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CALORIMETRIC AND SPECTROSCOPIC INVESTIGATION OF THE HELIX-TO-COIL TRANSITION OF THE SELF-COMPLEMENTARY DEOXYRIBONUCLEOTIDE ATGCAT ☆

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Differential scanning calorimetry and temperature-dependent uv spectroscopy are used to thermodynamically characterize the double-strand to single-strand transition of the self-complementary deoxyribo-oligonucleotide ATGCAT. The calorimetric experiments provide a value of 33.6 kcal (mol of double strand)$^{-1}$ for the transition between 10 and 90°C. In conjunction with available temperature-dependent nmr data (which reveals terminal base pair fraying), we attempt to define specifically those interactions to which the calorimetrically measured enthalpy change refers.

Values of $\Delta H_{V.H.}$ (van 't Hoff enthalpy change) are derived from the spectroscopic and calorimetric data and compared with the ΔH obtained directly from the calorimetric experiment. This comparison reveals that the part of the thermally-induced transition that occurs between 10 and 90°C is well represented by a two-state process. It is noted that in assessing the applicability of the two-state model it is best to compare the $\Delta H_{cal.}$ with $\Delta H_{V.H.}$ obtained from the calorimetric rather than the spectroscopic data.

1. Introduction

Ribo-oligonucleotides of defined sequence are useful models for the structural features found in naturally occurring ribonucleic acid polymers. The design, synthesis and thermodynamic investigation of the helix-to-coil transitions of these ribo-oligomers has greatly enhanced our understanding of the structures and conformational transitions of RNA molecules [1–3]. These studies have provided the basis for the thermodynamic characterization of the molecular forces that control RNA structures.

By contrast, relatively few corresponding thermodynamic studies have been carried out on DNA oligomers. Most of the early work examined natural and synthetic DNA polymers as well as deoxy-dinucleoside phosphates [4–10]. Investigations on oligomeric systems were usually limited to sequences that could be obtained from controlled degradation of available polymers and involved temperature-dependent optical studies from which van 't Hoff enthalpy data were indirectly calculated [11,12,13]. As a result, thermodynamic data on nearest-neighbor interactions in DNA molecules are relatively sparse when compared with the corresponding data on RNA molecules.

More recently, application of the di- and triester methods of chemical synthesis has resulted in the preparation of DNA oligomers possessing more diverse sequences and consequently greater information content for thermodynamic studies [14–18].

We have used a modified diester approach to synthesize deoxy-oligomers possessing sequences of special interest. In this paper we report the results obtained from an investigation of the hexanucleotide d-ATGCAT

This sequence was selected for several reasons. First, the sequence is self-complementary and can form a duplex consisting of four AT and two GC base pairs. Secondly, in addition to the calorimetric and spectroscopic results reported here, temperature-dependent nmr data are available on the order-disorder transition of this oligomer [14,19,20]. Thus, this study will represent the first example in which calorimetric, uv spectroscopic and nmr data are all available for the

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analysis of a helix-to-coil transition in a deoxyoligonucleotide. We shall show that possessing results from these three different physical techniques provides a unique opportunity to interpret observables in terms of specific molecular interactions.

2. Experimental

2.1. Materials

2.1.1. Synthesis of d-ATGCAT

Synthesis of the hexanucleotide was carried out by a diester procedure in which elongation is effected exclusively by addition of mononucleotides (Jones et al., in preparation). Appropriately protected mononucleotides are prepared in a one-flask reaction starting from the 5′-nucleotides via sequential derivatization of the 3′-hydroxyl with *tert*-butyldiphenylchlorosilane and reaction of the amino group with isobutyric anhydride. The 5′-terminal nucleoside used was 6-*N*-benzoyl-5′-*O*-pivalyl-2′-deoxyadenosine. The general procedure involved condensing the oligomer for six hours in pyridine solution with a two to four fold excess of mononucleotide, using triisopropylbenzenesulfonyl chloride (TPS) as the condensing agent. The reaction was terminated by addition of water. The product solution was then concentrated, dissolved in fifty ml of 0.1 M triethylammonium acetate (TEAA) buffer and extracted with a fifty ml portion of diethyl ether. The aqueous layer was applied directly to a column of Waters Frep PAK/C_{18}, eluted with a linear gradient of 10% to 70% acetonitrile in water, buffered with 0.1 M TEAA. The lipophilic 3′-*O*-*tert*-butyldiphenyl silyl group causes the product to be much more strongly retained on the reversed-phase column, giving excellent separation of oligonucleotides differing in size by only a single silylated nucleotide. Fractions containing product were combined, evaporated and treated with 1M tetra-n-butylammonium fluoride in pyridine to remove the 3′-*O*-*tert*-butyldiphenylsilyl group. The product was then dissolved in 0.1 M TEAA, extracted with ether and applied to the reversed-phase column as described above. After evaporation, the product was isolated by precipitation from ether. Yields for the five condensation steps were, respectively: 73% (dimer); 31% (trimer); 27% (tetramer); 38% (pentamer); 25% (hexamer).

The amino protecting groups were removed by treatment with concentrated ammonia, at room temperature, for twenty-four hours. This procedure does not remove the 5′-*O*-pivalyl group. The 5′-protected hexanucleotide was isolated and purified by chromatography on Sephadex G-10, to remove non-nucleotidic materials, and subsequently on a Waters C_{18} analytical column [21].

Completely deprotected hexanucleotide was obtained by treatment with tetraethyl ammonium hydroxide [22] after the ammonia reaction. Purification was effected as above.

2.1.2. Solvent

All measurements reported here were carried out in a buffer system consisting of 1 M NaCl, 0.01 M sodium phosphate and 10^{-4} M sodium EDTA, adjusted to pH 6.85.

2.2. Methods

2.2.1. Spectroscopy

The absorbance versus temperature profiles reported here were measured at 260 nm using a temperature-programmable, thermoelectrically controlled Perkin-Elmer 575 spectrophotometer interfaced to a Techtronix 4051 computer for data acquisition and analysis. The temperature was increased continuously at a rate of 0.5°C/min. For each melting curve, a total of 140 points were recorded and analyzed. Repetitive runs revealed the melting temperature, T_m, to be reproducible to better than ±0.5°C. The melting curves were determined over a concentration range of 2.45×10^{-5} M to 2.34×10^{-4} M in single strand.

2.2.2. Calorimetry

The differential scanning calorimetry was carried out on a Microcal-1 instrument similar to one previously described in detail [23]. In a typical experiment, the reaction and the reference platinum cells are each filled with 0.9 ml of solution and the temperature is scanned from 5 to 95°C at a rate of 0.94°C/min. For a thermally-induced endothermic transition, the temperature of the reaction cell will lag behind that of the solvent reference cell. In a given experiment, one continuously measures the additional electrical energy fed back to the reaction cell to maintain it at the same temperature as the solvent reference cell. The instrument

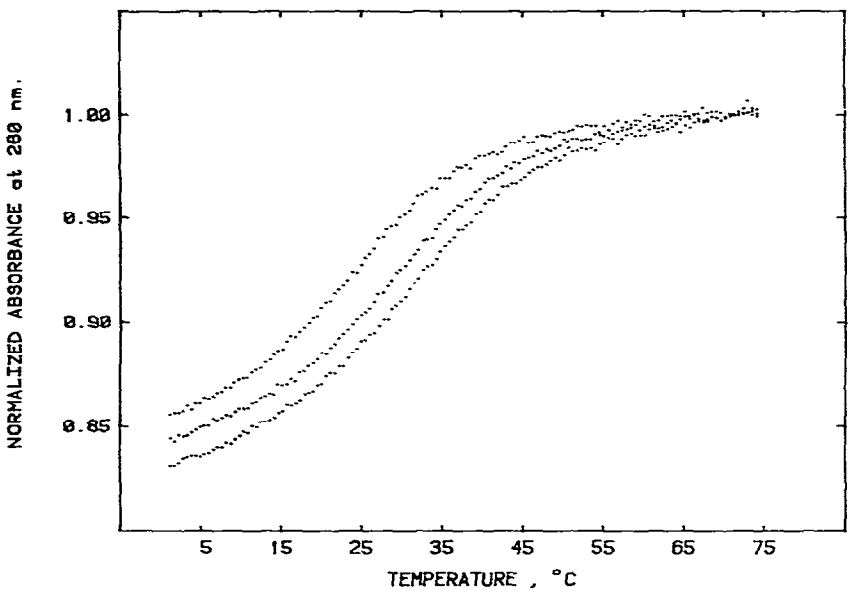

Fig. 1. Absorbance versus temperature profiles for d(ATGCAT) at different strand concentrations (normalized to an absorbance of 1.0) in 10 mM phosphate buffer, 1 M NaCl, 1 mM EDTA at pH 6.85.

is calibrated by measuring the area produced by a controlled electrical pulse. These data (along with the known concentration of the solute) permit the construction of an enthalpy mol^{-1} versus temperature curve as shown in fig. 4 and a specific heat versus temperature curve as shown in fig. 3. The data plotted in these figures are excess enthalpy and heat capacity values relative to buffer.

The concentration of the oligomer in the solutions used for calorimetry was spectrophotometrically determined to be 3.32×10^{-3} M using an extinction coefficient of 6.31×10^{4} at 55°C [24].

3. Results

3.1. Analysis of the spectroscopic data

The absorbance versus temperature profiles obtained for d-ATGCAT at different strand concentrations are shown in fig. 1. To extract thermodynamic information from such optical data, two approaches have been used. Both methods require that the experimental absorbance versus temperature curves be converted into a α versus temperature melting curves, where α represents the fraction in single strand. This conversion was accomplished by assuming that the fractional change in absorbance at any temperature is proportional to the extent of reaction at that temperature. For this analysis, upper baselines were drawn by least square fitting of the data in the linear, high temperature region. Lower baselines were established by normalizing the curves to melting profiles run at higher concentrations. Thus a value of α was obtained to any temperature simply by taking the ratio of the distance between the two baselines and the distance between the lower baseline and the experimental curve. In this manner, the experimental absorbance versus T curves were converted into α versus T "melting curves". The melting temperature (T_m) is simply defined as the temperature at which α equals 0.5.

To calculate a van 't Hoff enthalpy change from these data, one evaluates the slope for any one of the α versus T curves at T_m and applies the equation

$$\Delta H_{\mathrm{V.H.}} = 6RT_{\mathrm{m}}^{2}(\partial\alpha/\partial T)_{T_m}.$$

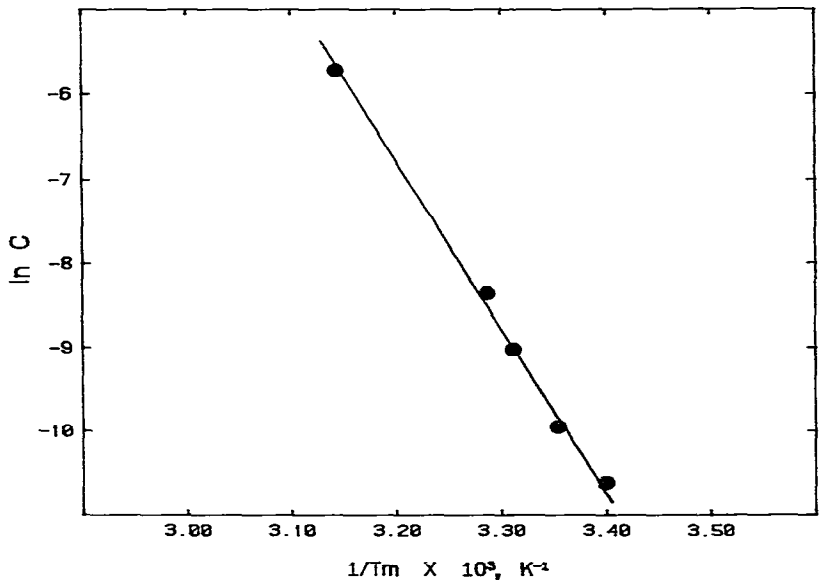

Fig. 2. ln c (total strand concentration) versus $1/T_m$ (reciprocal of the melting temperature) for d(ATGCAT) in 10 mM phosphate buffer, 1 M NaCl, 1 mM EDTA at pH 6.85.

The details of this treatment previously have been described [2].

Alternatively, one can obtain a value for the van 't Hoff enthalpy from the concentration dependence of melting temperature, T_m. The relevant relationship is described by the equation

$$\frac{1}{T_m} = \frac{R}{\Delta H} \ln C_T + \frac{\Delta S}{\Delta H}.$$

Thus, $\Delta H_{V.H.}$ can be determined from the slope of a plot of ln C_T versus the reciprocal of the melting temperature, $1/T_m$, as shown in fig. 2.

Application of these two methods yields the van 't Hoff enthalpy data listed in table 1. It should be noted that both these treatments of the optical data involve the assumption of a two-state (all-or-none) transition.

3.2. Analysis of the calorimetric data

The heat capacity, C_p versus T, and enthalpy, ΔH versus T curves obtained calorimetrically are shown in figs. 3 and 4. Table 2 summarizes the results obtained from these calorimetric experiments. These experiments provide a direct, model-independent measure of the enthalpy change accompanying the order–disorder transition. This contrasts with the model-dependent van 't Hoff enthalpy values obtained from analysis of the optical data.

A van 't Hoff enthalpy can also be obtained by analysis of the *shape* of calorimetric heat capacity curve [2,3] using the equation

$$\Delta H_{V.H.} = \frac{-4.37}{1/T_{1/2} - 1/T_{3/4}}.$$

Table 1
Calculated van 't Hoff enthalpy changes

Entry no.	Method of analysis	$\Delta H_{V.H.}$ (kcal mol^{-1})
1	Evaluation of slope at T_m of melting curve	39.2
2	Evaluation of slope of $1/T_m$ versus log c plot	39.0
3	Evaluation of *shape* of calorimetric heat capacity curve	34.8

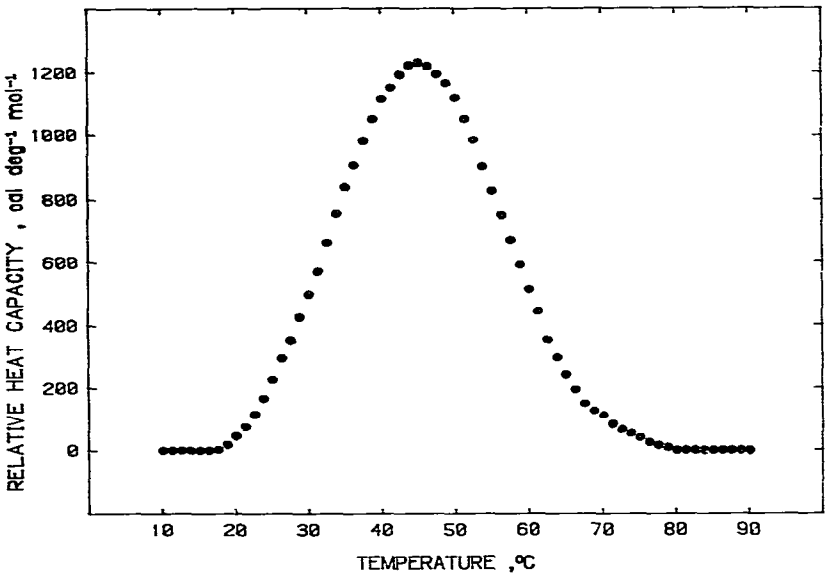

Fig. 3. Calorimetric heat capacity versus temperature for d(ATGCAT) in 10 mM phosphate buffer, 1 M NaCl, 1 mM EDTA at pH 6.85. The strand concentration is 3.32 mM. The heat capacity shown is the excess heat capacity relative to buffer.

Table 1 includes the result obtained by application of this approach.

4. Discussion

4.1. Interpreting the calorimetric data

The calorimetrically measured enthalpy change of 33.6 kcal reported here corresponds only to that *part* of the helix-to-coil transition of d-ATGCAT that occurs between 10 and 90°C. Thus, the crucial question is: what interactions exist at 10°C that are ruptured by 90°C? In previous calorimetric studies on oligonucleotides, the absence of such specific information resulted in the data being interpreted by *assuming* the helix to be *fully formed* at the low temperature limit and *fully disrupted* at the high temperature limit [2,25]. Thus, the total calorimetrically measured enthalpy was simply divided by the maximum number of possible base pairs (or base stacks) to arrive at an "average" enthalpy per interaction. In the case of the self-complementary oligomer d-ATGCAT studied here, one can envision the formation of a duplex consisting of a *maximum* of 6 base-pairs or 5 base-stacks. If all these interactions were manifest at the low temperature limit of the calorimetric scan (10°C) and fully broken at the high temperature limit (90°C), then the 33.6 kcal measured would simply correspond to an average base-pairing interaction of −5.6 kcal (or −6.7 kcal per base stack). However, nmr melting studies on d-ATGCAT [19] reveal that such a treatment of the calorimetric data would be in error since at 10°C the duplex is in fact *not fully formed*. The nmr data clearly show the terminal AT base-pairs to be partially open at 10°C while the remaining base-pairs are nearly fully formed. Thus, the calorimetrically measured enthalpy of 33.6 kcal must result from the rupture of *fewer* than

Table 2
Calorimetrically determined enthalpy

Experiment no.	ΔH (kcal mol^{-1})
1	34.2
2	33.1
Average	33.6

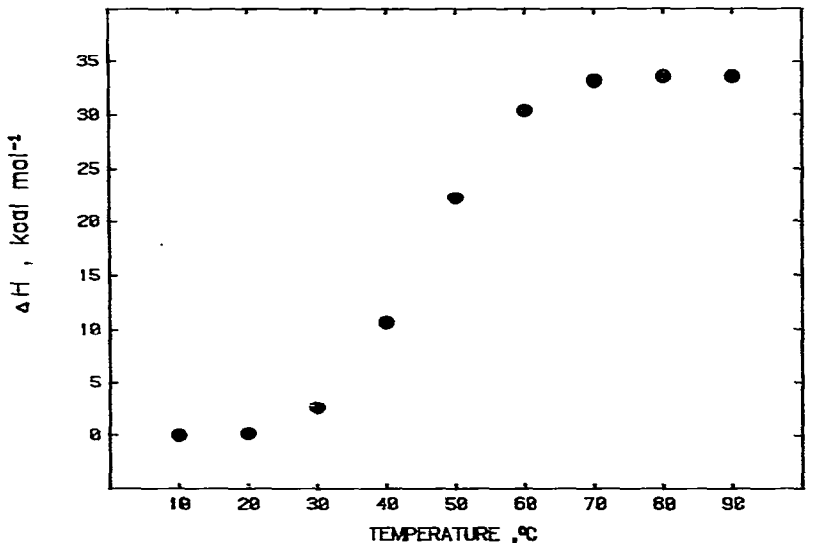

Fig. 4. Calorimetric enthalpy versus temperature curve for d(ATGCAT) in 10 mM phosphate buffer, 1 M NaCl, 1 mM EDTA at pH 6.85. The strand concentration is 3.32 mM. The enthalpy shown is the excess enthalpy relative to buffer.

6 base-pairs (or 5 base-stacks). This would yield an average base pairing interaction greater than −5.6 kcal and an average base-stacking interaction greater than −6.7 kcal.

Implicit in the use of nmr data to interpret calorimetric results is the assumption that a base pair defined as "open" by nmr is in fact ruptured from an energetic point of view. The validity of this assumption has not yet been established.

4.2. Dissecting the enthalpy data

Several approaches can be used to dissect the total enthalpy into fractional contributions from specific interactions. However, due to the scarcity of relevant data, these approaches are rather simplistic and require several explicit and implicit assumptions. Nevertheless, such efforts are useful in that they serve to focus on and define specific areas where additional data are needed.

One approach for dissecting the overall enthalpy change involves consideration of the base pairing interactions without regard to nearest-neighbor effects. In the self-complementary hexanucleotide ATGCAT, the relevant base pairs are: two internal GC base pairs, two internal AT base pairs and two frayed, terminal AT base pairs. In a recent calorimetric study on d-GCGCGC [18], a value of 9.9 kcal was determined for the GC base-pairing interaction. Thus, the two internal GC base pairs contribute 18.8 kcal to the overall enthalpy. Based upon previous calorimetric studies [2,29] an AT base pair contributes 7 kcal for a total of 14 kcal for the two internal AT base pairs. Thus, a total of 33.8 kcal is contributed by the four internal base pairing interactions, a number very close to the calorimetrically measured value of 33.6 kcal. As a consequence, one must conclude that the frayed, terminal AT base pairs are energetically ruptured at 10° and therefore do not contribute to the total enthalpy change.

A second more reasonable approach for dissecting the total enthalpy change takes nearest-neighbor interactions into account through consideration of the base-stacking rather than the base pairing interactions.

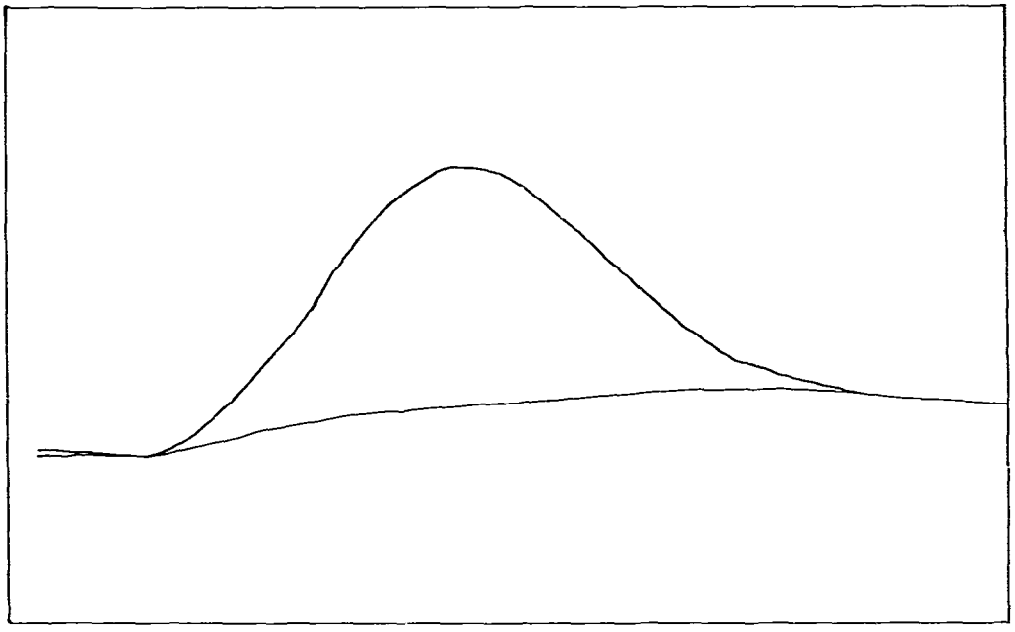

Fig. 5. Tracing of recorder output from differential scanning calorimeter for d(ATGCAT) in 10 mM phosphate buffer, 1 M NaCl, 1 mM EDTA at pH 6.85. The strand concentration is 3.32 mM. The *Y*-axis is compensating electrical energy required to maintain the sample cell at the same temperature as the reference cell and the *X*-axis is temperature.

The relevant base stacks are: one internal GC/CG stack, two internal CG/AT stacks and two frayed, terminal AT/TA stacks. Based on a calorimetric study of d-GCGCGC [18], a value of 11.9 kcal can be assigned to the GC/CG base stack. Unfortunately, no direct calorimetric data are available on the energetics of the CG/AT stacking interaction. However, using available van 't Hoff data on AU/CG, AU/GC, CG/AU and GC/AU, (derived from optical studies of ribo-oligomers), the deoxy base stacking interaction CG/AT can be assigned an approximate value of 5.9 kcal [26]. Thus, the three internal base stacks contribute a total of 23.9 kcal out of an overall enthalpy of 33.6 kcal. The remaining 9.9 kcal can be associated with the rupture of the two frayed, terminal AT/TA stacks. Thus, each frayed, terminal AT/TA stack contributes only 4.95 kcal to the total enthalpy change as compared with 7 kcal for a fully formed, interior AT/TA stack. If this energy difference is exclusively due to fraying, then one can calculate from a ratio of the observed and predicted enthalpies that each terminal AT/TA stack is approximately 70% formed (or 30% frayed). This result is qualitatively in agreement with the temperature-dependent nmr data [19] which reveal that the terminal AT base pairs are 27% frayed at 10°C. Such agreement supports the assumption made above that *in this system* a base pair defined as open by nmr is in fact ruptured from a energetic point of view. However, more systems must be studied by both nmr and calorimetry before the generality of this observation can be assessed.

Clearly, as mentioned earlier, these simplistic approaches for dissecting the overall enthalpy change involve a number of explicit and implicit assumptions which must and will be subjected to experimental tests. It is assumed that the deoxy CG/AT base-stack is energetically equivalent to the corresponding ribo CG/AU base stack. A direct measure of these stacking interactions is still not available and can only be estimated from optical data. More significantly, in dissecting the total ΔH in terms of molecular interactions we have assumed that the "dangling ends" that exist as a con-

sequence of fraying at the terminal A-T base-pairs have no effect on the energetics of the remaining duplex. However, studies in the ribo series suggest that an additional non-bonding base at the end of a helix stabilizes the duplex structure [27]. Furthermore, although the terminal AT base-pairs are partially "open" (not hydrogen bonded), the available nmr data [19] do not allow us to exclude the possibility that these bases nevertheless may still stabilize the helix by participating in base stacking interactions (both inter- and intrastrand). If part or all of these stabilizing effects were manifest, then the enthalpic contribution specifically assigned to the terminal AT/TA base stacks would further be reduced. Experiments on additional oligonucleotide sequences are currently being conducted that will allow evaluation of these secondary enthalpy sources.

4.3. Nature of the transition between 10 and 90°

In conjunction with the nmr data, we have concluded that the 33.6 kcal measured calorimetrically between 10 and 90°C corresponds to the disruption of fewer than 6 base pairs (5 base stacks). We may now ask: what is the nature of this transition? Does it occur in a cooperative, two-state manner, or are intermediate states significantly populated? The calorimetric data provide a means of answering this quetsion. If the van 't Hoff enthalpy (which is calculated here assuming two-state behavior) equals the directly measured calorimetric enthalpy, then the transition occurs in a two-state manner. If intermediate states are significantly populated then $\Delta H_{\text{V.H.}} < \Delta H_{\text{cal.}}$ [28]. Comparison of the data in tables 1 and 2 reveals the good agreement between the model-independent calorimetric enthalpy and the model-dependent van 't Hoff enthalpy change derived from the shape of the heat capacity curve. This observation allows the conclusion that the transition between 10 and 90°C is well represented by a two-state model.

One should note that the calorimetric enthalpy was compared with the van 't Hoff enthalpy derived from the same observable; namely, the $\Delta H_{\text{cal.}}$. This provides a more meaningful comparison than that obtained by comparing a calorimetric enthalpy with a van 't Hoff enthalpy derived from uv optical data.

Inspection of table 1 reveals that the van 't Hoff enthalpy values calculated from the temperature-dependent uv optical data are 10 to 14 per cent higher than the enthalpy values obtained either from the calorimetric or the nmr data. The origin of this disparity is not clear and may not be significant considering the errors inherent in the analysis of the optical data [2,25]. However, if the differences are real, they deserve further attention.

Clearly, different physical techniques monitor different windows of the transition. The nmr data of Patel monitor the base pairing interactions. The uv melting studies monitor the transition by following the hyperchromicity which reflects the base stacking interaction. Thus, it is of interest to note that the $\Delta H_{\text{V.H.}}$ obtained from the uv optical data (which monitors base stacking) is somewhat higher than the $\Delta H_{\text{V.H.}}$ calculated from the temperature-dependent nmr data (which monitors base pairing). By contrast, the calorimetric experiment monitors the total enthalpy change which includes both base pairing and base stacking as well as any additional enthalpy sources (e.g. differential ion binding to the helix and coil states). Thus, one should exercise caution when drawing conclusions about transitions based on data obtained from only one physical technique. On the other hand, as illustrated above, much can be learned about a transition by comparing results obtained from several complementary experimental techniques.

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