

## The Energetics of HMG Box Interactions with DNA: Thermodynamic Description of the Target DNA Duplexes

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The thermal properties and energetics of formation of 10, 12 and 16 bp DNA duplexes, specifically interacting with the HMG box of Sox-5, have been studied by isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC). DSC studies show that the partial heat capacity of these short duplexes increases considerably prior to the cooperative process of strand separation. Direct extrapolation of the pre and post-transition heat capacity functions into the cooperative transition zone suggests that unfolding/dissociation of strands results in no apparent heat capacity increment. In contrast, ITC measurements show that the negative enthalpy of complementary strand association increases in magnitude with temperature rise, implying that strand association proceeds with significant decrease of heat capacity. Furthermore, the ITC-measured enthalpy of strand association is significantly smaller in magnitude than the enthalpy of cooperative unfolding measured by DSC. To resolve this paradox, the heat effects upon heating and cooling of the separate DNA strands have been measured by DSC. This showed that cooling of the strands from 100 °C to –10 °C proceeds with significant heat release associated with the formation of intra and inter-molecular interactions. When the enthalpy of residual structure in the strands and the temperature dependence of the heat capacity of the duplexes and of their unfolded strands have been taken into account, the ITC and DSC results are brought into agreement. The analysis shows that the considerable increase in heat capacity of the duplexes with temperature rise is due to increasing fluctuations of their structure (e.g. end fraying and twisting) and this effect obscures the heat capacity increment resulting from the cooperative separation of strands, which in fact amounts to  $200(\pm 40) \text{ J K}^{-1} (\text{mol bp})^{-1}$ . Using this heat capacity increment, the averaged standard enthalpy, entropy and Gibbs energy of formation of fully folded duplexes from fully unfolded strands have been determined at 25 °C as  $-33(\pm 2) \text{ kJ} (\text{mol bp})^{-1}$ ,  $-93(\pm 4) \text{ J K}^{-1} (\text{mol bp})^{-1}$  and  $-5.0(\pm 0.5) \text{ kJ} (\text{mol bp})^{-1}$ , respectively.

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Abbreviations used: SOX-5, HMG box from mouse Sox-5; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry;  $\Delta H$ , enthalpy;  $\Delta S$ , entropy;  $\Delta S^\circ$ , standard entropy at 1 M concentration;  $\Delta G$ , Gibbs energy;  $\Delta G^\circ$ , standard Gibbs energy at 1 M concentration; DNA<sup>16bp</sup>, 16 bp DNA duplex; DNA<sup>12bp</sup>, 12 bp DNA duplex; DNA<sup>10bp</sup>, 10 bp DNA duplex.

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## Introduction

In the first report of this series (Crane-Robinson *et al.*, 1998) we commented that in order to determine the energetics of complex formation, in particular protein/DNA complexes, it is essential to characterise the thermal properties of all the components. The thermal properties of the HMG box from mouse Sox-5 have already been described (Crane-Robinson *et al.*, 1998) and here we describe the thermal properties and energetics of formation of three target DNA duplexes.

Notwithstanding the many publications devoted to the thermodynamics of DNA duplexes, there remain some unclear points of primary importance, particularly with regard to the short duplexes typically used to study protein/DNA interactions. The principal point of concern is the temperature dependence of the heat capacity of a duplex and its change upon strand dissociation. Knowledge of the change in heat capacity upon strand separation is important because it determines the temperature dependence of all the thermodynamic characteristics of the folded (functional) state of DNA, i.e. of the enthalpy, entropy and Gibbs energy of formation of the double helix. These parameters are usually estimated at the temperature at which the double helix melts, i.e. at high temperatures, but to extrapolate these values to lower (physiological) temperatures we need to know the heat capacity effect on strand separation. Previous attempts to calorimetrically measure this heat capacity effect have not been fully successful due to inherent limitations in instrument base line stability. Consequently, extrapolation of the pre and post-transition heat capacity functions into the transition zone did not reveal noticeable changes in the heat capacity upon DNA unfolding. Therefore, the general assumption has been that the enthalpy and entropy of DNA unfolding are not temperature dependent. This is surprising because separation of the strands results in the hydration of newly exposed groups and their hydration should result in a change of heat capacity. It is known that the heat capacity of non-polar groups increases upon their transfer into water (Edsall, 1935; Privalov & Gill, 1988; Makhatadze & Privalov, 1990, 1995; Livingstone *et al.*, 1991) and there are many non-polar groups which become exposed upon DNA strand separation. However, there are also many polar groups, and their hydration has an opposite heat capacity effect (Makhatadze & Privalov, 1990, 1995; Murphy & Gill, 1991; Spolar *et al.*, 1992). Therefore, either these two hydration effects balance one another upon DNA unfolding, or there is some systematic error in the extrapolation of the heat capacities of the native and unfolded DNA to the transition midpoint. The assumption of no change in heat capacity on melting also contrasts with the observation that the melting enthalpies of DNA and polynucleotides increase as their thermostability rises (Filimonov, 1986; Klump, 1990), particularly when thermostability is changed by

variation of the ionic strength (Privalov *et al.*, 1969; Chalikian *et al.*, 1999). The influence of ionic strength on the thermostability of DNA is assumed to be entropic (Manning, 1972) and if this assumption is true, then the enthalpy of DNA melting should increase with rise of the melting temperature if the dissociation of strands results in a heat capacity increment.

On the other hand, if the state of the double helix and the separated strands change with temperature, and correspondingly their heat capacities change, the simple extrapolation of these functions into the transition zone is not justified. Under such circumstances, the widely used isothermal titration calorimetric (ITC) studies of DNA/protein interaction at different temperatures should be conducted with caution, since they are normally interpreted assuming that the heat capacity of the duplex does not change with temperature.

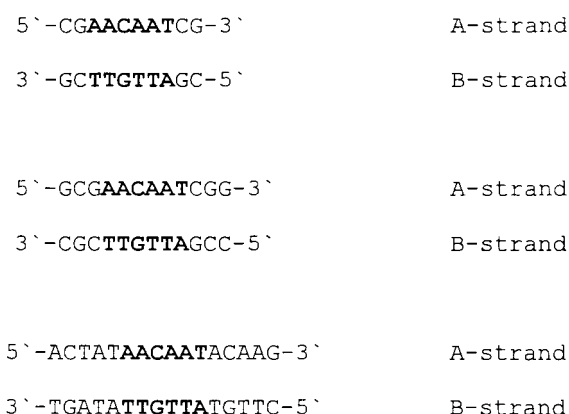
Unfortunately, we know almost nothing about the partial heat capacity of the DNA double helix or the separated oligonucleotide strands and their dependencies on temperature. The reason for this is that the differential scanning calorimeters (DSC) used for studying the thermal properties of macromolecules in dilute solution are sensitive instruments, but the base line in most contemporary models is rarely stable enough over a broad temperature range. These instruments are therefore used primarily for studying the relatively sharp heat effects of cooperative transitions but not for determinations of absolute values of the partial heat capacity of macromolecules over a broad temperature range. This is especially true for solutions of DNA and polynucleotides, since they are viscous and their viscosity changes upon heating, with consequent changes in convection in the cylindrical calorimetric cells, resulting in a redistribution of thermal gradients that induces artifacts in heating experiments. This was the main reason for developing the capillary Nano-DSC which is insensitive to viscosity changes and has an exceptionally stable base line (Privalov *et al.*, 1995). Using the Nano-DSC, Chalikian *et al.* (1999) have recently succeeded in measuring the heat capacity change upon melting of long double-stranded polynucleotides (see below) and report a  $\Delta C_p$  value similar to that which we determine in this study by a different method.

Here, we present the results of DSC and ITC studies of the thermodynamic characteristics of the three short DNA duplexes used in the accompanying paper (Privalov *et al.*, 1999) for studying the energetics of complex formation with the HMG box from mouse Sox-5. These results show that the apparent heat capacity of the duplex increases steeply with temperature due to increasing fluctuations of its structure and this effect obscures the heat capacity change associated with strand separation.

## Results and Discussion

### DNA duplexes

For this study we used 10, 12 and 16 bp DNA duplexes (Scheme 1) that were synthesized to investigate the DNA interactions with HMG pro-



Scheme 1.

teins which are described in the accompanying article (Privalov *et al.*, 1999), and therefore contain the specific DNA recognition site of the Sox-5 HMG box, AACAAT.

### Thermal properties of the DNA duplexes studied by DSC

The thermal properties of the DNA duplexes were studied by DSC at several concentrations in several different buffers, the standard buffer being 10 mM potassium phosphate (pH 6.0), 100 mM KCl, 1 mM EDTA. This particular buffer system was selected due to the necessity of obtaining the energetics of DNA duplex formation under identical conditions to those employed for HMG box/DNA interactions (Privalov *et al.*, 1999). Figure 1 shows a typical calorimetric recording of the apparent heat capacity of a DNA solution upon heating and subsequent cooling with a constant rate of 1 K/minute. The heat effects of unfolding and refolding of the DNA duplex appear as mirror images, albeit slightly shifted in temperature due to the slower refolding kinetics. The temperature-induced unfolding/refolding of the DNA is therefore highly reversible. In what follows we will primarily use the results obtained in cooling experiments since this permits one to reach lower temperatures (down to  $-10^{\circ}\text{C}$ ), which is important for obtaining an accurate measurement of the dependence of the heat capacity of a duplex in the temperature range below the region of its cooperative unfolding.

Figure 2 presents the partial molar heat capacity functions of the three duplexes examined in this study. It appears that the heat absorption peak of duplex melting develops from rather low temperatures and is somewhat asymmetric. There can be

several reasons for this asymmetry: (a) the transition is not two-state; (b) the dissociation of strands is a bi-molecular reaction; or (c) the melting of duplex results in significant heat capacity increase.

Linear extrapolation of the initial and final heat

capacities of the duplex into the transition zone (light lines in Figure 2), shows that at the temperature of maximum heat absorption (taken as the melting temperature,  $T_m$ , which to a first approximation is close to the transition midpoint  $T_i$ ; Privalov & Potekhin, 1986), the difference between the extrapolated heat capacities is very small. Since the heat capacity increment upon duplex unfolding appears to be small, the enthalpy of unfolding (the area of the excess heat absorption peak) can be determined simply by joining the initial and final heat capacity functions by a shallow line (broken

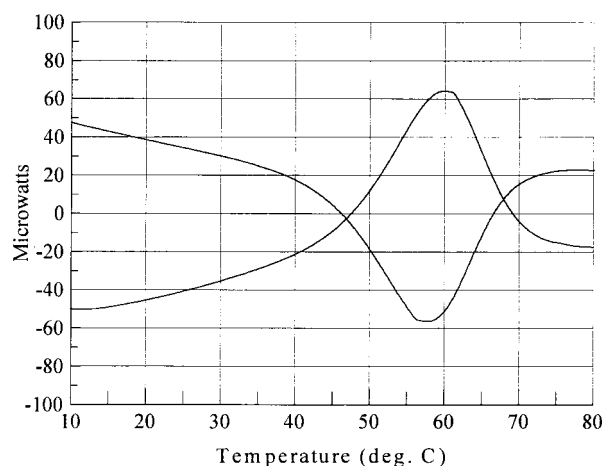
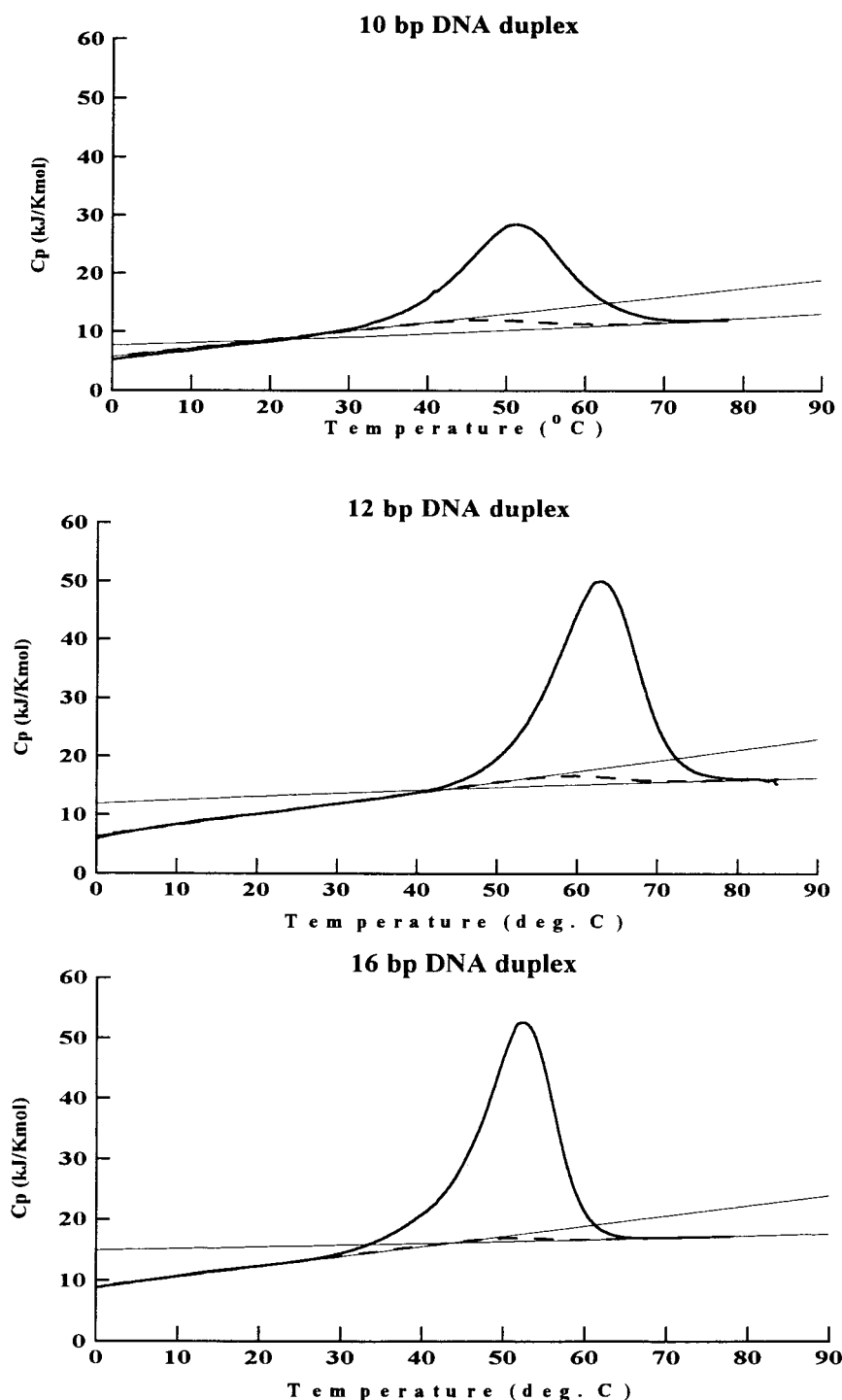


Figure 1. Original DSC recording on heating and cooling of the 10 bp DNA duplex at a rate of 1 K/minute showing excellent reversibility. Concentration of the duplex is 306  $\mu\text{M}$ ; solvent: 500 mM KCl, 10 mM phosphate, 1 mM EDTA (pH 6.0).



**Figure 2.** Measured heat capacity functions (continuous lines), for the 10 bp, 12 bp and 16 bp DNA duplexes at 204, 167 and 203  $\mu\text{M}$  concentrations, respectively. The light lines show linear extrapolations of the initial and final heat capacity functions into the transition zone. The difference in their values at the maximum of heat absorption, which is usually regarded as the heat capacity effect on unfolding, appears to be very small, if not negative. The broken lines represent the population-weighted average of these initial and final heat capacity functions.

lines in Figure 2). The corresponding enthalpies,  $\Delta H^{\text{peak}}$ , are listed in Table 1. These heat effects are in good correspondence with the van't Hoff enthalpies,  $\Delta H^{\text{vH}}$ , which can be determined from the shape of the heat absorption peak considering the

process as a bi-molecular two-state transition (see Materials and Methods). Such a correspondence for short DNA duplexes has been observed also by others (Vesnaver & Breslauer, 1991; Zieba *et al.*, 1991).

**Table 1.** Melting enthalpies of DNA duplexes measured by DSC in 100 mM KCl, 10 mM potassium phosphate (pH 6.0), 1 mM EDTA

| DNA   | Concentration<br>( $\mu\text{M}$ ) | $T_m$ ( $^{\circ}\text{C}$ ) | $\Delta H^{\text{vH}}$ <sup>a</sup> | $\Delta H^{\text{peak}}$ <sup>b</sup> | $\Delta H^{\text{tot}}$ <sup>c</sup> | $\Delta H^{\text{grad}}$ <sup>d</sup> | $\partial C_p/\partial T$ <sup>e</sup> |
|-------|------------------------------------|------------------------------|-------------------------------------|---------------------------------------|--------------------------------------|---------------------------------------|----------------------------------------|
| 10 bp | 214                                | 51.3                         | 300                                 | 316                                   | 360                                  | 44                                    | 0.13                                   |
|       | 204                                | 50.9                         | 313                                 | 327                                   | 392                                  | 65                                    | 0.12                                   |
|       | 169                                | 50.3                         | 290                                 | 305                                   | 353                                  | 48                                    | 0.15                                   |
|       | 107                                | 49.0                         | 310                                 | 322                                   | 373                                  | 51                                    | 0.14                                   |
|       | 6.7 <sup>f</sup>                   | 40.0                         | -                                   | -                                     | -                                    | -                                     | -                                      |
|       | mean                               | -                            | $303 \pm 10$                        | $318 \pm 15$                          | $370 \pm 30$                         | $51 \pm 5$                            | $0.13 \pm 0.02$                        |
| 12 bp | 403                                | 64.8                         | 430                                 | 447                                   | 536                                  | 89                                    | -                                      |
|       | 384                                | 64.6                         | 455                                 | 481                                   | 551                                  | 70                                    | 0.13                                   |
|       | 304                                | 63.8                         | 420                                 | 486                                   | 549                                  | 63                                    | 0.12                                   |
|       | 233                                | 63.2                         | 440                                 | 450                                   | 526                                  | 76                                    | 0.16                                   |
|       | 167                                | 62.6                         | 435                                 | 477                                   | 550                                  | 73                                    | 0.15                                   |
|       | 135                                | 61.3                         | 406                                 | 437                                   | 500                                  | 63                                    | 0.14                                   |
|       | 87                                 | 60.6                         | -                                   | -                                     | -                                    | -                                     | -                                      |
|       | 61                                 | 60.2                         | -                                   | -                                     | -                                    | -                                     | -                                      |
|       | 44                                 | 59.3                         | -                                   | -                                     | -                                    | -                                     | -                                      |
|       | 2.7 <sup>f</sup>                   | 54.7                         | -                                   | -                                     | -                                    | -                                     | -                                      |
|       | mean                               | -                            | $431 \pm 20$                        | $463 \pm 20$                          | $535 \pm 20$                         | $72 \pm 15$                           | $0.14 \pm 0.02$                        |
| 16 bp | 337                                | 53.3                         | 368                                 | 433                                   | 528                                  | 95                                    | 0.17                                   |
|       | 203                                | 52.0                         | 429                                 | 430                                   | 533                                  | 103                                   | 0.18                                   |
|       | 79                                 | 50.3                         | 384                                 | 464                                   | 546                                  | 82                                    | -                                      |
|       | 40                                 | 49.1                         | -                                   | -                                     | -                                    | -                                     | -                                      |
|       | 2.3 <sup>f</sup>                   | 43.8 <sup>f</sup>            | -                                   | -                                     | -                                    | -                                     | -                                      |
|       | mean                               | -                            | $394 \pm 20$                        | $441 \pm 20$                          | $535 \pm 20$                         | $93 \pm 15$                           | $0.17 \pm 0.02$                        |

<sup>a</sup>  $\Delta H$ , in  $\text{kJ mol}^{-1}$ .<sup>a</sup>  $\Delta H^{\text{vH}}$ , from analysis of the shape of the heat absorption peak.<sup>b</sup>  $\Delta H^{\text{peak}}$ , the area of the heat absorption peak above the lines corresponding to the heat capacities of the pre and post-transition regions extrapolated into the transition zone (Figure 2).<sup>c</sup>  $\Delta H^{\text{tot}}$ , the sum of the enthalpy accumulated in the fluctuating duplex upon heating from  $0^{\circ}\text{C}$  together with its cooperative dissociation. It is determined assuming that the heat capacity increment of duplex unfolding does not change with temperature (Figure 9).<sup>d</sup>  $\Delta H^{\text{grad}} = \Delta H^{\text{tot}} - \Delta H^{\text{peak}}$ , the enthalpy accumulated as a result of the gradual increase of heat capacity below the temperature of the cooperative transition.<sup>e</sup>  $\partial C_p/\partial T$ , the initial slope of the heat capacity function, in  $\text{kJ K}^{-2} \text{mol}^{-1}$ .<sup>f</sup> data from UV absorption at 260 nm.

Table 1 shows that  $T_m$  rises with increasing duplex concentration, as expected for a bimolecular reaction (see Figure 3). For a bimolecular reaction, the slope of the fitted line,  $\partial(1000/T_m)/\partial(\ln[N_0])$ , equals  $-R/\Delta H$  (Marky & Breslauer, 1987; Breslauer, 1995). The effective enthalpies calculated from these slopes for the three duplexes are equal to 270, 460 and  $490 \text{ kJ mol}^{-1}$  for the 10 bp, 12 bp and 16 bp duplexes, respectively. Their reasonable correspondence with the van't Hoff enthalpies determined from the sharpness of the excess heat absorption peaks and the calorimetric enthalpies,  $\Delta H^{\text{peak}}$ , found from the areas of these peaks (Table 1), is an additional argument that the heat absorbed in the peak is associated with the cooperative dissociation of the strands upon heating.

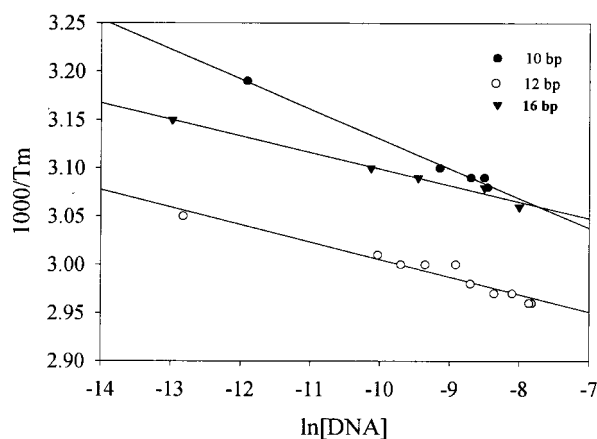
### Enthalpies of duplex formation from the separated strands measured in ITC experiments

To obtain direct information on the enthalpy of duplex formation and its dependence on temperature, we performed ITC experiments by mixing the isolated DNA strands of the three duplexes at several temperatures over the range of 8 to  $45^{\circ}\text{C}$ . The measured association enthalpies,  $\Delta H^{\text{ITC}}$ , shown in Figure 4, were found to be independent of the order of mixing of the strands (see, for example,

the two experimental points at  $24^{\circ}\text{C}$  for the 12 bp duplex). The observed  $\Delta H^{\text{ITC}}$  values of the enthalpies of strand association, which are listed in Table 2, were found to be much lower in magnitude than the melting enthalpies obtained using DSC (Table 1). The fact that the mixing enthalpies of strand association measured at room temperature are significantly smaller than the melting enthalpies of duplexes has also been observed by others (Vesnaver & Breslauer, 1991; Zieba *et al.*, 1991; Lu *et al.*, 1992; Ladbury *et al.*, 1994).

Another notable fact is that the enthalpies of strand association depend significantly on the temperature at which the mixing experiment is performed (Figure 4). The slope of these functions,  $\partial\Delta H/\partial T$ , is not identical for the three duplexes and varies between 0.26 and  $0.37 \text{ kJ K}^{-1} (\text{mol bp})^{-1}$  (Table 2). The temperature dependencies of the mixing enthalpies of DNA strands have previously been observed by others and was interpreted as due to the heat capacity decrement upon duplex formation (Lu *et al.*, 1992; Ladbury *et al.*, 1994). This interpretation, however, is in striking contrast to the DSC results, according to which melting of a DNA duplex does not result in any noticeable heat capacity increment, i.e. the enthalpy of duplex formation should not depend on temperature. There could be two reasons for this discrepancy, namely:



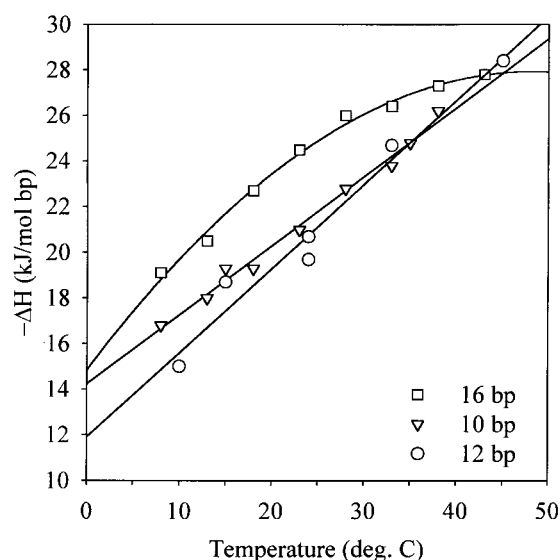


**Figure 3.** Plot of the inverse of the melting temperature,  $T_m$  (in Kelvin) of the 10 bp, 12 bp and 16 bp duplexes obtained from DSC scans and optical melting curves (the points at  $\ln[\text{DNA}]$  of  $-13$  and  $-12$ ), plotted against the logarithm of the molar duplex concentration. The slopes yield effective enthalpies of strand dissociation equal to 270, 460 and 490  $\text{kJ mol}^{-1}$  for the 10 bp, 12 bp and 16 bp duplexes, respectively.

(a) the observed temperature dependence of the association enthalpy does not truly represent the heat capacity difference between the folded duplex and unfolded separated strands; or (b) the apparent heat capacity of a duplex prior to its dissociation does not represent the heat capacity of fully folded duplex.

#### Analysis of the apparent heat capacity function of the duplexes

The remarkably steep temperature dependence of the heat capacity of the duplexes at temperatures below that of the cooperative transition is somewhat surprising. The initial slope of these functions for the considered duplexes,  $\partial C_p / \partial T$ , varies from 0.13 to 0.17  $\text{kJ K}^{-2} \text{mol}^{-1}$  (Table 1), i.e. the averaged specific value of the slope calculated on a weight basis is  $20 \times 10^{-3} \text{ J K}^{-2} \text{g}^{-1}$ . The latter value is more than three times larger than that observed for the temperature dependence of the specific heat capacity of stable globular proteins, which is about  $6 \times 10^{-3} \text{ J K}^{-2} \text{g}^{-1}$  (Makhatadze & Privalov, 1995). This raises doubts that the apparent heat capacity function observed for the duplexes can be considered as the intrinsic heat capacity function of fully folded duplexes, and thus that its linear extrapolation into the transition zone can provide us with the value of the heat capacity change on duplex unfolding. These doubts are enhanced by deconvolution analysis of the excess heat capacity function of the duplexes (i.e. the heat effect above the line connecting the initial and final points on the heat capacity curve at  $0^\circ\text{C}$  and  $80^\circ\text{C}$ , which assumes that at  $0^\circ\text{C}$  the



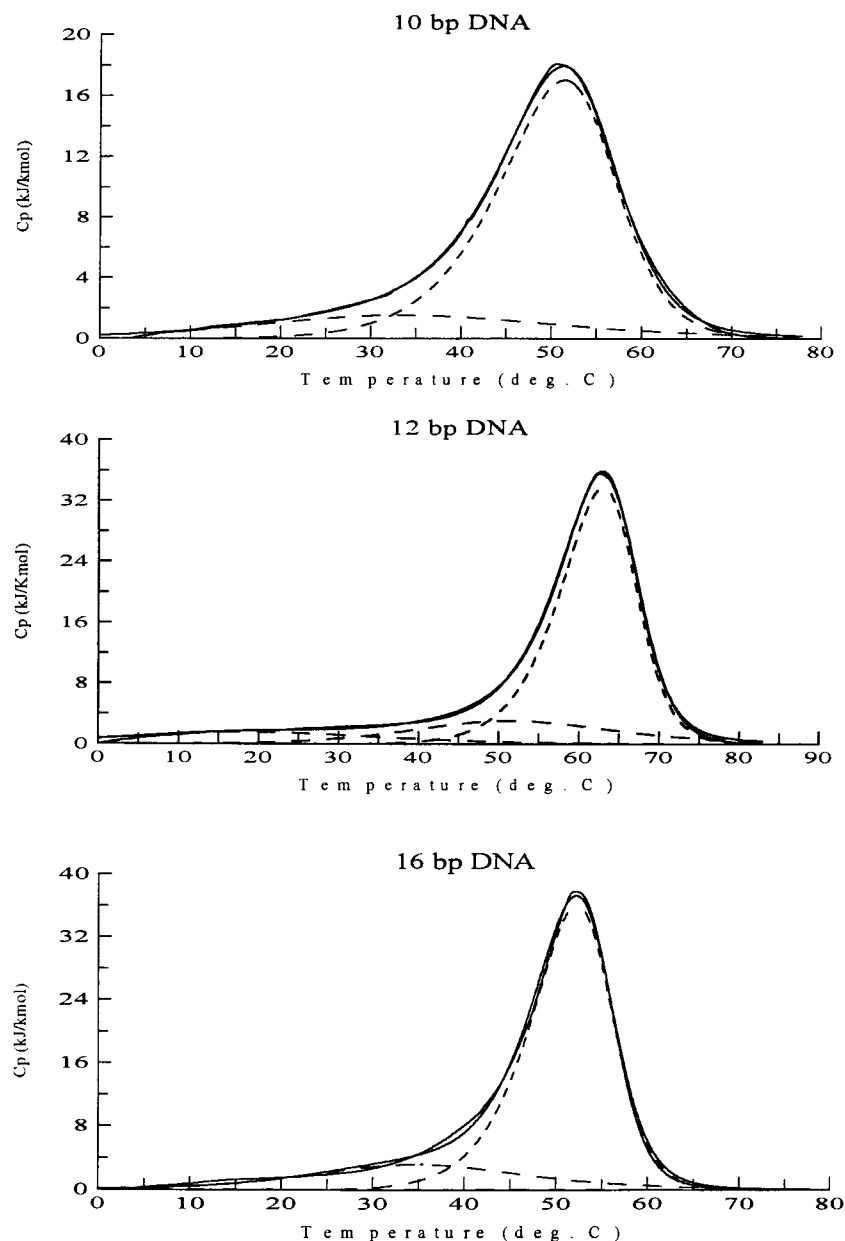
**Figure 4.** The measured enthalpies of duplex formation from single strands,  $\Delta H^{\text{ITC}}$ , expressed per base-pair, obtained by ITC measurements at different temperatures. The two values for the 12 nt strands at  $24^\circ\text{C}$  come from alternating the order of addition.

duplex is fully folded and at  $80^\circ\text{C}$  is fully unfolded). The results of this analysis are illustrated in Figure 5, which shows that the observed excess heat effect includes two qualitatively different phases: a phase of gradual heat absorption (which can also be regarded as two shallow, overlapping heat absorption peaks) and a phase of intensive heat absorption associated with cooperative bimolecular dissociation. It is clear that the slope of the initial heat capacity function is due to some temperature-induced process and cannot be used to represent the intrinsic heat capacity of the folded duplex at temperatures within the transition zone.

#### Heat effects in the separated strands

It is known that single-stranded polynucleotides can have residual structure, the extent of which depends on temperature. Melting of this residual structure is associated with significant heat effects (Filimonov & Privalov, 1978; Filimonov, 1986; Vesnaver & Breslauer, 1991; Zieba *et al.*, 1991; Davis *et al.*, 1998). The heat of association of complementary strands must therefore depend on temperature.

To investigate the contribution of residual structure in the strands to the enthalpy of duplex formation, we studied the separate strands of each of the three duplexes by DSC (Figure 6). Heating and cooling experiments showed that melting of residual structure upon heating and its formation upon cooling is a fully reversible process. Since residual structure in the strands disappears at tem-



**Figure 5.** Deconvolution analysis of the excess heat capacity function of the 10, 12 and 16 bp DNA duplexes at concentrations of 204, 167 and 203  $\mu\text{M}$ , respectively. The pre-transition and transition enthalpies are: for 10 bp, 75 and 294  $\text{kJ mol}^{-1}$ ; for 12 bp, 120 and 432  $\text{kJ mol}^{-1}$ ; and for 16 bp, 112 and 430  $\text{kJ mol}^{-1}$ .

peratures above 80°C and progressively reappears on cooling from this temperature down to even below 0°C, we used the state of the strands at 80°C as a reference and determined the heat effects associated with formation of residual structure upon cooling. In these experiments we used several strand concentrations comparable to those in the ITC experiments, which were 0.3–1.0 mg/ml ( $\sim 150$ –500  $\mu\text{M}$ ). It is notable that the heat effects of formation of residual structure proceed in two distinct stages and the low temperature stage, which appears below 10°C, depends significantly on concentration (Figure 7), showing that it is associated with the formation of intermolecular interactions (see also Davis *et al.*, 1998). Above 10°C the dependence on concentration almost

completely disappears at concentrations below 0.5 mg/ml.

Since the residual structure melts completely upon heating to 80°C, the linear heat capacity function above 80°C corresponds to the intrinsic heat capacity of unfolded strands. Assuming that this function (which probably reflects changes in non-cooperative interactions between bases in the strands) is linear also at lower temperatures, we can determine the enthalpy of formation of residual structure,  $\delta H^{\text{res}}$ , by integrating the area between this line and the observed heat capacity function of the strand from 80°C down to some specified lower temperature. These enthalpies and the slope of the heat capacity of unfolded strands are used for the corrections in Table 2. It should be

**Table 2.** Enthalpy of association of DNA strands measured by ITC and corrected for residual structure in the separated strands, for partial melting of the duplex and for the fluctuations

| T (°C)                                  | 8    | 10   | 13   | 15   | 18   | 23   | 24   | 28   | 33   | 35   | 38   | 43   | 45   | $\partial\Delta H/\partial T$ <sup>g</sup> |
|-----------------------------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|--------------------------------------------|
| <b>A. 10 bp</b>                         |      |      |      |      |      |      |      |      |      |      |      |      |      |                                            |
| $-\Delta H^{\text{ITC}}$ <sup>a</sup>   | 16.8 |      | 18.0 | 19.3 | 19.3 | 21.0 |      | 22.8 | 23.8 | 24.8 | 26.2 |      |      | 0.303                                      |
| $-\delta H^{\text{res}}$ <sup>b</sup>   | 13.7 |      | 12.6 | 12.1 | 11.3 | 9.7  |      | 8.0  | 6.3  | 5.6  | 4.7  |      |      |                                            |
| $-\delta H^{\text{melt}}$ <sup>c</sup>  | 0.0  |      | 0.0  | 0.0  | 0.0  | 0.2  |      | 0.5  | 1.0  | 1.5  | 2.4  |      |      |                                            |
| $-\Delta H^{\text{COR}}$ <sup>d</sup>   | 30.5 |      | 30.6 | 31.4 | 30.4 | 30.9 |      | 31.3 | 31.1 | 31.9 | 33.3 |      |      | 0.064                                      |
| $-\delta H^{\text{fluct}}$ <sup>e</sup> | 0.1  |      | 0.4  | 0.6  | 0.9  | 1.5  |      | 2.8  | 3.1  | 3.5  | 4.2  |      |      |                                            |
| $-\Delta H^{\text{NET}}$ <sup>f</sup>   | 30.8 |      | 31.0 | 32.0 | 31.3 | 32.4 |      | 33.1 | 34.2 | 35.4 | 37.5 |      |      | 0.197                                      |
| <b>B. 12 bp</b>                         |      |      |      |      |      |      |      |      |      |      |      |      |      |                                            |
| $-\Delta H^{\text{ITC}}$                |      | 15.0 |      | 18.7 |      |      | 19.7 |      | 24.7 |      |      |      | 28.4 | 0.369                                      |
| $-\delta H^{\text{res}}$                |      | 13.6 |      | 12.8 |      |      | 11.1 |      | 7.3  |      |      |      | 3.5  |                                            |
| $-\delta H^{\text{melt}}$               |      | 0.0  |      | 0.0  |      |      | 0.0  |      | 0.2  |      |      |      | 1.4  |                                            |
| $-\Delta H^{\text{COR}}$                |      | 28.6 |      | 31.5 |      |      | 30.8 |      | 32.2 |      |      |      | 33.3 | 0.109                                      |
| $-\delta H^{\text{fluct}}$              |      | 0    |      | 0.1  |      |      | 0.7  |      | 2.0  |      |      |      | 4.6  |                                            |
| $-\Delta H^{\text{NET}}$                |      | 28.6 |      | 31.6 |      |      | 31.5 |      | 34.2 |      |      |      | 37.9 | 0.240                                      |
| <b>C. 16 bp</b>                         |      |      |      |      |      |      |      |      |      |      |      |      |      |                                            |
| $-\Delta H^{\text{ITC}}$                | 19.1 |      | 20.5 |      | 22.7 | 24.5 |      | 26.0 | 26.4 |      | 27.3 | 27.8 |      | 0.256                                      |
| $-\delta H^{\text{res}}$                | 10.9 |      | 9.7  |      | 8.2  | 6.8  |      | 5.5  | 5.2  |      | 3.5  | 2.4  |      |                                            |
| $-\delta H^{\text{melt}}$               | 0.0  |      | 0.0  |      | 0.0  | 0.0  |      | 0.3  | 0.6  |      | 2.0  | 4.1  |      |                                            |
| $-\Delta H^{\text{COR}}$                | 30.0 |      | 30.2 |      | 30.9 | 31.3 |      | 31.8 | 32.2 |      | 32.8 | 34.3 |      | 0.113                                      |
| $-\delta H^{\text{fluct}}$              | 0.1  |      | 0.2  |      | 0.3  | 0.5  |      | 0.8  | 1.1  |      | 1.5  | 1.9  |      |                                            |
| $-\Delta H^{\text{NET}}$                | 30.1 |      | 30.4 |      | 31.2 | 31.8 |      | 32.6 | 33.3 |      | 34.5 | 36.2 |      | 0.167                                      |

$\Delta H$  in kJ (mol bp)<sup>-1</sup>.

<sup>a</sup>  $\Delta H^{\text{ITC}}$ , the enthalpy of strand association measured by ITC at the considered temperature.

<sup>b</sup>  $\delta H^{\text{res}}$ , the summed enthalpy of residual structure in both strands at the considered temperature.

<sup>c</sup>  $\delta H^{\text{melt}}$ , the area of the cooperative heat absorption peak up to the considered temperature.

<sup>d</sup>  $-\Delta H^{\text{COR}} = -\Delta H^{\text{ITC}} - \delta H^{\text{res}} - \delta H^{\text{melt}}$ .

<sup>e</sup>  $\delta H^{\text{fluct}}$ , the enthalpy accumulated in fluctuations of the duplex up to the considered temperature.

<sup>f</sup>  $-\Delta H^{\text{NET}} = -\Delta H^{\text{COR}} - \delta H^{\text{fluct}}$ .

<sup>g</sup>  $\partial\Delta H/\partial T$  in kJ K<sup>-1</sup> (mol bp)<sup>-1</sup>.

noted that the determined enthalpies of formation of residual structures in all the studied strands are almost three times larger than those reported by Holbrook *et al.*, (1999) for 14 nt strands. This discrepancy cannot be explained by differences in the sequences of the strands. A more probable explanation is that the temperature range from 10 to 50 °C used by these authors is insufficient to provide a base line for determination of the excess heat effect corresponding to the enthalpy of residual structure melting.

### The enthalpy of formation of fully folded duplex from the fully unfolded strands

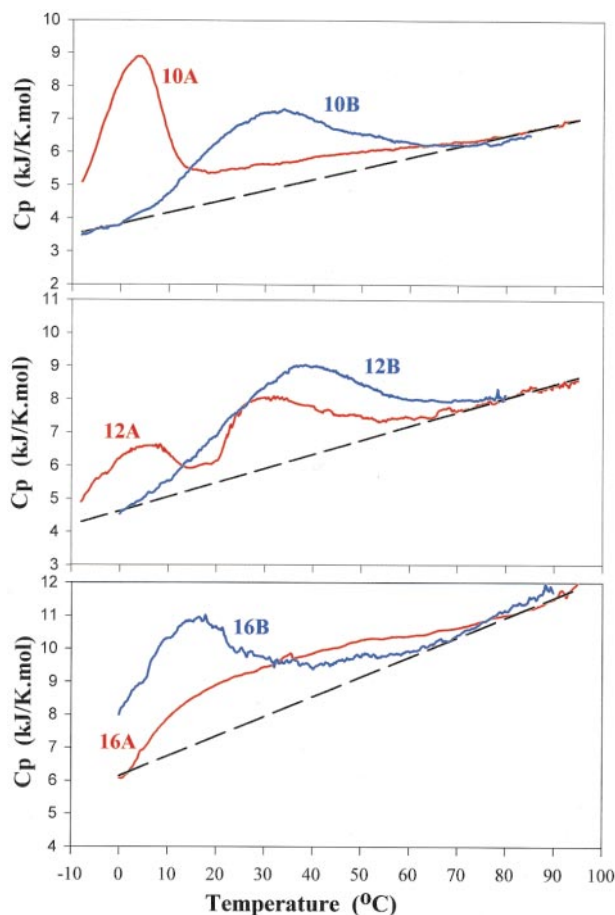
To determine the net enthalpy of duplex formation at some temperature,  $\Delta H^{\text{NET}}$ , we must first of all add to the ITC-measured enthalpy of strand association,  $\Delta H^{\text{ITC}}$ , see Table 2, the summed enthalpies of formation of residual structure in the two strands for that temperature ( $\delta H^{\text{res}}$ ) and also correct for the heat effect of partial melting of the duplex ( $\delta H^{\text{melt}}$ ) if the considered temperature is within the temperature range of cooperative duplex unfolding. This gives the values of  $\Delta H^{\text{COR}}$  in Table 2. Furthermore, since the slopes of the heat capacity functions of the separated strands and the real duplex are different, due to heat

accumulating in structural fluctuations of the duplex, we also have to correct for this effect. The correction for these accumulated energies is described formally as follows. Consider the association reaction of DNA strands at two different temperatures: at the reference temperature  $T_0$  and some other temperature  $T$ . The enthalpies of these strands and their duplex at these two temperatures can be written as:

$$\begin{aligned}
 H(T)^{\text{A-strand}} &= H(T_0)^{\text{A}} + \int_{T_0}^T C_p^{\text{A}} dT \\
 &= H(T_0)^{\text{A}} + (T - T_0)C_p(T_0)^{\text{A}} \\
 &\quad + \int_{T_0}^T [C_p(T) - C_p(T_0)]^{\text{A}} dT \quad (1)
 \end{aligned}$$

$$\begin{aligned}
 H(T)^{\text{B-strand}} &= H(T_0)^{\text{B}} + \int_{T_0}^T C_p^{\text{B}} dT \\
 &= H(T_0)^{\text{B}} + (T - T_0)C_p(T_0)^{\text{B}} \\
 &\quad + \int_{T_0}^T [C_p(T) - C_p(T_0)]^{\text{B}} dT \quad (2)
 \end{aligned}$$





**Figure 6.** Partial molar heat capacity functions for the DNA strands measured in cooling experiments at  $\sim 150 \mu\text{M}$  concentration. The excess heat effect, which can be assigned to the formation of residual structure on cooling, starts below  $80^\circ\text{C}$ . The heat capacity function above  $80^\circ\text{C}$  can therefore be regarded as corresponding to the fully unfolded strands. Extrapolations of these functions to lower temperatures are shown by the broken lines. The heat effect of formation of residual structure is determined by integration from  $80^\circ\text{C}$  down to the considered temperature, i.e. the area limited from above by the heat capacity function (continuous line) and from below by the extrapolated function of the unfolded state (broken line).

$$\begin{aligned}
 H(T)^{\text{AB-duplex}} &= H(T_0)^{\text{AB}} + \int_{T_0}^T C_p^{\text{AB}} dT \\
 &= H(T_0)^{\text{AB}} + (T - T_0)C_p(T_0)^{\text{AB}} \\
 &\quad + \int_{T_0}^T [C_p(T) - C_p(T_0)]^{\text{AB}} dT \quad (3)
 \end{aligned}$$

Therefore, for the enthalpy of association at these two temperatures we have:

$$\begin{aligned}
 \Delta H(T)^{\text{ass}} &= \Delta H(T_0)^{\text{ass}} + \Delta C_p(T_0)^{\text{ass}}(T - T_0) \\
 &\quad + \int_{T_0}^T \{[C_p(T) - C_p(T_0)]^{\text{AB}} \\
 &\quad - [C_p(T) - C_p(T_0)]^{\text{A}} \\
 &\quad - [C_p(T) - C_p(T_0)]^{\text{B}}\} dT \quad (4)
 \end{aligned}$$

where:

$$\Delta C_p(T_0)^{\text{ass}} = C_p(T_0)^{\text{AB}} - C_p(T_0)^{\text{A}} - C_p(T_0)^{\text{B}}$$

In the case where the heat capacities of all components and their complex do not depend on temperature, or depend in such a way that the temperature induced change of the complex equals that of the sum of the heat capacity changes of the components, the integral part of equation (4) is zero and we have for the temperature dependence of the association enthalpy:

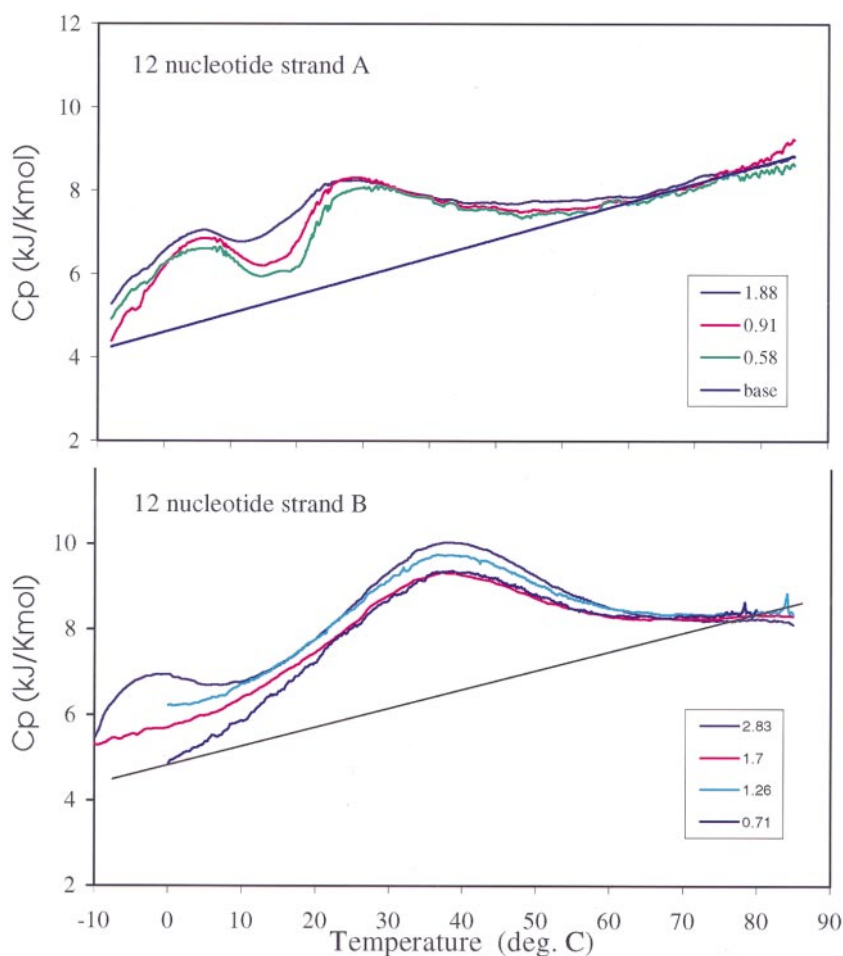
$$\Delta H(T)^{\text{ass}} = \Delta H(T_0)^{\text{ass}} + \Delta C_p(T_0)^{\text{ass}}(T - T_0) \quad (5)$$

Correspondingly, the heat capacity effect of association can be determined as:

$$\Delta C_p(T_0)^{\text{ass}} = [\Delta H(T)^{\text{ass}} - \Delta H(T_0)^{\text{ass}}]/(T - T_0) \quad (6)$$

However, if the integral term of equation (4) is not zero (as in the present case), it must be taken into account if we are interested in the net enthalpy and the net heat capacity effect of association of fully unfolded strands into a fully folded duplex without frayed ends. The summed slopes of the heat capacities of two separated strands is about  $7.5 \text{ J K}^{-2} (\text{mol bp})^{-1}$  (Figure 6). The slopes of the heat capacity functions,  $\partial C_p / \partial T$ , for the 10, 12 and 16 bp duplexes are 13, 12 and  $11 \text{ J K}^{-2} (\text{mol bp})^{-1}$ , respectively (Table 1). The corresponding corrections for thermal fluctuations,  $\delta H^{\text{fluct}}$ , are given in Table 2. Application of this third correction to  $\Delta H^{\text{COR}}$  yields the net enthalpy of association,  $\Delta H^{\text{NET}}$ .

The corrected values of the association enthalpy are significantly larger than those obtained directly from the ITC measurements (Figure 8). Furthermore, these enthalpies calculated per base-pair show a marked similarity for the three duplexes: at  $25^\circ\text{C}$  the averaged value is  $-32(\pm 2) \text{ kJ} (\text{mol bp})^{-1}$ . Most notably, their temperature dependence is much lower: after correction for residual structure in the strands and partial cooperative melting,  $\Delta H^{\text{COR}}$ , the mean slope of the enthalpy functions for all three duplexes becomes  $95(\pm 30) \text{ J K}^{-1} (\text{mol bp})^{-1}$ ; after further correction for fluctuations in the duplex,  $\Delta H^{\text{NET}}$ , the slope averages to  $200(\pm 40) \text{ J K}^{-1} (\text{mol bp})^{-1}$ . The actual values of the  $\Delta H^{\text{NET}}$  temperature dependencies are 197, 256 and  $167 \text{ J K}^{-1} (\text{mol bp})^{-1}$  for the 10, 12 and 16 bp duplexes, respectively. We note that these values correlate with the G + C content of the duplexes



**Figure 7.** The partial heat capacity of the 12 nt strands A and B measured at several concentrations. Concentrations (mg/ml) are given in the boxes.

(50 %, 58 % and 25 %), suggesting that hydration of a G·C base-pair contributes the larger heat capacity effect. This correlation is to some extent confirmed by the earlier observation that the heat capacity increment on melting of an  $(\text{AU})_n$  duplex is  $125 \text{ J K}^{-1} (\text{mol bp})^{-1}$  (Filimonov & Privalov, 1978). It is interesting that using high-precision densitometry and ultrasonic measurements, Chalikian *et al.* (1994) also came to the conclusion that G·C base-pairs are more strongly hydrated than A·T base-pairs.

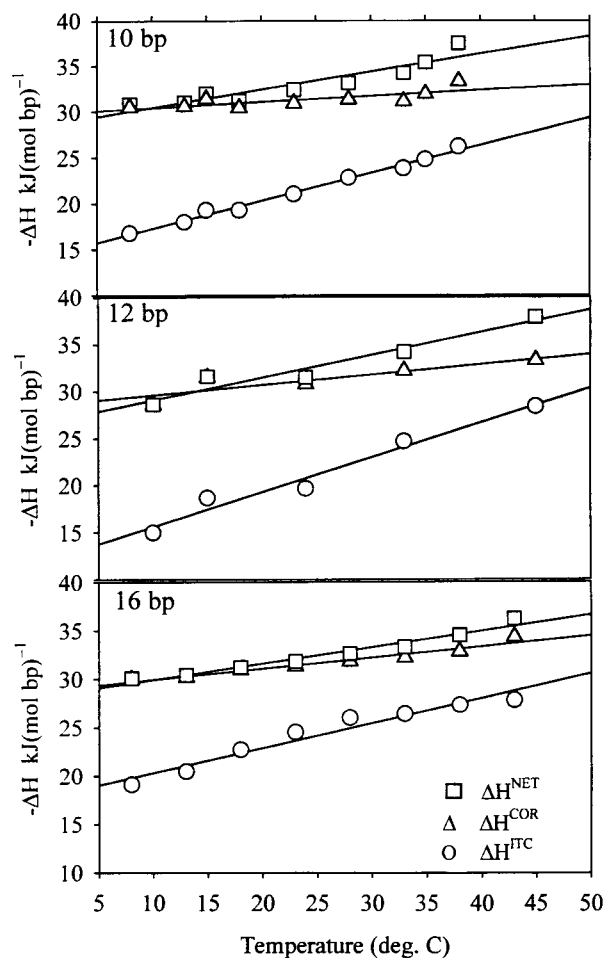
Since  $\Delta H^{\text{NET}}$  does not include the effects of end fraying (fluctuations of the structure), its temperature dependence can be considered as the heat capacity increment upon unfolding of long DNA helices, for which end effects are insignificant. The melting of long polynucleotides was recently studied calorimetrically by Chalikian *et al.* (1999) and the heat capacity increment was observed to be  $275(\pm 60) \text{ J K}^{-1} (\text{mol bp})^{-1}$ . It should be noted that Holbrook *et al.* (1999) have reported that the value of the heat capacity increment on melting of a 14 bp duplex is  $236 \text{ J K}^{-1} (\text{mol bp})^{-1}$ , however this value was obtained using completely arbitrary extrapolations of the heat capacities of the initial and final states of the duplex into the transition

zone. As we have shown, the heat capacity function of a short duplex below its cooperative transition zone is not the intrinsic heat capacity of the fully folded duplex and cannot therefore be used to determine the heat capacity increment on duplex unfolding.

It is notable that the melting enthalpies of poly[d(A)] poly[d(T)] and poly[d(AT)] poly[d(AT)] duplexes, measured in solutions of different ionic strengths in which they melt at different temperatures, were found by to have a temperature dependence of  $203 \text{ J K}^{-1} (\text{mol bp})^{-1}$  (Chalikian *et al.*, 1999). The correspondence of this slope to the  $\Delta C_p$  value determined here on short oligonucleotides is striking. This correspondence is in fact the first experimental verification of the theoretical prediction (Manning, 1972) that the influence of ionic strength on the stability of DNA is entropic in nature.

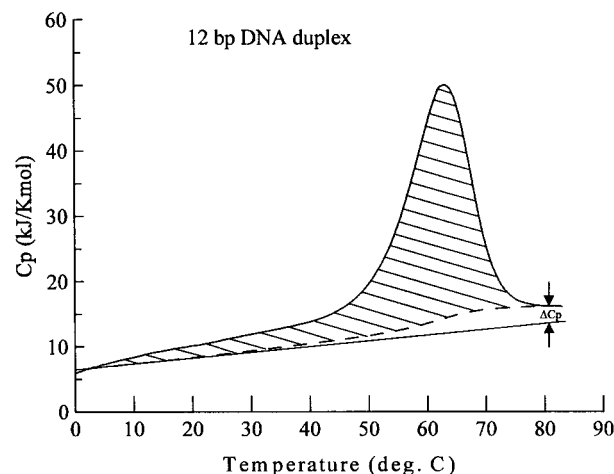
#### The heat capacity function of the folded duplex and the total enthalpy of its dissociation

If we subtract the value of  $\Delta C_p$  calculated per mol of duplex, i.e.  $2.9 \text{ kJ K}^{-1} \text{ mol}^{-1}$  for the 12 bp duplex) from the heat capacity at  $80^\circ\text{C}$ , where the duplex is assumed to be fully unfolded, we obtain



**Figure 8.** Temperature dependencies of the measured enthalpies of association of the duplexes,  $\Delta H^{\text{ITC}}$ , corrected for residual structures in the strands and for partial melting of the duplex,  $\Delta H^{\text{COR}}$ , and also for the enthalpy of fluctuations,  $\Delta H^{\text{NET}}$ .

the heat capacity of the folded state at that temperature. The line connecting this point with the heat capacity of the duplex at 0°C, where the duplex is assumed to be fully folded, can be considered as the heat capacity function of the fully folded duplex (Figure 9). The slope of this function,  $\partial C_p / \partial T$ , for the 12 bp duplex is  $87 \text{ J K}^{-2} \text{ mol}^{-1}$ , i.e. the specific slope is  $7.1 \text{ J K}^{-2} (\text{mol bp})^{-1}$  or  $11.7 \times 10^{-3} \text{ J K}^{-1} \text{ g}^{-1}$ . Correspondingly, using the above  $\Delta C_p$  values for the 10 bp and 16 bp duplexes we find that their slopes are  $66 \text{ J K}^{-2} \text{ mol}^{-1}$  and  $74 \text{ J K}^{-2} \text{ mol}^{-1}$ , respectively. The average for the specific slope per base-pair is  $6.1(\pm 1.5) \text{ J K}^{-2} (\text{mol bp})^{-1}$  or per gram is  $10(\pm 2) \times 10^{-3} \text{ J K}^{-2} \text{ g}^{-1}$ . It should be emphasized that this is the first experimental determination of the temperature dependence of the intrinsic partial heat capacity of an unfrayed duplex. This specific value is close to, though somewhat larger than the value for rigid globular proteins ( $6 \times 10^{-3} \text{ J K}^{-2} \text{ g}^{-1}$ ; Makhatadze



**Figure 9.** Partial molar heat capacity function of the DNA<sup>12 bp</sup> duplex. The lower light line corresponds to the heat capacity of the fully folded state, plotted assuming that  $\Delta C_p = 2.9 \text{ kJ K}^{-1} \text{ mol}^{-1}$ . The hatched area represents the total enthalpy of dissociation,  $\Delta H^{\text{tot}}$ .

& Privalov, 1995). This difference indicates that the DNA double helix is more flexible than compact globular proteins.

Knowledge of the temperature dependence of the partial specific heat capacity of a fully folded duplex is important, since it can be used for extrapolation of the heat capacity of other folded duplexes into the transition zone, rather than using some unjustified extrapolation of the apparent low temperature heat capacity function.

The broken line in Figure 9 represents the weighted average heat capacity of the folded and unfolded states and the area above it corresponds to the total enthalpy of duplex unfolding ( $\Delta H^{\text{tot}}$  in Table 1). We can determine the energy which has accumulated in the heated duplex prior to strand dissociation by subtracting the enthalpy of the cooperative transition from the total enthalpy:  $\Delta H^{\text{grad}} = \Delta H^{\text{tot}} - \Delta H^{\text{peak}}$  (Table 1). It is notable that the values of the corrected enthalpy of formation of the fully folded helix, extrapolated to the temperature of duplex melting,  $\Delta H(T_m)^{\text{NET}}$ , are larger than that of  $\Delta H^{\text{peak}}$  and rather close to  $\Delta H^{\text{tot}}$ . For example, in the case of the 12 bp duplex at a concentration of  $300 \mu\text{M}$ , the net enthalpy of unfolding at  $T_m = 63.8^\circ\text{C}$  is about  $510 \text{ kJ mol}^{-1}$ ,  $\Delta H^{\text{peak}}$  is about  $460 \text{ kJ mol}^{-1}$ , and  $\Delta H^{\text{tot}}$  is  $530 \text{ kJ mol}^{-1}$ . Correspondingly,  $\Delta H(T_m)^{\text{NET}}$  values for the 10 bp and 16 bp duplexes are  $401$  and  $594 \text{ kJ mol}^{-1}$ , while the  $\Delta H^{\text{peak}}$  enthalpies are  $318$  and  $441 \text{ kJ mol}^{-1}$ , respectively. It thus appears that in the case of the 10 and 16 bp duplexes, the difference between the extrapolated net enthalpy and the observed enthalpy of the cooperative transitions is somewhat larger. This is to be expected, since it is not the fully folded duplex that melts at  $T_m$  but a duplex with partly unfolded ends. It

appears that the weaker are the terminal parts of the duplex, the larger is the difference between the extrapolated net enthalpy and the enthalpy of the cooperative transition. At both ends of the 12 bp duplex there are three G·C base-pairs, but in the 16 bp duplex there is only one and it is the least stable duplex.

It is interesting to compare the  $\Delta H(T_m)^{\text{NET}}$  values obtained with those predicted using literature values of the enthalpies of base-base interactions:  $-30.5 \text{ kJ (mol bp)}^{-1}$  for the A·T base-pair and  $-53.6 \text{ kJ (mol bp)}^{-1}$  for the G·C base-pair (Breslau, 1986). For the considered 10, 12 and 16 bp duplexes, the following enthalpy values are calculated: 420, 528 and  $580 \text{ kJ mol}^{-1}$ , respectively. These values are in very good correspondence with our  $\Delta H(T_m)^{\text{NET}}$  values. The fact that predicted enthalpies of melting of short duplexes are larger than the calorimetrically measured enthalpies of the cooperative transitions has been noted previously by Breslau (1986) and was attributed to end effects.

### The entropy and Gibbs energy of duplex formation

It follows from the above discussion that the unfolding of short duplexes is a highly cooperative process which can be regarded, at least to a first approximation, as a two-state transition. The equilibrium constant for dissociation of a heterodimer is expressed by the equation:

$$K(T) = \frac{NF(T)}{1 - F(T)} \quad (7)$$

Here  $N = N^0/N^{\text{st}}$  is the dimensionless relative initial concentration of the complex,  $N^0$  is the initial concentration,  $N^{\text{st}}$  is the standard concentration and  $F(T)$  is the fraction of the molecules that have undergone the transition at the given temperature  $T$  (Privalov & Potekhin, 1986; Tamura & Privalov, 1997). On melting of a duplex the temperature of maximum heat absorption,  $T_m$ , is close to the half transition temperature,  $T_v$ , differing on the absolute scale by less than 1% (Tamura & Privalov, 1997). At this temperature  $F = 1/2$ ,  $K = N$  and:

$$\Delta G(T_m) = -RT_m \ln(N) \quad (8)$$

i.e.  $\Delta G(T_m)$  is not zero as it is in the case of monomolecular reactions; it becomes zero at a signifi-

cantly higher temperature than  $T_m$ . Therefore, for the standard entropy of unfolding/dissociation of a dimer at  $T_m$  we have:

$$\Delta S^0(T_m) = \frac{\Delta H(T_m) - \Delta G(T_m)}{T_m} = \frac{\Delta H(T_m)}{T_m} + R \ln(N) \quad (9)$$

At any other temperature  $\Delta S^0$  can be determined by the equation:

$$\Delta S^0(T) = \Delta S(T_m) - \Delta C_p \ln(T_m/T) \quad (10)$$

assuming that  $\Delta C_p$  does not depend on temperature.

Extrapolating the  $\Delta H^{\text{NET}}$  functions of Figure 8 to the temperatures of duplex melting,  $T_m$ , we can evaluate the enthalpy of strand association at this temperature. For the 12 bp duplex at  $167 \mu\text{M}$  concentration,  $T_m = 62.6^\circ\text{C} = 335.8 \text{ K}$  (Table 2); correspondingly,  $\Delta H(T_m)^{\text{NET}} = -41.7 \text{ kJ (mol bp)}^{-1}$ , i.e.  $-501 \text{ kJ mol}^{-1}$ . For the standard association entropy (at 1 M concentration) we then obtain  $-1.420 \text{ kJ K}^{-1} \text{ mol}^{-1}$  at this temperature. Extrapolating to  $25^\circ\text{C}$ , the standard association entropy becomes  $-1.078 \text{ kJ K}^{-1} \text{ mol}^{-1}$ , and since the association enthalpy at this temperature is  $-392 \text{ kJ mol}^{-1}$ , the Gibbs energy of association is  $-71 \text{ kJ mol}^{-1}$ . This corresponds to a dissociation constant of the order of  $10^{-13} \text{ M}$ . Dividing these values by the number of base-pairs, we obtain the average contribution of a base-pair to the stabilization of the duplex at  $25^\circ\text{C}$ . Table 3 gives these values for all three duplexes. The data show that although all three duplexes are stabilized by the enthalpy factor, it is mainly the entropy which determines the differences between their stabilities.

How reliable are these calculations of the thermodynamic parameters for the formation of folded duplexes, particularly the Gibbs energy? A point of particular concern is that to determine the entropy of association of fully unfolded strands into the fully folded duplex, we extrapolate the enthalpy to the observed  $T_m$ , i.e. to the temperature of dissociation of a fluctuating duplex. This, however, should not introduce a large error in estimating the Gibbs energy of association. Indeed, at the  $T_m$  almost all residual structure in the strands is melted and thus residual structure in the strands will not introduce a significant change into the magnitude of the Gibbs energy of duplex dissociation/association at the  $T_m$ . Even if not all of

**Table 3.** Standard thermodynamic parameters for the formation of the fully folded DNA duplexes from denatured single strands at  $25^\circ\text{C}$

| DNA   | $\Delta H^0$ | $\Delta S^0$ | $\Delta G^0$ |
|-------|--------------|--------------|--------------|
| 10 bp | -33.4        | -95.4        | -4.9         |
| 12 bp | -32.7        | -89.8        | -5.9         |
| 16 bp | -32.4        | -94.4        | -4.2         |

$\Delta H$  and  $\Delta G^0$  in  $\text{kJ (mol bp)}^{-1}$ ;  $\Delta S^0$  in  $\text{J K}^{-1} \text{ (mol bp)}^{-1}$ .



the residual structure is melted at  $T_m$ , its contribution to the Gibbs energy of duplex unfolding at  $T_m$  will be very small due to the enthalpy/entropy compensation effect. As for fluctuations of the duplex, although these result in accumulation of considerable enthalpy, their contribution to the Gibbs energy of dissociation at  $T_m$  is minimal, because fluctuations also result in a considerable increase of the compensating entropy factor:

$$\delta\Delta G(T_m) = \int_0^{T_m} (C_p^{\text{fluct}} - C_p^{\text{fold}})dT - T_m \int_0^{T_m} (C_p^{\text{fluct}} - C_p^{\text{fold}})d\ln T \approx 0 \quad (11)$$

The melting temperature of a fully folded duplex should therefore not differ significantly from that of a fluctuating duplex, and the entropy and Gibbs energy of duplex formation determined by the above method is thus quite reliable. It should be noted that this method is in fact the only way to determine these parameters that have principal importance for understanding the mechanism of stabilization of the double helix. This is because experimentally one cannot associate fully unfolded strands lacking residual structure into a fully folded duplex without end fraying. If one measures an association enthalpy or binding constant at 25 °C, the contribution of residual structure in the strands will be considerable. If the enthalpy of dissociation is measured at a relatively high  $T_m$ , where the contribution of residual structure is small, the contribution of fluctuations will be significant. Even if fluctuation effects could be ignored, one cannot extrapolate the measured enthalpy and entropy to 25 °C without knowledge of  $\Delta C_p$ .

## Conclusion

Although the main purpose of this study is to describe thermodynamically the three target DNA duplexes specifically interacting with the HMG box from mouse Sox-5 (Privalov *et al.*, 1999), the method used and the conclusions drawn have general application and significance for all DNA duplexes, particularly short ones. The analysis shows that temperature-induced changes in short DNA duplexes are a complex process, consisting of two qualitatively different phases: (a) a low temperature phase characterized by a gradual increase in heat capacity with a slope that is relatively high for a folded, rigid macromolecule; and (b) the phase of cooperative strand dissociation which proceeds with intensive heat absorption. The first phase results from increasing fluctuations of the double helix, e.g. twisting and end fraying, that are especially pronounced in short duplexes. The observed heat capacity function in the first phase cannot therefore be considered as intrinsic to the double helix and its extrapolation to the midpoint

of the cooperative transition does not therefore provide the heat capacity increment on melting. Cooperative dissociation of a duplex in the second phase can be envisaged as starting when thermal fluctuations of its structure reach some critical level.

## Materials and Methods

### DNA duplex preparation

Custom-synthesized DNA oligonucleotides were purchased from Biosynthesis Inc. and purified by anion exchange FPLC on a Mono-Q column using a linear 0.1 M to 1 M gradient of NaCl in 10 mM Tris-HCl, 1 mM EDTA, 20% (v/v) acetonitrile (pH 7.0). After precipitation with ethanol, the samples were vacuum-dried, dissolved in water and dialyzed against working buffer. To prepare duplex DNA, equimolar amounts of the complementary strands were mixed, heated to 70 °C and slowly cooled. Non-denaturing 8% (w/v) polyacrylamide gels were used to check for the absence of any excess single strands. Concentrations were determined from  $A_{260}$  of the nucleotides after complete digestion of single strand oligonucleotides and duplexes by phosphodiesterase I (Sigma) in 100 mM Tris-HCl (pH 8.0). In all cases, duplex and single-stranded oligonucleotide concentrations were determined after dialysis and repeated after calorimetric experiments.

For calorimetric studies, solutions of duplexes were dialysed for 24 hours with three changes of buffer using a 6000-8000 molecular mass cut-off membrane (Spectra/Por). Solutions of single-stranded oligonucleotides for calorimetric studies were dialyzed under the same conditions using 3500 molecular mass cut-off membranes. The buffer used, unless otherwise stated, was 10 mM phosphate (pH 6.0), 100 mM KCl, 1 mM EDTA.

### Spectroscopy

UV absorption was measured using a Hitachi spectrophotometer equipped with thermostated cuvettes. Thermostabilisation was achieved using a water-bath and the sample temperature was measured with a thermistor inserted directly in the sample cuvette. The heating rate was 1 K/minute.

### Isothermal titration calorimetry

ITC was performed on an OMEGA titration calorimeter (MicroCal, Inc., Northampton, MA). The calorimeter was calibrated with electrically generated heat pulses and by the interaction of 2'-CMP with RNase A. All solutions were thoroughly degassed by evacuation. Samples of DNA were prepared with the same batch of buffer to minimize artifacts due to minor differences in buffer composition. The stirring rate was 400 rpm during both equilibration and experiment. Injections were started after equilibration to baseline stability. Experiments used strand concentrations of about 0.3-0.5 mg/ml, which corresponds to molar concentrations of about  $10^{-4}$ .

### Differential scanning calorimetry

Scanning calorimetric experiments were carried out on the new version of the Nano-DSC calorimeter (CSC,



Utah) with cylindrical calorimetric cells of 0.3 ml volume, with the advantage that less material was required for the experiments. Details of the instrument's performance and data acquisition are given elsewhere (Privalov *et al.*, 1995). The heating and cooling rate was 1 K/minute. The DNA duplexes were studied over the concentration range of 0.15 to 4 mg/ml and the separated strands over the concentration range of 0.5 to 3 mg/ml. Partial specific volumes for duplexes and single strands were taken as  $0.54 \text{ cm}^3 \text{ g}^{-1}$ . The partial specific heat capacity was estimated as described by Privalov & Potekhin, (1986).

The heat capacity of each duplex was measured upon heating from  $0^\circ\text{C}$  to  $100^\circ\text{C}$  and subsequent cooling to  $0^\circ\text{C}$ . The heat capacity of the separated strands was measured upon heating to  $100^\circ\text{C}$  and subsequent cooling to  $-10^\circ\text{C}$ . The latter was required since formation of residual structure continues upon cooling below  $0^\circ\text{C}$ . Since the heat effects observed in heating and cooling experiments were essentially reversible, the enthalpy of formation of residual structure in the strands was determined from the cooling experiments, which permit heat capacity measurements down to lower temperatures.

The results were analyzed using the CpCALC program included with the Nano-DSC. The van't Hoff enthalpy  $\Delta H^{\text{vH}}$ , was determined by the equation for an n-meric transition:

$$\Delta H^{\text{vH}} = (\sqrt{n} + 1)T_m \sqrt{R \left( \langle \Delta C_p \rangle_{\text{max}} - \frac{\Delta C_p \sqrt{n}}{\sqrt{n} + 1} \right)} \quad (12)$$

where  $\langle \Delta C_p \rangle_{\text{max}}$  is the height of the peak,  $\Delta C_p$  is the unfolding heat capacity change and  $n$  equals 2 for a bimolecular reaction (Privalov & Potekhin, 1986).

## Acknowledgments

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