Temperature Dependence and Sequence Specificity of DNA Triplex Formation: An Analysis Using Isothermal Titration Calorimetry

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Abstract: We have investigated the thermodynamics and specificity of DNA triplex formation with isothermal titration calorimetry (ITC). The triplex formation between a 23-mer double-stranded homopurine−homopyrimidine and a 15-mer single-stranded homopyrimidine oligonucleotide forming T·AT and C^+·GC triads at pH 4.8 is driven by a large negative calorimetric enthalpy change, ΔH_{cal}, of the order of −80 kcal/mol. ΔH_{cal} is strongly temperature dependent, yielding a heat capacity change, ΔC_p, of about −1 (kcal/mol)K^{-1}. The equilibrium association constant, K, obtained from the titration curve is about 9×10^7 M^{-1} at 25 °C (binding free energy change, ΔG, is about −11 kcal/mol). Thus, the triplex formation is accompanied by a negative entropy change ($\Delta S = -245$ (cal/mol)K^{-1} at 25 °C). We found that K is insensitive to temperature near room temperature, leading to an apparently small van't Hoff enthalpy change (ΔH_{vH}), in sharp contrast with the large negative ΔH_{cal}. Together, the analyses of the observed temperature dependences of K and ΔH and the large negative ΔC_p suggest that the triplex formation is a coupled process between conformational transitions in single-stranded DNA and its binding with double-stranded DNA. The examination of single mismatches in the triplex formation has shown that K and ΔG are not strongly affected by the particular combination of triad sequences (differences in ΔG are within 1.2 kcal/mol). In contrast, single mismatches affected ΔH_{cal} to a greater extent (up to 7-kcal/mol differences). We discuss possible means to enhance specificity in triplex formation, implied by the present findings.

Introduction

Interest in triplex DNA has grown recently because of its possible biological function and its potential applications as antisense drugs.[1] Triplex formation has been applied to specific cleavage of DNA,[2] repression of gene expression,[3] and genome screening.[4] Triplex is usually formed between a double-stranded homopurine−homopyrimidine and a single-stranded homopyrimidine tract[2a,5] or a homopurine tract.[6] The third homopyrimidine single-stranded DNA (ss-DNA) binds to the major groove of the double-stranded DNA (ds-DNA), in parallel with the purine strand, via Hoogsteen hydrogen bonding,[7] whereas the third homopurine strand binds to the major groove antiparallel to the other purine strand.[6] Typical triplexes are formed between thymine (T) and adenine·thymine (AT) base pairs (T·AT triad), and between protonated cytosine (C) and guanine·cytosine (GC) base pairs (C^+·GC triad).[1,2a,5,8] However, other base combinations have been suggested to form stable triplexes, e.g., G·TA, G·GC, and T·CG triplets.[9,10] The stability of a triplex is affected by various environmental conditions such as pH, ionic strength, temperature, and supercoil density.[9b,11,12]

In order to understand the mechanism of triplex formation and to assess the effects of sequence and environment on its specificity and stability, it is necessary to obtain detailed thermodynamic and kinetic data for the binding process under various conditions. The dissociation process of triplex has been widely investigated by the measurements with UV melting, CD

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melting,[13] and differential scanning calorimetry (DSC).[14] On the other hand, the association process of triplex has not been well characterized nor has its mechanism been understood. Furthermore, there are some variations in the thermodynamic and kinetic data of dissociation and association of triplex obtained by different methods, e.g., in the enthalpy change for the association process measured by filter-binding assay[12,15] and affinity-cleavage assay[11g] and for the dissociation process derived from UV, CD, and DSC measurements.[13,14] There are several pieces of evidence indicating that triplex formation is not a simple two-state reaction.[12] Thus, it is important to investigate the details of the thermodynamic properties for the triplex formation. Measurements by isothermal titration calorimetry (ITC) enable us to obtain detailed information on thermodynamic quantities such as enthalpy and heat capacity changes of molecular associations directly from the heat produced by the reaction.[16] Also, the binding free energy change and the entropy change can be estimated at the same time. The evaluation of these quantities should inform us about the mechanism of triplex formation. In this paper, we examine detailed thermodynamic properties for the triplex formation between a double-stranded homopurine−homopyrimidine and a single-stranded homopyrimidine oligonucleotide containing T·AT and C^+·GC triads at pH 4.8, and its sequence specificity by using ITC. Based on the results, we discuss the mechanism of triplex formation and suggest possible means for enhancing sequence specificity.

Results

Thermodynamic Quantities of Triplex Formation. Figure 1a shows the titration profile for triplex formation between the 15-mer single strand (Pyr15Z) and 23-mer double strand (Pur23Y/Pyr23X) with Z·YX = T·AT (T·AT triad) measured by ITC at 25 °C and pH 4.8. An exothermic heat pulse is observed upon each injection of the 15-mer third strand into the reaction cell containing the 23-mer double strand. The magnitude of the peak decreases progressively upon subsequent injections, and residual peaks at saturation correspond to the heat of dilution for placing the third strand into the buffer. The area of each peak was integrated and corrected for the heat of dilution, which was estimated by a separate experiment by injecting the 15-mer strand into the dialysis buffer. The corrected heat was divided by the moles of the injectant and the values were plotted in Figure 1b as a function of the molar ratio of 15-mer/23-mer. The titration passes a half-saturation point near the equimolar ratio between the single and double strands, indicating a one-to-one binding to form a triplex. By fitting the titration curve with a nonlinear least-squares method, the enthalpy change, ΔH, and the equilibrium association constant, K, of triplex formation can be estimated with the assumption of a two-state model for triplex formation. Averaging over multiple experiments, we estimate ΔH and K to be -83.8 ± 0.5 kcal/mol and $(9.08 \pm 0.67) \times 10^7$ M^{-1}, respectively, at 25 °C. Thus, the triplex formation is driven by a large negative enthalpy change. The association constant obtained by calorimetry is similar to the values estimated by the filter-binding assay.[15] From the estimated association constant, the value of the binding free energy change, ΔG, is calculated to be -10.8 ± 0.1 kcal/mol. Thus, the entropy change, ΔS, for triplex formation is -245 ± 2 (cal/mol)K^{-1}; that is, the entropy change is unfavorable for the triplex formation.

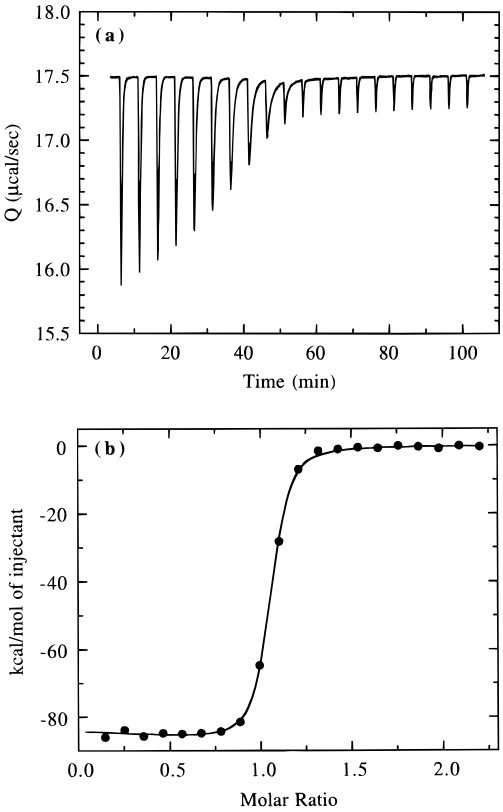

Figure 1. (a) The titration profile of the binding process between the 15-mer single strand, Pyr15T (5′-CTCTTCTTTTCTTTC-3′), and the 23-mer duplex DNA, Pur23A/Pyr23T (5′-GCGCGAGAAGAAAA-GAAAGCCGG-3′/5′-CCGGCTTTCTTTTCTTCTCGCGC-3′), by ITC at pH 4.8. Temperature is 25 °C. Buffer contains 10 mM sodium acetate/10 mM cacodylic acid, pH 4.8, 0.2 M NaCl, and 20 mM $MgCl_2$. Each pulse corresponds to a 5-μL injection containing 120 μM of the 15-mer third strand into the cell containing 5 μM of the 23-mer duplex. (b) The area of each peak in (a) was integrated and corrected for the heat of dilution, which was estimated by a separate experiment by injecting the 15-mer strand into the dialysis buffer. The corrected heat was divided by the moles of injectant and values were plotted as a function of the molar ratio of 15-mer/23-mer. The titration curve was fit with a nonlinear least-squares method.

Temperature Dependence of Triplex Formation. We have examined the temperature dependence of the enthalpy change in the temperature range between 15 and 35 °C, in order to evaluate the heat capacity change, ΔC_p, associated with the triplex formation. The results are shown in Table 1 and Figure 2. ΔH decreases with increasing temperature, indicating that the triplex formation is accompanied by a negative ΔC_p. The plot of ΔH vs T shows a nearly linear dependence in this temperature range. If the plot of ΔH vs T is fit with a straight line, the estimated value of ΔC_p is -914 ± 47 (cal/mol)K^{-1}.

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Table 1. Thermodynamic Parameters for Triplex Formation, Obtained by ITC Measurements at pH 4.8[a]

T	K (M^{-1})	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS ((cal/mol)K^{-1})
15	$(7.97 \pm 1.97) \times 10^7$	-10.2 ± 0.2	-73.1 ± 0.8	-223 ± 7
20	$(8.37 \pm 0.88) \times 10^7$	-10.6 ± 0.1	-78.1 ± 0.2	-230 ± 1
25	$(9.08 \pm 0.67) \times 10^7$	-10.8 ± 0.1	-83.8 ± 0.5	-245 ± 2
30	$(8.20 \pm 0.69) \times 10^7$	-11.0 ± 0.1	-87.2 ± 0.8	-252 ± 3
35	$(8.21 \pm 0.12) \times 10^7$	-11.2 ± 0.1	-91.4 ± 0.6	-261 ± 2

[a] Concentration of the 15-mer third strand is 120 μM in the syringe. 5 μL is injected into the cell. Concentration of the 23-mer duplex is 5 μM in the cell (total 23-mer duplex in the cell is at a 10-fold excess over the single 15-mer injection). Corrected for the heat of dilution by the 15-mer.

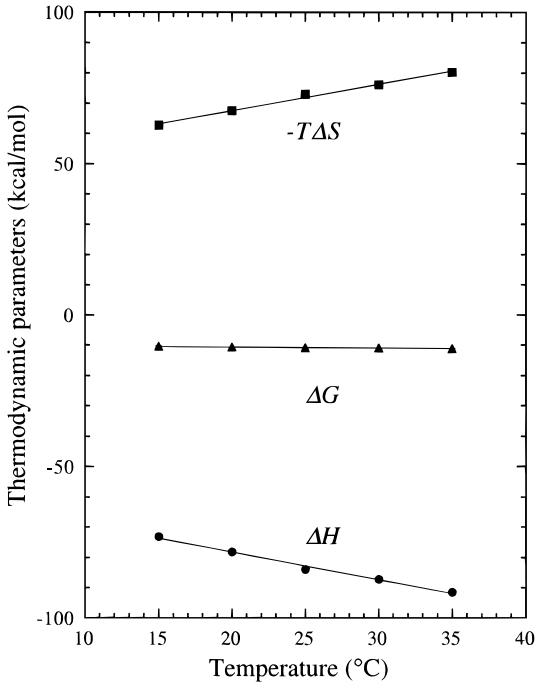

Figure 2. Temperature dependence of ΔH, ΔG, and $-T\Delta S$ for the T·AT 15-mer/23-mer triplex formation. Conditions are the same as in Figure 1.

Since the magnitude of ΔC_p is much larger than that of ΔS, $-T\Delta S$ shows a similar temperature dependence with a slope of opposite sign (see Figure 2). Because of this "enthalpy−entropy compensation", the temperature dependence of ΔG is weak (Figure 2). When the values of ln K are plotted against $1/T$, the curve is nearly flat (see Figure 5). The linear least-squares fitting of the curve yields an apparent van't Hoff enthalpy change of -0.35 ± 0.79 kcal/mol. This is in sharp contrast to the large negative calorimetric enthalpy change. However, the plot of ln K vs $1/T$ is not strictly linear, and could be fit with more complicated functions (see Discussion).

CD Melting of Triplexes. In order to make sure that the triplex is stable in this temperature range, we also measured by circular dichroism the temperature-induced denaturation of triplex. As shown in Figure 3, the triplex with T·AT triads begins melting at around 50 °C, with a T_m of about 70 °C. Since there is no significant denaturation between 15 and 35 °C, the temperature dependence of ΔH measured by ITC can be attributed to the triplex formation. We have also checked the temperature dependence of pH of the buffer used, and found it to be negligible (the pH variation should be less than 0.01 in our temperature range, based on the enthalpy changes of protonation for the sodium acetate/cacodylic acid buffer). Figure 3 also shows the melting of other triplexes for the different triads, Z·YX = T·TA, A·TA, and A·AT. These triplexes with

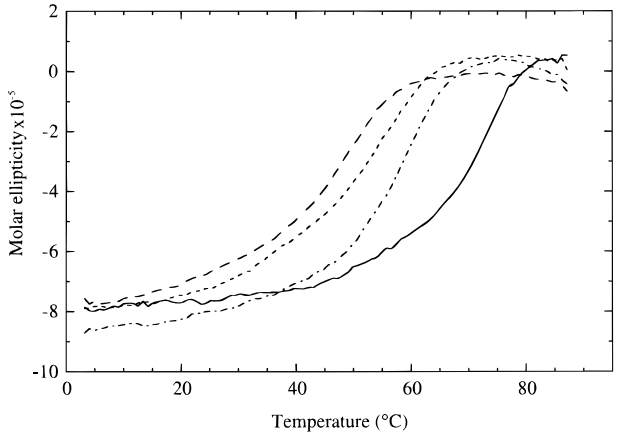

Figure 3. Melting profile of the 15-mer/23-mer triplexes, T·AT (solid line), T·TA (dash line), A·TA (dot line), and A·AT (dash−dot line), measured by CD. The melting profiles were recorded at 220 nm under the same conditions as in Figure 1. The solution contains 10 μM of each strand in the same buffer as in Figure 1. The heating rate is 20 °C per h.

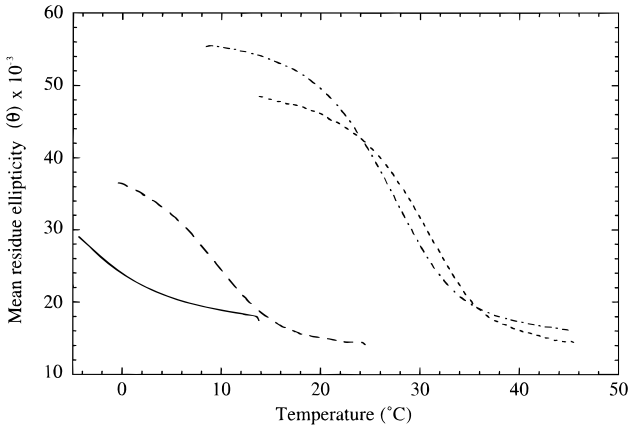

Figure 4. Melting profile of the single strands, Pyr15T (solid line), Pyr19C (dash line), TC15 (dash-dot line), and TC19 (dot line). Measurements were made at 285 nm under the same conditions as in Figure 1. The sample solution contains 50 μM of each strand in the same buffer as in Figure 1. The heating rate is 5 °C per h.

one mismatch at the center are less stable than the T·AT triad; their T_m's are 58 (A·AT), 53 (A·TA), and 48 °C (T·TA) in decreasing order of stability. However, these molecules are indicated to be dominantly in the triplex state at room temperature for these triads.

CD Melting of Single Strands. We have also carried out CD melting experiments for the single strands, Pyr15T, Pyr19C, TC15, and TC19 (Figure 4), in order to determine whether a temperature-dependent conformational transition of the single strand might play a role in the triplex formation (see Discussion). As shown in Figure 4, all single strands exhibit conformational transitions with different melting temperatures (T_m's for Pyr15T, Pyr19C, TC15, and TC19 are −5, 10, 27, and 30 °C, respectively). The transitions were almost reversible as the transition curves in both directions were almost identical. Longer DNA's tend to melt at higher temperatures. Furthermore, alternating T/C sequences (TC15 and TC19) are much more stable than irregularly mixed T/C sequences (Pyr15T and Pyr19C), indicating that the alternating T/C single strands may assume a specific conformation in solution.

Sequence Dependence of Thermodynamic Quantities in Triplex Formation. We have measured thermodynamic quantities for four different triad sequences, Z·YX = T·AT, T·TA,

Table 2. Effect of Single Mismatches on Thermodynamic Parameters for Triplex Formation, Measured by ITC[a]

triad	K (M^{-1})	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS ((cal/mol)K^{-1})
T·AT	$(9.08 \pm 0.67) \times 10^7$	-10.8 ± 0.04	-83.8 ± 0.5	-245 ± 2
T·TA	$(1.23 \pm 0.10) \times 10^7$	-9.66 ± 0.05	-76.9 ± 1.7	-225 ± 6
A·TA	$(1.73 \pm 0.11) \times 10^7$	-9.87 ± 0.04	-82.3 ± 0.4	-203 ± 2
A·AT	$(4.42 \pm 0.22) \times 10^7$	-10.4 ± 0.03	-77.6 ± 0.6	-218 ± 2

[a] Temperature is 25 °C. Other conditions are the same as in Table 1. Mismatches are made at the center of the triplex with Z·YX, where Z, Y, and X are in Pyr15, Pur23, and Pyr23 strands, respectively.

A·TA, and A·AT, by ITC, as shown in Table 2. From this result, the value of ΔG varies within 1.2 kcal/mol for the single mismatches. The T·AT triad is the most stable triplex, and A·AT is next most stable. The A·TA and T·TA triads are significantly less stable than the T·AT by 0.9 and 1.2 kcal/mol, respectively. This order of relative stability agrees with that obtained from our melting experiments (Figure 3) and also by others.[13c,17] On the other hand, ΔH exhibits a very large sequence dependence, with up to 7 kcal/mol variation per single mismatch among the four different triads. In terms of ΔH, the order of stability is T·AT, A·TA, A·AT, and T·TA. Among these, the T·TA triad is the least stable in terms of ΔG or ΔH.

Discussion

The present results have shown that triplex formation is driven by a large negative enthalpy change of -73 to -91 kcal/mol in the temperature range examined. Since the present system contains cytosines, which are protonated to form a triplex, the calorimetric enthalpy change, $\Delta H_{\mathrm{cal}}^{\mathrm{assoc}}$, measured by ITC contains a contribution from the protonation of cytosine and the deprotonation of buffer. Here, "assoc" is used to distinguish the association process from the dissociation process. At pH 4.8, approximately 33% of cytosine will be protonated in solution (pK_a of cytosine is 4.5). Thus, about 3.3 protons need to be taken up from the buffer. After correcting for the deprotonation enthalpy change of buffer (-0.18 kcal/mol) and the protonation enthalpy change of cytosine (-4.5 kcal/mol), the corrected $\Delta H_{\mathrm{cal}}^{\mathrm{assoc}}$ is about -69 kcal/mol (-4.6 kcal/mol per base) for the T·AT triad at 25 °C.

Although reports on the enthalpy change of the association between ss-DNA and ds-DNA are relatively scarce,[11g,12,18] the dissociation process has been analyzed by various methods. The van't Hoff enthalpy change, $\Delta H_{\mathrm{vH}}^{\mathrm{dissoc}}$, measured by UV melting at different pH, salt concentrations, DNA lengths, and sequences ranges from 4.5 to 6.6 kcal/mol per base.[13] On the other hand, the calorimetric enthalpy change, $\Delta H_{\mathrm{cal}}^{\mathrm{dissoc}}$, measured by DSC for the same process is 2−5.8 kcal/mol per base,[14] which tends to be smaller than $\Delta H_{\mathrm{vH}}^{\mathrm{dissoc}}$. The magnitude of the present calorimetric enthalpy change for the association process, $\Delta H_{\mathrm{cal}}^{\mathrm{assoc}}$, falls at the lower end of $\Delta H_{\mathrm{vH}}^{\mathrm{dissoc}}$, and the higher end of $\Delta H_{\mathrm{cal}}^{\mathrm{dissoc}}$. The present $\Delta H_{\mathrm{cal}}^{\mathrm{assoc}}$ is also at the lower end of the denaturation enthalpy change of double helical DNA's.[19]

The van't Hoff enthalpy change for the association process, $\Delta H_{\mathrm{vH}}^{\mathrm{assoc}}$, measured by affinity cleavage titration for a 15-mer of mixed C/T oligonucleotides and a 339-bp plasmid fragment at pH 7.0 and 0.1 M NaCl is about -2 kcal/mol per base.[11g] This value is much smaller in magnitude than the present calorimetric enthalpy change. On the other hand, the present results show K to be insensitive to temperature (Table 1). If we assume that ΔH and ΔS are independent of temperature, the van't Hoff enthalpy change can be calculated by

$$\Delta H_{\mathrm{vH}}^{\mathrm{assoc}} = -R\frac{\partial}{\partial(1/T)} \ln K \qquad (1)$$

For a linear fit of this slope in the present temperature range, $\Delta H_{\mathrm{vH}}^{\mathrm{assoc}}$ was found to be -0.35 ± 0.79 kcal/mol. It might be thought that this temperature insensitiveness of K could be caused by an instrumental limit of ITC, since the K value is relatively large. The "c value" defined by $M_{\mathrm{tot}}K$, where M_{tot} is the concentration of molecule in the calorimetry cell, needs to be smaller than 1000 for the accurate measurement of K.[16b] In the present case, c is below 450. And, indeed, we observed a variation in K for the triplex formation with different sequences (see Table 2) around this c value, and were able to measure an even larger association constant than the present values. Furthermore, in the previous analyses by filter-binding assay, we also found that the binding constant of the triplex formation is similar to the values obtained by ITC and is insensitive to temperature at low pH.[12] Thus, all of this evidence precludes the possibility of any instrumental limit as the cause of the temperature insensitiveness of K. The $\Delta H_{\mathrm{vH}}^{\mathrm{assoc}}$ measured by filter binding assay varies from 0 to -0.8 kcal/mol per base as pH increases from 5 to 6.8.[12] Thus, the discrepancy between the van't Hoff enthalpy changes obtained by the affinity cleavage[11g] and the present ITC measurements appears to result from the difference in pH.

The discrepancies between ΔH_{vH} and ΔH_{cal} have been recognized, and possible causes of the discrepancies have been discussed.[20] Weber[20a] has emphasized the importance of including the temperature dependence of ΔH and ΔS, and in several model calculations on protein subunit association, the calculated enthalpy changes differ significantly from the simple van't Hoff enthalpy changes. In the present case, the assumption that ΔH and ΔS are independent of temperature is not valid (Figure 2), i.e., we observed a large negative ΔC_p. Thus, we cannot use a linear least-squares fit to eq 1 to calculate the true enthalpy change for the triplex formation. If ΔC_p is assumed to be independent of temperature and to originate in a simple two-state binding process, we would expect the temperature dependence of K to be[20b]

$$\ln \frac{K}{K_0} = \frac{\Delta H_0 - T_0 \Delta C_p}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right) + \frac{\Delta C_p}{R} \ln \frac{T}{T_0} \qquad (2)$$

Inserting the observed ΔC_p (-914 (cal/mol)K^{-1} by the linear fitting) and the values of $\Delta H_{\mathrm{cal}}^{\mathrm{assoc}}$ (-83.8 kcal/mol) and K_0 (9.08×10^7 M^{-1}) at $T_0 = 25$ °C into this equation, the predicted temperature dependence of ln K would be the dashed curve shown in Figure 5. This expected temperature dependence of ln K based on the constant ΔC_p model deviates significantly from the experimental data. Although the titration curves in ITC measurements appear to be fit well by a two-state transition (Figure 1), the discrepancy in the temperature dependence of ln K suggests that triplex formation may be more complex than

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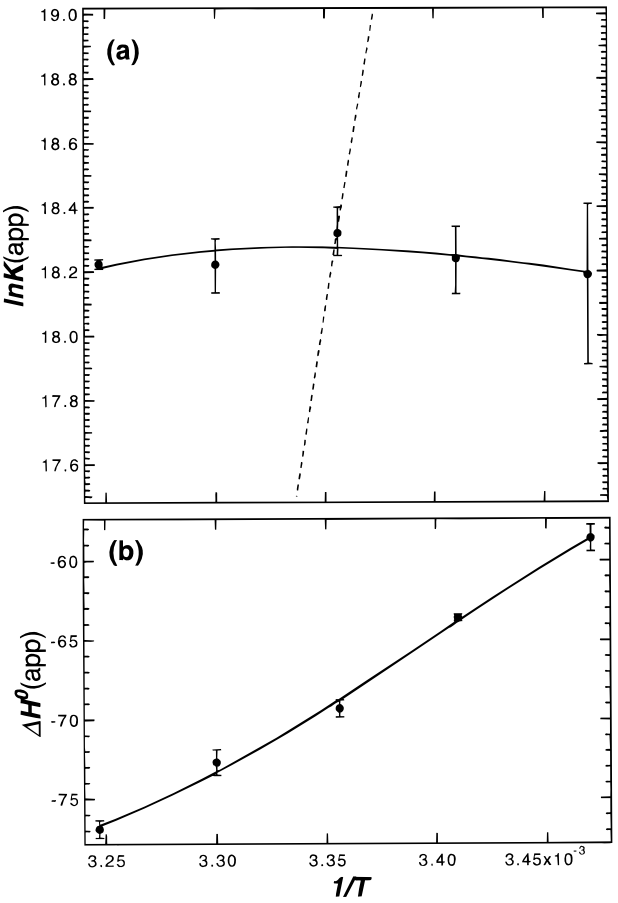

Figure 5. (a) Temperature dependence of experimentally observed K(app) (solid circles). The dashed line is the temperature dependence predicted by eq 2 with the experimentally observed values: $\Delta C_p = -914$ (cal/mol)K^{-1}, $\Delta H_0^\circ = -83.8$ kcal/mol, and $K_0 = 9.08 \times 10^7$ M^{-1} at $T_0 = 25$ °C. The solid line is the curve fitted by using eq 7 with the fitted parameters: $\Delta S_0^\circ = -229$ (cal/mol)K^{-1}; $\Delta H_0^\circ = -87.3$ kcal/mol; $\Delta H_u^\circ = 6.1$ kcal/mol; and $T_m = 53.7$ °C. n was set to 15. (b) Temperature dependence of experimentally observed ΔH°(app) (solid circles). The solid line is the curve fitted by using eq 8 with the fitted parameters: $\Delta H_0^\circ = -87.3$ kcal/mol; $\Delta H_u^\circ = 2.9$ kcal/mol; and $T_m = 21.6$ °C. n was set to 15.

expected from a simple two-state binding process. In this respect, the origin of the observed large negative ΔC_p is interesting. ΔC_p is usually considered to indicate an involvement of hydrophobic effect in protein folding and molecular association. Thus, we evaluated the accessible surface area buried upon the triplex formation, in order to examine the contribution of any hydrophobic effect in the triplex formation. As shown in Table 3, the estimated ΔC_p values range between 6 and -120 (cal/mol)K^{-1}, depending on whether the free ss-DNA is completely stacked or unstacked. The magnitude of the calculated ΔC_p is far less than the experimental value, and at least -830 (cal/mol)K^{-1} of ΔC_p in the triplex formation remains to be explained by some factors other than a hydrophobic effect. Table 3 also shows the calculated ΔS, which contains hydrophobic effect and other contributions in the binding process. The observed ΔS(app) values are negative, opposite to what would be expected for a hydrophobic effect. The discrepancies in the observed and expected values of ΔC_p or ΔS have been reported for other association processes.[23,26]

Table 3. Estimation of ΔC_p and ΔS due to Hydrophobic Effect[a]

		ΔA_{np}	ΔA_p	ΔA_{tot}	ΔC_p	ΔS_{HE}
C$^+$·GC	(unstacked)	−118	−49	−69	−6.1	1.8
	(stacked)	−80	−22	−58	1.2	4.1
T·AT	(unstacked)	−118	−55	−63	−8.8	1.9
	(stacked)	−76	−23	−53	0	4.6
Total	(unstacked)	−797	−972	−1769	−119	28
	(stacked)	−341	−820	−1161	5.6	66

[a] ΔA_{np}, ΔA_p, and ΔA_{tot} in units of Å^2 represent changes in nonpolar, polar, and total solvent accessible surface areas due to the triplex formation. These values were calculated by rolling a probe size of a water molecule[21] over the surface. A probe size of 1.4 Å was used for the effective radius of water. We took three adjacent base pairs and a third strand with three matching bases[22] to calculate the surface of the central base triad, either T·AT or C$^+$·GC. Then, we calculated the surface areas for the triplex, ds-DNA, and ss-DNA. We considered two extreme stacking states for free ss-DNA: one with fully stacked bases, which is the same as in the triplex form; and the other with completely exposed bases, where surfaces of aromatic planes are fully exposed. In both cases, we calculated the accessible surfaces of the central bases, and that of triplex was subtracted from those of ds-DNA and ss-DNA. Then, ΔA for the 15-mer/23-mer triplex was calculated by adding each triad contribution. ΔC_p and ΔS_{HE} due to the hydrophobic effect were calculated by the following empirical relations:[23] $\Delta C_p = 0.32\Delta A_{np} - 0.14\Delta A_p$; $\Delta S_{HE} = 0.32\Delta A_{np} \ln (T/386)$ in (kcal/mol)K^{-1}. In the case of triplex formation, exchanges of counterions and protons could make the effect of hydration more complicated. The values of ΔS should be corrected by the loss of translational and rotational degrees of freedom upon the association between ss-DNA and ds-DNA, which is approximately -50 (cal/mol)K^{-1}.[24] The entropy change should also be corrected by a cratic entropy, which reflects the entropy difference between a 1 M standard state and approximately infinite dilution, where the experiments are done. It is approximately -8 (cal/mol)K^{-1}.[25] The net entropy change corrected for these factors becomes 9 to -30 (cal/mol)K^{-1} for stacked and unstacked ss-DNA. The contribution of a conformational entropy of the ss-DNA may be estimated roughly by assuming three stable rotational states (*trans*, *gauche*+ and *gauche*-) for torsion angles, α, β, γ, ϵ, and ζ, and two states for the sugar ring ($C_{2'}$-*end* and $C_{3'}$-*end*) and χ (*anti* and *syn*), and that these degrees of freedom are frozen upon triplex formation. The net entropy change for a 15-mer ss-DNA will be -207 (cal/mol)K^{-1} ($15(5R \ln 3 + 2R \ln 2)$). In spite of the oversimplification, the estimation leads to a value close to the experimental value of -245 (cal/mol)K^{-1} at 25 °C.

Several factors such as vibrational[26c,27] and conformtional transitions[23] have been proposed to contribute to the ΔC_p and ΔS accompanying a binding process.

Considering the above circumstance, the assumption of the temperature independence of ΔC_p in eq 2 may not be valid for interpreting the present data of association constant and enthalpy change. Eftink *et al.*[28] built a general thermodynamic model of protein−ligand interaction which involves the coupling between multiple states of a protein and the binding processes. Such coupled processes can result in a heat capacity change even if the binding process is not accompanied by an intrinsic heat capacity change. Their model is worth considering for the present triplex formation because the free ss-DNA can assume multiple conformations. Although there is no detailed information on the conformational states of ss-DNA, we have observed conformational transitions of ss-DNA by CD melting, as shown in Figure 4; ss-DNA's exhibit conformational transitions with different melting temperatures depending on sequence and buffer conditions such as pH. Similar conformational transitions of ss-DNA have been observed by differential scanning calorimetry (DSC),[29] and it has been suggested that the conformational transition may contribute to the thermodynamics of melting of

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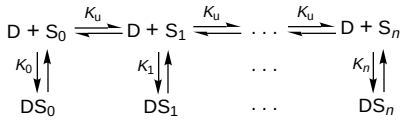
ds-DNA. Thus, it is quite likely that the conformational transition of ss-DNA can also affect the thermodynamics of triplex formation. The model of Eftink *et al.*[28] can be represented by the following scheme for the triplex formation:



where S_i represents the various conformational states of ss-DNA such as those with different extents of stacked bases, and D denotes ds-DNA. K_u represents the equilibrium constant for the stacking and unstacking between the microstates of ss-DNA. $K_0 = \exp(\Delta S_0^\circ/R - \Delta H_0^\circ/RT)$, and K_i represents the equilibrium association constant between ds-DNA and the *i*th microstate of ss-DNA. Each DS_i represents a complex between ss-DNA and ds-DNA, which will lead to a triplex. According to Eftink *et al.*,[28] the apparent association constant, K(app), and apparent enthalpy change, ΔH°(app), are given by

$$K(\text{app}) = K_0 \frac{1 + \sum_{i=1}^{n} \gamma_i p_i}{1 + \sum_{i=1}^{n} p_i} \tag{3}$$

$$\Delta H^\circ(\text{app}) = \Delta H_0^\circ + \frac{\sum_{i=1}^{n} i\gamma_i \epsilon p_i}{1 + \sum_{i+1}^{n} \gamma_i p_i} - \frac{\sum_{i=1}^{n} i\epsilon p_i}{1 + \sum_{i=1}^{n} p_i} \tag{4}$$

where p_i is the probability of the *i*th microstate of ss-DNA and is assumed to be expressed by $n!\exp(-i\epsilon/RT)/(n-i)!i!$, where ϵ represents the average energy of intramolecular interaction (in the case of ss-DNA, this will be mostly the stacking energy). γ_i is the ratio K_i/K_0. The apparent heat capacity change, ΔC_p(app), is given by the derivative of eq 4 with respect to T. When γ_i is assumed to decrease with i as e^{-i}, the peak and width of the microstate distribution p_i would change. The apparent heat capacity change can result from such a ligand-induced shift in the microstate distribution, even if the intrinsic heat capacity change is absent in the ligand binding process. The coupled process can also result in a temperature-insensitive region in the ln K(app) vs $1/T$ plot, such as we have observed in the triplex formation.

Ferrari and Lohman[30] applied the model of Eftink *et al.*[28] to a particular case of the binding of an *n*-mer ss-DNA to single-strand-binding protein (SSB). An apparent large negative ΔC_p(app) has been observed for the binding between SSB and $dA(pA)_n$.[30] By assuming independent base stacking among all the base combinations within a single strand, and that only the completely unstacked ss-DNA can bind to SSB (i.e., $\gamma_i = 0$, $i = 1, ..., n$), K(app) and ΔH°(app) are given by

$$K(\text{app}) = K_0\left(\frac{K_u}{1 + K_u}\right)^n \tag{5}$$

$$\Delta H^\circ(\text{app}) = \Delta H_0^\circ + \frac{n\Delta H_u^\circ}{1 + K_u} \tag{6}$$

(30) Ferrari, M. E.; Lohman, T. M. *Biochemistry* **1994**, *33*, 12896−12910.

where K_u and ΔH_u° represent the equilibrium constant and enthalpy change of unstacking of ss-DNA, respectively. $K_u = \exp((-\Delta H_u^\circ/R)(1/T - 1/T_m))$, where T_m is the melting temperature of base stacking in ss-DNA. On the other case, where the base stacking is assumed to take place only sequentially along the strand, i.e., each microstate consists of only a single state, eqs 5 and 6 become

$$K(\text{app}) = K_0 \frac{K_u^n}{1 + \sum_{i=1}^{n} K_u^i} \tag{7}$$

$$\Delta H^\circ(\text{app}) = \Delta H_0^\circ + \Delta H_u^\circ \left(n - \frac{\sum_{i=1}^{n} iK_u^i}{1 + \sum_{i=1}^{n} K_u^i} \right) \tag{8}$$

Equations 3 and 4 contain rather complicated distributions of the conformational states of ss-DNA and more adjustable parameters. Thus, we applied simpler eqs 5 through 8 to fit the temperature dependences of the experimentally observed ln K(app) and ΔH°(app), by considering ΔS_0°, ΔH_0°, ΔH_u°, T_m, and n as variables. The experimental ln K(app) and ΔH°(app) could be fit well with reasonable ranges of parameters even with these simplest models (Figure 5). By nonlinear least-squares fitting of ln K(app), the estimated values of ΔS_0° range from −210 to −230 (cal/mol)K^{-1} with either eq 5 or 7. On the other hand, the values of ΔH_0° range from −80 to −100 kcal/mol, and eq 5 tends to yield a larger magnitude of ΔH_0°. The ΔH_u° value is 6−7 kcal/mol, and T_m is 50−90 °C. On the other hand, the fitting of experimental ΔH°(app) by eq 6 or 8 yielded similar values of ΔH_0° and ΔH_u° but the range of estimated T_m is 3−20 °C. The larger T_m obtained by the fitting of ln K(app) could be due to larger experimental errors in ln K(app) within narrow temperature range. Smaller n did not improve the fitting. The fitting with the above coupled process yielded ΔC_p(app) near the experimental values (−914 (cal/mol)-K^{-1} obtained by a linear fitting), even if there is no heat capacity change associated with either the conformational equilibrium of ss-DNA or the binding process. This calculated ΔC_p(app) varies from −700 to −1100 (cal/mol)K^{-1} as temperature changes from 35 to 15 °C. The above coupled model of triplex formation can explain the temperature dependences of ln K(app) and ΔH°(app), and the magnitude of ΔC_p(app) as observed experimentally. However, we cannot uniquely specify the particular model and evaluate all parameters accurately based on the present data alone. More careful analyses of the conformational transitions of ss-DNA would be necessary to evaluate more accurately values of the parameters such as ΔH_u°, T_m, and n.

The present results strongly suggest that the conformational states of ss-DNA play an important role in the thermodynamics of triplex formation. We have found that the conformational equilibria of the ss-DNA shown in Figure 4 are pH dependent, i.e., increasing pH lowers T_m. Thus, the pH dependence of the ΔH_{vH}^{assoc} observed by the filter-binding assay[12] could be partly attributed to the conformational transition of ss-DNA. We also found that the stability of ss-DNA is strongly sequence dependent (Figure 4). Alternating T/C sequences, TC15 and TC19, are stable at room temperature, whereas randomly mixed T/C sequences, Pyr15T and Pyr19C, are much less stable. The

coupling of the conformational equilibrium of disordered ss-DNA to the triplex formation is similar to the protein folding problem, in which the disordered unfolded state of proteins can significantly contribute to the thermodynamics of protein folding. This suggests that more attention should be paid to the conformational states of ss-DNA for analyzing the mechanism of triplex formation.

The present analyses of sequence dependence show that single mismatches in the triplex do not have strong effects on K and thereby ΔG. On the other hand, ΔH_{cal} exhibits more sequence-dependent variations, as shown in Table 2. This result may be rationalized by assuming a nucleation-elongation process in the triplex formation; in the nucleation step only a few base contacts of the Hoogsteen type may be formed between ss-DNA and ds-DNA, and this may be followed by a slower elongation step, in which base pairing progresses to form a complete triplex. The observed K and ΔG, which may mostly reflect a rapid equilibrium of the nucleation step, would be relatively insensitive to single mismatches, since the nucleation step may contain only limited numbers of base tripling between ss-DNA and ds-DNA. On the other hand, the observed ΔH_{cal} would be mainly contributed by the elongation step, which may accompany enthalpically favorable base stacking and hydrogen bonding. The present results also show that triplex formation is accompanied by a large negative ΔS, which counteracts the effect of a large negative ΔH_{cal}. As a result, the values of ΔG are rather small.

These results offer some hints for designing modified oligonucleotides with enhanced sequence specificity. We suggest here that the design of a highly specific triplex DNA would require attention not only to the interaction between ss-DNA and ds-DNA but also to the state of the free ss-DNA. One such strategy is to amplify the contribution of ΔH_{cal}, which is highly sequence dependent, to ΔG by suppressing the negative ΔS. A major source of the negative ΔS may come from flexibility of ss-DNA (see the legend of Table 3), which becomes substantially restricted in the triplex structure. If a modification could make the chain more rigid without changing the required conformation of nucleotides, the magnitude of ΔG might become larger and more sensitive to sequence. Such oligonucleotides with enhanced specificity as well as stability could be more readily applied as antisense drugs.

Experimental Section

Preparation of DNA. We synthesized 15-mer oligo-DNA's, 5′-CTCTTCTZTTCTTTC-3′ (denoted as Pyr15Z), and complementary 23-mer's, 5′-GCGCGAGAAGAYAAGAAAGCCGG-3′ (Pur23Y) and 5′-CCGGCTTTCTTXTCTTCTCGCGC-3′ (Pyr23X), by DNA synthesizer (ABI 381-A), where X, Y, and Z were varied to give different combinations of base triad, and purified them by HPLC with an C18 reverse phase column (WAKO-SIL DNA). The sample concentrations were determined by UV absorption. After annealing the complementary strands, the sample was passed through a hydroxyapatite column (KOKEN) to remove any unpaired single strands. We also prepared other single strands, 5′-TCTCTCTCTCTCTCT-3′ (TC15), 5′-TCCTCT-TCTCTTCTTTCTT-3′ (Pyr19C), and 5′-TCTCTCTCTCTCTCTCTCT-3′ (TC19), for the CD melting study. The purified oligo-DNA's were dialyzed against the binding buffer (10 mM sodium acetate/10 mM cacodylic acid, pH 4.8, 0.2 M NaCl and 20 mM $MgCl_2$).

Calorimetry. For the measurement of heat produced by the triplex formation, we used the ITC system, OMEGA, and MCS (Microcal, Inc.).[16b] The concentrated samples of the 15-mer oligo-DNA's were injected at a constant interval into the cell containing the 23-mer duplex. The heat pulse produced by the mixing decreases as the injection progresses. The residual heat that remains after the saturation is due to the heat of dilution by the injectant, and this effect was corrected by measuring the titration against the buffer. The integrated heat pulses were plotted against the total ligand injected into the cell, and the titration curve was fit by nonlinear least-squares analysis using the Origin software (Microcal, Inc.) to determine the enthalpy change and binding constant.

Circular Dichroism. The denaturation of triplex was measured by circular dichroism (CD) spectropolarimeter JASCO J-720. The melting profiles of triplex were recorded at 220 nm, whereas 285 nm was used to monitor the melting of single strands. Data were collected in the temperature range between −5 and 85 °C at a heating rate of 5−50 °C per h. The measurements were made using a square quartz cell with a 1 mm path length. Temperature was controlled by a Periche controller. The sample solution for triplex contains 10 μM of each strand in the same buffer as in the calorimetric measurement.

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