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Probing the energetics of oligo(dT)·poly(dA) by laser cross-linking

(DNA structure/DNA replication/processivity/pyrimidine dimers/thermodynamics)

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ABSTRACT **Experimentally determined changes in free energy ($\Delta G°$) for thymine–thymine interactions occurring in oligo(dT)·poly(dA) are dependent on the method used for preparation of the double-stranded template. A rapid laser cross-linking technique was used to examine the equilibrium between oligomers of (dT) bound to either poly(dA) or poly(rA). The single-pulse (4–6 nsec) ultraviolet laser excitation of these polynucleotides causes pyrimidine dimer formation between contiguous oligo(dT) molecules, resulting in a "ligation" of the oligomers. Analysis of the resulting data using standard binding isotherms allowed determination of the degree of cooperativity existing between oligomers. Using the cooperativity, $\Delta G°$, $\Delta H°$, and $\Delta S°$ are calculated, thereby providing thermodynamic parameters for this interaction. The measured cooperativity of oligo(dT) molecule interactions allows direct calculation of the number of 3′ ends available as nicked structures or the number of 3′ ends associated with gaps for oligo(dT)·poly(dA) when used as a substrate for DNA synthesis.**

An accurate determination of the change in free energy ($\Delta G°$) associated with stabilization of double-stranded DNAs is essential to a fundamental understanding of the structure of those DNAs. Stacking interactions between adjacent bases of nucleic acids have been a prominent consideration in the evaluation of the stability and conformation of double-stranded polynucleotides (1, 2). Crystallographic analysis of double-stranded oligonucleotides indicates that other factors such as bifurcated hydrogen bonds and a spine of hydration must be considered for proper evaluation of nucleic acid structure, especially in oligo(dT)·oligo(dA) stretches (3, 4). Various theoretical calculations have been performed to estimate the $\Delta G°$ for the thymine–thymine base stacking interaction in oligo(dT)·oligo(dA) stretches but, as a result of different assumptions used for the models, the approximations of the stacking energies made by various investigators differ significantly from each other (1, 5, 6). In addition, none of the models has incorporated the contribution of either the bifurcated hydrogen bonds or the spine of hydration. Relevant thermodynamic parameters have been derived from calorimetric and melting temperature studies (7, 8) and we are able to confirm the $\Delta G°$, $\Delta H°$, and $\Delta S°$ for thymine–thymine interactions through our studies of oligo(dT)·poly(dA).

Oligo(dT)·poly(dA) has been used extensively as a primer-template in studies of DNA polymerase mechanisms (9–12). The use of this nucleic acid is predicated on the concept that the oligo(dT) molecules will be distributed randomly along the poly(dA) lattice (13). Consequently, at low thymidine to adenosine ratios all 3′ termini are presumed to have gaps adjacent to them and are considered usable for DNA synthesis. This concept is based on the presumption that the favorable enthalpic parameters for thymine–thymine interactions are more than offset by entropic factors resulting in the random distribution of the thymidine oligomers. We provide definitive enthalpic and entropic parameters that can be used to determine the distribution of the oligo(dT) molecules and that allow calculation of the number of 3′ termini not directly adjoining another oligomer and hence potentially available for DNA synthesis.

MATERIALS AND METHODS

Reaction Conditions. All reaction mixtures, unless otherwise indicated, contained 60 mM potassium acetate, 25 mM Tris acetate (pH 7.4), 6 mM magnesium acetate, 6 mM 2-mercaptoethanol, 250 nM (nucleotide) 5′ ^{32}P-labeled oligodeoxyribothymidines [^{32}P-oligo(dT)] and various concentrations of polydeoxyribodenosine (dA) or polyriboadenosine (rA). The oligo(dT) was 5′ ^{32}P-end-labeled according to the method of Maxam and Gilbert (14). Since nucleotide concentrations in excess of 0.5 mM reduce the cross-linking efficiency (15), total nucleotide concentrations were maintained below this level. Samples (10 μl) were prepared as described in Table 1 and were irradiated with 3.2×10^{16} photons from a single 4- to 6-nsec pulse of 266-nm light (15, 16).

Identification of Ligated Products. Irradiated samples were denatured and electrophoresis was performed (17). Gels were dried, equilibrated at −80°C, and exposed to Kodak X-Omat XAR-5 film in the presence of a DuPont Cronex Lightning Plus intensifying screen for various lengths of time such that the bands corresponding to the separated products were within the linear range of our standard absorbance curve (16, 18).

Quantitation of Reaction Products. The absorbance of each band (ligated or unligated reaction products) was determined by densitometric scanning of the autoradiographs using a Dage VC68 video camera linked through an image capture board (Frame Grabber) to an AT&T 6300 microcomputer, previously calibrated with a series of neutral density filters. The relative number of ligations (fractional associations, f_a) for a given sample were calculated from the relationship:

$$f_a = \sum_{i=1}^{N} [(A_i/A)(i - 1/i)],$$

where i is the number of oligonucleotides in a given product (e.g., for T_{12}, $i = 1$; for T_{24}, $i = 2$), N is the number of oligonucleotides in the largest ligated species we could quantify, A is the summation of standardized absorbencies from all oligonucleotides for a given sample, and A_i is the standardized absorbance of the ith product. We thus express the amount of ligated species formed (associated oligomers) as a fraction of the total possible oligomer associations (f_a), disregarding end effects of the lattice. Since detection of the multimers would be a direct function of the cross-linking efficiency, with multimers that require multiple cross-links for detection being rarer than the dimers that only require a

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single cross-link (Fig. 1), this analysis does not depend on the relative number of monomers, dimers, trimers, etc., of the oligo(dT) that are formed. Instead, we assume that the efficiency of pyrimidine dimer formation does not change as a function of the number of contiguous oligo(dT) molecules and, consequently, the same fraction of the total number of associations will be measured regardless of whether the cluster is a dimer, trimer, or longer.

Analysis of Oligomer Association. The b_nb_1 and b_nf forms (for $\omega > 1$) of binding equation 13 from McGhee and von Hippel's model for ligand binding to a one-dimensional homogeneous lattice (19) were used for fitting data and calculation of the number of available 3' ends, respectively:

$$(b_nb_1) = \frac{1 - (n - 2\omega + 1)v - R}{2v(\omega - 1)}, \quad (b_nf) = \frac{(n - 1)v - 1 + R}{2v(\omega - 1)}$$

where

$$R = \sqrt{[1 - (n + 1)v]^2 + 4\omega v(1 - nv)}.$$

The parameter b_nb_1 is defined as the probability that the nth residue of a ligand (free 3'-hydroxyl) lies on the immediate left of the first residue (free 5'-phosphate) of a second bound ligand. The parameter b_nf is the probability that the nth residue of a bound ligand lies to the left of a free lattice residue. The site size (n) or number of lattice residues covered corresponds to the number of thymidine residues in the oligonucleotide. The binding density (v) is defined as the mol of bound ligand [oligo(dT) molecules] per mol of total lattice residues (dA nucleotides). Therefore at a 1:1 nucleotide ratio of dT·dA, $v = 1/n$. The cooperativity parameter (ω) is defined as the equilibrium constant for the process of moving a bound ligand from an isolated site to a singly contiguous site or from a singly contiguous site to a doubly contiguous site. Fractional associations (f_a) at various binding densities (v) were used to fit f_a (at $v = 1/n$) and ω using the nonlinear function minimization program NONLIN (20). Thus, the parameter b_nb_1, at a given v_i, is the f_a (at v_i) divided by the f_a at $v = 1/n$. Each binding isotherm was composed of 15–54 data points.

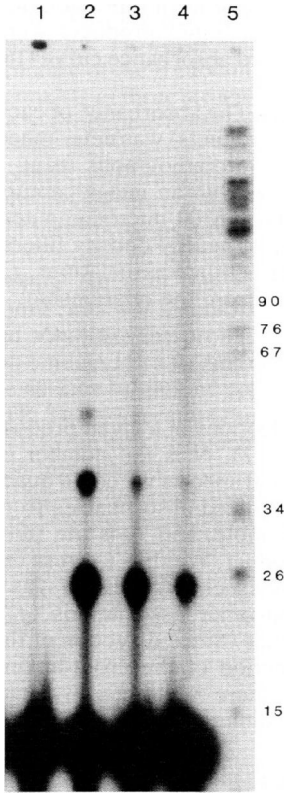

FIG. 1. Polyacrylamide gel (10%) electrophoresis showing ligation of $(dT)_{12}$ by rapid-pulse ultraviolet irradiation. Poly-$(dA)_{4000-6000}$ was added in the following amounts: lane 1, none; lane 2, 2.5 μM (in nucleotide); lane 3, 25 μM; and lane 4, 250 μM. Lane 5, 5' ^{32}P-*Msp* I-digested pBR322 DNA.

RESULTS

A rapid, ultraviolet laser cross-linking method (16) was used to induce cyclobutane pyrimidine dimer formation between adjacent oligo(dT) molecules on a poly(dA) lattice, with the "ligation" of the oligonucleotides presumably occurring between the first and last thymidines of adjacent oligo(dT) molecules (21) (Fig. 1). Initial studies with $(dT)_{12}$·$(dA)_{5000}$ yielded cross-linked multimers of $(dT)_{12}$ corresponding to $(dT)_{24}$, $(dT)_{36}$, $(dT)_{48}$, etc. The cross-linking process has a quantum yield of 2.6×10^{-2} for the induction of the pyrimidine dimer between adjacent oligo(dT) macromolecules, which is consistent with the previously reported quantum yield for pyrimidine dimers formed in poly(dT) by either continuous low-intensity ultraviolet irradiation (low-pressure mercury lamp, 254 nm) or nanosecond laser irradiation (laser, 266 nm) (22). After quantitation, the data were fit using the b_nb_1 form of binding equation 13 of McGhee and von Hippel (19) and plotted (Fig. 2). The initial data resulted in a cooperativity parameter (ω) of 4700. Using the relationship, $\Delta G° = -RT\ln K_{eq} = -RT\ln\omega$, we find a $\Delta G°$ of −4.6 kcal/mol (1 cal = 4.184 J) for the end-to-end interactions of $(dT)_{12}$ on $(dA)_{5000}$ (Table 1). These data indicate that there is significant clustering of the oligo(dT) molecules on the poly(dA). The equilibrium binding curve for $(dT)_{20}$ is essentially identical to the $(dT)_{12}$ curve, yielding an identical free energy for the oligo(dT)–oligo(dT) interactions and indicating that the cooperativity and cross-linking are unrelated to any properties, such as length, peculiar to the shorter oligomer (Table 1).

The McGhee–von Hippel model used for fitting our data is based on a ligand binding to an infinitely long lattice (19). Table 1 shows data for lattice lengths that vary over 25-fold in length, thereby demonstrating that the ligand–ligand interaction of the oligo(dT) molecules is independent of length for lattices of poly(dA) in excess of 200 nucleotides in length. No ligand–ligand interactions could be detected in experiments with oligo(dT) in the absence of a complementary lattice (Fig. 1).

Concluding that such experiments are feasible over a 25-fold change in lattice length, additional experiments using $(dT)_{12}$·$(rA)_{500}$ yielded an ω of 160 and a $\Delta G°$ value of −2.8 kcal/mol (Fig. 2 and Table 1). This value is very similar to the base stacking free energy predicted by theoretical models (1) and indicates a much lower level of clustering of the oligo(dT) on poly(rA) than occurs on poly(dA). The $\Delta G°$ values were confirmed with $(dT)_{20}$·$(rA)_{500}$ (Table 1).

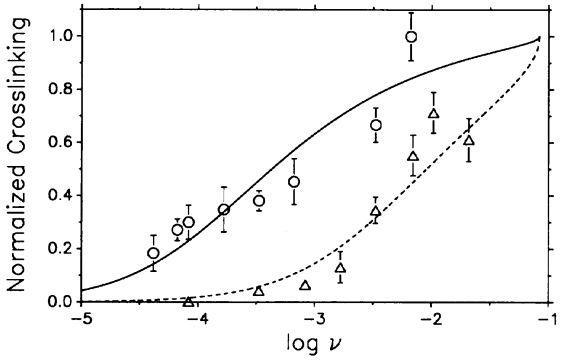

FIG. 2. Laser ligation of oligo(dT) at various poly(dA) (○) or poly(rA) (△) concentrations. The oligo(dT) was held constant at 250 nM (nucleotide). The data points shown are the mean values derived from triplicate data points. All data (not the means) were fit to a model for binding of ligands to one-dimensional homogeneous lattices (19) using a simple non-linear least-squares fitting routine.

Table 1. Free energy of interaction

Nucleic acid	Temp., K	Method*	Conc., nM dT	$\Delta G°$,† kcal/mol
$(dT)_{12}$·$(dA)_{5000}$	273	A	250	−4.6 (−4.4, −4.8)
$(dT)_{20}$·$(dA)_{5000}$	273	A	250	−4.6 (−4.3, −4.8)
$(dT)_{12}$·$(dA)_{5000}$	277	B	250	−5.1 (−4.1, −5.6)
$(dT)_{12}$·$(dA)_{1000}$	277	B	250	−4.7 (−3.3, −5.1)
$(dT)_{12}$·$(dA)_{200}$	277	B	250	−5.4 (−4.6, −6.2)
$(dT)_{12}$·$(dA)_{5000}$	273	C	250	−4.6 (−4.2, −4.9)
$(dT)_{12}$·$(dA)_{5000}$	273	A	25	−4.0 (−3.8, −4.2)
$(dT)_{20}$·$(dA)_{5000}$	273	A	25	−3.9 (−3.8, −4.0)
$(dT)_{12}$·$(rA)_{500}$	273	A	250	−2.8 (−2.7, −3.0)
$(dT)_{20}$·$(rA)_{500}$	273	A	250	−2.7 (−2.6, −2.9)
$(dT)_{12}$·$(dA)_{5000}$	273	D	250	−1.9 (−1.8, −2.0)
$(dT)_{12}$·$(rA)_{500}$	273	D	250	−1.9 (−1.7, −2.0)

*Methods of preparation: A, samples were mixed, incubated 30 min, and irradiated at the indicated temperature; B, samples were mixed at 293 K, incubated ≈18 hr at 277 K, and irradiated at 277 K; C, samples were mixed at 273 K, heated to 348 K, placed at 294 K for 15 min, incubated at 273 K for 30 min, and irradiated at 273 K; D, samples were mixed at 273 K, heated to 348 K, cooled at a controlled rate of 0.5 degree/min to the indicated temperature, and held there for 30 min prior to irradiation at that temperature.

†Values in parentheses are the lower and upper confidence limits derived from the fit of the data.

Much of the physical data available for oligo(dT)·poly(dA) complexes has been derived from spectroscopic studies (23). Such studies typically require nucleotide concentrations that are ≈1000-fold higher than those used in typical assays of polymerization activity. Furthermore, these spectroscopic studies have confirmed that the melting temperature of a duplex is dependent on the oligonucleotide concentration. Since polymerization and processivity assays are frequently performed at concentrations 10-fold lower than our experiments, we chose to repeat the experiments at a total thymidine concentration of 25 nM. Working at this concentration, we were successful in detecting dimer formation at a dT:dA ratio of 1:20,000, which means that for every dimer of $(dT)_{12}$ bound there must be at least 95 single-stranded $(dA)_{5000}$ molecules without an annealed $(dT)_{12}$. The results shown in Table 1 reveal that the $\Delta G°$ remains large even at significant dilutions. Hence, the laser cross-linking provides relevant physical constants under the constraints normally used to measure enzymatic synthesis of polynucleotides.

We have performed mixing experiments (23, 24) that demonstrate that none of the nucleic acid complexes is in a triple-stranded form but that all are duplexes (data not shown). We have also attempted to perturb the equilibrium by long incubations or by heating. In particular, Table 1 shows that up to 18 hr of incubation at 4°C does not significantly alter the cooperative interaction nor does melting followed by relatively quick cooling (preparation method C). However, we have successfully reached a new equilibrium by using a controlled slow cooling step. The samples were heat denatured and subsequently cooled in a water bath at a controlled rate of 0.5 degree/min. This slow cooling results in a lower level of cooperativity between the oligo(dT) molecules and yields a $\Delta G°$ that is identical for the poly(dA) and poly(rA) lattices at 273 K.

We have repeated our titrations at various temperatures in order to develop appropriate empirically derived thermodynamic parameters. A van't Hoff plot (Fig. 3) demonstrates a number of interesting observations. (*i*) As shown for the poly(dA) lattice, the method of mixing is important with respect to the final equilibrium and the degree of cooperativity. (*ii*) Table 1 shows that the poly(dA) and poly(rA) lattices can have identical free energies of interaction for the oligo(dT) molecules, but Fig. 3 reveals that the identity only takes place at a very discrete temperature (273 K). (*iii*) The linear relationship for the mixing and incubation of the $(dT)_{12}$ with the $(dA)_{5000}$ at temperatures ranging from 273 K to 310 K demonstrates that significant cooperativity continues to persist even as the midpoint of the thermal denaturation is approached (23). (*iv*) oligo(dT)–oligo(dT) interactions on the poly(rA) lattice become virtually noncooperative at 284 K. A summary of the thermodynamic parameters is shown in Table 2.

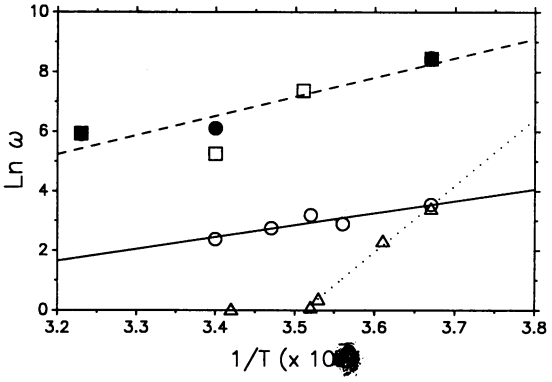

FIG. 3. van't Hoff plot. For each datum point shown, $(dT)_{12}$·$(dA)_{5000}$ (□) or $(dT)_{20}$·$(dA)_{5000}$ (●) was prepared by method A (Table 1), and a binding isotherm consisting of 10 thymidine:adenosine ratios (triplicates) was produced and fit for ω. The dashed line is the best fit by linear regression. In a similar fashion, $(dT)_{12}$·$(dA)_{5000}$ (○) and $(dT)_{12}$·$(rA)_{500}$ (△) were prepared by method D, resulting in the solid and dotted regression lines, respectively. The 293 K datum point was not included in the fit of the poly(rA) data since the oligo(dT) interaction has reached a noncooperative state by 284 K.

Since we could measure the oligomer–oligomer interaction, we were also interested to determine whether poly(dA) could compete for the binding of oligo(dT) molecules previously bound. Fig. 4 shows a $(dA)_{5000}$ titration of oligo(dT) and also a similar titration of a 1:2 complex of $(dT)_{12}$·$(dA)_{5000}$. The two titrations demonstrate that $(dA)_{5000}$ can effectively compete for free $(dT)_{12}$ but cannot compete for $(dT)_{12}$ that is already bound. This observation confirms an earlier report that oligo(dT) molecules are not free to exchange between poly(dA) lattices (13).

DISCUSSION

Our interest in this problem was stimulated by our studies of protein–nucleic acid interactions (15, 16). We noticed that a single ≈5-nsec pulse of ultraviolet light produced a single radiolabeled cross-linked product for a protein·^{32}P-oligo(dT) mixture but produced two radiolabeled products for a protein·^{32}P-oligo(dT)·poly(dA) mixture. The second photoproduct varied in size when the length of the oligo(dT) was varied and appeared in the absence of protein when only the ^{32}P-oligo(dT)·poly(dA) was present, leading us to conclude that the photoproduct was a multimer of the oligo(dT) molecule (Fig. 1). We have used our previously reported rapid laser cross-linking technique to "freeze" the existing equilibrium (15, 16) in this oligo(dT)·poly(dA) system, thereby allowing us to establish thermodynamic parameters for the system. Our reported thermodynamic parameters were ob-

Table 2. Thermodynamic parameters of oligo(dT)–oligo(dT) interactions

Nucleic acid	$\Delta H°$, kcal/mol	$\Delta S°$, cal/mol·degree	Preparation method*
$(dT)_{12}$·$(dA)_{5000}$	−12.8	−30	A
$(dT)_{12}$·$(dA)_{5000}$	−8.0	−22	D
$(dT)_{12}$·$(rA)_{500}$	−44.4	−156	D

*See footnote * in Table 1.

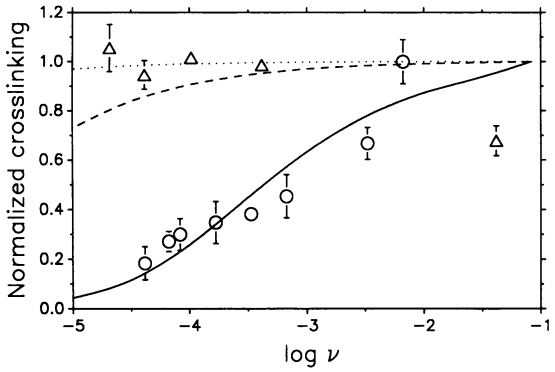

FIG. 4. Lattice challenge with poly(dA)$_{5000}$. Serial dilutions of poly(dA)$_{5000}$ were either mixed with (dT)$_{12}$ to yield a final concentration of 250 nM thymidine (○) or added in equal volumes to 250 nM (dT)$_{12}$ previously mixed and incubated at 273 K for 30 min in a 1:2 complex with (dA)$_{5000}$ (△). After addition of the challenging lattice, the samples were incubated for 30 min at 273 K prior to cross-linking. Lines represent ω values of 10^8 (dotted), 10^6 (dashed), and 4650 (solid).

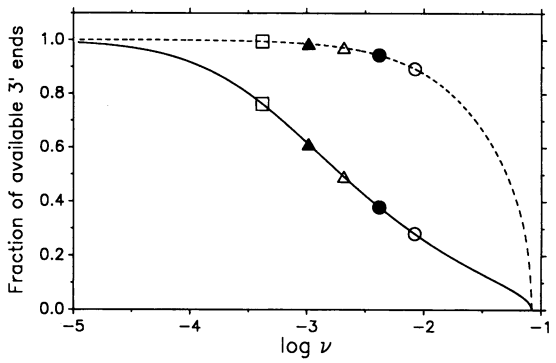

FIG. 5. Available 3′ ends for (dT)$_{12}$·(dA)$_{5000}$ at 20°C. The b_{nf} form of equation 13 from McGhee and von Hippel (19) was used to calculate the number of 3′ ends that are not energetically involved with the 5′ end of another oligomer. Data were calculated for each of the two methods of mixing described in Table 1: A (solid line); and D (dashed line). The symbols denote specific thymidine:adenosine ratios of 1:10 (○), 1:20 (●), 1:40 (△), 1:80 (▲), and 1:200 (□).

tained under conditions of pH and salt concentration that are typical of protein–nucleic acid enzymological studies.

We interpret our data to indicate that oligo(dT)·poly(dA) can exist in at least two states depending on the method of preparation. When mixed at temperatures below the thermal transitions for melting, the oligomers bind to the poly(dA) adjacent to each other in a cooperative fashion, yielding a $\Delta G^\circ = -3.9$ kcal/mol (25°C). Such clustering of the oligo(dT) molecules is consistent with previous reports arising from observations by electron microscopy (25). However, annealing of the oligo(dT) to the poly(dA) by total denaturation with subsequent slow cooling yields a much lower level of clustering and a correspondingly lower ΔG° of -1.4 kcal/mol (25°C), which is consistent with the approximated free energy resulting from a single base stacking interaction of thymine on thymine (1, 7). We conclude that the method of preparation of the oligo(dT)·poly(dA) has a significant effect on the clustering of the oligonucleotides on the polymer. The clustering has specific relevance for studies of DNA polymerase mechanisms and especially measurements of polymerase processivity. Our results indicate that traditional methods of preparation of oligo(dT)·poly(dA) templates (9) lead to a clustering of oligo(dT) molecules. The high frequency of end-to-end interactions for thymidine oligomers bound to poly(dA) requires a critical look at the use of this primer–template DNA for studies of DNA polymerase mechanisms. All 3′-hydroxyl ends of the oligo(dT) will only be accessible to the DNA polymerase if the polymerase has a binding free energy that is greater than the free energy of the thymine–thymine interaction, thereby allowing the oligomer ends to be "shoved" apart. This explains why some DNA polymerases cannot efficiently use an oligo(dT)·poly(dA) template (10, 26), even though they may use an oligo(dT)-tailed poly(dA) template, and may also explain why putative polymerase stimulatory factors, such as proliferating cell nuclear antigen (PCNA), have their greatest effect when the DNA polymerase is using an oligo(dT)·poly(dA) template (10, 11). In addition, it should be noted that not all 3′ ends will be equally accessible to enzymes that do not bind at nicks, and hence the number of available 3′ termini may not be the same as the number of 3′ termini added to the reaction mix—a point critical to estimates of processivity based on polymerase kinetics. In Fig. 5 we show the results of our calculations of the number of 3′ ends not directly adjoining another oligomer in each of the two states that we have characterized.

Poly(dA) and poly(rA) have been shown to have ordered structures that can be removed by melting. The midpoint of the melting curve is ≈55°C (23, 27, 28). We suggest that at temperatures below this level, the first oligo(dT) molecule must melt the existing poly(dA) structure for proper hybridization to take place. After hybridization of the first oligo(dT) molecule, additional oligo(dT) molecules hybridize directly adjacent to the first one because the free energy of the oligo(dT)–oligo(dT) interaction is favorable and therefore acts to offset the cost of melting the ordered poly(dA) structure. Alternatively, the oligo(dT) molecule that hybridizes to the poly(dA) may initially result in removal of any proximal structure in the polymer through nearest-neighbor effects (29) or alterations in the local solvent structure. Once bound, the oligo(dT) molecules will not be "free" to spread randomly on the poly(dA) because the loss of free energy from the cooperative interactions coupled with the additional free energy required to melt the ordered structure of the poly(dA) will be energetically prohibitive. In Table 1 we demonstrate that much of the clustering of the oligo(dT) molecules can be overcome by a total thermal denaturation followed by slow cooling. Thermal denaturation removes the secondary structure of the poly(dA) and at the same time diminishes the cooperativity of the oligo(dT) interaction because the $T\Delta S$ term becomes unfavorable. The result is that the oligo(dT) molecules bind in a more dispersed mode as a direct consequence of the entropic effects and concomitant formation of duplex structure and secondary structure in the poly(dA). Our data suggest that this model may also be appropriate for oligo(dT)·poly(rA).

One interpretation for the differences between binding of the oligo(dT) to poly(dA) and poly(rA) is to attribute the additional favorable free energy found for thymine–thymine interactions in oligo(dT)·poly(dA) to the additional bifurcated hydrogen bond that may occur (3, 4) and the three additional water molecules that may be added to the spine of hydration (30) when two oligo(dT) molecules are abutted one to another. Other interpretations are possible; perhaps the additional free energy results from the organization of an entire A-T base pair, which might include the effects noted above and also reorganization of the poly(dA) strand. Regardless of the interpretation, these data provide a clear thermodynamic basis for the observed rigidity (3, 4, 31) and the increased base-pair lifetimes (32) observed in oligo(dT)·oligo(dA) tracts. Attempts have been made to use theoretical base stacking energies to predict the conformation of a DNA oligomer with the conclusion that either the calculated base-stacking energies are in error or that other factors play a role (33). We have provided data that lend credence to those

conclusions and that provide fundamental thermodynamic values for future predictions of nucleic acid conformations.

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