Temperature Dependence of the Energetics of Oligonucleotide-Directed Triple-Helix Formation at a Single DNA Site

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Abstract: The influence of temperature on the energetics of oligonucleotide-directed triple-helix formation has been investigated in mixed valence salt solutions at pH 7.0. Equilibrium constants for formation of the local pyrimidine·purine·pyrimidine structure afforded by binding of the oligonucleotide 5′-d(T*TTTTCTCTCTCTCT)-3′ to a single 15-bp site within a 339-bp plasmid fragment were measured using quantitative affinity cleavage titrations between 8 and 37 °C. In three different solutions buffered by 10 mM Bis-Tris at pH 7.0, BTNS [100 mM NaCl and 1 mM spermine tetrahydrochloride ($SpmCl_4$)], BTP1 (10 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, and 1 mM $SpmCl_4$), and BTP4 (10 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, and 4 mM $SpmCl_4$), the equilibrium association constants decreased at least 100-fold (from $>10^7$ M^{-1} to ca. 10^5 M^{-1}) as the temperature was increased from 8 to 37 °C. Least squares analysis of van't Hoff plots (ln *K versus* 1/*T*) of the data revealed that in each solution the triplex is enthalpically stabilized by ca. 2 kcal per mol of base triplets. This average value for single-site triplex formation on large DNA at pH 7.0 in mixed valence salt solutions, which reflects contributions from both T·AT and C+GC base triplets, is consistent with those reported previously for a number of oligonucleotide triplexes in solutions containing single cationic species and in excellent agreement with the calorimetrically determined enthalpy for binding of this 15mer to a 21-bp oligonucleotide DNA duplex.

The local triple-helical complexes formed upon binding of pyrimidine oligonucleotides parallel to purine-rich strands in the major groove of double-helical DNAs or RNAs offer a versatile structural motif for designing sequence-specific duplex DNA- and RNA-binding molecules.[1-4] The sequence specificity of complex formation is imparted by Hoogsteen-type hydrogen bonds between T in the third strand and AT base pairs[5-8] and between *N*3-protonated C in the third strand and GC base pairs[6-10] to afford T·AT and C+GC base triplets, respectively (Figure 1). The high stabilities of the local triple-helical complexes,[11-13] the sensitivity of triplex stability to single base triplet mismatches,[1,13-17] and the broad range of potential DNA target sequences[18-26] provide the basis for the power of this approach.

A goal of our research efforts is the determination of the kinetic[11] and thermodynamic[12,13,27,28] parameters that characterize the formation and stabilities of local triple-helical complexes at single sites on relatively large double-helical DNA (>200 bp) near physiological solution conditions. Triple-helical nucleic acids are enthalpically stabilized, and the dissociation of a single-stranded nucleic acid from polymeric and oligomeric triple helices is therefore strongly promoted by increasing temperature.[29] Differential scanning calorimetric (DSC) measurements at pH 6.5 showed an oligonucleotide triple helix, containing both T·AT and C+GC base triplets and identical in sequence to the one described herein, to be enthalpically stabilized by an average of 2.0 kcal per mol of base triplets (kcal·(mol bt)$^{-1}$).[12]

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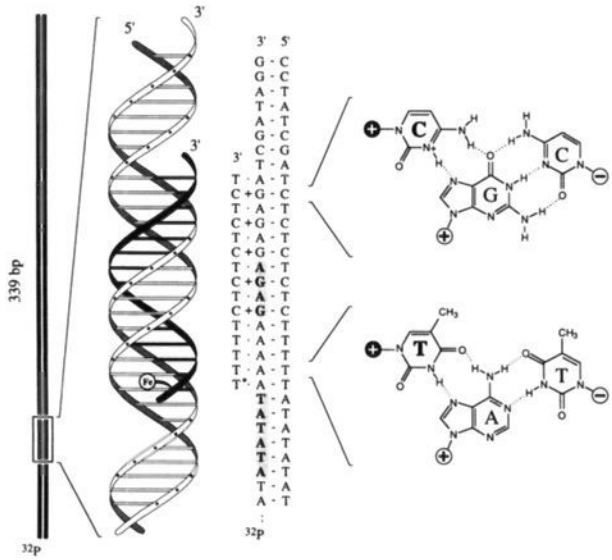

Figure 1. (Left) Ribbon model of the local triple-helical structure formed by the binding of a 15mer oligonucleotide to a 15-bp target sequence within a 339-bp end-labeled duplex. The Watson−Crick duplex strands are depicted as white ribbons while the oligonucleotide−EDTA·Fe is depicted as a dark ribbon. The sequences modeled by the ribbons are shown in the center. The sequence of the oligonucleotide-EDTA used in this study is 5′-d(T*TTTTCTCTCTCTCT)-3′. Gray boxes are drawn around the ten nucleotide positions of the duplex cleaved most efficiently by the oligonucleotide−EDTA·Fe conjugate and used to obtain I_{site} values. (Right) Two-dimensional models depicting the C+GC and T·AT base triplets formed by Hoogsteen-type hydrogen bonding of N3-protonated C to a Watson−Crick GC base pair (top) and by Hoogsteen hydrogen bonding of T to a Watson−Crick AT base pair (bottom), respectively. The bases of the third strand are labeled with bold type and the bases of the Watson−Crick duplex are labeled with normal type. The circles attached to N1 of the pyrimidines and N9 of the purines indicate the position of attachment to the sugar−phosphate backbone, while the plus and minus signs designate the relative 5′-to-3′ polarity of the strands.

In contrast, the corresponding Watson−Crick duplex was stabilized by 6.3 kcal per mol of base pairs. Subsequent measurements on triplexes of different sequence compositions using a number of techniques, including analyses of both the oligonucleotide concentration dependence of melting temperatures and the shapes of optical melting curves, under a variety of conditions indicated the triplex to be enthalpically stabilized by a broader range of values, 2.5 −8.1 kcal·(mol bt)$^{-1}$ (*vide infra*). Because these values are average enthalpies for T·AT, protonated C+GC, and unprotonated C·GC base triplets, different sequence compositions would yield different average values. Alternatively, reports of triple-helix formation enthalpies may vary as a result of differences in the models and assumptions used to relate temperature-dependent experimental observables to the equilibrium constant. Direct application of the van't Hoff equation to the temperature dependence of equilibrium association constants measured using quantitative affinity cleavage titrations would provide an independent estimate of the enthalpic stabilization of a local triple-helical complex identical in sequence to that studied previously.[12]

To assess the influence of temperature on the association free energy for an oligonucleotide binding a single site in large duplex DNA near intracellular solution conditions, we determined the temperature dependence of the equilibrium association constant for binding of the oligonucleotide−EDTA conjugate 5′-d(T*TTTTCTCTCTCT-CT)-3′ to a single 15-bp site within a 339-bp plasmid fragment (Figure 1) at pH 7.0. Specifically, we varied the temperature from 8 to 37 °C in three different Bis-Tris-buffered solutions characterized by differences in their concentrations of NaCl, KCl, $MgCl_2$, and spermine tetrahydrochloride ($SpmCl_4$) and measured the equilibrium association constant under each set of conditions. Van't Hoff analysis of the observed temperature dependence in each case allowed the extraction of enthalpy and entropy changes during oligonucleotide-directed triple-helix formation. The observed thermodynamic parameters for single-site triple-helix formation are compared to prior energetic analyses of oligomeric triplexes under simple solution conditions.

Results

Affinity Cleavage Titrations. The equilibrium association constant (K_T) for the binding of an oligonucleotide−EDTA·Fe to an individual DNA site to form a local triple-helical structure can be measured using quantitative affinity cleavage titration.[13] In general, the technique affords apparent association constants which are accurate to within a factor of 2−3, and free energies are accurate to ≤0.6 kcal·mol^{-1}.[30] Recent work has demonstrated the utility of affinity cleavage titration for measuring oligonucleotide-directed triple-helix formation constants as a function of cation concentrations[28] and in systems of cooperatively binding oligonucleotides.[31−33] Theoretical considerations and experimental details for this method have been reported previously.[13,30]

At a given temperature, ^{32}P-5′-end labeled DNA (<50 pM) and various concentrations of oligonucleotide−EDTA·Fe (1 nM−20 μM) were mixed in Bis-Tris-buffered salt solutions at pH 7.0. The time required to reach equilibrium depended on the temperature at which the association reactions occurred. As expected based on the heat of helix initiation,[16] lower temperatures yielded faster apparent association rate constants (k_{on}); however, this effect was mitigated by increased equilibrium association constants (*vide infra*) which required binding at lower oligonucleotide concentrations. Hence, the apparent triplex formation rates, which are controlled by $k_{on}[\mathbf{O}]_{tot}$, were only modestly influenced by temperature. After minimal equilibration times at each temperature were established,[13] equilibration times of 24 h at 8 and 22 °C and 48 h at 29 and 37 °C were chosen for experimental convenience.

After the association reactions reached equilibrium DTT was added (1 mM final concentration) to initiate the thymidine−EDTA·Fe-mediated cleavage chemistry. The reactions were allowed to proceed to a maximum site-specific cleavage yield of 10−15%. The time required for the cleavage reactions was also temperature dependent: cleavage times were 8, 6, 4, or 3 h at 8, 22, 29, and 37 °C, respectively. Cleavage was stopped by ethanol precipitation and subsequent denaturation of the DNA strands, and the products were separated from uncleaved strands using urea−PAGE. The signal intensities of the bands at the target cleavage site (Figure 1) and at a reference site[34] were measured from storage phosphor autoradiograms, and the site-specific cleavage (I_{site}) was determined for each $[\mathbf{O}]_{tot}$. The ($[\mathbf{O}]_{tot}$, I_{site}) data points were fitted using a nonlinear least-squares method and eq 3, with K_T and I_{sat} as adjustable parameters (see Experimental Section).[13,27,28]

Equilibrium Association Constants as a Function of Temperature. The sets of ($[\mathbf{O}]_{tot}$, I_{site}) data obtained for the

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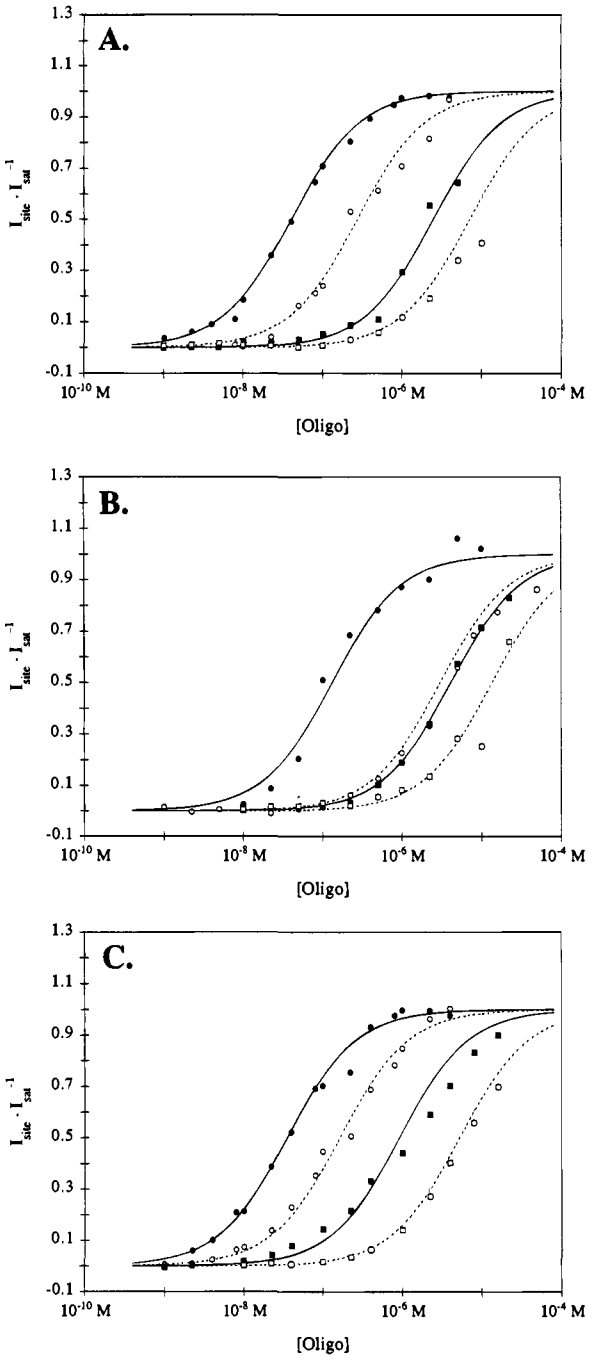

Figure 2. Data for quantitative affinity cleavage titrations performed in 10 mM Bis-Tris (pH 7.0) solutions at either 8 (●), 22 (○), 29 (■), or 37 °C (□). The data points represent the average site-specific cleavage signal intensities from three to five experiments. The sigmoidal curves show the titration binding isotherms plotted using the mean values of K_T (Table 1) and eq 3. The data points were normalized using I_{sat} from each experiment, and the isotherms were subsequently normalized using $I_{sat} = 1$ for eq 3. (A) The experimental binding titrations were performed in BTNS buffer at pH 7.0 (10 mM Bis-Tris, 100 mM NaCl, and 1 mM $SpmCl_4$). (B) The experimental binding titrations were performed in BTP1 buffer at pH 7.0 (10 mM Bis-Tris, 10 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, and 1 mM $SpmCl_4$). (C) The experimental binding titrations were performed in BTP4 buffer at pH 7.0 (10 mM Bis-Tris, 10 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, and 4 mM $SpmCl_4$).

15mer oligonucleotide at 8, 22, 29, and 37 °C in BTNS buffer at pH 7.0 (10 mM Bis-Tris, 100 mM NaCl, and 1.0 mM $SpmCl_4$) were averaged from three to five independent titrations and are plotted in Figure 2A. The mean K_T values obtained from analyses of these data (Table 1) were used to construct binding isotherms which are plotted along with the averaged data points in Figure 2A. The value of K_T measured at 22 °C is identical within experimental uncertainty to that reported previously for the same triple helix at the same pH, temperature, and salt concentrations in the presence of 50 mM Tris–acetate.[13] The room temperature value is 7.6-fold lower than that measured at 8 °C, and 24-fold higher than that at 37 °C. Overall, the measured K_T values demonstrate that raising the temperature from 8 to 37 °C causes a 180-fold decrease in the association constant. This trend of decreasing triple-helix stability with increasing temperature is consistent with the negative enthalpies of triple-helix formation reported under a number of solution conditions.

In order to explore this result further, the temperature dependence of the equilibrium association constant was measured under two new solution conditions that differed from each other and from BTNS buffer in their salt compositions. The first buffer, BTP1 (10 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, and 1 mM $SpmCl_4$ in 10 mM Bis-Tris at pH 7.0), was selected to provide a minimum of likely intracellular cation concentrations and was found to reduce the stability of the local triple-helical complex 10-fold relative to that in BTNS buffer because of its higher concentration of monovalent cations.[28] The second buffer, BTP4 (10 mM NaCl, 140 mM KCl, and 4 mM $SpmCl_4$ in 10 mM Bis-Tris at pH 7.0), contains the same concentrations of mono- and divalent cations but a higher concentration of the spermine tetracation, at which the equilibrium association constant is increased to a value similar to that found in BTNS buffer.[28] The results of the titrations in which the temperature was varied in BTP1 buffer are shown in Figure 2B. The observed equilibrium association constant (Table 1) increased when the temperature was cooled below 22 °C and decreased as the temperature was raised to 37 °C. Overall, as the temperature is increased from 8 to 37 °C, the association constant decreases 100-fold (Figure 3B). Similarly, the result of increasing the temperature from 8 to 37 °C in BTP4 buffer is a 190-fold decrease in the observed association constant (Figure 2C and Table 1).

Thermodynamic Parameters from van't Hoff Analyses. The observed trends of increasing triple-helix stability with decreasing temperature in BTNS, BTP1, and BTP4 are clearly indicated by the van't Hoff plots (ln K_T *versus* T^{-1}) displayed in Figures 3, parts A, B, and C, respectively. Linear least-squares analyses of the data allowed the enthalpies to be extracted from the slopes of the lines based on a form of the van't Hoff expression

$$\ln K_{eq} = (-\Delta H^\circ/R)\cdot T^{-1} + (\Delta S^\circ/R) \qquad (1)$$

and the entropies to be calculated from the intercept. Estimated uncertainties in each K_T were used to weight the linear fits, and the uncertainties were propagated through the fitted parameters to the thermodynamic parameters. The linear fits are of high quality, having correlation coefficient values >0.96 and χ^2 values <0.8 in every case. The thermodynamic parameters derived in this manner are compiled in Table 2.

For each set of salt conditions at pH 7.0, the enthalpic contribution to the free energy of triple-helix formation is relatively large and negative, in agreement with previous measurements using oligonucleotide triplexes. In contrast, the entropic contribution from the $-T\cdot\Delta S$ term is large and positive, also as found in previous investigations of oligonucleotide triplexes. Under the solution conditions employed here, the entropy and enthalpy changes are indistinguishable within experimental uncertainty. Although affinity cleavage titrations provide accurate free energies, the small uncertainties in each K_T are magnified to produce larger relative uncertainties in ΔH_T°

Table 1. Temperature Dependence of the Equilibrium Association Constant for Oligonucleotide-Directed Triple-Helix Formation at pH 7.0[a]

	100 mM NaCl, 1 mM $SpmCl_4$[b]		10 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, 1 mM $SpmCl_4$[c]		10 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, 4 mM $SpmCl_4$[d]	
temp	K_T	ΔG_T	K_T	ΔG_T	K_T	ΔG_T
8	$2.5\ (\pm0.7) \times 10^7$	$-9.5\ (\pm0.2)$	$7.6\ (\pm0.6) \times 10^6$	$-8.8\ (\pm0.1)$	$3.7\ (\pm0.7) \times 10^7$	$-9.7\ (\pm0.1)$
22	$3.3\ (\pm0.4) \times 10^6$	$-8.8\ (\pm0.1)$	$3.3\ (\pm1.4) \times 10^5$	$-7.6\ (\pm0.2)$	$6.3\ (\pm1.0) \times 10^6$	$-9.2\ (\pm0.1)$
29	$4.1\ (\pm1.4) \times 10^5$	$-7.8\ (\pm0.2)$	$2.4\ (\pm0.4) \times 10^5$	$-7.4\ (\pm0.1)$	$1.7\ (\pm1.2) \times 10^6$	$-8.6\ (\pm0.5)$
37	$1.4\ (\pm0.8) \times 10^5$	$-7.3\ (\pm0.4)$	$7.4\ (\pm1.3) \times 10^4$	$-6.9\ (\pm0.1)$	$1.9\ (\pm0.7) \times 10^5$	$-7.5\ (\pm0.2)$

[a] The K_T values in the table are mean values (±SEM) of three to five independent measurements in 10 mM Bis-Tris·HCl buffer (pH 7.0) at the indicated temperatures (°C) and dalt concentrations. Standard errors in the free energies are propagated from the SEM of each K_T value. The K_T and derived ΔG_T values are reported in units of M^{-1} and kcal·mol^{-1}, respectively. [b] BTNS buffer. [c] BTP1 buffer. [d] BTP4 buffer.

and $\Delta S_T°$. On average, binding of the 15mer oligonucleotide to the duplex target site under the range of conditions studied here is accompanied by an enthalpy change of −2.0 kcal per mol of base triplets and an entropy change of −4.8 cal·K^{-1} per mol of base triplets. It is important to point out that these *average* values contain contributions from both T·AT and C+GC base triplets, whose individual stabilities may be distinct.

Discussion

To date, most thermodynamic data have been reported for oligomeric triplexes in solutions containing single cationic species at low pH. We have extended the affinity cleavage titration method to the measurement of enthalpic and entropic contributions to the stabilities of single-site local triple-helical complexes formed within large DNA at neutral pH in the presence of physiologically important salt concentrations. In each set of salt conditions at pH 7.0, the enthalpic contribution to the free energy of triple helix formation is relatively large and negative (Table 2), in accord with expectations based on the formation of hydrogen bonds and $\pi-\pi$ interactions between the bases. In contrast, the entropic contribution to the free energy is large and positive as would be anticipated for the loss of translational and rotational degrees of freedom for the oligonucleotide[35,36] and for the condensation of cations around the triple helix.[28,37] These results are consistent with previous investigations of oligonucleotide triplexes. Changes in salt composition among the three buffers were expected to be reflected in the entropic contribution from counterion condensation.[28,37] Moreover, by increasing the concentrations of mono- and divalent cations (from BTNS buffer to BTP1 buffer) or by increasing the concentration of Spm^{4+} (from BTP1 to BTP4), we anticipated small changes in $\Delta H_T°$ based on differential changes in the charge screening parameter.[28,38,39] In the three solutions studied here, these small differences in $\Delta H_T°$ and $\Delta S_T°$ are indistinguishable within the experimental uncertainties.

Comparison with Enthalpies Measured for Other Triplexes. A minimal equilibrium expression for the formation of a triple helix ($S_1 \cdot S_2 \cdot S_3$) from single-stranded (S_1) and double-stranded ($S_2 \cdot S_3$) nucleic acid components in a solution containing buffer (**B**) and mono-, di-, and multivalent cations (M^+, N^{2+}, and Q^{z+}, respectively) can be written as

$$S_1 + S_2 \cdot S_3 + iM^+ + jN^{2+} + kQ^{2+} + lBH^+ \rightleftharpoons S_1H_l S_2 \cdot S_3 + lB \quad (2)$$

where i, j, and k represent the number of M^+, N^{2+}, and Q^{z+} ions, respectively, thermodynamically bound per phosphate during the association reaction,[28] and l is the number of protons transferred from the buffer in the formation of any protonated C+GC base triplets.[27,40] Evidence obtained by a number of laboratories indicates that the factors influencing the stability of triplexes include the following: (i) the nature of S_1, including its length,[1,13] sequence composition,[39-41] and functional groups on both the heterocycles[12,42,43] and backbone;[3,4,44,45] (ii) the 2′-substituents on the ribosyl moieties of S_2 and S_3;[3,4,45] (iii) the identities and concentrations of cations;[1,11,12,16,28,39,46] (iv) the solution pH;[1,11,12,27,36,40,42,46] and (v) the identity and ionization heat of **B**.[40] Furthermore, the effect of each factor described above is likely coupled to other factors so that thermodynamic data depend on the nature and concentration of each species. In particular, the influences of cations, pH, and the number of C+GC base triplets are not independent of each other.

In order to compare the $\Delta H_T°$ measured here with values reported in the literature for oligonucleotide triple helices, we considered that there are at least three different kinds of oligodeoxyribonucleotide triplexes based on their sequence composition and the solution pH (eq 2). First, triplexes containing only T·AT base triplets are distinct from those containing both C+GC and T·AT triplets for the following reasons: (i) enthalpies for T·AT-only triplexes result from only a single type of base triplet or nearest neighbor triplet pair (T·AT/T·AT) rather than an average over several types; (ii) oligo(dA)·oligo(dT) duplexes may have unusual conformations and/or hydration states;[47] (iii) the protonation of C+GC triplets contributes to the observed enthalpy of triple-helix formation, and ionized cytosine heterocycles likely alter the enthalpies

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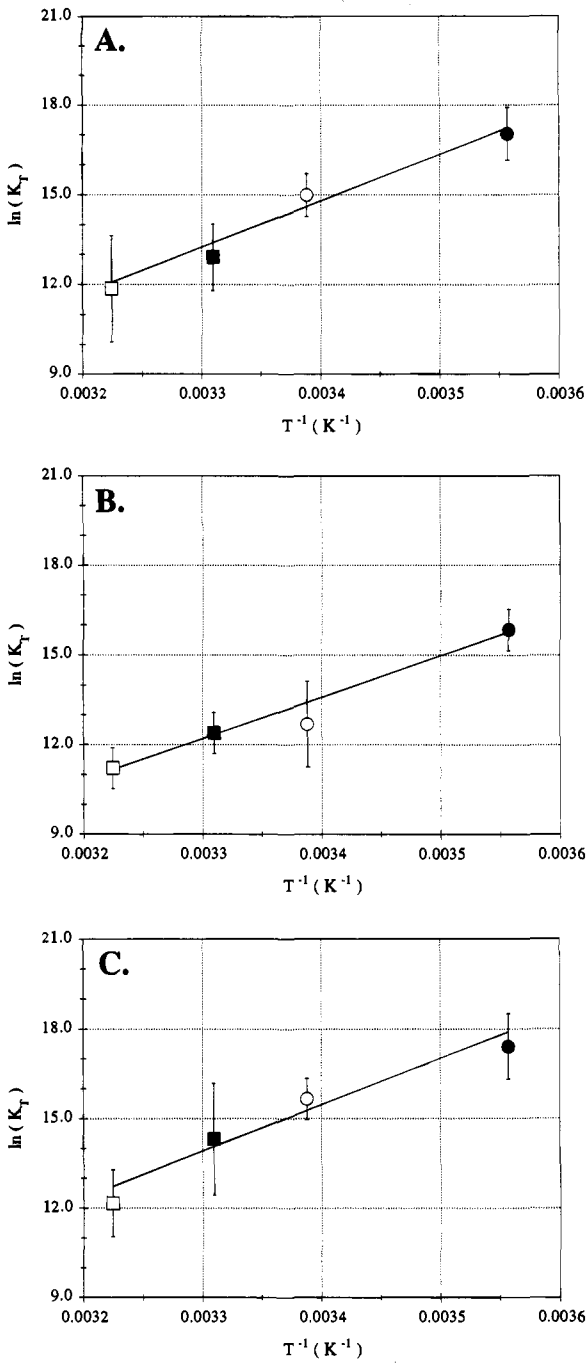

Figure 3. The natural logarithms of the mean association constants (Table 1) are plotted as a function of the reciprocal of the absolute temperature. The symbols represent the same temperatures as in Figure 2. Error bars represent estimated confidence limits. (A) Apparent equilibrium association constants measured in BTNS buffer. (B) Apparent equilibrium association constants measured in BTP1 buffer. (C) Apparent equilibrium association constants measured in BTP4 buffer.

associated with hydrogen-bonding and base-stacking as well as reduce the charge density of the triple-helical complex. Based on reason (iii), triplexes containing both C+GC and T·AT triplets were further divided into two categories depending on the pH used in the measurement. Over the pH range 4.5−6.0, the C+GC triplets are mostly protonated, while over the range 6.5−7.0, the protonation state likely varies among different sequence and solution compositions. The ranges of reported *dissociation* enthalpies per base triplet are compiled in Table 3.

The magnitude of the average *association* enthalpy change

Table 2. Apparent Thermodynamic Parameters for Oligonucleotide-Directed Triple-Helix Formation in 10 mM Bis-Tris·HCl at pH 7.0[a]

	100 mM NaCl, 1 mM $SpmCl_4$[e]	10 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, 1 mM $SpmCl_4$[f]	10 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, 4 mM $SpmCl_4$[g]
ΔG_T[b]	−8.8 (±0.4)	−7.6 (±0.9)	−9.2 (±0.2)
$\Delta H_T°$[c]	−31 (±9.1)	−28 (±5.6)	−31 (±9.2)
$\Delta S_T°$[d]	−75 (±31)	−67 (±19)	−74 (±31)

[a] The ΔG_T and $\Delta H_T°$ values are reported in units of kcal·mol^{-1}, while those of $\Delta S_T°$ are given in cal·mol^{-1}·K^{-1}. Estimated uncertainties in the thermodynamic parameters are given in parentheses. [b] The ΔG_T values are the values derived at 22 °C and the indicated salt concentrations from Table 1. [c] The $\Delta H_T°$ values are derived from the slopes of the van't Hoff plots (Figure 2). [d] The $\Delta S_T°$ values are calculated from the intercepts of the van't Hoff plots. [e] BTNS buffer. [f] BTP1 buffer. [g] BTP4 buffer.

Table 3. Literature Values of Apparent Dissociation Enthalpies for Oligodeoxyribonucleotide Triplexes[a]

		calorimetric		non-calorimetric	
Nts[b]	pH[c]	$\Delta H°_{app}$[d]	ref	$\Delta H°_{app}$[d]	ref
T	5.5−7.1	2.5[58]	*e*	1.9−4.5	*f*
C,T	4.5−6.0	4.7−6.2	*g*	3.4−8.1	*h*
	6.5−7.0	2.0−4.2	*i*	4.8−5.9	*j*

[a] The ranges of enthalpies previously measured using calorimetric or non-calorimetric methods. [b] The nucleotide compositions of the third strands in the triplexes studied. "T" indicates the triplex contained T·AT base triplets only; "C,T" indicates the triplex contained both C+GC and T·AT base triplets. [c] The pH range indicated contains each solution pH used for measurements of the tabulated enthalpy changes. [d] The apparent dissociation enthalpy changes are reported in kcal per mol of base triplets. [e] References 40 and 48. [f] References 40, 49, and 50. [g] References 36, 40, and 49. [h] References 15, 36, 39, 52, and 53. [i] References 12, 36, and 40. [j] References 16 and 36.

per base triplet measured here (2.0 kcal per mol of base triplet) is on the low end of the range of values reported for triplexes containing C+GC and T·AT triplets from pH 6.5 to 7.0 (2.0−5.9 kcal·(mol bt)$^{-1}$). One potential contributing source of the relative placement is the requirement for the formation of junctions between double- and triple-helical regions. The triple-helical complex reported here and previously[12] was designed to model the structures formed by oligonucleotide-directed triple-helix formation *in vivo* and thus occurs within a larger double-helical DNA. The conformational differences between the two domains result in the formation of two duplex−triplex junctions, each of which is characterized by an enthalpic penalty.[54−57] In addition to differences resulting from differences in the identities of cation species, cation concentrations, pH, and buffering species, the sequence of base triplets influences the triple-helix enthalpy. The arrangement of cytosine residues in the sequence studied here, in which each cytosine is separated from the next by a single thymidine residue (Figure 1), may render the complex particularly sensitive to the protonation state and stability of the individual C+GC triplets. A systematic investigation of sequence composition effects on triplex thermodynamics has not been reported.

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Comparison with the Enthalpy Measured Previously for a Triplex of the Same Sequence. Each of the variables discussed above contributes to the enthalpic stabilization of triple-helical complexes and to differences between individual complexes, making comparisons among experiments difficult. In collaboration with Breslauer and co-workers, we characterized the thermodynamic stability of a triplex formed by the same oligonucleotide sequence as that studied here using differential scanning calorimetry and optical spectroscopy.[12] In the previous work, the enthalpy determined by direct integration of DSC excess heat capacity curves (ΔH_{cal}) for the triple helix formed by binding of the oligonucleotide to 15 bp within a 21-bp duplex at pH 6.5 (phosphate buffer) in the presence of 200 mM Na^+ was 30.4 (±2) kcal·mol^{-1}. In contrast, enthalpies estimated by van't Hoff analysis of the shapes of calorimetric or optical thermal denaturation curves (ΔH_{vH}) were a factor of 3 higher. The apparent enthalpy measured in each salt solution used in the present study is identical within experimental uncertainty to that measured directly by DSC for the triplex of the same sequence. While there are undoubtedly differences in the true enthalpies based on differences in the length and sequence of the duplex neighboring the target site, the salt compositions,[39] the pH,[36,40] and the buffering species,[40] the magnitudes of the differences are apparently small and the observed enthalpies are consistent with that of the DSC measurement rather than those extracted from melting curves.

Similar to the 15mer intermolecular triplex described above, ΔH_{cal} was measured to be significantly less than ΔH_{vH} for a 10mer intramolecular triplex formed by folding of a 38mer.[36] It is important to note that this behavior is observed at pH 6.7, but not at pH 4.5, for the intramolecular triplex. The observation that $\Delta H_{vH} > \Delta H_{cal}$ is usually interpreted as evidence for aggregation of the complex undergoing the transition; however, in the cases of the intermolecular triplex, there is evidence inconsistent with aggregate formation,[30] and there is no corroborating experimental evidence for aggregation in either case.[12,36] Alternatively, the assumption that the conformational transition is a true two-state process, which is required for the extraction of thermodynamic parameters from the analysis of melting curve shapes, may not hold. Resolution of the discrepancy between these two methods must await the further application of more precise biophysical techniques. However, the results of the direct van't Hoff analyses described herein provide independent corroboration of the enthalpy measured using calorimetry for one triple-helical complex and suggest the use of caution in the interpretation of enthalpy values obtained indirectly from the shapes of thermal denaturation profiles near neutral pH.

Conclusions

In summary, we have measured the temperature dependence of the equilibrium constant for oligonucleotide-directed triple-helix formation at neutral pH in the presence of several physiologically important cations, including spermine. Direct application of the van't Hoff equation allowed the extension of the affinity cleavage titration method to the determination of the enthalpic and entropic contributions to local triple-helix stability. The significance of this work is that it allows comparisons between thermodynamic parameters for triplexes formed within larger duplex DNA near likely intracellular proton and salt concentrations with those for oligomeric triplexes formed at acidic pH in the presence of single salt species. Under the solution conditions investigated, the local triple-helical complex is enthalpically stabilized by an average of 2 kcal per mol of base triplets at pH 7.0. These data provide independent corroboration of the enthalpy measured previously for a triple helix of the same sequence using differential scanning calorimetry. However, the average value reflects contributions from T·AT, protonated C+GC, and unprotonated C·GC base triplets, each of whose enthalpic stablizations are likely distinct. Future biophysical measurements will reveal enthalpies for each base triplet, which will undoubtedly depend on sequence context effects as well as the exact solution conditions.

Experimental Section

DNA Preparation. The oligonucleotide 5′-d(T*TTTTCTCTCT-CTCT)-3′ was synthesized and purified as previously described.[13] The 5′-^{32}P-labeled 339-bp linear duplex DNA was prepared and purified as previously described,[13] resuspended in 5 mM Tris−HCl buffer (pH 8.0) at a final activity of 30 000 cpm per μL, and stored at 4 °C.

Stock Solutions of Buffer and Salts. Bis-Tris [bis(2-hydroxyethyl)-aminotris(hydroxymethyl)methane; Fluka BioChemika MicroSelect], sodium chloride (Fluka BioChemika MicroSelect), potassium chloride (Mallinckrodt), magnesium chloride (Mallinckrodt), and spermine tetrahydrochloride (Fluka BioChemika MicroSelect) were used as obtained from commercial suppliers. In order to obtain three pH 7.0-buffered solutions containing various salt compositions at each of four different temperatures, twelve different stock solutions were required. First, six stock solutions containing 5× concentrations of the desired salts and 5× concentration of *either* Bis-Tris free base or its conjugate acid were prepared. These six stock solutions (and the concentration of solutes) were BTNSa (50 mM Bis-Tris·H^+, 500 mM NaCl, 5 mM $SpmCl_4$), BTNSb (50 mM Bis-Tris, 500 mM NaCl, 5 mM $SpmCl_4$), BTP1a (50 mM Bis-Tris·H^+, 50 mM NaCl, 700 mM KCl, 5 mM $MgCl_2$, 5 mM $SpmCl_4$), BTP1b, (50 mM Bis-Tris, 50 mM NaCl, 700 mM KCl, 5 mM $MgCl_2$, 5 mM $SpmCl_4$), BTP4a (50 mM Bis-Tris·H^+, 50 mM NaCl, 700 mM KCl, 5 mM $MgCl_2$, 20 mM $SpmCl_4$), and BTP4b (50 mM Bis-Tris, 50 mM NaCl, 700 mM KCl, 5 mM $MgCl_2$, 20 mM $SpmCl_4$). The solutions containing the conjugate acid of Bis-Tris were prepared identically to those containing the free base except that a few drops of 1 M HCl were added to a pH ≈ 4. Based on the known temperature dependence of the pK_a of Bis-Tris, the pairs of stock solutions were mixed at room temperature to give 12 solutions whose pH's were a few tenths of a unit above the room temperature value corresponding to pH 7.0 at the desired temperature. Each of the solutions was then equilibrated in a water bath at the appropriate temperature and the pH adjusted to 7.0 at that temperature using 1 M HCl. The pH of the solutions was measured using a digital pH/millivolt meter (Orion Research, Model No. 611) and a ROSS semimicro combination pH electrode (Orion Research, Model No. 81−15). The final 12 buffers ready for use at the appropriate temperature were BTNS-08, -22, -29, and -37 (50 mM Bis-Tris·HCl, 500 mM NaCl, and 5 mM $SpmCl_4$), BTP1-08, -22, -29, and -37 (50 mM Bis-Tris·HCl, 50 mM NaCl, 700 mM KCl, and 5 mM $SpmCl_4$), and BTP4-08, -22, -29, and -37 (50 mM Bis-Tris·HCl, 50 mM NaCl, 700 mM KCl, and 20 mM $SpmCl_4$).

Temperature Control. Triple-helix formation and affinity cleavage reactions performed at low temperature were carried out in 0.6-mL tubes suspended in a circulating cooling bath (Lauda) containing ≈20% aqueous ethylene glycol thermostated at 8.0 (±0.2) °C. Reaction tubes for high-temperature titrations were placed in aluminum heating blocks (VWR) at 29.0 (±3.0) or 37.0 (±2.0) °C. Room temperature experiments were performed on the laboratory bench top at a temperature of 22.0 (±2.0) °C.

Quantitative Affinity Cleavage Titrations. Because the experimental protocol for the titrations has been published,[13] only changes are reported here. The oligonucleotide−EDTA·Fe and the [^{32}P]DNA were allowed to equilibrate in solutions containing buffer, salts, and carrier DNA for a length of time dependent on the temperature (24 h at 8 or 22 °C, and 48 h at 29 or 37 °C). The cleavage reactions were initiated by the addition of aqueous DTT to each tube and incubated for a length of time dependent on the temperature (8, 6, 3, or 2 h at 8, 22, 29, and 37 °C, respectively). Final reaction conditions in 40 μL total volume were 10 mM Bis-Tris buffer at pH 7.0, 10−100 mM NaCl, 0−140 mM KCl, 0−1 mM $MgCl_2$, 1−4 mM $SpmCl_4$, 20 μM Fe, 0.1 mM bp calf thymus DNA, 1 mM DTT, and approximately 15 000 cpm

labeled duplex (the specific activity of the DNA varied slightly from experiment to experiment but was the same for each reaction within a given experiment, and always resulted in a final target site concentration of less than 50 pM). The reactions were quenched and the products separated electrophoretically as described.

At low concentrations of oligonucleotide, iron loading in the thymidine−EDTA can limit the observed cleavage intensity and, thus, the apparent fraction of duplex sites occupied. Therefore, mean K_T values $\geq 3 \times 10^6\ M^{-1}$ were confirmed by performing titrations in which 20 μM Fe^{2+} was included in the reaction solutions to ensure maximal oligonucleotide−EDTA loading.[30] Moreover, quantitative DNase I footprint titrations, which do not rely on the EDTA·Fe cleavage chemistry, were employed to independently confirm these values.

As reported above, all titrations included carrier DNA (from calf thymus) at a final concentration of 100 μM in base pairs. The inclusion of a relatively high concentration of potential binding sites can, in general, reduce the true concentration of free ligand significantly below the total concentration of ligand, and it is thus not recommended for all ligand-binding studies of this type. However, affinity cleavage titrations in the absence of carrier, in the presence of various concentrations of calf thymus DNA (10−100 μM bp), or in the presence of tRNA all give mean equilibrium association constants that are identical within experimental uncertainty.[30] Likely, the presence of duplex carrier DNA does not affect the observed equilibrium association constant because the oligonucleotide binding site is relatively long and triple-helix formation occurs with high sequence selectivity. We chose to include carrier DNA in order to maximize the quality and reproducibility of cleavage results from individual experiments.

Construction of Equilibrium Titration Isotherms. Cleavage data (I_{site}) were obtained using storage phosphor autoradiography as previously described.[13] A theoretical binding curve was fit to the experimental data using the apparent maximum cleavage (I_{sat}) and K_T as adjustable parameters:

$$I_{fit} = I_{sat}\frac{K_T[O]_{tot}}{1 + K_T[O]_{tot}} \quad (3)$$

The difference between I_{fit} and I_{site} for all data points was minimized using the nonlinear least-squares fitting procedure of KaleidaGraph (version 3.0.1; Synergy Software) running on a Macintosh IIfx computer. Titration isotherm fits were performed without weighting the data points. All data points were included in the fitting procedure unless one of the following conditions was met: (i) visual inspection of the computer image from a storage phosphor screen revealed a flaw at either the target site or reference blocks; (ii) the I_{site} value for a single lane was greater than two standard errors away from both values from the neighboring lanes; or (iii) the I_{site} values at high oligonucleotide concentrations decreased because the DTT was depleted.

Each K_T reported in the text or tables is the mean of three to five experimental observations plus or minus the standard error of the mean. All measured K_T values were included in determining mean values unless one of the following conditions was met: (i) fewer than 80% of the data lanes were usable (*vide supra*); (ii) the reduced χ^2 criterion for judging the fit of the titration curve to the data points was >1.5; (iii) the measured K_T value failed the Q-test. Experiments using a particular set of conditions were performed using different serial dilutions of oligonucleotide prepared from at least two different aliquots of the original solution, at least two different preparations of 5′-labeled duplex DNA, and at least two uniquely prepared buffer solutions.

For graphical representation, I_{site} values are normalized to the range representing apparent fractional occupancy of the duplex binding site (θ_{app}) by dividing I_{site} by I_{sat}. Values of $I_{site}{\cdot}I_{sat}^{-1}$ from repeat experiments under the same conditions were averaged and are plotted along with the isotherms constructed using mean K_T values and eq 3 with $I_{sat} \equiv 1$.

Quantitative DNase I Footprint Titrations. Using the solution conditions described above, footprint titration experiments and data reduction and analysis were performed according to a published protocol.[13]

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