

Triple-helix formation of DNA oligomers with methylthiourea-linked nucleosides (DNmt): A kinetic and thermodynamic analysis

(antisense/antigene/kinetics/triplex/cationic oligonucleotide)

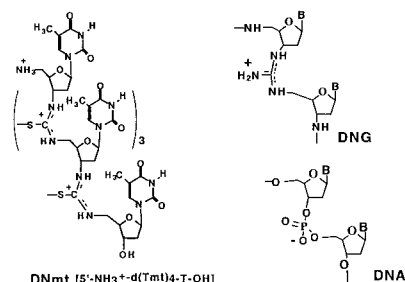
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ABSTRACT Complementary short-strand DNA homooligomers and methylthiourea-linked mononucleosides associate and form triplexes in solution. The melting temperatures, T_m , the association and dissociation kinetic and thermodynamic parameters, and activation energies were determined by UV thermal analysis for the triplexes of short-strand DNA homooligomers [d(pA)₁₀-d(pA)₂₃] and poly(dA) with the methylthiourea-linked nucleoside [5'-NH₃⁺-d(Tmt)₄-T-OH {DNmt₅}]₃. Circular dichroism studies show evidence of triple-helical association dependent on the length of the target homooligomer. The melting and cooling curves exhibit hysteresis behavior in the temperature range of 10–95°C at 0.13 deg/min thermal rate. From these curves, the rate constants and the energies of activation for association (k_{on} , E_{on}) and dissociation (k_{off} , E_{off}) were obtained. T_m decreases with the ionic strength and increases with increase in length of the monomers. The rate constants k_{on} and k_{off} at a given temperature (288 K–310 K) are dependent on the DNA strand length and also decrease and increase respectively with the ionic strength. The energies of activation for the association and dissociation processes are in the range of -18 to -38 kcal/mol and 3 to 18 kcal/mol, respectively. The equilibrium constant for the formation of the triplexes [5'-NH₃⁺-d(Tmt)₄-T-OH]₂d(pA)_x, $x = 10$ –23] is several orders of magnitude greater when compared with the triplexes of DNA. The number of base triplets in the nucleus of the DNmt₂-DNA triple-helix (nucleation-zipping model) increases with decreased DNA oligomer length and with increased ionic strength. The values of ΔH° calculated from the activation parameters are between -30 and -50 kcal/(mol base) and the values of ΔG° are between -6 and -11 kcal/(mol base) for short-strand DNA.

The negatively charged phosphodiester linkages of double- and triple-stranded DNA and RNA reside side by side, causing considerable charge-charge electrostatic repulsion. This is particularly so at the low ionic strength that is physiological. This feature, as well as the susceptibility of DNA and RNA to nuclease activity, limits the usefulness of RNA and DNA as antisense or antigene drugs. We have reported the replacement of the phosphate linkages in DNA and RNA by achiral guanido groups, which we identify as DNG and RNG (1–3) and more recently have designed the polycationic nucleotide linkage with methylisothiourenium salts, or methylated thioureas, abbreviated as DNmt (Scheme 1) (4, 5). In this study, we report the melting temperatures, T_m and circular dichroism curves, as well as the association and dissociation kinetic and thermodynamic parameters for the formation of triplexes of short-strand DNA homooligomers [d(pA)₁₀, d(pA)₁₅, d(pA)₂₀, d(pA)₂₃] with 5'-NH₃⁺-d(Tmt)₄-T-OH.



SCHEME 1

MATERIALS

The concentrations of nucleotide solutions were determined by using the extinction coefficients (per mol of nucleotide) calculated according to the nearest neighboring effects (6). For d(Tmt)₅ we used $\epsilon_{268} = 8700 \text{ M}^{-1} \text{ cm}^{-1}$. All experiments were conducted in either (i) 0.015 M phosphate buffer, pH 7–7.5 or (ii) 0.008 M phosphate buffer at pH 6.85. The ionic strength, μ , was adjusted with KCl and is presented with the corresponding concentration of KCl. The concentration of nucleosides, expressed in M/base, was $4.0 \times 10^{-5} \text{ M}$, and the ionic strength ranged from 0.03 to 0.12 M KCl. The nucleoside concentration referred to is the limiting component forming the triplex (e.g., a concentration of $4.0 \times 10^{-5} \text{ M}$ /base in the reaction of A + 2 T means [A] = $4.0 \times 10^{-5} \text{ M}$ /base and [T] = $8.0 \times 10^{-5} \text{ M}$ /base). All stock solutions were kept at 4°C between experiments.

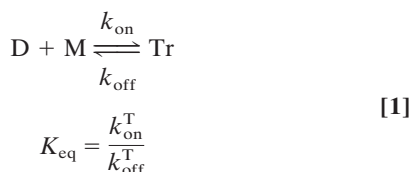
CD, UV Spectroscopy, and Data Collection. CD spectra were obtained on an OLIS (Jefferson, GA) RSM circular dichroism spectrophotometer. Scans were run from 320 nm to 190 nm. Measurements were recorded at every nanometer. Ten scans were recorded, averaged, and smoothed for each curve. Samples were held in a 1-cm path length cuvette at 25°C. UV spectra were recorded at $\lambda = 260 \text{ nm}$ on a Cary 1E UV/vis spectrophotometer equipped with temperature programming. Spectrophotometer stability and λ alignment were checked before initiation of each melting point experiment. For the T_m determinations, hypochromicity was used. Data were recorded every 1.0 degree. The samples were heated from 25°C to 95°C at 5 deg/min (Scheme 1), the annealing (95–10°C) and the melting (10–95°C) were conducted at 0.13 deg/min, and the samples were brought back to 25°C at a rate of 5 deg/min. The reaction solutions were equilibrated for 15 min at the highest and lowest temperatures.

Analysis of Kinetic Data for Triplex Formation. The equation for triplex formation that was used (Eq. 1) describes the formation of the triplex (Tr) from the duplex (D) and the monomer (M), as individual strands. This equation could be interpreted as the reaction of double-strand D

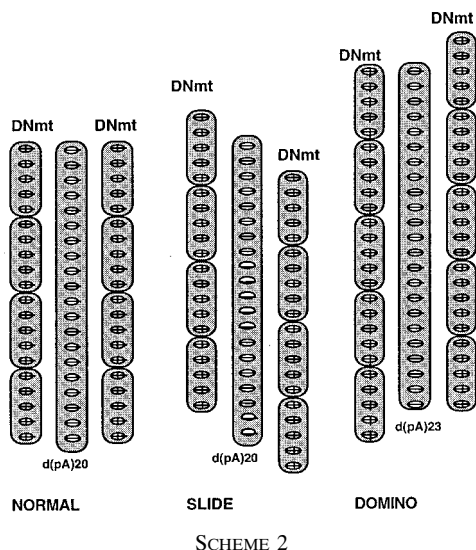
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with single-strand M. However, the process of triplex formation may take place in different ways that we shall refer to as domino, normal, and slide (or dangling ends) (Scheme 2) (2).



The expression of kinetic equations for the reactants and products (Eq. 2) can be accomplished by expressing the concentrations of strands in molar per base (M/base). The theory behind the “on” and “off” rates of the dissociation/association of the triplexes and the derivation of kinetic equations has been described in detail (2, 7). In Eq. 1, a triplex is formed from and dissociates to a duplex and a third monomer strand with rate constants k_{on} and k_{off} . The associated rate for this reaction is given in Eq. 2.

$$\frac{d[\text{D}]}{dt} = k_{\text{off}}^T[\text{Tr}] - k_{\text{on}}^T[\text{D}][\text{M}] \quad [2]$$

Letting $D_{\text{tot}} = [\text{D}] + [\text{Tr}]$ and $M_{\text{tot}} = [\text{M}] + [\text{Tr}]$, where the subscript “tot” stands for the total concentration and superscript T stands for temperature, the monitored absorbance is a weighted combination of the absorbances of the trimer, dimer, and monomer (Eq. 3), where $\alpha = [\text{Tr}]/D_{\text{tot}}$.

$$A = \alpha A_{\text{Tr}} + (1 - \alpha)A_{\text{D+M}} \quad [3]$$

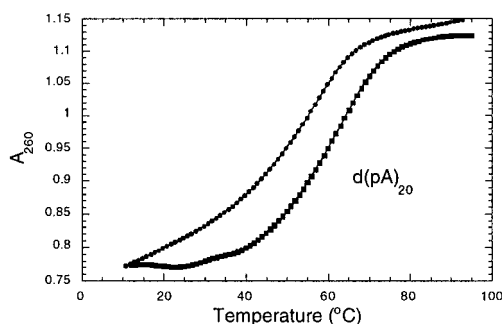


FIG. 1. Hysteresis curve for the triplex denaturation and renaturation of $d(\text{pA})_{20}$ with $5\text{'-NH}_3^+\text{-d}(\text{Tmt})_4\text{-T-OH}$ ($\text{Tmt}\cdot\text{Ap}\cdot\text{Tmt}$) at 4.0×10^{-5} M/base of single-strand DNA respectively at $\mu = 0.03$ and 0.13 deg/min heating (lower curve)/cooling (upper curve) rate.

The expressions for k_{on} and k_{off} were solved as previously reported (2, 7). The rate constants k_{on} and k_{off} are functions of temperature and, therefore, can be expressed as Arrhenius equations (2, 7):

$$k_{\text{on}} M_{\text{tot}} = k_{\text{on}}^{\text{ref}} M_{\text{tot}} \exp\left\{-\frac{E_{\text{on}}}{R} \left(\frac{1}{T} - \frac{1}{T_{\text{ref}}}\right)\right\} \quad [4a]$$

$$k_{\text{off}} = k_{\text{off}}^{\text{ref}} \exp\left\{-\frac{E_{\text{off}}}{R} \left(\frac{1}{T} - \frac{1}{T_{\text{ref}}}\right)\right\} \quad [4b]$$

where $R = 1.98$ cal/mol·K, and T_{ref} is the reference temperature at which the rate constant k^{ref} applies. In a plot of $\ln(k_{\text{on/off}})$ vs. $1/T - 1/T_{\text{ref}}$, the thermodynamic parameters $E_{\text{on/off}}/R$ are obtained as the slope and the kinetic $k_{\text{on}}M_{\text{tot}}$, k_{off} parameter as the intercept. The limitations of this approach have been reported previously (2, 7–9).

Data Analysis. The raw data from the melting point determinations was subjected to Gaussian smoothing by using MATLAB 4. Calculations of thermodynamic parameters were performed by using the resulting database.

RESULTS

Melting Curves and Their Hysteresis Behavior. Melting studies of triplexes formed from short-strand DNA homooligomers and $5\text{'-NH}_3^+\text{-d}(\text{Tmt})_4\text{-T-OH}$ were carried out by using UV spectroscopy at 260 nm. The ratio between $5\text{'-NH}_3^+\text{-d}(\text{Tmt})_4\text{-T-OH}$ and $d(\text{pA})_x$ was 2:1. We have found that all melting curves exhibit hysteresis (Fig. 1). The divergence of the heating and cooling curves are functions of the rates of heating and cooling.

Stoichiometry of the Binding. Job plots were constructed from absorbances of solutions containing $5\text{'-NH}_3^+\text{-d}(\text{Tmt})_4\text{-T-OH}$ and the DNA homooligomers $d(\text{pA})_{10-23}$ and poly(dA) to assess whether the hysteresis generally seen in the heating and cooling curves (Fig. 2) are associated with triplex denaturation. As experienced with our previously reported studies of DNmt binding to poly(dA) and poly(rA) (4), the Job plots clearly establish a minimum at ca. 67% $5\text{'-NH}_3^+\text{-d}(\text{Tmt})_4\text{-T-OH}$, which corresponds to the formation of a 2:1 ($5\text{'-NH}_3^+\text{-d}(\text{Tmt})_4\text{-T-OH}$)₂(DNA) complex. The same results were obtained at three wavelengths (202, 260, and 284 nm) (4).

Circular Dichroism Spectroscopy of Complementary DNA Oligomers with DNmt. The CD spectrum of an oligomer solution can give valuable information about the conformation of the oligomers as single strands or in association with other DNA oligomers. The CD spectrum of the complex formed between pentameric thymidyl DNA oligomer $5\text{'-NH}_3^+\text{-d}(\text{Tmt})_4\text{-T-OH}$ and poly(dA) is shown in Fig. 3. When DNmt is associated with complementary DNA, the CD spectrum of the complex does not match the spectrum calculated from the weighted sums of the CD spectra for the constituent oligomers. Fig. 4 shows the difference CD spectrum of the triple helical complex of DNmt with $d(\text{pA})_x$ subtracted from the spectrum obtained for $d(\text{pA})_x$ alone.

The Effect of Ionic Strength on T_m . At 4.0×10^{-5} M concentration in base A and a 2:1 ratio between the T bases and A bases (in 10 mM phosphate buffer pH 7.0), we saw only one T_m transition (at 260 nm) after annealing $5\text{'-NH}_3^+\text{-d}(\text{Tmt})_4\text{-T-OH}$ with $d(\text{pA})_{10}$, $d(\text{pA})_{15}$, $d(\text{pA})_{20}$, and $d(\text{pA})_{23}$ (Fig. 1). We assigned this transition to the triplex denaturation. In contrast, interaction of $5\text{'-NH}_3^+\text{-d}(\text{Tmt})_4\text{-T-OH}$ with poly(dA) exhibits the two transitions corresponding to triplex and duplex melting, as described previously (4). With increase in ionic strength (μ), the value of T_m decreases (Table 2). Above $\mu = 0.12$, the melting curves become shallow (i.e., as ΔA_{260} becomes smaller), and the T_m determination is less accurate.

Kinetics of the Association and Dissociation. An expression that provides the temperature dependence of the rate constants for association (k_{on}) and dissociation (k_{off}) is provided in Eq. 4. As the rate of the heating and cooling increases, the rate of equilibration of the species lags such that the hysteresis becomes more marked. The four ramps depicted in Fig. 5 show the

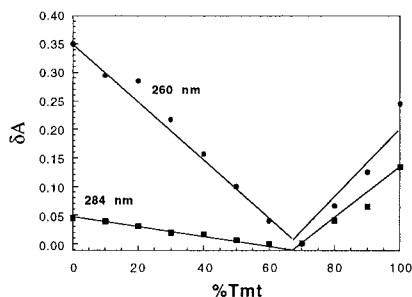


FIG. 2. Job plots of d(pA)₂₀ with 5'-NH₃⁺-d(Tmt)₄-T-OH in a concentration of 4.0×10^{-5} M/base at 260 nm (●) and 284 nm (■).

variation of the absorbance (A_{260}) vs. temperature. At equilibrium, both heating and cooling curves coincide, satisfying the mathematical condition $d\alpha/dt = 0$ (α = fraction of duplex engaged in the triplex). Eq. 4 provides k_{on}^{ref} and k_{off}^{ref} as well as the activation E_{on} and E_{off} parameters at a given reference temperature. Examination of plots of $\ln(k_{on})$ and $\ln(k_{off})$ vs. $1/T$ show that in each case there is a linear and a scattered region. The scattered region is associated with the initial and final portions of the triplex melting curve where there is little change in the absorbance, i.e., $d\alpha/dt = 0$. For our studies, plots of $\ln(k)$ vs. $1/T$ were found to be linear between the temperature range of 14°C–40°C. An exception was d(pA)₁₀, which showed linearity before 35°C because of the lower T_m compared with longer DNA oligomers. From the intercepts of plots of $\ln(k_{on})$ and $\ln(k_{off})$ vs. $1/T - 1/T_{ref}$, we obtain $k_{on}^{ref}M_{tot}$ and k_{off} , respectively. E_{on}/R and E_{off}/R are determined from the respective slopes from Fig. 6 (Table 1). Numerical values calculated from Fig. 6 by using Eqs. 4a and 4b are:

$$\begin{aligned} k_{on}^{15}M_{tot} &= 36.8 \times 10^{-4} \text{ s}^{-1} & k_{off}^{15} &= 1.451 \times 10^{-6} \text{ s}^{-1} \\ E_{on}/R &= -9555 \text{ K} & E_{off}/R &= 9,454 \text{ K} \\ E_{on} &= -18.92 \text{ kcal/mol} & E_{off} &= 18.72 \text{ kcal/mol} \end{aligned}$$

Inspection of Table 1 shows that the values of $k_{on}^{15}M_{tot}$, for short-strand DNA oligonucleotides, vary between 7.52×10^{-4} and $49.04 \times 10^{-4} \text{ s}^{-1}$ and those of k_{off}^{15} vary between 1.45×10^{-6} and $20.01 \times 10^{-6} \text{ s}^{-1}$. The energies of activation E_{on}^{15} for short-strand oligonucleotides were between -18 and -38 kcal/mol and E_{off}^{15} between 3.1 and 18.7 kcal/mol. Table 1 shows the values of k_{off}^{15} , k_{on}^{15} , and K_{eq} at different ionic strengths and temperatures. Reference temperatures (15°C, 25°C, and 37°C) were chosen to make relevant comparisons with other published works (15°C, 25°C) (2, 3, 7, 10) and their biological relevance (37°C).

Influence of the DNA Length on k_{on} , k_{off} , and K_{eq} . The rate constant for the association (k_{on}) of 5'-NH₃⁺-d(Tmt)₄-T-OH to d(pA)_x increases with increase in chain length ($x = 10$ –20), while the rate constant for the dissociation (k_{off}) decreases (Table 1 and Fig. 7). On going from d(pA)₂₀ to d(pA)₂₃, there is a slight drop

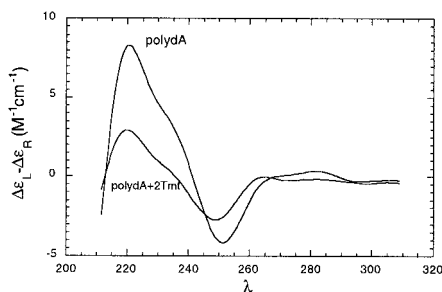


FIG. 3. CD spectra of poly(dA) compared with DNmt₂:poly(dA) triplex. Solution conditions: 1.0×10^{-5} M in oligomer, 0.03 M KCl, 10 mM phosphate buffer, pH 6.8 at 25°C.

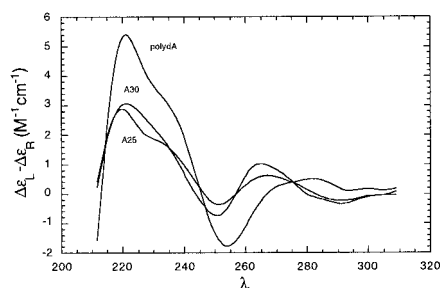


FIG. 4. Difference CD spectra of poly(dA), d(pA)₃₀ and d(pA)₂₅ binding to DNmt. The ratio of d(pA)_x to DNmt was 1:2. Spectrum of poly(dA) refers to the difference spectra [poly(dA) - DNmt₂:poly(dA) triplex]. Solution conditions: 1.0×10^{-5} M in oligomer, 0.03 M KCl, 10 mM phosphate buffer, pH 6.8 at 25°C.

in the value of k_{on} (except at $\mu = 0.03$, 15°C), whereas k_{off} increases slightly. This is best explained by looking at the K_{eq} values. At every ionic strength and temperature, K_{eq} increases from d(pA)₁₀ to d(pA)₂₀ and decreases slightly from d(pA)₂₀ to d(pA)₂₃ (Table 1, Fig. 7).

Dependence of Kinetic and Activation Parameters on Ionic Strength. By increasing the ionic strength, k_{on} increases while k_{off} decreases (Table 1). For d(pA)₁₀ at 4.0×10^{-5} M/base concentration using 15°C as T_{ref} , $\Delta k_{on}M_{tot} = 13 \times 10^{-4} \text{ s}^{-1}$ and $\Delta k_{off} = 8.2 \times 10^{-6} \text{ s}^{-1}$. For d(pA)₁₀, with the ionic strength decrease (0.08 to 0.03), E_{on} decreases from -37 to -26 kcal/mol, whereas E_{off} increases from 3.12 to 10.37 kcal/mol (Table 2).

Dependence of Activation Parameters on DNA Length. At $\mu = 0.03$, an increase in DNA length from d(pA)₁₀ to d(pA)₂₀ while maintaining the 2:1 ratio between 5'-NH₃⁺-d(Tmt)₄-T-OH and DNA results in an increase in E_{off} from 10.37 to 18.73 kcal/mol, whereas E_{on} decreases from -26 kcal/mol to -18 kcal/mol or becomes less negative at $\mu = 0.03$ (Table 2). E_{off} values decrease (similar to increase in k_{off}) on going from d(pA)₂₀ to d(pA)₂₃.

DISCUSSION

Our previous work on DNmt binding (4, 5) to polynucleotides has shown that (i) thymidyl DNmt (5'-NH₃⁺-d(Tmt)₄-T-OH) has much stronger affinity for DNA and RNA, because of electrostatic attractions, than DNA for RNA or *vice versa*; (ii) thymidyl DNmt is specific for its complementary tracts of adenine bases and does not interact with guanylic, cytidylic, or uridylic tracts; (iii) the thermal stability of DNmt:RNA and DNmt:DNA structures is attenuated by increasing salt concentrations; (iv) DNmt forms triple helical structures from 15–60°C that are very stable under physiological ionic strength conditions. The effect of ionic strength on melting is more pronounced with 5'-NH₃⁺-d(Tmt)₄-T-OH interacting with d(pA)_x and has an opposite effect as compared with DNA complexes with DNA or RNA. This is because of electrostatic interactions being attenuated by increasing salt concentrations.

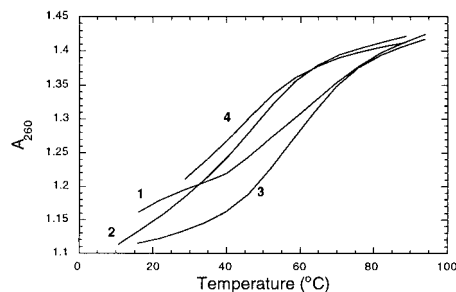


FIG. 5. Effect of the heating/cooling rate on the extent of association/dissociation of the triplex of d(pA)₂₀ with 5'-NH₃⁺-d(Tmt)₄-T-OH at 4.0×10^{-5} M/base monomer concentration. 1, fast heating (5 deg/min); 2, slow annealing (0.13 deg/min); 3, slow melting (0.13 deg/min); 4, fast cooling (5 deg/min).

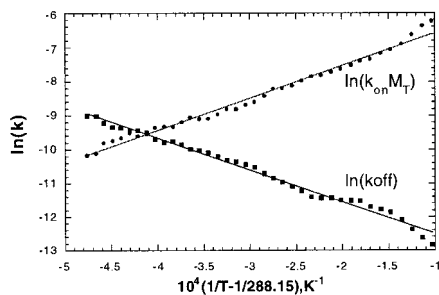


Fig. 6. Dependence of $\ln(k_{on}M_T)$ and $\ln(k_{off})$ on $1/T$ for the denaturation/renaturation of triplex of $(5'-NH_3^+-d(Tmt)_4-T-OH)_2 \cdot d(pA)_{20}$ at 4.0×10^{-5} M/base and $\mu = 0.03$ and $dT/dt = 0.13$ deg/min.

The melting studies of complexes formed from short-strand DNA homooligomers and $5'-NH_3^+-d(Tmt)_4-T-OH$ showed that all melting curves exhibit hysteresis (Fig. 1) at the rate of heating-cooling used (0.13 deg/min). This points to the formation of triplexes between $5'-NH_3^+-d(Tmt)_4-T-OH$ and $d(pA)_x$. Previous melting studies with poly(dA) and $5'-NH_3^+-d(Tmt)_4-T-OH$ established the existence of two transitions corresponding to melting of triplex and duplex (4). The triplex formation between $5'-NH_3^+-d(Tmt)_4-T-OH$ and $d(pA)_{10-23}$ under our experimental conditions leads to only one transition between 5°C and 95°C, similar to results obtained with DNG melting curves with DNA homooligomers (2). Further evidence for triplex formation comes from the use of the continuous variation method. The minimum observed in Fig. 2 occurs at ca. 67% $5'-NH_3^+-d(Tmt)_4-T-OH$, demonstrating the existence of the $(5'-NH_3^+-d(Tmt)_4-T-OH)_2 \cdot (DNA)$ complex. To further study the nature of $(5'-NH_3^+-d(Tmt)_4-T-OH)_2 \cdot (DNA)$ triplex, we used circular dichroism (Figs. 3 and 4). Fig. 3 shows the binding of $5'-NH_3^+-d(Tmt)_4-T-OH$ to poly(dA) in a 2:1 fashion at a concentration of 4.0×10^{-5} M/base poly(dA). As expected, the molar ellipticity of poly(dA) is substantially changed on formation of the triplex. Fig. 4 shows the difference spectra of $5'-NH_3^+-d(Tmt)_4-T-OH$ binding to poly(dA), $d(pA)_{30}$, and $d(pA)_{25}$. Clear differences can be seen indicating that different degrees of structural changes have taken place in the three oligomers because of their association. For the complementary poly(dA), the difference spectra show the largest changes of all the samples.

Table 1. Kinetic parameters for the triplex melting of $d(pA)_x$ with DNmt

T, °C	μ	$d(pA)_x, x =$	$k_{on} \cdot M_T \cdot 10^4$	$k_{off} \cdot 10^6$	$K_{eq} \cdot 10^{-5}$
15	0.03	10	20.23	11.88	42.572
	0.03	15	38.21	9.54	100.13
	0.03	20	36.68	1.451	631.98
	0.03	23	49.04	2.176	563.42
	0.08	10	7.52	20.01	9.3953
	0.08	15	32.14	12.2	65.861
	0.08	20	36.23	4.382	206.7
	0.08	23	14.56	11.93	30.511
25	0.03	10	2.73	19.95	3.4211
	0.03	15	9.108	18.06	12.608
	0.03	20	11.95	4.36	68.521
	0.03	23	13.6	5.79	58.722
	0.08	10	1.7	27.7	1.5343
	0.08	15	3.39	24.04	3.5254
	0.08	20	5	11.975	10.438
	0.08	23	5.79	23.72	6.1024
37	0.03	15	1.838	36.84	1.2473
	0.03	20	3.45	14.94	5.7731
	0.03	23	3.246	17.284	4.6951
	0.08	15	0.27	51.44	0.13122
	0.08	20	0.547	36.832	0.37128
	0.08	23	0.28	51.1	0.13699

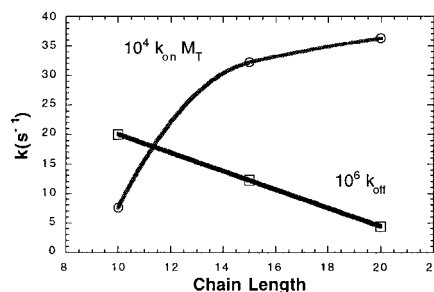


Fig. 7. Dependence of k_{on}^{15} and k_{off}^{15} with the DNA length for the dissociation of the triplex $(5'-NH_3^+-d(Tmt)_4-T-OH)_2 \cdot d(pA)_x$ at 4.0×10^{-5} M/base monomer concentration, $\mu = 0.03$ and $dT/dt = 0.13$ deg/min.

As the length of the oligomer drops, difference spectra drop in amplitude, indicating that the degree of association is weakening. For the complementary oligomer $d(pA)_{10}$ (data not shown), the difference spectra are very flat, indicating little association that reorganizes the bases of the nucleosides.

At 15°C, the second order rate constant, k_{on} , for the triplex formation with short-strand (10–23) oligonucleotides is $17-122 M^{-1} s^{-1}$ (the molarity is expressed in per base) and the first-order rate constant k_{off} is $0.14-2.54 \times 10^{-5} s^{-1}$ in the range of $\mu = 0.03-0.08$. These k_{on} values for DNmt₅, like DNG₅ ($k_{on} = 30-200 M^{-1} s^{-1}$, $k_{off} = 0.10-3.19 \times 10^{-5} s^{-1}$) are, on average, one order of magnitude larger than those found for a DNA triplex having the length of 22 bp at $[NaCl] = 0.02-0.30$ M. On the other hand, the k_{off} values are of the same order of magnitude as seen with a DNA triplex having the length of 22 bp (7). This comparison is qualitative only because the ionic strength acts in the opposite direction for DNmt relative to DNA, and the composition of the 22-mer DNA oligonucleotide differs from the short-strand DNA oligonucleotides in this study.

As in the case of DNA duplexes and triplexes, the association of the strands decreases as the oligomer strand becomes shorter. The second order rate constant for reaction $5'-NH_3^+-d(Tmt)_4-T-OH$ with $d(pA)_x$, k_{on} , increases with the increased length of the DNA strand, while the first order rate constant, k_{off} , decreases (Fig. 7 and Table 1). This is not so for the transition from $d(pA)_{20}$ to $d(pA)_{23}$. At every ionic strength and temperature, K_{eq} increases from $d(pA)_{10}$ to $d(pA)_{20}$ and decreases slightly from $d(pA)_{20}$ to $d(pA)_{23}$. This is best explained by the domino effect of DNmt₅ binding to $d(pA)_{23}$ (Scheme 2). The kinetic equations get very complicated if the presence of unoccupied sites is considered [e.g., $d(pA)_{23} + 5 \times DNmt_5$, Scheme 2]. Even in the case of $d(pA)_{10}, 15, 20$, where we expect to have fully occupied (–) sites by two molecules of $5'-NH_3^+-d(Tmt)_4-T-OH$, there can be slides of the strands (Scheme 2) (2). Species other than full associations are neglected (all-or-none model) in the kinetic and thermodynamic studies. Whereas studies of DNG-DNA triplexes showed an increase in k_{on} values with an increase in DNA length (5 through 7) (2), increase in the length of triplex from

Table 2. Activation parameters for the triplexes of $d(pA)_x$ with DNmt

μ	$d(pA)_x, x =$	$E_{on}, kcal/mol$	$E_{off}, kcal/mol$	$\Delta H, kcal/mol^{\dagger}$	$T_m, ^\circ C^{\ddagger}$
0.03	10	-26.87	10.37	-37.24	36.03
0.03	15	-24.37	10.85	-35.22	52.74
0.03	20	-18.92	18.73	-37.65	64.22
0.03	23	-21.7	16.65	-38.35	60.31
0.08	10	-37.73	3.129	-40.859	34.7
0.08	15	-38.01	11.52	-49.53	45.16
0.08	20	-33.67	17.087	-50.757	49.6
0.08	23	-31.34	11.68	-43.02	49.7

Margin of errors: $k_{on} \cdot M_{tot} = \pm 0.25 \times 10^{-4} s^{-1}$; $k_{off} \pm 0.25 \times 10^{-6} s^{-1}$; E_{on} and $E_{off} = \pm 0.5$ kcal/mol.

[†]From $E_{on} - E_{off}^{\ddagger}$; [‡] ± 0.2 kcal/mol; [‡] $\pm 1^\circ C$.

DNmt₂:d(pA)₂₀ to DNmt₂:d(pA)₂₃ shows a decrease in k_{on} and increase in k_{off} values (Table 1), illustrating, a little more selectivity in the binding of DNmt to its target. In contrast to DNG-DNA triplexes, DNmt complexes with oligomers d(pA)₅₋₈ did not show appreciable hypochromicity on binding. This shows that DNmt binds less strongly than DNG, as previously reported.

As expected, by decreasing the ionic strength k_{on} increases, while k_{off} decreases (Table 1). We find that the sensitivity of k_{on} and k_{off} to μ is similar to that observed for DNG-DNA triplexes (2). On the other hand, k_{off} values for DNA-DNA triplexes are largely independent of ionic strength (7).

The same trend has been found for the energies of activation, E_{on} and E_{off} . For d(pA)₁₀, with decrease in ionic strength, E_{on} decreases from -37.73 to -26.87 kcal/mol, whereas E_{off} increases from 3.12 to 10.37 kcal/mol (Table 2, Fig. 8). A more negative E_{on} at high ionic strength and a less positive value of E_{off} favors a better association at low μ values. The negative values for E_{on} (activation energy for k_{on}) are obtained since the rate of triple helical formation (k_{on}) decreases with temperature leading to a positive slope ($-E_{on}/R$) of $\ln(k_{on})$ vs. $1/T$ (Fig. 6). This is similar to the negative activation energies obtained for association of double and triple helical DNA complexes (2, 7). However, an elementary kinetic step cannot have an activation energy less than zero. Therefore, k_{on} (and k_{off}) must represent composites of rate constants for individual steps. As proposed for DNA-DNA complexes, the negative activation energies rule out the formation of the first base pair as rate limiting (7, 11). The development of the nucleation-zipping model, as applied previously to triple-helical DNAs, can be used to explain this large negative value of E_{on} (7, 11). The helix formation begins with two or three bases pairing and unpairing in rapid but unfavorable equilibrium. On formation of the critical intermediate, a helix nucleus is formed, which zips up to form the fully bonded helix more rapidly than it dissociates to single strands. The equilibrium constant $K = k_{on}/k_{off} = \beta s^n$, where β is the equilibrium constant for nucleation of the triplex (formation of the first base triplet). The chain growth parameter $s = k_f/k_b$, where k_f and k_b are the first order rate constants for the formation and breakage of the base triplet at the end of a triplex segment, and n is the number of base triplets being formed. If ν is the number of base triplets in the nucleus, which is in rapid equilibrium with the separated duplex + third strand, the activation energy E_{on} equals the sum of one activation energy, E_{kf} , and $\nu + 1$ reaction enthalpies for base triplet reactions, $\Delta H_\beta + \nu \Delta H_s$:

$$E_{on} = E_{kf} + \Delta H_\beta + \nu \Delta H_s \quad [5]$$

The first term is small and positive but the enthalpies are negative, such that E_{on} becomes negative with its magnitude increasing with ν , and E_{off} ($E_{off} = E_{kb} - (n - \nu) \Delta H_s$) is largely positive. As a first approximation ($E_{kf} + \Delta H_\beta \approx 0$), if the temperature dependence of kinetic parameters rests on the growing chain factor s , we obtain Eq. 6 (from $\Delta H^\circ = E_{on} - E_{off}$).

$$\nu = nE_{on}/\Delta H^\circ \quad [6]$$

By using Table 2 and $n = 5$, the following values of ν are obtained:

d(pA) _x	d(pA) ₁₀	d(pA) ₁₅	d(pA) ₂₀	d(pA) ₂₃
ν ($\mu = 0.03$)	3.6	3.4	2.5	2.8
ν ($\mu = 0.08$)	4.6	3.8	3.3	3.6

ν must be an integer value, so it increases from 2 to 4 bases [almost to 5 in d(pA)₁₀]. In other words, more than 50% of the DNmt₂ strand is required for nucleation with these homooligomers, and yet we are able to form stable triplexes that exceed the association rates of DNA triplexes of 22 bp ($\nu = 2-4$) and longer. The drop in ν from d(pA)₂₀ to d(pA)₂₃ is anticipated because of the incomplete association of DNmt in the domino fashion, as previously explained. The lower value of ν at lower ionic strength again reinforces the role of ionic attractions of oppositely charged

backbones in the formation and stability of the helices. We notice from Table 2 that with an increase in ionic strength, E_{on} values become more negative [for d(pA)₂₀, E_{on} goes from -18.9 kcal/mol ($\mu = 0.03$) to -33.6 kcal/mol ($\mu = 0.08$)]. This is in stark contrast to previous studies with DNG-DNA triplexes, where E_{on} was found to increase with decreased ionic strength (2). Whereas ΔH_s /base pair (Eq. 5) would tend to decrease with an increase in ionic strength for DNG as well as DNmt, the value of ν increases with increased ionic strength. It is the balance between ΔH_s and ν that determines the overall value of E_{on} and further the value of ΔH° . In the case of DNmt₂-DNA triplexes, the increase in ν compensates for the decrease in ΔH_s . In other words, the number of bases required for nucleation in DNmt₂-DNA triplexes increases with increase in μ and decreases with increase in the size of d(pA)_x. On the other hand, for DNG₅ binding to d(pA)₁₀, the value of ν (2.8, calculated from ref. 2) remains virtually unchanged in going from an ionic strength of 0.03 to 0.22. The decrease of ν with length of d(pA)_x studied also explains why the E_{on} values become less negative as we go from d(pA)₁₀ to d(pA)₂₀, since the extent of ΔH_s /base pair would be expected to remain constant with the change in length of d(pA)_x. The difference in DNG and DNmt binding to DNA can be ascribed to the relative independence of ν to μ and d(pA)_x length in case of DNG-DNA triplexes.

The thermodynamic parameters can be extracted from the kinetic data or experimentally determined by calorimetric methods (2, 8, 9). In most cases, these data obtained by different methods are in good agreement. Eq. 7 shows the relationship between the thermodynamic parameters ΔH° and ΔS° and the equilibrium constant K_{eq} at a given temperature.

$$\Delta H^\circ - T\Delta S^\circ = -RT \ln K_{eq}(T) \quad [7]$$

At the melting temperature, T_m , $K_{eq} = 2/M_{tot}$ and at 288 K, K_{eq} is given by $k_{on}^{ref}/k_{off}^{ref}$. From Eq. 7 one can calculate the standard molar entropies for the triplex formation and the free energies of formation, ΔG° (Table 2, Fig. 8).

The standard molar enthalpies have larger negative values at higher ionic strength than at low ionic strength in accord with the observation that at lower μ values the value of ν of 5'-NH₃⁺-d(Tmt)₄-T-OH binding with d(pA)_x is lower. The standard molar enthalpies $\Delta H^\circ(288) = E_{on} - E_{off}$ are between -35 and -50 kcal/(mol base) for short strands (10-23 base pairs, Table 2) as compared with -120 kcal/mol for a 22 base pair DNA triplex (7), and -40 to -60 kcal/(mol base) for DNG₅-DNA triplexes (2). The thermodynamic parameters for the RNA/DNA hybrid duplexes have been studied and pentameric hybrid duplexes with different mixed base sequences have been shown to have ΔH° of ca. -45 kcal/(mol strand) (12, 13). These large and negative values of $\Delta H^\circ(288)$ indicate a better stabilization of the (5'-NH₃⁺-d(Tmt)₄-T-OH)₂:(DNA) complex as compared with the DNA triplexes with at least one order of magnitude on the enthalpy scale even with the entropy compensation. With an increase in temperature, the entropic contribution, ΔS° , of formation of the triplexes gets more negative as the triplex begins to dissociate, leading to an increase in entropy (Table 3). Triplex formation of 5'-NH₃⁺-d(Tmt)₄-T-OH with d(pA)_x is associated with values of $\Delta G^\circ(288)$ ranging between -8 and -11 kcal/mol with large negative values at low ionic strengths (Table 3). The free energies of triplex formation have also been calculated at 25°C and 37°C and show a gradual decrease as the temperature is raised (Table 3, Fig. 9). (For 5'-NH₃⁺-d(Tmt)₄-T-OH₂-d(pA)₂₀ triplex, ΔG° decreases from -10.2 kcal/mol (288 K) to -8.9 kcal/mol (298 K) and further to -7.5 kcal/mol (310 K). This denotes the decreasing stabilization of triplex with increase in temperature, which is expected.

CONCLUSIONS

Replacement of the phosphodiester linkages of DNA with methylthiourea linkages provides a polycation deoxyribonucleic meth-

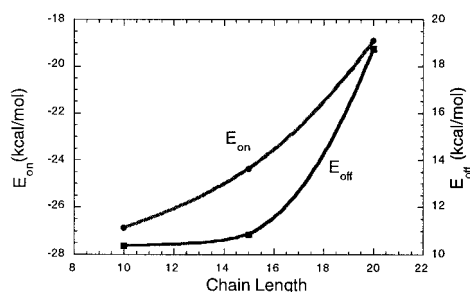


FIG. 8. Dependence of E_{on} and E_{off} on chain length for denaturation/renaturation of the triplex formed from $d(pA)_x$ (4.0×10^{-5} M/base) with $5'-NH_3^+-d(Tmt)_4-T-OH$ at $dT/dt = 0.13$ deg/min.

ylthiourea (DNmt). The polycation $5'-NH_3^+-d(Tmt)_4-T-OH$ binds to homooligomers $d(pA)_x$ with high affinity and with base pair specificity to provide triple-stranded helices. The electrostatic attraction between polycation $5'-NH_3^+-d(Tmt)_4-T-OH$ and polyanion $d(pA)_x$ stabilizes the triple helical hybrid structures. At a concentration of 4.0×10^{-5} M/(base A) and at a stoichiometry of 2:1 T/A, one observes triplexes with $d(pA)_{15}$ and $d(pA)_{20}$ possessing T_m values of 52.74°C and 64.22°C, respectively.

The triplex forms and dissociates to a duplex and a third monomer strand with rate constants k_{on} and k_{off} . At 15°C and in the range of ionic strengths of 0.03–0.12, the second order rate constant (k_{on}) for the triplex formation with short-strand oligonucleotides [$d(pA)_x$, where $x = 10, 15, 20$, and 23] is $17\text{--}22 \text{ M}^{-1} \text{ s}^{-1}$ (the molarity is expressed in per bases) and the first order rate constant (k_{off}) is $0.14\text{--}2 \times 10^{-5} \text{ s}^{-1}$. The value of k_{on} for reaction of $5'-NH_3^+-d(Tmt)_4-T-OH$ with $d(pA)_x$ increases with increase in length of DNA strand while the rate constant k_{off} decreases. Similar trends have been found for the activation parameters, E_{on} and E_{off} . For $d(pA)_{10\text{--}23}$, there is a decrease in E_{on} from -38.0 to -18.9 kcal/mol with decrease in ionic strength, whereas E_{off}

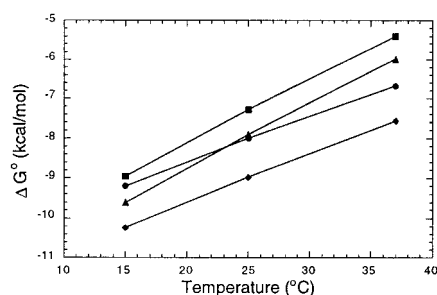


FIG. 9. Dependence of ΔG° on temperature for the triplex ($5'-NH_3^+-d(Tmt)_4-T-OH$) $_2d(pA)_{20}$ at $\mu = 0.03$ (◆), $\mu = 0.08$ (●) and triplex ($5'-NH_3^+-d(Tmt)_4-T-OH$) $_2d(pA)_{15}$ at $\mu = 0.03$ (▲), $\mu = 0.08$ (■).

increases from 3.1 to 18.7 kcal/mol. A less negative E_{on} at low ionic strength and a more positive value of E_{off} favors association, since ν decreases with increase in μ . The difference in DNG and DNmt binding to DNA is ascribed to the dependence of ν (number of base triplets in the nucleus of the forming helix) to μ (ionic strength) and length of oligomer $d(pA)_x$ in case of DNmt-DNA triplexes. The standard molar enthalpies, ΔH° (288) = $E_{on} - E_{off}$ are between -30 and -50 kcal/(mol base) and the standard free energies ΔG° (288) are between -8 and -11 kcal/(mol base) for short strands (10–23 base pairs). In comparison, the standard free energies ΔG° (288) reported for DNG binding to short-strand DNA (5–15 base pairs) is between -9 and -13 kcal/mol. The entropies ΔS° and free energies ΔG° of triplex formation show a gradual decrease as the temperature is raised. The ΔH° (288) and ΔG° (288) values indicate a better stabilization of the [$5'-NH_3^+-d(Tmt)_4-T-OH$] $_2d(pA)_x$ complex as compared with triplexes of DNA, but less than that of DNG triplexes to DNA. A better understanding of physical parameters responsible for the equilibration of these positively charged triple helices (DNmt $_2$ -DNA and DNG $_2$ -DNA) is thus obtained. DNmt, a positively charged analogue of DNG, is unique in its strong binding strength (k_{on} , k_{off} comparable to DNG $_2$ -DNA triple helix) yet resembling DNA $_2$ -DNA triple helices in their nucleation behavior. Further studies of DNmt $_2$ -DNA triple helices involving complementary mismatch sequences coupled with synthetic efforts to design longer DNmt sequences should help in the development of a novel class of putative antisense/antigene agents.

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Table 3. Thermodynamic parameters for the triplexes of $d(pA)_x$ with DNmt

$T, ^\circ\text{C}$	μ	$d(pA)_x, x =$	$\Delta S^\circ, \S \text{ cal/mol}\cdot\text{K}$	$\Delta G^\circ, \P \text{ kcal/mol}$		
15	0.03	10	-99.015	-8.7087		
		15	-90.312	-9.1967		
		20	-95.097	-10.248		
		23	-97.753	-10.182		
	0.08	10	-114.57	-7.8467		
		15	-140.8	-8.9577		
		15	-140.8	-8.9577		
		20	-142.8	-9.6102		
	0.08	23	-119.73	-8.5187		
		25	0.03	10	-104.01	-7.2703
				15	-94.414	-8.0145
				20	-99.496	-8.9803
23	-102.23			-8.8922		
0.08	10		-118.15	-6.8128		
	15		-146.6	-7.2874		
	20		-148.71	-7.9067		
	23		-122.92	-7.6005		
37	0.03		10	—	—	
			15	-98.995	-6.6946	
			20	-104.39	-7.5688	
			23	-107.23	-7.4509	
	0.08	10	—	—		
		15	-153.12	-5.4099		
		20	-155.31	-6.0033		
		23	-130.44	-5.4344		

$\S \pm 1 \text{ cal/mol}\cdot\text{K}$; $\P \pm 0.2 \text{ kcal/mol}$.

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