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A thermodynamic study on the formation and stability of DNA duplex at transcription site for DNA binding proteins GCN4

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Abstract

Using isothermal titration calorimetry (ITC), we studied the thermodynamic parameters of the 15-mer duplex dsDNA [d(GAGATGACTCATCTC)]·[d(GAGATGAGTCATCTC)] formation from its two complementary single strands (S1 and S2) over a range of temperatures. The two complementary single strands d(GAGATGACTCATCTC) (herein called S1) and d(GAGATGAGTCATCTC) (herein called S2) containing palindromic sequences may assume ordered structures at low temperatures, which made the duplex dsDNA formation rather complicated. The thermodynamic parameters for the duplex formation, such as the binding constants (K_b), the enthalpies (ΔH^0), the free energies (ΔG^0), the entropies (ΔS^0) are strongly temperature-dependent. The thermally-induced disruptions of the duplex and its two complementary single strands, S1 and S2, were measured using differential scanning calorimetry (DSC) and CD spectroscopy, the results demonstrate that the DNA duplex is very stable, and its component single strands have an ordered structure at low temperature. This 15-mer specific sequence DNA may act as recognition site for DNA binding proteins GCN4 and plays a key role in transcription regulation of gene expression. Our analyses of the thermodynamic data suggest that the duplex formation is a coupled process between conformational transitions in the two single strands and their binding to form duplex dsDNA.

Keywords: DNA duplex formation; Thermodynamic; Thermal stability; Enthalpy; ITC; DSC; CD

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1. Introduction

It is both important and valuable to understand the stability of DNA secondary structures under a variety of conditions in many significant biochemical applications. A number of studies of thermodynamic stability of various sequences of DNA have been presented [1–6]. These studies confirm that the overall stability and the melting behavour of a DNA duplex structure depend on its base sequence. Marky et al. [1] demonstrated that DNA duplex structures can thermodynamically be considered to be the sum of their nearest-neighbor interactions. Recently, differential scanning calorimetry (DSC) and UV spectroscopy have been employed to characterize thermally-induced helix-to-coil transitions in specially designed and synthesized oligomeric and polymeric DNA molecules [7,8]. The thermodynamic function (ΔH^0) upon duplex formation has also been investigated by isothermal titration calorimetry (ITC) for some DNA oligomers with known sequence [9,10].

Specific sequence DNA structures are of interest from a biological point of view, because they may act as recognition sites for DNA binding and modifying proteins. DNA molecules containing palindromic sequences have been implicated to be involved in gene regulation, protein–DNA recognition and drug–DNA binding affinity and specificity [11–14]. The complementary single strands with a specific sequence can form ordered structures, which can significantly influence the duplex formation [15]. However, because of the difficulties in quantitatively analyzing equilibrium data associated with these single strands forming duplexes at room temperature, the thermodynamic characterization (ΔH^0, ΔG^0, ΔS^0) of the formation of these sequence-specific DNA duplexes from complementary ordered single strands are conspicuously lacking. Furthermore, the affinities that the complementary single strands exhibit in forming the unhybridized or hybridized DNA duplexes are also deficient. This deficiency is serious because such data are required for learning their many biological functions such as the control of transcription and recognition of regulatory proteins.

Our objective is to investigate the formation of double helical DNA duplexes from two complementary single strands and the effects of temperature on DNA duplex formation and stability. In this article, we have conducted calorimetric investigations and CD measurements on the two complementary single strands shown below. They differ only from the central base, one is C (referred to as S1) and the other is G (referred as to S2).

Strand 1: 5′GAGATGACTCATCTC3′

Strand 2: 5′GAGATGAGTCATCTC3′

This 15-mer specified sequence DNA is of interest because it plays a key role in transcription regulation of gene expression and may act as recognition sites for DNA binding proteins such as yeast transcriptional activator GCN4 in vivo [16,17]. To be specific, we have measured the duplex formation enthalpy (ΔH^0) and binding constant (K_b) at various temperatures using isothermal titration calorimetry. The isothermal titration calorimetric results reported here provide a unique ability to determine detailed information on thermodynamic quantities such as enthalpy and binding constants of molecular associations directly from the heat produced by the reaction. At the same time, the standard free energies of binding and the standard entropies of binding can also be obtained. The data derived from these studies enable us to evaluate quantitatively the influence of single strands containing palindromic sequences on the formation of duplex, the stability and the melting behavior of a DNA duplex, and the influence of temperature on the thermodynamic properties of the duplex dsDNA formation. The interaction process of DNA binding proteins GCN4 and DNA has not been well characterized nor has its mechanism been understood, thus, it is important to investigate the details of thermodynamic properties for this specific sequence DNA duplex formation. The DNA designed for this study enable us to further investigate the mechanism and the conformational change of their interaction with proteins or peptides.

2. Material and methods

2.1. Solution preparation

The dried individual component strands were purchased from Sheng Gong Bioengineer Company, Shanghai, China. They were synthesized on an automatic DNA synthesizer Model 391 (PE company, USA) using standard phosphoramidite chemistry. Each strand synthesized was purified by C18 reverse-phase HPLC, and purity of the strands was checked by polyacrylamide gel electrophoresis and analytical reverse-phase HPLC. All solutions were prepared by directly dissolving dry and desalted oligomers. The buffer solution consisted of 10 mM sodium phosphate and 100 mM NaCl, adjusted to pH 7.4. The duplex was formed by mixing equal amounts of complementary strands and temperature annealing by heating at 80°C for 10 min, followed by slow cooling. The annealed samples were allowed to equilibrate at 4°C for 24 h before analysis. Oligonucleotide concentrations were determined spectrophotometrically by measuring the absorbance at 260 nm, the extinction coefficients (ε): 1.420×10^{-5} for S1 and 1.422×10^{-5} $M^{-1}cm^{-1}$ for S2. The ε values were determined directly by using a nearest-neighbor analysis [18].

2.2. Isothermal titration calorimetry (ITC)

The measurements of the heat of mixing a single strand with its corresponding complementary strand at various temperatures were carried out with an isothermal titration calorimeter (ITC) from CSC Company, USA. For DNA duplex, solutions of one strand were used to titrate the complementary strand to form a duplex. A 250-μl syringe was used for the titrant, mixing was affected by stirring this syringe at 300 rev./min. Typically 25 injections of 10 μl each were performed in a single titration at temperatures of 25, 35, 40, 45, and 50°C, respectively, wherein duplexes are formed, that is, temperatures approaching that where the single strands started to melt. The reference cell of the calorimeter acts as a thermal reference to the sample cell, and was filled with buffer. To correct for single strand heats of dilution, the control experiments were also performed at various temperatures using similar conditions with buffer solution only. All solutions were degassed to reduce the noise. At each temperature, the instrument was electrically calibrated by means of a standard electric pulse. The concentrations of the two complementary single strands for titration are 1.4×10^{-5} and 8.8×10^{-5} M, respectively. The buffer contains 100 mM NaCl, 10 mM sodium phosphate, pH 7.4.

The isothermal titration calorimetry (ITC) measurements were designed to obtain primarily the enthalpy of each complex formation and their stoichiometries. The heats of each reaction were determined by integration of the peaks observed. After the contribution from the heat of dilution of each injection was subtracted, the heat was plotted against the molar ration of the two complementary single strands. The binding constants (K_b), enthalpy of binding (ΔH^0), and stoichiometry (N) of the formation of duplex were determined by fitting the binding isotherm against the binding equation described by Freire et al. [19] using the independent binding model.

2.3. High-sensitivity differential scanning calorimetry (DSC)

Differential scanning calorimetry can be used to detect and follow thermally-induced order–disorder transitions in oligonucleotides and other molecules. Calorimetric heat flow (mW) vs. temperature (T) profiles were directly measured for the duplex and the two component single strands using Micro DSC III, Setaram, France. The calorimeter is connected with the CS32 controller and interfaced with a compatible IBM computer, using the software package for data acquisition and analysis. The instrument was calibrated with a standard electrical pulse. A scan rate of 1°C/min was employed from 10 to 110°C throughout. An extra pressure of 1 atm was maintained during all DSC runs to prevent possible degassing of the solutions on heating. In all experiments, the reference cell was filled with the buffer and all samples were degassed to reduce the noise. The concentrations of the oligomers for the calorimetric studies in single strand were

2.9×10^{-4} M for dsDNA, S1 and S2. The buffer solution used for the calorimetric measurements contained 100 mM NaCl, 10 mM sodium phosphate, pH 7.4. The area of the resulting curve is proportional to the transition heat, which when normalized for the number of moles is equal to the transition enthalpy (ΔH_{cal}).

2.4. Circular dichroism (CD) spectroscopy

CD spectroscopy for the complementary single strands S1, S2 was measured using a CD6 Spectropolarimeter, Jobin-Yvon Company, France. This instrument is computerized and is equipped with a programmable thermoelectrically controlled cell holder. The 0.1-cm cells were used and the concentration of all samples were 18 μM. All solutions contained 10 mM sodium phosphate buffer, pH 7.4, 0.1 mM EDTA and 100 mM NaCl.

3. Results

3.1. Isothermal titration calorimetric experiments

We carried out isothermal titration calorimetric experiments on the duplex dsDNA formation from the two complementary single strands at 25, 35, 40, 45, and 50°C, respectively. Fig. 1 shows experimental data for a typical titration for duplex formation between the two complementary single strands, S1 and S2, at 40°C. The upper panel shows the trace recorded for each of 25 10-μl injections made at 600-s intervals. The significant observation is that we measured exothermic enthalpies for the formation of the dsDNA. The area of each peak was integrated and corrected for the heat of dilution, which was estimated by a separate experiment by injecting the titrant strand into the buffer. By fitting the titration curve with a non-linear least-squares method, the enthalpy change ΔH^0, and the equilibrium binding constant K_b of DNA duplex formation can be estimated with the assumption of an independent binding site model for duplex formation. The lower panel shows the fit of each integrated heat to a titration curve calculated on the basis of one binding site model. The results

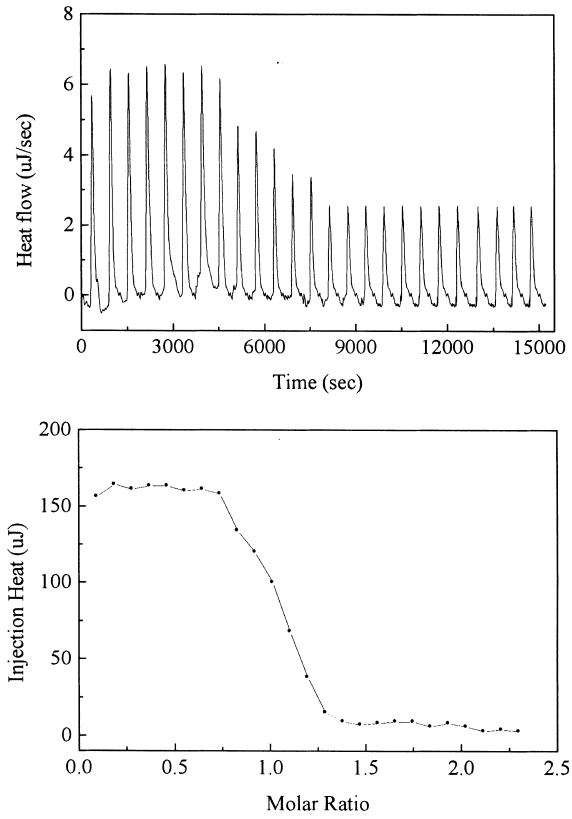

Fig. 1. (a) Typical ITC profile of the binding process between the 15-mer single strands S1 and S2 at 40°C. Buffer contains 10 mM sodium phosphate, pH 7.4, and 100 mM NaCl. Each peak corresponds to a 10-μl injection containing 89 μM S2 into the cell containing 14 μM S1. (b) The results of curve fitting to a single-site model by the software supplied with the calorimeter.

obtained by this curve fitting were $N = 0.98 \pm 0.02$ binding sites per molecule, $K_b = 1.34\ (\pm 0.12) \times 10^6\ M^{-1}$ and $\Delta H^0 = -158.9 \pm 0.6$ kJ/mol.

The equilibrium properties of double helical duplex formation at various temperatures are summarized in Table 1. These properties depend on temperature. The N values for DNA duplex formation are slightly lower than 1. However, the deviations of this magnitude decrease as the temperature rises. The possible reasons for the deviations we think are that the two complementary single strands assume multiple conformations (see Section 4). After correction for dilution heats at each temperature, the molar binding enthalpies

Table 1
Thermodynamic parameters of dsDNA formation at different temperatures

T (°C)	$K_b \times 10^{-6}$ (M^{-1})	ΔH^0 (kJ/mol)	ΔG^0 (kJ/mol)	ΔS^0 (J/K·mol)
35	1.38 ± 0.06	-128.2 ± 3.7	-36.2 ± 0.1	-298 ± 12
40	1.34 ± 0.12	-158.9 ± 0.6	-36.7 ± 0.2	-392 ± 4
45	0.93 ± 0.16	-228.0 ± 0.3	-36.3 ± 0.4	-607 ± 4
50	0.28 ± 0.05	-281.4 ± 0.9	-33.7 ± 0.4	-767 ± 4

and binding constants K_b for dsDNA duplex formation were calculated by fitting calorimetric titration data using the calorimetric software DataWorks and BindWorks supplied with the calorimeter. The standard free energies of dsDNA formation (ΔG^0) were obtained from the equation $\Delta G^0 = -RT\ln K_b$, in which the K_b is the binding constant at the corresponding temperature. The ΔS^0 function was calculated from the standard thermodynamic relation $\Delta G^0 = \Delta H^0 - T\Delta S^0$. We obtained favorable free energies that result from partial compensation of favorable enthalpies with unfavorable entropy.

The temperature dependence of the enthalpy change in the temperature range studied here enabled us to evaluate the heat capacity change, ΔC_p, associated with the duplex formation from the complementary palindromic single strands. From Table 1, we observed that the apparent ΔH^0 decreases with increasing temperature, indicating that the duplex formation is accompanied by a negative ΔC_p. The plot of ΔH vs. T shows a nearly linear dependence in the temperature range from 35 to 50°C. Fitting the plot of ΔH^0 vs. T with a straight line, we obtained the estimated value of ΔC_p of -10.6 J/mol/K in the temperature range studied here.

3.2. Calorimetric melting of S1 and S2 single strands

In order to make sure that the duplex dsDNA is stable in the temperature range studied and determine whether a temperature-dependent conformational transition of the two complementary single strands might play a role in the duplex formation, we measured their thermal disruption by DSC. Fig. 2 shows a typical calorimetric trace (heat flow vs. temperature) for the thermally-induced, order–disorder transition of S1 (curve a), S2 (curves b) and dsDNA in 100 mM NaCl, 10 mM sodium phosphate, pH 7.4 buffer, which provide us with the characterization of their thermal transition. Each of three curves shown in Fig. 2 was obtained under identical conditions for the heating scan. Note that the dsDNA and the two single strands exhibit large endothermic effects that reflect the thermally-induced disruption of the ordered structures. However, comparison of these three curves in Fig. 2 reveals a dramatic difference. The shape of the dsDNA thermal transition with the T_m of 71.3°C is sharp, which is the typical thermodynamic profile for double helical nucleic acid structures. However, by contrast, the thermal disruptions of the two single strands are rather broad, covering a temperature range

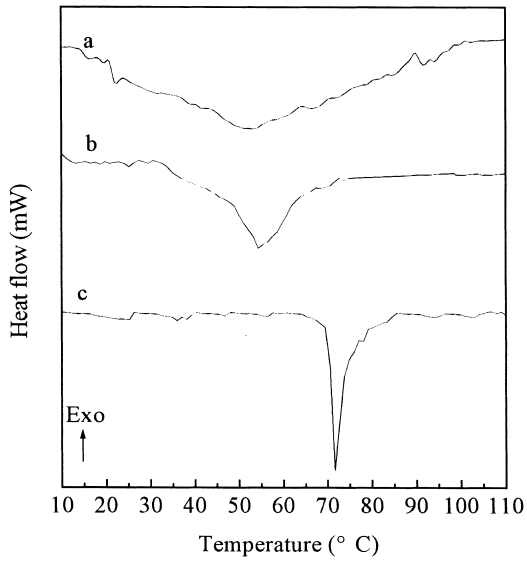

Fig. 2. Differentail scanning calorimetry curves for dsDNA and its two complementary single strands S1, and S2 in 10 mM sodium phosphate buffer containing 100 mM NaCl at pH 7.4. (a) single strand S1; (b) single strand S2; (c) duplex dsDNA.

from 14 to 80°C for S1 and from 35 to 70°C for S2. The results from our experiments are in agreement with other temperature-dependent studies on equilibrium properties of single-stranded oligonucleotides [15].

For each transition, the area under the experimental heat flow vs. temperature curve is proportional to the total endothermic heat needed for random-coil disruption. By averaging multiple runs, the transition enthalpy ΔH_{cal} was obtained directly when these heats were normalized for the total number of moles of strands. Transition temperatures T_m and the corresponding calorimetric enthalpies (ΔH_{cal}) are listed in Table 2. The enthalpy of dsDNA disruption is 380.6 kJ/mol at 71.3°C, which indicates that the dsDNA is considerably stable thermally. The value we obtained is in excellent agreement with the value of 387.7 kJ/mol calculated from the sum of its nearest-neighbor interactions according to the method reported by Breslauer et al. [1].

3.3. CD spectroscopy

In order to determine the conformational transition of the single strands with increasing temperature, we measured temperature-dependent CD spectra for the two single strands S1 and S2. Fig. 3 shows the results, the upper and lower panels are for S1 and S2, respectively. The shapes of the CD spectra shown in Fig. 3 indicate that at low temperature, both single strands exhibit CD spectra characteristic of an ordered structure. At approximately 80°C, the CD spectra for S1 and S2 exhibit altered shapes and reduced intensities, reflecting that the ordered structure present at low temperatures has been thermally disrupted.

Table 2
Calorimetrically measured enthalpies for the melting (ΔH_{cal}) and melting temperatures (T_m) for dsDNA and its component single strands

DNA	T_m (°C)	ΔH_{cal} (kJ/mol)
S1	52.5	347.8
S2	54.5	282.4
dsDNA	71.3	380.6

4. Discussion

The objective of these studies was to investigate the thermodynamic parameters of the DNA duplex [d(GAGATGACTCATCTC)] · [d-(GAGATGAGTCATCTC)] formation from two complementary single strands (S1 and S2) and the influence of the temperature on its formation and stability. The results obtained here form the basis for our further studies on its interaction with DNA binding proteins. Isothermal titration calorimetry (ITC) is a general method for measuring binding interactions [20–22]. Since it measures heat directly, it is the only technique [23] which allows simultaneous determination of all

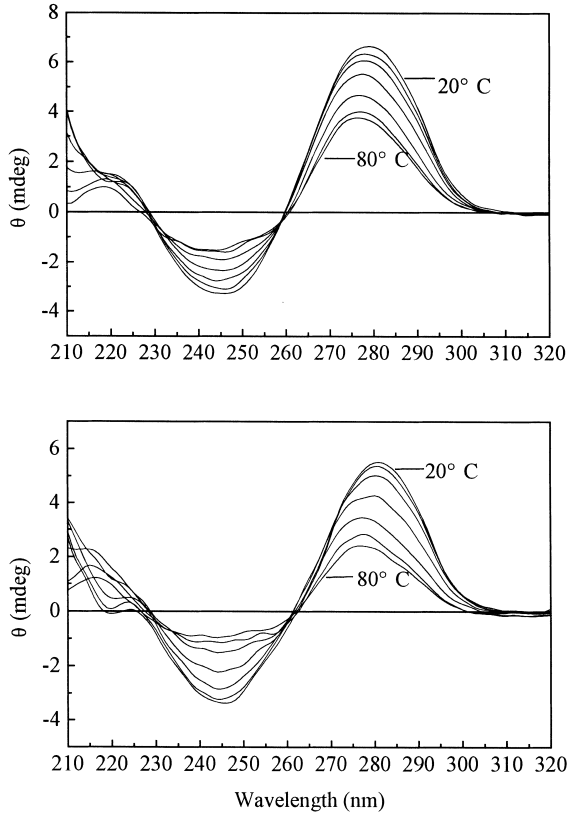

Fig. 3. Family of temperature-dependent CD spectra for the single strands S1 and S2 at $T = 20$, 30, 40, 50, 60, 70, and 80°C. The concentration is 18 μM for both single strands and the buffer contains 10 mM sodium phosphate, 100 mM NaCl, and 0.1 mM EDTA, at pH 7.4. The upper and lower panels are for S1 and S2, respectively.

binding parameters (K_b, ΔH^0, ΔG^0, ΔS^0). In our studies, binding interaction between the two complementary single strands can be characterized in the general form as below,

$$S1 + S2 \rightleftharpoons dsDNA \quad (1)$$

At each temperature, the thermodynamic equilibrium system in solution has two processes, the dsDNA formation and the dsDNA disruption, which are reversible and form a dynamic state equilibrium process. The binding constant K_b and the heat of binding ΔH^0 are the independent variables of thermodynamic interest.

4.1. Enthalpies of duplex formation

The observed binding enthalpies for the duplex formation from two complementary strands at various temperatures are listed in Table 1. The results show that the binding enthalpies are all endothermic in the temperature range studied herein, but decrease with increasing temperature. This indicates that the duplex formation is accompanied by an apparently large negative ΔC_p. The ΔH^0 is nearly linearly dependent on the temperatures studied. If we assume ΔC_p is independent of temperature in the temperature range studied, and linearly fit this plot, the value of ΔC_p is calculated to be -10.6 J/mol/K in the temperature range 35–50°C.

Comparing with the enthalpy for duplex disruption measured by DSC melting at the duplex thermal transition temperature 71.3°C, the enthalpy differences between duplex formation and duplex disruption are observed. The enthalpy for duplex disruption (380.6 kJ/mol) is larger than the duplex formation enthalpies of -128.2, -158.9, -228.0 and -281.4 kJ/mol at 35, 40, 45 and 50°C, respectively. This disparity can be interpreted in terms of the differences in the enthalpy states of the single strands at different temperatures [15]. The enthalpies for duplex formation comprise endothermic contributions from the disruption of the single-strand stacking interactions prior to duplex formation, and exothermic contributions from the duplex formation, which include base–stacking interactions, hydrogen-binding, Van der Waals interactions, hydration interactions, etc. [9].

Breslauer [15] has constructed a thermodynamic cycle to interpret the enthalpy difference between duplex formation at 25°C and duplex disruption at 74°C. The following model is worth considering for the present duplex formation and enables us to rationalize the disparity and evaluate the contributions of the single strands stacking interactions to the duplex formation.

$$\begin{array}{ccc} & \xrightarrow{\Delta H_1} & \\ \text{Duplex } (10^\circ\text{C}) & \longrightarrow & S_1\ (\approx 80^\circ\text{C}) + S_2\ (\approx 80^\circ\text{C}) \\ \downarrow \Delta H_2 & & \uparrow \Delta H_4 \end{array}$$

$$\text{Duplex } (T) \xrightarrow[\Delta H_3]{} S_1(T) + S_2(T)$$

ΔH_1 corresponds to ΔH_{cal}, the transition enthalpy was obtained from DSC melting, which also depends on the temperature range used to integrate. ΔH_3 corresponds with $-\Delta H^0$, the negative of the duplex formation enthalpy measured by ITC at 35, 40, 45 and 50°C. ΔH_2 is equal to zero since our DSC profile for dsDNA reveals no enthalpy change in heating the duplex from 10°C to the temperature range studied here. ΔH_4 is the sum of enthalpy changes associated with the heating of each single strand from T to 80°C.

For the two single strands S1 and S2, we have measured the enthalpy changes of their thermal disruption using DSC (Fig. 2). The values, ΔH_{cal} and ΔH_{VH} for S1 and S2, are listed in Table 2. Inspection of Fig. 3 reveals that the transitions of S1 and S2 begin approximately 20°C lower than the temperature range studied in ITC. Thus, at different temperatures, the ΔH_4 values are different, i.e. the contributions of the ordered structure of S1 and S2 to the duplex formation are different. At low temperatures, the single strands can possess more ordered structures which should need more energy for random-coil transition first, and then enthalpically drive them to form a duplex. The enthalpy for duplex disruption at 71.3°C can be considered as the enthalpy for duplex formation in the overall random-coil states of the single strands. Thus, based on the thermodynamic

cycle shown above, $\Delta H_1 = \Delta H_2 + \Delta H_3 + \Delta H_4$, we can calculate the magnitudes of contribution of S1 and S2 to the duplex dsDNA formation at various temperatures. The results are 66.3%, 58.2%, 40.1%, 32.0% at 35, 40, 45, and 50°C, respectively.

From these ITC, DSC and CD experiments, we can conclude that at low temperatures (lower than T_m), the single strands can possess considerable ordered structure that can significantly influence the thermodynamic driving forces for duplex formation. In the present cases, there are two equilibria (as below) which may make significant contributions to the simple binding equilibrium [1] varying with temperature.

S1 (ordered structure) $\rightleftharpoons$ S1 (random coil) (2)

S2 (ordered structure) $\rightleftharpoons$ S2 (random coil) (3)

At equilibrium, the two single strands might have multiple conformational transitions, which make the duplex dsDNA formation studied here rather complicated. This suggests that more attention should be paid to the conformational states of single strands for analyzing the mechanism of duplex formation. Other interactions in this binding equilibrium system, such as hairpin formation in single strands and the intermolecular bulge duplex formation from same single strands must be considered. Although there is no detailed information on the conformational states of the single strands, the CD spectra of S1 and S2 (Fig. 3) confirm that they have ordered structures.

Furthermore, we have carried out similar experiments for duplex formation at lower temperatures (e.g. 25°C). The curve of Fig. 4 shows the heat produced in each of 25 10-μl injections of 7.2×10^{-5} M S2 into 0.5 ml of 1.3×10^{-5} M S1 at 25°C with 500-s intervals between an injections. It is evident that this binding process at 25°C is quite complicated, strongly suggesting that the single strands possess stable ordered structures at low temperature which cannot make them overcome the energy barrier of transitions to a random-coil state. Therefore, they cannot form the duplex dsDNA in the same form as that at high temperatures. The two palindromic single strands

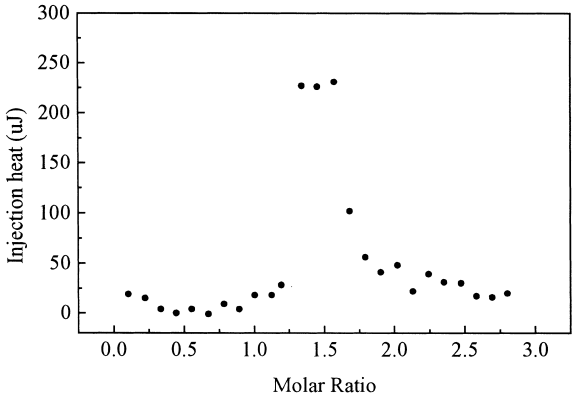

Fig. 4. Calorimeter data observed in the titration of 72 μM S2 into 0.5-ml of 13 μM S1 at 25°C under the same conditions as in Fig. 1. The injections were made at 500-s intervals.

are self-complementary and could adopt secondary structures such as hairpins or bulge duplex formed by intermolecular interaction below their thermal transition temperature (T_m) [24,25]. The secondary structure features of these two complementary single strands should significantly influence the duplex dsDNA formation.

4.2. Binding affinities

The binding constants for interaction between the two complementary single strands at several temperatures are listed in Table 1. While the binding free energy only increases slightly with temperature, the enthalpy of duplex formation decreases dramatically. The observed binding constant is lower at 50°C than that at lower temperatures (e.g. 35°C). This result indicates the duplex is more stable at low temperatures, which can be easily rationalized in the form of the equilibrium movement varying with temperatures. In solutions, the binding equilibrium (as shown in equilibrium 1) moves to duplex disruption transition with increasing temperature, so the binding affinities decrease with increasing temperature. The experimental K_b values obtained from ITC experiments are in the range of 10^5–10^6 M^{-1} at the temperature range studied herein. At lower temperatures, the binding constants K_b should be larger.

This binding equilibrium system is complex,

which may include other binding interactions, such as the binding between the same single strand forming the bulge duplex and the intra-molecular binding in the single strand forming hairpins. Thus, we have also carried out the ITC experiments under similar conditions to verify whether these binding interactions exist. The results indicate these interactions have not been observed in the binding process of the two single strands in the temperature range studied.

4.3. Free energy and entropy of duplex formation

Table 1 lists the free energy and entropy of DNA duplex formation from single strands S1 and S2 at several temperatures. The ΔG^0 are derived from the experimental values of K_b according to $\Delta G^0 = -RT\ln K_b$. The overall free energies of duplex formation will include contributions from exothermic enthalpy terms and entropy terms. The observed ΔG^0 changes slightly with the increasing temperature, which results from the partial compensation of exothermic enthalpies and unfavorable entropy that counteracts the effect of a large negative ΔH^0. According to Marky's work [9], the entropy contribution is due to the following contributions: the bimolecular association reaction, the complementary nature of the oligomers, changes in the oligomer configuration in going from single strands to duplex, uptake or release of counterions, uptake of water molecules, etc. The entropy contribution for the DNA duplex formation is more unfavorable at high temperatures. A major source of the negative ΔS^0 may come from the flexibility of the two single strands.

5. Conclusions

We have thermodynamically characterized DNA duplex formation at several temperatures and the thermal disruptions of its two complementary single strands. Our results show that at low temperatures, single strands can possess considerable stable secondary structures, which can influence the duplex DNA formation, especially those single strands containing palindromic sequences are very stable at room temperature. Consequently, due to this single strand base–stacking interaction, the mechanism of the DNA duplex formation is complicated, and the thermodynamic parameters for the duplex formation, such as the binding affinities, the formation enthalpies, the formation free energies, may change with temperature. Thus, potential contributions from the conformational states of single strands must be recognized and accounted for to study the formation of duplexes and high-order structures (such as triplex) from their component single strands.

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