdot\left(\frac{1-(n-1)r+R}{2(1-nr)}\right)^{2}$$
$$R = ((1-(n+1)r)^2+4\omega r(1-nr))^{1/2} \quad (3)$$

where ω is the cooperativity factor.

3. Results and discussion

3.1. Characteristics of fluorescence of EtBr and AO complexes with parARB and antiATT

Quantum yields q of EtBr and AO complexes with parARB and antiATT were measured to be $q_{EtBr} = 0.16 \pm 0.01$ and $q_{AO} = 0.88+0.05$, thus they practically coincided with those for DNA. The q values appeared to be the same for both types of triplexes studied (Table 1). The lifetime of fluorescence $\tau_{EtBr} = 25 \pm 1$ ns of EtBr bound to parARB was shown to be the same within experimental error as that for EtBr bound to antiATT and DNA (Table 1). Similarly, $\tau_{AO} = 5.0 \pm 0.2$ ns was found to be the same for AO complexes with parARB, antiAT and DNA. EtBr and AO binding to the DNA double helix by an intercalative mechanism is known to bring about an ~10-fold increase in q_{EtBr} [34,35] and ~3-fold increase in q_{AO} in comparison with free dyes [37]. The obtained values for q_{EtBr} and q_{AO} for parARB and antiATT complexes with dyes testify to the EtBr and AO intercalations in these triplexes, which is in agreement with our previous data [15,23,27] and with the results of EtBr binding to antiparallel triplexes [5,7].

3.2. Affinities of EtBr and AO for parARB and antiATT triplexes and homologous duplexes

The EtBr binding curves plotted in Scatchard coordinates for triplexes parARB, antiATT, and duplex dsARB were fitted to Eq. 2, while the cooperative curve for antiA/T was fitted to Eq. 3 (Fig. 1). The obtained binding constants ${}^bK$ and the numbers ${}^bn$ of base pairs or triplets per bound ethidium are given in Table 1. Remarkably, both triplexes have higher affinities for EtBr than their homologous duplexes. As for the EtBr binding to poly(dA)·2poly(dT) and to poly(dA)·poly(dT) this was observed earlier [5]. Thus poly(dA)·poly(dT) (and similarly antiA/T) is known to have a non-standard structure strongly stabilized with a water spine located in the minor groove that does not favor the intercalation (see, for example, [35]). The 2-fold greater ${}^bK$ for parARB than that for the homologous dsARB seems to be original and is fundamental for this triplex stabilization with intercalators. The parallel triplex parARB has been shown to be stabilized with propidium iodide [23], the triplex-specific intercalator bearing two positive charges [6]. The binding constant for parARB is found to be similar to that for the antiparallel triplex antiATT, but the number of base triplets per bound ligand ${}^bn$ for parARB is close to 2 and is clearly smaller than ${}^bn$ for antiATT. This might be relevant to the structural peculiarity of the parallel (recombinant) triplexes [14]. The data on AO binding are concordant, $K = (2.6 \pm 0.1) \times 10^5$ M^{-1} for the parARB triplex and $(1.7 \pm 0.1) \times 10^5$ M^{-1} for the dsARB duplex (curves not shown). Under the experimental conditions used the observed ${}^bK = (1.7 \pm 0.1) \times 10^5$ M^{-1} for EtBr binding to antiATT is 10-fold larger than that for duplex antiA/T, whereas the corresponding value for poly(dA)·2poly(dT) was about 30-fold larger than that for poly(dA)·poly(dT) [5]. The ${}^bn$ values for antiATT and antiA/T are somewhat larger than those in the above cited study. This fact may be explained by the differences in experimental conditions and/or by the finite length of the short oligonucleotides. To take into account the length of the triplexes and duplexes, we analyzed the non-

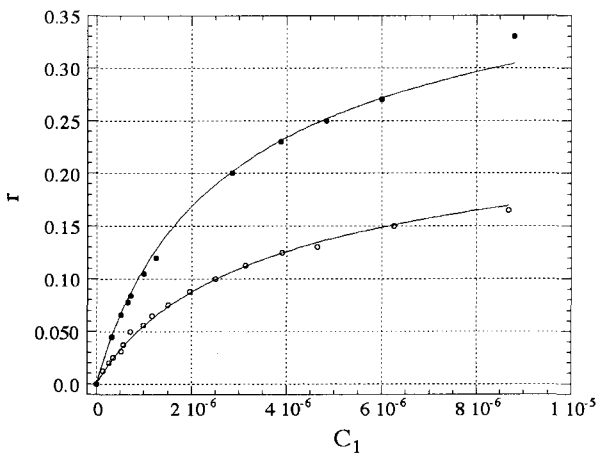

Fig. 2. Binding curves of ethidium bromide to parARB (●) and dsARB (○) fitted to equations

$$r = 0.1K\left(\frac{9 + 56KC_1^2 + 105K^2C_1^3 + 60K^3C_1^4 + 5K^4C_1^5}{1 + 9KC_1 + 28K^2C_1^2 + 35K^3C_1^3 + 15K^4C_1^4 + K^5C_1^5}\right)$$ and

$$r = 0.1K\left(\frac{8 + 30KC_1^2 + 12K^2C_1^3}{1 + 8KC_1 + 15K^2C_1^2 + 4K^3C_1^3}\right)$$, respectively (curves). The derived association constants ${}^cK$ are given in Table 1.

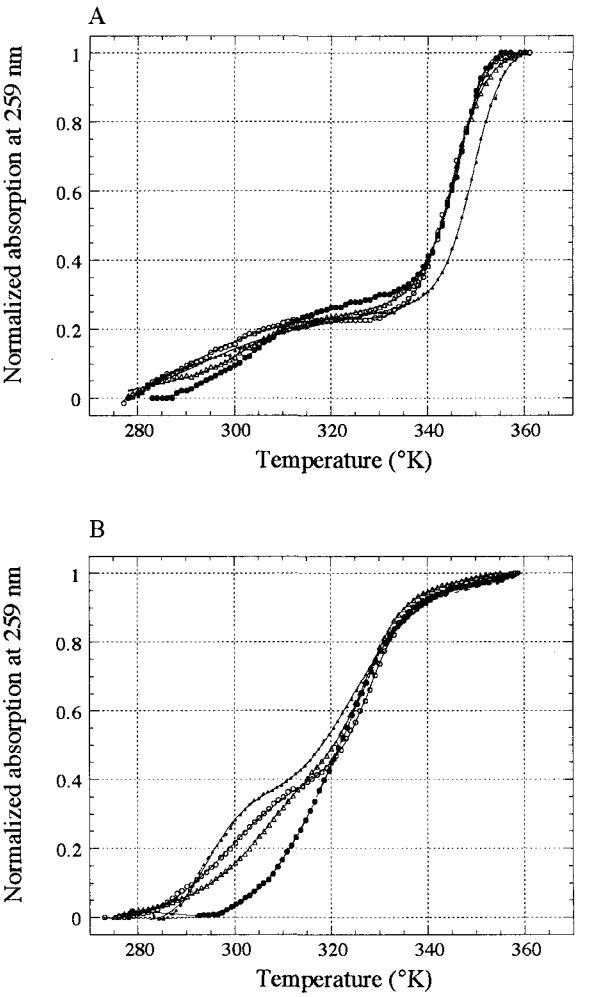

Fig. 3. Thermal denaturation profiles of parARB complexes wiht EtBr (A) and antiATT complexes with AO (B). Free oligonucleotide (○), C_2/triplex = 1 (△), C_2/triplex = 2 (●), C_2/triplex = 3 (▲). Curves are the fits with program *helix_fit_14* [33]; for details see text.

cooperative curves with a model for binding ligands to a finite lattice [30]. In order to evaluate binding constants, we simplified the equation derived in the above cited study

$$r = N^{-1}\frac{\delta\ln\left(\sum_{q=0}^{\frac{q}{n}}\frac{[N-q(n-1)]!}{[N-qn]!q!}(KC_1)^q\right)}{\delta\ln C_1}$$

inputting the length of the lattice $N=10$ bp or base triplets (bt) and integer exclusion lengths, or number of bp or bt per bound ligand, $n=2$ for parARB and $n=3$ for dsARB (Fig. 2). The obtained ${}^cK=(1.80\pm0.05)\times10^5$ M^{-1} for parARB and $(0.97\pm0.02)\times10^5$ M^{-1} for dsARB (Table 1) are surprisingly close within experimental error to the binding constants derived using the theory of McGhee and von Hippel (Fig. 1) without taking into account the finite triplex and duplex lengths.

3.3. Stabilization and destabilization of triplexes with intercalators binding

Thermal denaturation of oligonucleotides complexed at 3°C in the average to one, two or three intercalated EtBr or AO was performed. The added dye concentrations C_0 for these experiments were calculated from binding curves so that the ratios of bound dye concentrations per oligonucleotide were equal to 1, 2 or 3. Melting profiles of parARB:EtBr and antiATT:AO complexes with different numbers of intercalated dyes are shown in Fig. 3A,B, respectively. The biphasic shape of the curves reflects two successive monomolecular transitions, triplex-to-duplex with the dangling third strand and duplex-to-open oligonucleotide. The former transition occurs at lower temperatures, while the latter proceeds in a higher temperature range. Melting parameters of free parARB and antiATT are in agreement with previous estimations [15,27]. For the given sequences the transition enthalpy of the parallel triplex is about two times lower than that for the denaturation of the Hoogsteen strand from the antiATT triplex. The first and second intercalated dye molecules cause right shifts of the triplex melting curve (open triangles and filled circles), but the third one shifts it to the left (filled triangles). Solid curves are the best fits obtained with a formalism for the two successive

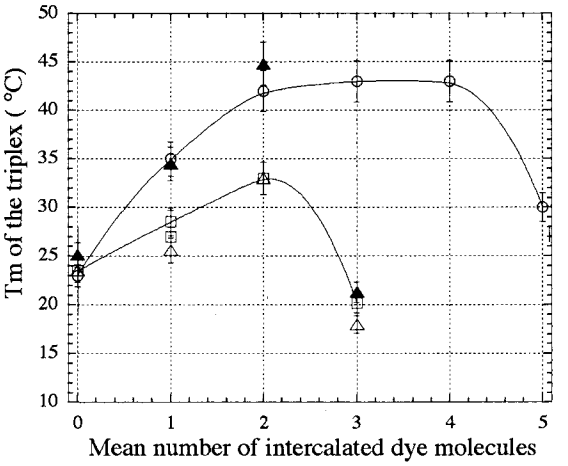

Fig. 4. Melting temperatures for triplex-to-duplex transition at different C_2/triplex ratios for complexes parARB:EtBr (□), parARB:AO (△), parARB:PI (○), antiATT (▲).

concerted reactions [33]. Derived parameters, melting temperatures T_m and van't Hoff enthalpies ΔH for both transitions are given in Table 2. T_ms of the triplexes are plotted versus average number of intercalated dye molecules (Fig. 4). Effects of EtBr and AO on parARB melting temperature are close due to similar binding constants (Tables 1 and 2). The greater difference of intercalator affinity for antiATT as compared to that for antiA/T leads to a more significant increase of T_m (Fig. 4, black triangles). Propidium iodide (PI), which has a greater affinity for parARB than EtBr due to an additional positively charged group bound with a flexible linker, provides a significant stabilizing effect (open circles, data for the number of bound PI from 0 to 4 are taken from [23]). The destabilization of both triplexes on binding the third EtBr or AO and of parARB binding the fifth PI is dramatic (Fig. 4). It is much more pronounced than could be expected for the triplex-to-duplex melting with ligands of the observed affinities according to the theory of melting of DNA complexes with low molecular ligands [38]. The observed destabilization effect may be explained by the intercalator-induced conformational changes of the triplex structure which might occur upon too high a degree of triple helix extension. Possible local changes in oligonucleotide hydration at the relatively high total con-

Table 2
Thermodynamic parameters of thermal dissociation

Oligonucleotide	Triplex-to-duplex transition			Dupex-to-open-strand transition	
	T_m (°C)	ΔH (kJ M^{-1})	ΔH^* (kJ M^{-1})	T_m (°C)	ΔH (kJ M^{-1})
parARB	23.5±8	40±10	39±10	72.5±0.5	236±10
parARB+1EtBr	28.5±0.5	195±32		72.6±0.2	291±9
parARB+2EtBr	33.1±0.5	148±16		75.3±0.3	290±10
parARB+3EtBr	20.2±1.7	108±28		76.5±0.4	319±13
parARB+1AO	25.6±0.3	157±15		74.1±0.3	290±8
parARB+2AO	33 ±0.9	NA		73.9±0.2	NA
parARB+3AO	18.2±0.4	162±5		74.4±0.1	347±5
ATT	25.9±1.0	78±11	80±10	54.7±0.3	250±6.0
ATT+1AO	35.3±0.3	110±8		54.9±0.1	246±5.0
ATT+2AO	44.2±3.7	104±18		56.2±2.8	213±19
ATT+3AO	21.4±0.3	189±45		51.4±1.3	162±16

T_m and ΔH values for triplex and duplex meltings were obtained using the three-state mode (see Section 2). The triplex-to-duplex transition enthalpies ΔH^* were estimated also using Eq. 4; δT_m were calculated from T_m values, $\delta\Delta T$ were taken from the melting profiles in Fig. 3A,B (open circles and triangles).

centrations of dyes might also contribute to this phenomenon. In the case of PI, triplex stability up to C_2/oligonucleotide = 4 may be brought about by additional stabilization by the positively charged side group of PI [23].

It is worth noting that melting of DNA complexes with ligands provides an independent estimate of the transition enthalpy ΔH for the ligand-free DNA [38]. We consider the triplex-to-duplex transition. According to the theory for $C_2 \ll 1$

$$\Delta H = \frac{\delta \Delta T}{(\delta T_m)^2} \cdot T_0^2 C_2 \quad (4)$$

where T_0 is the transition melting temperature of ligand-free DNA, δT_m is the shift of transition melting temperature at bound ligand concentration C_2, $\delta \Delta T$ is the change of the width of the transition. Considering the melting curves in Fig. 3A,B for $C_2 = 0$ and $C_2 = 0.1$ dye/base triplet (one dye molecule per triplex) we estimated the triplex-to-duplex enthalpies ΔH^* for parARB and antiATT (Table 2) that appeared to be similar within the experimental error to the corresponding ΔH values obtained with a three-state model.

4. Conclusions

Ligands intercalation in DNA triple helices may stabilize as well as destabilize triplex structure. The effect depends on the concentration of bound intercalators per base triplet. One or two intercalated EtBr or AO molecules stabilize 10 nucleotide long triplexes, whereas intercalation of the third dye molecule destabilizes the triplexes.

The above effects are similar for both classical antiparallel triplexes and for parallel (recombinant) triplexes of the nucleotide sequences studied.

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