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A thermodynamic study on rA_7U_7

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Ultraviolet absorbance spectroscopy and differential scanning calorimetry were employed to study the heat-induced helix-to-coil transition of the oligoribonucleotide rA_7U_7. The analysis of concentration-dependent ultraviolet 'melting' profiles was used to derive the van't Hoff transition enthalpy ΔH_{vH}^{UV} (−458 kJ/mol cooperative unit). From the DSC data we calculated the calorimetric transition enthalpy ΔH_{cal} (−412.6 kJ/mol duplex) as well as ΔH_{vH}^{cal} (−447.9 kJ/mol cooperative unit). For the size of the cooperative unit we obtained $\lambda \approx 1$. In contrast to this result, by means of statistical numerical deconvolution we show that intermediate states are significantly populated; at the maximum the fraction of these states reaches 25.4% of the total population. Therefore, this DSC-deconvolution technique offers a more appropriate way to register amounts of populated intermediate states which are not sufficient to obtain a value of λ which is essentially lower than unity.

1. Introduction

The heat-induced helix-to-coil transition of short oligoribonucleotides with a high A/U content has been the subject of extensive studies since the beginning of the seventies. Thermodynamic parameters on helix formation for these oligoribonucleotides were obtained by analysis of the melting behavior derived from hypochromicity vs. temperature profiles [1,2]. However, thermodynamic data derived in this way only yield correct results in the case of two-state transitions. A more careful analysis of thermal denaturation processes needs model-independent data which can be obtained by using differential scanning calorimetry (DSC). The first, and thus far, only calorimetric study on rA_xU_y systems was described by Breslauer et al. in 1975 [3]. Combining DSC and UV data and calculating the size of the cooperative unit

$$\lambda = \Delta H_{vH}/\Delta H_{cal} \tag{1}$$

these authors identified the helix-to-coil transition of rA_7U_7 as being a multi-state process.

In recent years, much progress has been made in the field of numerical treatment of DSC data [4]. In this paper, we use partition functions as described by Freire and Biltonen [5–7] to deconvolute DSC data on the melting of rA_7U_7. Compared to calculating λ, this deconvolution technique offers a more accurate way to decide whether a helix-to-coil transition of oligonucleotides proceeds in a two-state or multi-state manner.

2. Materials and methods

2.1. Synthesis and purification of rA_7U_7

Synthesis of rA_7U_7 was carried out as described by Breslauer et al. [3]. All biochemicals needed for the synthesis were purchased from Boehringer (Mannheim). For isolation of $r(Ap)_7$ and rA_7U_7, we used a Fractogel TSK DEAE-650(S) (Merck) MPLC column [8]. Purified $r(Ap)_7$ was obtained using a linear gradient of 0–0.5 M NaCl (2 × 500

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ml). The desired $r(Ap)_7$ peak was identified by means of the 5′-AMP marker peak at the beginning of the chromatogram. rA_7U_7 was isolated by means of a linear gradient at pH 10.4 in 10 mM Tris from 0.3 to 0.6 M KCl (2×1000 ml). Determination of chain length was accomplished by means of the relative band intensities in the infrared spectra of the random coil at 80 ° C.

Desalting was executed in two steps. For preliminary desalting, the 10-fold diluted MPLC eluate was loaded on a Fractogel LC column. After washing with 0.05 M TEAB in order to remove salt, the oligonucleotide was eluted with 1 M TEAB. TEAB was removed by drying the eluate using a rotary evaporator, re-dissolving in water and re-drying three times. Final desalting was achieved by subsequent passage through a Sephadex G-10 column (Pharmacia) using 0.05 M TEAB. Rotary evaporation finished the desalting.

2.2. Nucleotide solution

Measurements reported here were performed in a buffer system consisting of 1 M NaCl, 0.01 M sodium phosphate and 10^{-4} M EDTA, adjusted to pH 7. For DSC measurement, the double-strand concentration was spectroscopically determined. The extinction coefficient ϵ at 25 ° C was calculated using a nearest-neighbor analysis [9]. For rA_7U_7 we obtained $\epsilon = 11014\ M^{-1}\,cm^{-1}$.

2.3. Ultraviolet absorbance spectroscopy

Determinations of nucleotide concentrations as well as ultraviolet melting profiles were accomplished with a Perkin-Elmer Lambda 7 UV/VIS spectrometer at the maximum absorbance at 260 nm. The temperature was controlled by means of a digital controller. In denaturation experiments changes of temperature and extinction were registered on an x-y recorder. In order to extract thermodynamic data from optical melting curves we performed a series of experiments over a range of strand concentrations. The heating rate in each experiment was 1 degree/min between 5 and 80 ° C.

2.4. Differential scanning calorimetry

The DSC experiments were carried out with a DASM-4 microcalorimeter (V/O Mashpriborintorg, U.S.S.R.) [10] equipped with a serial-input-output system for data collection developed in our laboratory. The temperature was scanned from 5 to 80 ° C at a rate of 1 degree/min.

3. Results

3.1. Thermodynamic analysis of optical melting data

The formation of the double helix from two separate self-complementary single strands rA_7U_7 is a bimolecular process. Therefore, melting temperature data are concentration-dependent and can be interpreted using the equation [1,11]

$$\frac{1}{T_m} = \frac{R}{\Delta H_{vH}} \ln C_T + \frac{\Delta S}{\Delta H_{vH}} . \qquad (2)$$

The T_m data of our series of concentration-dependent melting curves were used to construct a $1/T_m$ vs. $\ln C_T$ plot, which is shown in fig. 1. Here, $1/T_m$ denotes the reciprocal melting temperature (in K) and C_T equals the total concentration in single strands. The van't Hoff transition enthalpy ΔH_{vH} can be calculated from the slope of this plot. By means of this method we obtained $\Delta H_{vH}^{UV} = -458$ kJ/mol cooperative unit.

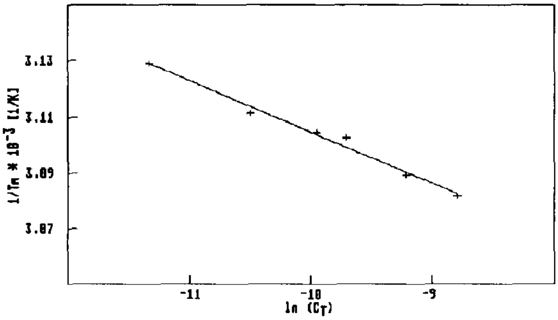

Fig. 1. $1/T_m$ (reciprocal melting temperature) vs. $\ln C_T$ (total strand concentration) for rA_7U_7 in 1 M NaCl, 0.01 M sodium phosphate and 10^{-4} M EDTA at pH 7.

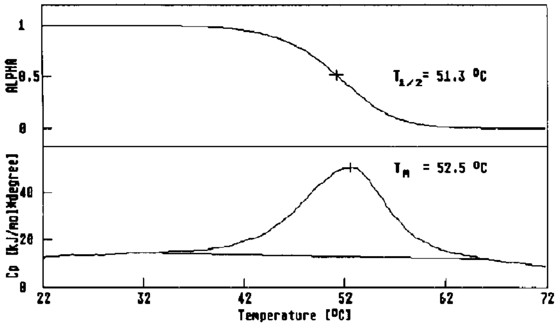

Fig. 2. Heat-induced helix-to-coil transition curves of rA_7U_7 in 1 M NaCl, 0.01 M sodium phosphate and 10^{-4} M EDTA at pH 7. Strand concentration, 1.41×10^{-4} M. Lower curve: calorimetric molar heat capacity vs. temperature. Upper curve: fraction of double-stranded molecules. It is shown that $T_{1/2} < T_m$.

3.2. *Conventional analysis of the calorimetric data*

The result of heating rA_7U_7 in the DSC experiment is shown in fig. 2. The lower curve shows the molar heat capacity vs. temperature, while the upper sigmoidal curve represents the fraction α of strands in the double-stranded state calculated from the experimental calorimetric recorder trace as a function of temperature.

The area under the heat absorption peak is proportional to the enthalpy change accompanying the thermal transition of rA_7U_7. The main problem in calculating the enthalpy arises from the determination of upper and lower limits of integration. For this reason, with the same nucleotide solution we monitored the denaturation by means of ultraviolet spectroscopy in parallel with the DSC experiment. From this melting curve limits were taken in order to accomplish the integration of the DSC heat absorbance peak.

Calculation of the van't Hoff transition enthalpy ΔH_{vH} from calorimetric data can be achieved by means of the following equation [12]

$$\Delta H_{vH}^{cal} = -\frac{17.73}{\dfrac{1}{T_{1/2}} - \dfrac{1}{T_{3/4}}}$$

$$\text{(J/mol cooperative unit)}, \qquad (3)$$

where $T_{1/2}$ and $T_{3/4}$ represent the half and three quarters temperatures at half height. In symmetrical DSC plots $T_{1/2}$ can be identified with the temperature at which the molar heat capacity attains its maximum value ('T_m'). However, as shown in fig. 2 our curve is asymmetrical, i.e. here we find $T_{1/2} < T_m$. Therefore, eq. 3 cannot be applied directly in our DSC plots and we used the equation

$$\Delta H_{vH}^{cal} = 6RT_{1/2}^2(\partial\alpha/\partial T)_{T_{1/2}}$$

$$\text{(J/mol cooperative unit)}. \qquad (4)$$

$T_{1/2}$ can be determined according to the condition

$$\alpha = 0.5 \text{ at } T = T_{1/2}. \qquad (5)$$

Values for ΔH_{cal} and ΔH_{vH}^{cal} from two DSC experiments at different strand concentrations are listed in table 1. On average, we obtained $\Delta H_{cal} = -412.2$ kJ/mol duplex and $\Delta H_{vH}^{cal} = -438.2$ kJ/mol cooperative unit. The agreement between corresponding results is satisfactory. From these data, we conclude that the maximum uncertainty in the calorimetric results should be not higher than 5%.

Using eq. 1, the size of the cooperative unit λ can be calculated by means of ΔH_{vH}^{cal} as well as ΔH_{vH}^{UV}. The values of λ obtained are set out in table 2. In both cases, we find $\lambda \approx 1$, which can be interpreted in such a way that the thermal denaturation of rA_7U_7 occurs in a two-state

Table 1

Calorimetric results from the thermal denaturation of rA_7U_7

	$-\Delta H_{cal}$ (kJ/mol duplex)	$-\Delta H_{vH}^{cal}$ (kJ/mol cooperative unit)	λ	$F_{1,max}$
Expt. 1	420.0	447.9	1.07	0.242
Expt. 2	405.1	428.5	1.06	0.266
Average	412.6	438.2	1.07	0.254

Table 2

Size of the cooperative unit

	λ
$\Delta H_{cal}/\Delta H_{vH}^{UV}$	1.09
$\Delta H_{cal}/\Delta H_{vH}^{cal}$	1.07

manner. If intermediate states are significantly populated the melting curve becomes broader, for ΔH_{vH} a lower result is obtained and therefore $\lambda < 1$ [4,11,13].

3.3. Statistical numerical treatment of the calorimetric data

According to the deconvolution formalism given by Freire and Biltonen [5–7], it is possible to calculate the fractions of molecules in multi-state transitions which are fully double-stranded (F_0), fully single-stranded (F_n) and the fraction of those which populate intermediate states (F_I). Using the partition function for double strands

$$Q = \exp\left[\int_{T_0}^{T} \frac{\langle H \rangle}{RT^2} \mathrm{d}T\right] \qquad (6)$$

the fraction F_0 can be calculated

$$F_0 = \frac{1}{Q} = \exp\left[-\int_{T_0}^{T} \frac{\langle H \rangle}{RT^2} \mathrm{d}T\right]. \qquad (7)$$

For this calculation values of the excess enthalpy relative to the initial state, $\langle H \rangle$, are needed, which can be derived by integrating the DSC plot from T_0, the lower limit of integration at which all molecules exist in the initial total helical state, to the actual temperature T. In this way the sigmoidal transition curve shown in fig. 3 was obtained.

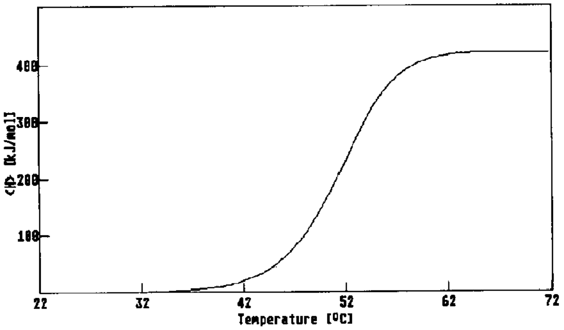

Fig. 3. Calorimetric excess enthalpy relative to the initial state vs. temperature. Strand concentration, 1.41×10^{-4} M; buffer, 1 M NaCl, 0.01 M sodium phosphate, 10^{-4} M EDTA.

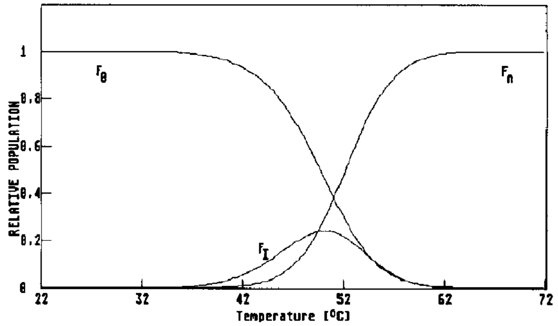

Fig. 4. Relative populations of states associated with the thermal denaturation of rA_7U_7. F_0, fraction of molecules populating the initial state; F_n, fraction populating the final state; F_I, summed population of all intermediates.

The fraction F_n of single-stranded molecules can be obtained using the equation

$$F_n = \exp\left[-\int_{T}^{T_n} (\Delta H_n - \langle H \rangle) \frac{1}{RT^2} \mathrm{d}T\right], \qquad (8)$$

where at T_n, the upper limit of integration, all molecules exist in the final coil state and ΔH_n represents the enthalpy change of the whole process. If intermediate states are present their fraction F_I can be evaluated by noting that

$$F_I = 1 - F_0 - F_n. \qquad (9)$$

Application of eqs. 7–9 to the data of one of our DSC measurements on rA_7U_7 yielded the result shown in fig. 4. The relative populations F_0, F_n and F_I have been calculated and are plotted vs. temperature. The maximum of intermediate states $F_{I,\max}$ can be obtained by using the condition

$$(\partial F_I / \partial T) = 0 \text{ at } F_I = F_{I,\max}. \qquad (10)$$

The results from deconvolution of our two DSC curves are shown in table 1. On average, 25.4% of the total population at maximum occupy intermediate states which clearly demonstrates that the thermal denaturation of rA_7U_7 does not proceed in a two-state fashion as suggested by the result of calculating λ from ΔH_{cal} and ΔH_{vH}. The numerical values of $F_{I,\max}$ from these two independent measurements are in good agreement. Therefore, we estimate that the uncertainty in $F_{\mathrm{I,max}}$ values does not exceed 10%.

4. Discussion

4.1. The 'λ criterion'

In table 3 our calorimetric results are compared with those reported by Breslauer et al. [3,11]. It is clear that good agreement exists in the calorimetric transition enthalpy ΔH_{cal}. Our values for the van't Hoff enthalpy $\Delta H_{\mathrm{vH}}^{\mathrm{cal}}$ as well as $\Delta H_{\mathrm{vH}}^{\mathrm{UV}}$, which are in reasonably good agreement, are significantly higher as compared to the value for $\Delta H_{\mathrm{vH}}^{\mathrm{UV}}$ reported by the above authors. As a consequence of this discrepancy, we obtain $\lambda \approx 1$ instead of $\lambda = 0.76$ for the size of the cooperative unit.

According to this λ criterion, the denaturation of rA_7U_7 should proceed in a two-state manner. However, the question is whether this helix-to-coil transition is indeed a real two-state process or an apparent two-state process with a relative population of intermediate states which does not lower ΔH_{vH} sufficiently to obtain $\lambda < 1$.

4.2. The $F_{I,max}$ criterion

From the deconvolution of our DSC curves, it is evident that rA_7U_7 melts in a multi-state manner. This result can be explained by means of the generally accepted concept of frayed terminal base-pairs in small oligonucleotides. The self-complementary rA_7U_7 consists only of A-U base-pairs which undergo fraying at the ends of the helix. Consequently, the base-pair stability becomes gradually higher from the end toward the middle of the helix. For this reason, we suppose that the melting of the helix starts at its ends and proceeds to its centre. Therefore, intermediate states which occur during the melting process should contain intact base-pairs at the middle and molten base-pairs at the end of the helix.

Table 3

Comparison of previously published data with the present results

	$-\Delta H_{\mathrm{cal}}$ (kJ/mol duplex)	$-\Delta H_{\mathrm{vH}}^{\mathrm{UV}}$ (kJ/cooperative unit)	$-\Delta H_{\mathrm{vH}}^{\mathrm{cal}}$	λ
Refs. 3, 11	415	317		0.76
This work	412.6	458		438.21.07

Moreover, by calculating $F_{\mathrm{I,max}}$ it becomes possible to obtain information about the degree of approximation to a real two-state transition of the system under examination. Oligonucleotides which in terms of the λ criterion undergo a two-state transition can now be analysed in a more accurate way and therefore involve differing considerations.

4.3. Conclusions

Our theoretical understanding of helix-to-coil transitions of oligonucleotides essentially depends on the ability to derive sequence-dependent thermodynamic parameters by means of model-independent methods. The deconvolution formalism used above does not rely on a defined model except that a transition between a double-stranded and a single-stranded form is assumed.

The numerical value of $F_{\mathrm{I,max}}$ should become lower if the degree of cooperativity decreases. For this reason, the $F_{\mathrm{I,max}}$ criterion provides an interesting method for comparing the cooperativity of oligonucleotides with different sequence and length.

In recent years, thermodynamic parameters for a series of oligoribonucleotides have been published which have been determined by analysis of ultraviolet melting profiles according to the two-state model [14–17]. In order to check the reliability of these data, it would be desirable to calculate $F_{\mathrm{I,max}}$ based on DSC data. Knowledge of $F_{\mathrm{I,max}}$ would resolve the question of whether intermediate states are indeed not significantly populated or if there is a population in such states which cannot be neglected. In order to obtain correct thermodynamic results, only in the first case is an analysis of ultraviolet melting profiles allowed. Otherwise DSC is a more appropriate method for the analysis of such phenomena [18].

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