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Articles

Thermodynamics of Double- and Triple-Helical Aggregates Formed by Self-Complementary Oligoribonucleotides of the Type rA_xU_y[†]

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Received November 6, 1989; Revised Manuscript Received February 14, 1990

ABSTRACT: The thermal denaturation of a series of oligoribonucleotides of the form rA_xU_y ($x = 5$ or 7 and $y = 3-11$) has been characterized by means of IR spectroscopy, UV spectroscopy, and DSC. IR spectra proved the occurrence of double- and triple-helical regions at various contents of uracil residues in the nucleotide. From DSC measurements transition enthalpies, entropies, and free enthalpies were derived. The effect of fraying in terminal base pairs of symmetrical nucleotides ($x = y$) was quantified. Thermodynamic excess parameters due to dangling ends (5′A and 3′U), terminal AU base pairs, and UAU base triplets were obtained by comparing DSC results from different nucleotides. Empirical values for contributions of base stacking and pairing to the stability of terminal AU base pairs have been estimated: for nucleotides under study with a high degree of fraying at the ends of the helix the major stabilization effect comes from base stacking. The size of the cooperative unit λ in most nucleotides under study is larger than 1; i.e., in these cases intermolecular cooperation takes place. Through deconvolution of DSC data maximum populations of intermediate states $F_{1,\max}$ were obtained. On the basis of these results all nucleotides under study were proved to melt in multistate manner. $F_{1,\max}$ increases with the number of base pairs, decreases through dangling ends, and shows approximately constant values for triple-helical aggregates of the series rA_5U_y as well as rA_7U_y.

The knowledge of thermodynamic parameters for RNA[1] secondary structure stability is an essential base for better understanding of the function of different RNA structures like tRNA, mRNA, or rRNA. Typically, these structures contain short helical segments which are interrupted by internal loops, mismatches, or bulge nucleotides and terminated by hairpins or dangling ends (Cantor & Schimmel, 1980; Crawford & Stauffer, 1980; Gutell et al., 1985). The contribution of these various structural features to the stability of RNA has been subject to intensive research since the beginning of the seventies (Martin et al., 1971; Gralla & Crothers, 1973; Uhlenbeck et al., 1973; Tinoco et al., 1973; Borer et al., 1973, 1974; Breslauer et al., 1975). By means of improved synthetic techniques more interesting sequences could be realized and became the subject of different studies in the last few years (Petersheim & Turner, 1983; Freier et al., 1983, 1985, 1986a,b; Hickey & Turner, 1985; Sugimoto et al., 1987a,b; Turner et al., 1987; Groebe & Uhlenbeck, 1988, 1989; Longfellow et al., 1990). As a main result of these studies it is generally accepted that for a wide range of various sequences the prediction of RNA stabilities has become possible.

For most oligoribonucleotides the excess stabilization of double helices by dangling ends is well-known as an effect due to enthalpic contributions (Petersheim & Turner, 1983; Freier et al., 1983; Sugimoto et al., 1987a). Favorable entropic contributions have only been obtained for nucleotides in which 5′ dangling ends are added next to GC pairs (Freier et al., 1985, 1986a). However, self-complementary oligoribonucleotides of the general type rA_xU_y (Martin et al., 1971) seem to be an exception: the reason for stabilization of a symmetrical core helix by dangling ends appears to be an unusual entropic effect even in cases of a 3′ dangling U base due to these results.

Data which lead to this surprising conclusion have been determined by analysis of UV melting profiles and therefore depend on the assumption of a two-state process governing the helix to coil transition of the oligoribonucleotide. However, oligoribonucleotides with a high content of AU base pairs actually do not melt in a two-state manner (Breslauer et al., 1975). Therefore, a more careful analysis of thermal denaturation processes of oligoribonucleotides like rA_xU_y needs model-independent data which can be obtained from calorimetric measurements (Breslauer, 1986; Marky & Breslauer, 1987; Sturtevant, 1987; Ackermann, 1989).

[†] This work was supported by the Deutsche Forschungsgemeinschaft through SFB 60 (Projects A-4 and H-1).

[1] Abbreviations: UV, ultraviolet; DSC, differential scanning calorimetry; FTIR, fourier transform infrared spectroscopy; IR, infrared; A, adenine; U, uracil; RNA, ribonucleic acid; tRNA, transfer ribonucleic acid; mRNA, messenger ribonucleic acid; rRNA, ribosomal ribonucleic acid; bp, base pair(s).

0006-2960/90/0429-5237$02.50/0

For this reason, we started a program in order to determine thermodynamic parameters of thermal denaturation of the self-complementary oligoribonucleotides rA_5U_y (y = 3–10) and rA_7U_y (y = 5–11) by means of DSC. For the purpose of gaining better insight into the degree of cooperativity DSC curves have been deconvoluted (Freire & Biltonen, 1978a–c). The deconvolution parameter $F_{\mathrm{I,max}}$ has been compared with the length of the cooperative unit λ (Ohms & Ackermann, 1989). Additionally, for comparing our results with the former ones we present temperature-dependent UV melting data.

In cases of $x < y$ the formation of triple-stranded (A + 2U) regions becomes possible (Martin et al., 1971). The excess stabilization due to this effect has been quantified. IR spectra of nucleotides with different U contents verify the assumption of triple-helical regions in those associates of rA_xU_y.

The theoretical calculation of the free enthalpy of complex RNA structures like 5S rRNA yields values that are sufficiently lower than those obtained from melting experiments. Therefore, additional tertiary interactions are assumed to play a major part in the maintenance of RNA structures (Appel et al., 1979; Digweed et al., 1986). In this context from the analysis of IR spectra it has been suggested that triple-helical structural elements like CGC or UAU occur in the B form of *Escherichia coli* 5S rRNA (Böhm et al., 1981). Furthermore, triple base pairs are assumed to exist in various tRNA molecules (Shulman et al., 1975; Schernau & Ackermann, 1977). Currently, triple-stranded DNA helices cause great interest due to their potential role in the regulation of the eukaryotic genome (Wells et al., 1988; de los Santos et al., 1989). Thermodynamic data concerning UAU triplets reported here give further insight into stabilization effects of triple-helical regions.

MATERIALS AND METHODS

Synthesis and Purification of Oligoribonucleotides. rA_5U_y (y = 3–10) and rA_7U_y (y = 5–11) have been synthesized and purified according to procedures described previously (Rockstroh et al., 1988; Ohms & Ackermann, 1989). Chain lengths were determined by means of relative band intensities in IR spectra of the random coil at 80 °C.

Nucleotide Solutions. Measurements reported here were performed in 1 M NaCl, 0.01 M sodium phosphate, and 10^{-4} M EDTA, adjusted to pH 7. For DSC measurements double-strand concentrations were spectroscopically determined. The 25 °C extinction coefficients ϵ were calculated by using a nearest-neighbor analysis (Fasman, 1975). In units of 10^4 M^{-1} cm^{-1} they are the following: rA_5U_3, 1.14; rA_5U_4, 1.12; rA_5U_5, 1.11; rA_5U_6, 1.10; rA_5U_7, 1.09; rA_5U_8, 1.08; rA_5U_9, 1.07; rA_5U_{10}, 1.06; rA_7U_5, 1.12; rA_7U_6, 1.11; rA_7U_7, 1.10; rA_7U_8, 1.09; rA_7U_9, 1.09; rA_7U_{10}, 1.08.

UV Absorbance Spectroscopy. Determinations of nucleotide concentrations as well as UV melting profiles were accomplished with a Perkin-Elmer Lambda 7 UV/vis spectrometer at the nucleotide's maximum absorbance at 260 nm. The temperature was controlled by means of a digital controller. In denaturation experiments the heating rate was 1 deg/min between 5 and 80 °C. For each RNA molecule six melting profiles over a 10-fold range of concentrations were measured. Changes of temperature and extinction were registered with an x–y recorder and subsequently translated into digital data by means of a CRP digitizer. In this way 600 data points were obtained and analyzed for each transition curve.

Differential Scanning Calorimetry (DSC). For DSC experiments a DASM-4 microcalorimeter (V/O Mashpriborintorg, USSR) (Privalov, 1980) was used. In each experiment the temperature was scanned from 5 to 80 °C at a rate of 1 deg/min. Collection of 1500 data points per run was accomplished by means of a serial input–output system developed in our laboratory.

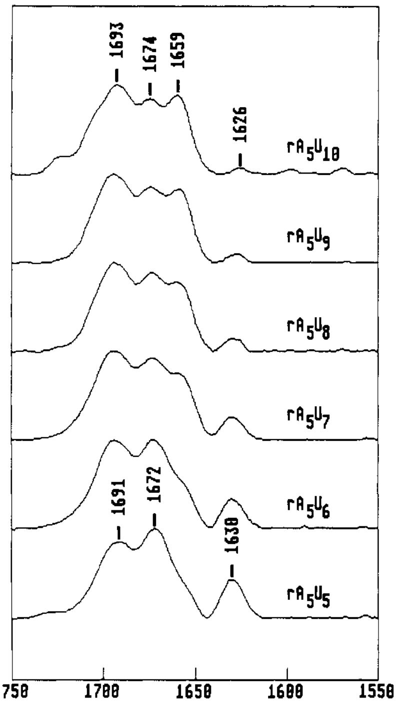

FIGURE 1: FTIR spectra of rA_5U_y (y = 5–10) in 1 M NaCl, 0.01 M sodium phosphate, and 10^{-4} M EDTA at 10 °C.

For each RNA molecule at least two independent DSC experiments at various strand concentrations were performed. Furthermore, measurements with the same probe were repeated twice. Therefore, DSC values reported here result from at least six measurements.

FTIR Spectroscopy. For preparation the nucleotide–buffer solutions were lyophilized, three times resolved in deuterium oxide, and again lyophilized. Probes resolved in deuterium oxide were filled in a CaF_2 cell of 100-μm path length. The cell was thermostated by an external water bath. Spectra were recorded at 2-cm^{-1} resolution with a Bruker IFS 113v Fourier transform infrared spectrometer equipped with a DTGS detector and an Aspect 2000 computer. A total of 512 interferograms were collected, apodized, and Fourier transformed after one level of zero filling.

RESULTS

Structural Features from IR Spectra. Double- and triple-helical regions in ordered structures of self-complementary oligoribonucleotides like rA_xU_y can be proved by means of charactertistic bands in their IR spectra. For this purpose Figure 1 shows a series of IR spectra of rA_5U_y (y = 5–10) obtained at 10 °C.

The 10 °C spectrum of rA_5U_5 shows strong coupled U carbonyl bands (1673 and 1691 cm^{-1}) (Lewis et al., 1984) and an A-ring vibration band (1630 cm^{-1}). The appearance of U carbonyl bands at 1673 and 1691 cm^{-1} is characteristic of two-stranded AU helices and persists through structural changes of both A and U bases (Miles & Frazier, 1964a; Howard et al., 1966; Tsuboi et al., 1973). The band at 1673 cm^{-1} is mainly assigned to the vibration of the hydrogen-bonded $C_4{=}O$ group of the U residue. During melting of the double helix this band is shifted to 1659 cm^{-1}. In the random coil the A-vibration mode is observed at 1623 cm^{-1}.

The spectrum of rA_5U_{10} recorded at 10 °C shows a quite different spectral pattern due to the triple-helical structure of

Table I: Thermodynamic Parameters of Helix to Coil Transitions of Self-Complementary Oligoribonucleotides rA_xU_y from DSC Measurements at 1 M NaCl, 0.01 M Sodium Phosphate, and 10^{-4} M EDTA, Adjusted to pH 7

	$-\Delta H$ (kJ/mol of duplex)	$-\Delta\Delta H$ (kJ/mol of duplex)	$-\Delta S$ [kJ/(deg·mol of duplex)]	$-\Delta\Delta S$ [kJ/(deg·mol of duplex)]	$-\Delta G_{37}$ (kJ/mol of duplex)	$-\Delta\Delta G_{37}$ (kJ/mol of duplex)
rA_5U_3	171.9	54.3	0.557	0.165	-0.77	3.15
rA_5U_4	226.2	45.1	0.722	0.137	2.38	2.63
rA_5U_5	271.3	14.9	0.859	0.04	5.01	2.5
rA_5U_6	286.2	48.1	0.899	0.15	7.51	1.6
rA_5U_7	334.3	41.3	1.049	0.126	9.11	2.24
rA_5U_8	375.6	36.2	1.175	0.108	11.35	2.72
rA_5U_9	411.8	48	1.283	0.15	14.07	1.5
rA_5U_{10}	459.8		1.433		15.57	
rA_7U_5	314.7	49.5	0.977	0.151	11.83	2.69
rA_7U_6	364.2	48.4	1.128	0.145	14.52	3.45
rA_7U_7	412.6	13.8	1.273	0.039	17.97	1.71
rA_7U_8	426.4	49	1.312	0.159	19.68	-0.29
rA_7U_9	475.4	44.1	1.471	0.14	19.39	0.7
rA_7U_{10}	519.5	34.6	1.611	0.1	20.09	3.5
rA_7U_{11}	554		1.711		23.59	

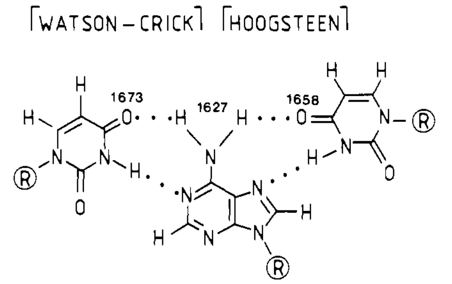

FIGURE 2: Base-pairing scheme for the triplex UAU. Both U residues contribute their $C_4{=}O$ group to hydrogen bonds. The figure also contains frequencies for IR bands of the major corresponding groups.

this system (Miles & Frazier, 1964b) in which A is engaged in both antiparallel Watson–Crick and parallel Hoogsteen base pairs (Saenger, 1984) (Figure 2). A suspicious difference in comparison to the rA_5U_5 spectrum is the presence of a strong 1658-cm^{-1} band which in contrast to the random coil spectrum corresponds primarily to the Hoogsteen-bonded $C_4{=}O$ group in the triple-helical base pair. For the Watson–Crick-bonded $C_4{=}O$ group the same vibration as in double-helical complexes at 1673 cm^{-1} appears. Furthermore, the intensity of the A band at 1627 cm^{-1} is lower than in double-helix spectra. On heating, the helix to coil transition occurs, and at high temperature the typical random coil spectrum is obtained (Figure 3).

In summary, the spectrum of rA_5U_5 represents a "pure" double helix whereas the rA_5U_{10} spectrum shows 100% triple helix. Growing intensity of the 1658-cm^{-1} band as well as decreasing intensity of the 1627-cm^{-1} band from rA_5U_7 to rA_5U_{10} results from the increase of triple-helical regions in these RNA molecules (Figure 1).

Thermodynamic Analysis of RNA Stability. Helix to coil transitions of oligoribonucleotides can be characterized by model-independent ΔH, ΔS, and ΔG values which can be derived directly from DSC experiments. The area under the heat absorption peak (Figure 4) is proportional to the enthalpy change ΔH_{cal} accompanying the thermal transition of the nucleotide:

$$\Delta H_{cal} = \int_{T_0}^{T_n} C_p \, dT \qquad (1)$$

whereas integration of the corresponding C_p/T versus T curve yields the transition entropy ΔS:

$$\Delta S = \int_{T_0}^{T_n} \frac{C_p}{T} \, dT \qquad (2)$$

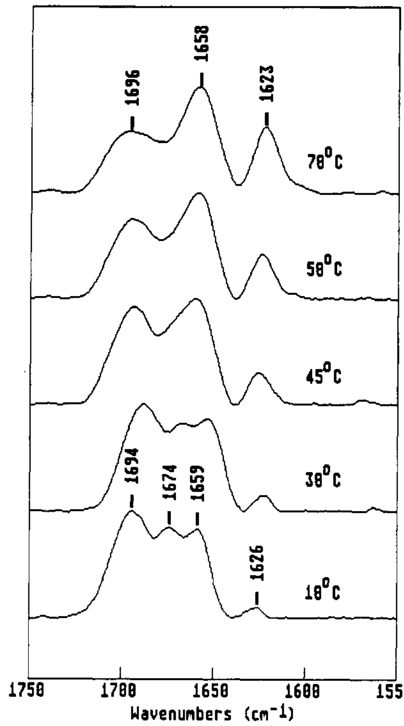

FIGURE 3: FTIR spectra of rA_5U_9 in 1 M NaCl, 0.01 M sodium phosphate, and 10^{-4} M EDTA recorded at different temperatures.

From ΔH_{cal} and ΔS the free enthalpy change ΔG can be calculated by means of

$$\Delta G = \Delta H_{cal} - T\Delta S \qquad (3)$$

Averaged data from at least six independent DSC measurements at different strand concentrations are given in Table I. The agreement of data concerning the same nucleotide is satisfactory. From our data we conclude that the maximum uncertainty of these calorimetric results should not be higher than 5%. For rA_7U_7, our ΔH_{cal} of -412.6 kJ/mol of duplex is in good agreement with the result of -415 kJ/mol of duplex reported by Breslauer et al. (1975).

Calorimetric results shown in Table I are related to the bimolecular formation of ordered structures (i.e., they are given *per mole of duplex*) and are not affected by possible intermolecular aggregation. Primarily, we are interested in thermodynamic excess parameters due to dangling ends, terminal AU base pairs, and UAU base triplets in systems with a high degree of AU base pairs. In consequence of our data being self-consistent these parameters can be calculated by taking

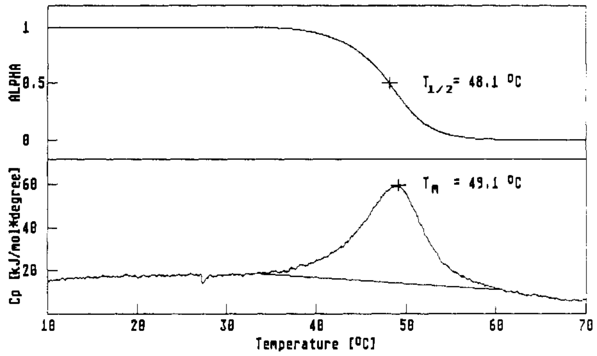

FIGURE 4: Heat-induced helix to coil transition curves of rA_5U_9 in 1 M NaCl, 0.01 M sodium phosphate, and 10^{-4} M EDTA at pH 7. Strand concentration: 0.988×10^{-4} M. Lower curve: Calorimetric molar heat capacity versus temperature. Upper curve: Fraction of helical molecules. It is shown that $T_{1/2} < T_m$.

Table II: Excess Stabilization through Dangling Ends Next to AU Base Pairs

	$-\Delta\Delta H$ (kJ/mol of base pair)	$-\Delta\Delta S$ [kJ/(deg·mol of base pair)]	$-\Delta\Delta G_{37}$ (kJ/mol of base pair)
5′A	10.85	0.03	1.71
3′U	7.18	0.02	1.05

differences between DSC results of nucleotides under study.

Excess Stabilization through Dangling Ends Next to AU Base Pairs. The difference of $\Delta H_{\rm cal}$ values from rA_5U_5 and rA_7U_5 represents the excess enthalpy due to a double A stack at the 5′-end of the helix. Assuming that these two stacks are of equal value, the excess enthalpy per A stack should be

$$\Delta\Delta H_{\rm S,5'A} = 0.25[\Delta H_{\rm cal}(rA_7U_5) - \Delta H_{\rm cal}(rA_5U_5)]$$

In the same way the excess entropy $\Delta\Delta S_{\rm S,5'A}$ and the excess free enthalpy $\Delta\Delta G_{\rm 37,S,5'A}$ can be calculated. Furthermore

$$\Delta\Delta H_{\rm S,3'U} = 0.5[\Delta H_{\rm cal}(rA_5U_6) - \Delta H_{\rm cal}(rA_5U_5)]$$

and

$$\Delta\Delta H_{\rm S,3'U} = 0.5[\Delta H_{\rm cal}(rA_7U_8) - \Delta H_{\rm cal}(rA_7U_7)]$$

represent the excess enthalpy caused by a 3′ dangling U. The values of $\Delta\Delta H_{\rm S,3'U}$ and $\Delta\Delta S_{\rm S,3'U}$ from rA_5U_6/rA_5U_5 and rA_7U_8/rA_7U_7 are in good agreement, which leads to the thesis that stacking parameters of dangling bases do not depend on the chain length. Results obtained from this data treatment are shown in Table II.

Influence of Chain Length. By means of comparing transition enthalpies of the symmetrical nucleotides rA_5U_5 and rA_7U_7 the influence of chain length becomes obvious: −27.1 and −29.5 kJ per base pair are obtained. The influence of less stable terminal AU base pairs ("fraying ends") on $\Delta H_{\rm cal}$ becomes higher if the chain length decreases. Therefore, the transition enthalpy per base pair increases with the chain length.

With the assumption that the enthalpy increment of dangling A bases (Table II) does not depend on the chain length, transition enthalpies of rA_3U_3, rA_4U_4, and rA_6U_6 can be calculated by means of

$$\Delta H_{\rm cal}(rA_3U_3) = \Delta H_{\rm cal}(rA_5U_3) - 4(\Delta\Delta H_{\rm S,5'A})$$

$$\Delta H_{\rm cal}(rA_4U_4) = \Delta H_{\rm cal}(rA_5U_4) - 2(\Delta\Delta H_{\rm S,5'A})$$

$$\Delta H_{\rm cal}(rA_6U_6) = \Delta H_{\rm cal}(rA_7U_6) - 2(\Delta\Delta H_{\rm S,5'A})$$

Results obtained in this way are summarized in Table III.

Empirical Base-Stacking and Base-Pairing Contributions to Thermodynamic Excess Parameters from Terminal AU Base Pairs. Thermodynamic excess parameters $\Delta\Delta H_{\rm AU}$, $\Delta\Delta S_{\rm AU}$, and $\Delta\Delta G_{\rm 37,AU}$ of terminal AU base pairs can be obtained from differences between corresponding calorimetric results obtained for symmetrical nucleotides rA_5U_5, rA_6U_6, and rA_7U_7 (Table IV). From these excess parameters and dangling end increments (Table II) empirical base-stacking and -pairing contributions can be estimated. For terminal base pairs by means of

$$\Delta\Delta G_{\rm P,AU} = \Delta\Delta G_{\rm 37,AU}(rA_xU_y) - (\Delta\Delta G_{\rm S,5'A} + \Delta\Delta G_{\rm S,3'U}), \quad x = y$$

the empirical base-pairing contribution $\Delta\Delta G_{\rm P,AU}$ can be calculated. Results obtained by this procedure are assumed to be valid if stacking contributions do not depend on the chain length and if stacking parameters are equal for nucleotides forming a dangling end or being part of a base pair. In the same way as described above $\Delta\Delta H_{\rm P,AU}$ and $\Delta\Delta S_{\rm P,AU}$ can be obtained (Table V).

Table III: Transition Enthalpies of Symmetrical AU Cooligomers

	$-\Delta H$ (kJ/mol of bp)		$-\Delta H$ (kJ/mol of bp)
rA_3U_3	21.4	rA_6U_6	28.5
rA_4U_4	25.6	rA_7U_7	29.5
rA_5U_5	27.1		

Table IV: Excess Stabilization through Terminal AU Base Pairs

	$-\Delta\Delta H_{\rm AU}$ (kJ/mol of terminal bp)	$-\Delta\Delta S_{\rm AU}$ [kJ/(deg·mol of terminal bp)]	$-\Delta\Delta G_{\rm 37,AU}$ (kJ/mol of terminal bp)
rA_6U_6	35.6	0.105	3.05
rA_7U_7	35.05	0.102	3.43

Table V: Estimated Values for Contributions of Empirical Stacking and Pairing to Thermodynamic Excess Parameters through Terminal AU Base Pairs

	$\Delta\Delta H_{\rm AU}$ (kJ/mol of terminal bp)		$\Delta\Delta S_{\rm AU}$ [kJ/(deg·mol of terminal bp)]		$\Delta\Delta G_{\rm 37,AU}$ (kJ/mol of terminal bp)	
	stacking	pairing	stacking	pairing	stacking	pairing
rA_6U_6	18.02	17.58	0.049	0.056	2.76	0.29
rA_7U_7	18.02	17.03	0.049	0.053	2.76	0.67

Triple-Helical Contributions to Helix Stability. Enthalpy increments $\Delta\Delta H_{\rm cal}$ (Table I) from nucleotides with high U content ($y > x + 1$) show values that are too high to result from simple U stacking. The values

$$\Delta\Delta H_{\rm triple} = \Delta H_{\rm cal}(rA_5U_7) - \Delta H_{\rm cal}(rA_5U_6) \approx \Delta H_{\rm cal}(rA_7U_9) - \Delta H_{\rm cal}(rA_7U_8)$$

in the range of −24.1 kJ/mol of base pair can be explained through triple-helical excess stabilization as suggested by means of IR spectra (Figure 1). For $\Delta\Delta H_{\rm triple}$ increments up to rA_5U_{10} and rA_7U_{11} an average per base pair of −21.5 kJ/mol of base pair excess enthalpy due to triple base pairs is found.

A partition of transition enthalpies $\Delta H_{\rm cal}$ in contributions for double helix, dangling ends, and triple helix in the series rA_5U_y is shown in Figure 5.

Nature of the Transition. The size of the cooperative unit

$$\lambda = \Delta H_{\rm vH}/\Delta H_{\rm cal} \qquad (4)$$

is generally used as a criterion for the decision whether a helix to coil transition occurs in two-state ($\lambda = 1$) or multistate manner ($\lambda < 1$). In cases of intermolecular interaction λ becomes higher than 1 (Tsong et al., 1970; Sturtevant, 1987).

The van't Hoff transition enthalpy $\Delta H_{\rm vH}^{\rm cal}$ can be derived from DSC plots (Figure 4) by means of the equation

$$\Delta H_{\rm cal}^{\rm vH} = 6RT_{1/2}^{2}(\partial\alpha/\partial T)_{T_{1/2}} \qquad (5)$$

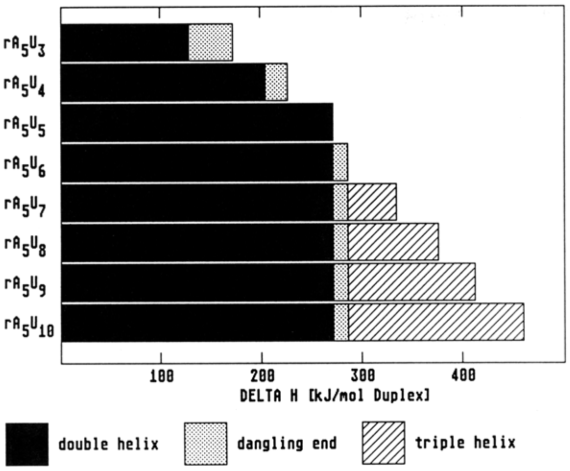

FIGURE 5: Partition of transition enthalpies from nucleotides of the series rA_5U_y into parts of double helix, dangling ends, and triple helix.

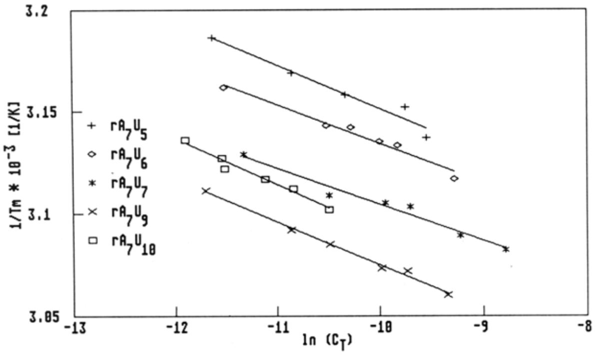

FIGURE 6: $1/T_m$ (reciprocal of the melting temperature) versus ln C_T (total strand concentration) plots for rA_7U_5, rA_7U_6, rA_7U_7, rA_7U_9, and rA_7U_{10} at 1 M NaCl, 0.01 sodium phosphate, and 10^{-4} M EDTA, adjusted to pH 7.

$T_{1/2}$ can be determined from the fraction of helical state α according to the condition

$$\alpha = 0.5 \qquad \text{at } T = T_{1/2} \tag{6}$$

In order to become sure about the reliance of our $\Delta H_{\mathrm{vH}}^{\mathrm{cal}}$ data for most of the nucleotides we performed UV experiments. From concentration-dependent UV data for bimolecular duplex formations $\Delta H_{\mathrm{vH}}^{\mathrm{UV}}$ can be calculated by using the equation

$$\frac{1}{T_m} = \frac{R}{\Delta H_{\mathrm{vH}}^{\mathrm{UV}}} \ln C_T + \frac{\Delta S}{\Delta H_{\mathrm{vH}}^{\mathrm{UV}}} \tag{7}$$

where C_T represents the total strand concentration (Figure 6). The melting temperature T_m has been determined from α versus temperature plots according to eq 6. The fraction of helical states α was calculated from normalized UV melting curves which were obtained by subtraction of lower and upper base lines (Albergo et al., 1981). The results for $\Delta H_{\mathrm{vH}}^{\mathrm{cal}}$ and $\Delta H_{\mathrm{vH}}^{\mathrm{UV}}$ are in good agreement (Table VI). For this reason, systematic errors in calculating the van't Hoff transition enthalpy by means of DSC can be excluded. From values of $\Delta H_{\mathrm{vH}}^{\mathrm{cal}}$ sizes of the cooperative unit λ are calculated (Table VII). With the exception of rA_7U_y (y = 9–11) λ is higher than 1; i.e., in these cases the size of the cooperative unit is larger than the duplex. This result can be explained by means of intermolecular cooperation between double strands.

In consequence of this cooperation the size of the cooperative unit is higher than 1 even if the nucleotides melt in a multistate manner. Only in cases of highly populated intermediate states the effect of aggregation is overcompensated and λ becomes lower than 1. For this reason we performed deconvolution of DSC curves (Biltonen & Freire, 1978a–c; Ohms & Ackermann, 1989) in order to calculate the maximum fraction of intermediate states $F_{\mathrm{I,max}}$. In this context it should be mentioned that $F_{\mathrm{I,max}}$ is related to the core duplex and for this reason is not affected from intermolecular aggregation. The fraction of fully helical molecules F_0 is given by

$$F_0 = \exp\left(-\int_{T_0}^{T} \frac{\langle H \rangle}{RT^2}\,\mathrm{d}T\right) \tag{8}$$

where $\langle H \rangle$ represents the excess enthalpy per mole of duplex relative to the initial state. The fraction F_n of single-stranded molecules can be obtained by using the equation

$$F_n = \exp\left[-\int_{T}^{T_n} (\Delta H_{\mathrm{cal}} - \langle H \rangle)\frac{1}{RT^2}\,\mathrm{d}T\right] \tag{9}$$

At T_n all molecules exist in the final random coil state and ΔH_{cal} represents the enthalpy change of the whole process (compare eq 1). The fraction of intermediate states F_{I} can be evaluated by noting that

$$F_{\mathrm{I}} = 1 - F_0 - F_n \tag{10}$$

Application of eqs 8–10 to DSC data yields a plot of F_{I} versus temperature (Figure 7) from which the maximum of intermediate states $F_{\mathrm{I,max}}$ (Table VII) can be obtained by means of the condition

$$\partial F_{\mathrm{I}}/\partial T = 0 \tag{11}$$

Results for $F_{\mathrm{I,max}}$ given in Table VII are average values from the same DSC measurements on which the DSC results shown in Table I are based. The numerical values of $F_{\mathrm{I,max}}$ obtained by independent experiments with the same nucleotide are in good agreement. We estimate that the uncertainty of $F_{\mathrm{I,max}}$ values does not exceed 10%.

Table VI: van't Hoff Transition Enthalpies of rA_5U_y and rA_7U_y from UV and DSC Measurements in Comparison with Former Results (Martin et al., 1971)[a]

	$-\Delta H_{\mathrm{vH}}^{\mathrm{cal}}$ (kJ/mol of cooperative unit)	$-\Delta H_{\mathrm{vH}}^{\mathrm{UV}}$ (kJ/mol of cooperative unit)	$-\Delta H_{\mathrm{vH}}^{\mathrm{UV}}$ (Martin et al., 1971) (kJ/mol of cooperative unit)
rA_5U_3	248.8		
rA_5U_4	302.7		184.1
rA_5U_5	327.9	299.9	301.2
rA_5U_6	394.9	376.2	[234.3]
rA_5U_7	401.9	385.9	
rA_5U_8	505.4	500.2	
rA_5U_9	522.9	515.2	
rA_5U_{10}	550.9		380.7
rA_7U_5	396.8	392.1	234.3
rA_7U_6	442.4	439.3	338.9
rA_7U_7	437.5	458	447.7
rA_7U_8	448.5	463.6	
rA_7U_9	384.2	393.7	
rA_7U_{10}	354.3	368.8	
rA_7U_{11}	380.3	336.8	

[a] Data reported by Martin et al. have been obtained from concentration-dependent UV measurements with the exception of the value in brackets.

Table VII: Maximum Population of Intermediate States $F_{\mathrm{I,max}}$ from DSC Measurements and Size of the Cooperative Unit λ

	$F_{\mathrm{I,max}}$	λ		$F_{\mathrm{I,max}}$	λ
rA_5U_4	10.6	1.34	rA_7U_5	15.7	1.26
rA_5U_5	21.2	1.21	rA_7U_6	20.5	1.21
rA_5U_6	17.4	1.38	rA_7U_7	25.4	1.06
rA_5U_7	19.2	1.20	rA_7U_8	32.5	1.05
rA_5U_8	24.3	1.35	rA_7U_9	50.9	0.81
rA_5U_9	24.2	1.27	rA_7U_{10}	59.8	0.68
rA_5U_{10}	26.5	1.20	rA_7U_{11}	55.2	0.69

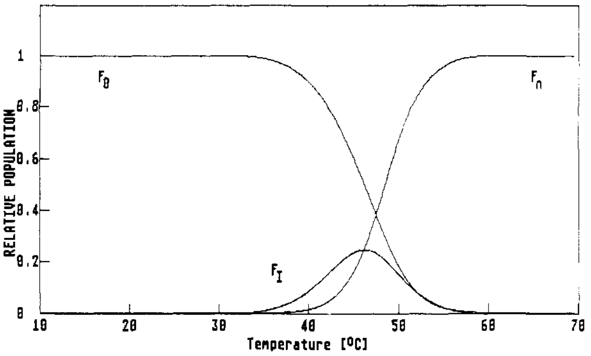

FIGURE 7: Relative populations of states associated with the thermal denaturation of rA_5U_9. F_0 is the fraction of molecules populating the initial state, F_n represents those that populate the final state, and F_I is the summed population of all intermediates.

The values of $F_{I,max}$ clearly show that generally in all AU oligonucleotides under study a multistate helix to coil transition occurs on melting. From these results it can be stated that an increase in the number of base pairs leads to higher populations in intermediate states. Comparison of $F_{I,max}$ values from rA_5U_5 with those of rA_7U_5 and rA_5U_6 shows that $F_{I,max}$ is lowered by dangling ends. A considerable difference in the magnitude of $F_{I,max}$ turns out in triple-helical systems of rA_5U_y (y = 7–10) with an average $F_{I,max}$ of 23.6% and of rA_7U_y (y = 9,10,11) with populations of intermediate states above 50%.

DISCUSSION

The goals of this work are (1) to derive model-independent thermodynamic parameters for helix to coil transitions of self-complementary AU oligoribonucleotides, (2) to quantify enthalpic, entropic, and stabilization effects due to double-helical and triple-helical base pairs as well as dangling ends, (3) to show that fraying of terminal AU base pairs is an effect that should not be neglected, (4) to estimate empirical values for contributions to base stacking and pairing to excess parameters through terminal AU base pairs, and (5) to discuss the extent of deviation from two-state transitions.

Conclusions from IR Spectra. The interpretation of thermodynamic results is based on the knowledge of the helical state of the system under study as it can be derived from IR spectra. The IR spectra of rA_5U_5 and rA_5U_{10} (Figure 1) clearly show considerable differences due to the helical state of the nucleotide. Frequencies of bands in both cases show agreement with those that already have been attached to double- and triple-helical aggregates of AU polyribonucleotides (Miles & Frazier, 1964a,b; Howard et al., 1966; Tsuboi et al., 1973). IR spectra of nucleotides with increasing U excess show increasing contributions of triple-helical regions. In nucleotides of the type rA_5U_y the existence of UAU triplets becomes evident if $y \geq 7$. This conclusion is confirmed by DSC results. From IR spectra of rA_5U_9 recorded at different temperatures (Figure 3) it becomes clear that the helix–coil transition of triple-stranded systems occurs in one step ($3 \rightarrow 1$ transition). An alternative possible strandwise $3 \rightarrow 2 \rightarrow 1$ dissociation can be excluded for this reason.

Stacking of Dangling Ends. van't Hoff transition enthalpies from UV data concerning some of the nucleotides that we have studied have already been reported by Martin et al. (1971) (Table VI). There is a good agreement of results for the symmetrical nucleotides rA_5U_5 and rA_7U_7 but also a considerable difference for other systems. As mentioned above, a survey over all data published by Martin et al. leads to conflicting conclusions concerning stabilization effects through dangling ends.

Intensive research concerning excess-stabilization effects through dangling ends in the last few years has yielded some general trends which are important for the interpretation of our results: (1) 3′ dangling purines add more stability than 3′ dangling pyrimidines. (2) For GC pairs, a 3′ dangling end provides more excess stability than a corresponding 5′ dangling end due to a better possibility of cross strand stacking at the 3′ terminus of the A-RNA helix. 5′ dangling ends cannot stack in such a favorable manner on the helix, and therefore the small excess stability is associated with a small favorable entropy term which overcomes an unfavorable enthalpy term. (3) This generalization is not true for dangling ends on AU pairs where only purines add more stability if they occur at the 3′ terminus (Sugimoto et al., 1987a).

In order to calculate the effect of a 5′ dangling A, we used thermodynamic parameters from rA_7U_5 and rA_5U_5. This procedure depends on the assumption that thermodynamic excess parameters of the two A stacks have the same values. This is true if intrastrand interactions between adjacent A bases at the 5′ terminus play a dominant role in these nucleotides. We believe this assumption to be valid because the geometry of the A-RNA does not allow interstrand stacking for 5′ dangling ends. Therefore, in order to reach the most favorable duplex conformation, intrastrand stacking probably will take place.

From our results we conclude that excess stabilization of 3′ dangling U as well as 5′ dangling A is associated with a favorable enthalpy term and unfavorable entropy term (Table I). Stabilization through dangling ends due to entropic contributions as stated by Martin et al. (1971) could not be confirmed. Furthermore, we state that the stabilization contributed by a 5′ dangling A is larger than the effect of a 3′ dangling U. This result is in agreement with the conclusion drawn by Sugimoto et al. (1987a), who found that effects of 3′ dangling pyrimidines are smaller than those of 5′ dangling purines if the dangling end is added next to an AU pair.

Stability of Frayed Terminal AU Base Pairs: Contributions of Stacking and Pairing. Calculation of transition enthalpies per base pair for symmetrical AU cooligomers rA_xU_y (x = y = 3–7) clearly shows the destabilization effect caused by fraying of terminal AU pairs (Table III).

For rA_6U_6 and rA_7U_7 the excess parameters due to terminal AU base pairs have been calculated (Table IV). For rA_7U_7 a larger excess stabilization $\Delta\Delta G_{37,AU}$ than for rA_6U_6 has been obtained which results from a smaller entropy term $\Delta\Delta S_{AU}$. This increase of the stabilization increment parallel to the chain length is a consequence of an increased stability of internal base pairs due to a decreased degree of fraying in the center of the helix.

The difference between thermodynamic excess parameters ($\Delta\Delta H_{AU}$, $\Delta\Delta S_{AU}$, and $\Delta\Delta G_{37,AU}$) for terminal AU pairs and stacking parameters ($\Delta\Delta H_S$, $\Delta\Delta S_S$, and $\Delta\Delta G_{37,S}$) for 5′A and 3′U dangling ends can be identified with empirical pairing contributions (Table V). In this context pairing contribution is defined as the thermodynamic effect associated with having two complementary bases opposite each other, minus the sum of the effects each base has separately (Petersheim & Turner, 1983). However, this estimation contains several uncertainties which are discussed in detail below.

Stacking contributions as the sum of dangling end parameters neglect the objection that stacking of a terminal base is not necessarily the same in a base pair as it is when a base is not paired, because a dangling end presumably has more freedom to adopt an optimum stacking geometry. Therefore, stacking used in this sense is only an empirical measure for

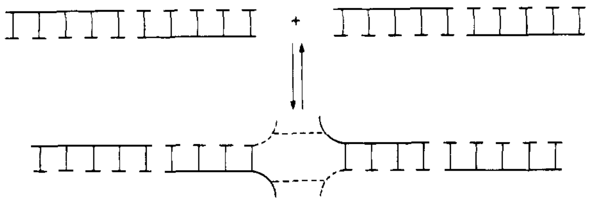

FIGURE 8: Model of aggregation between two self-complementary AU oligoribonucleotides. Population of such aggregates would increase the size of the cooperative unit λ.

the upper limit for the stacking contribution to the base pair formation (Turner et al., 1986). Further objections are concerned with the estimation of pairing contributions. Freier et al. (1986a) discuss the significance of an unfavorable configurational free enthalpy which is associated with the fixing of the flexible 5′-terminal nucleotide. Subtracting this term from the empirical pairing term, an increased excess stabilization due to pairing is obtained. However, on the basis of our results we believe 5′ dangling A bases in nucleotides of the type rA_xU_y to be fixed in intrastrand interaction, and therefore, this objection should be of minor bearing. At least the same significance should be ascribed to the effect of fraying. Assuming that stacking of helical bases does not depend on their position in the helix, we state that empirical pairing contributions calculated above (Table V) contain parts of pairing for terminal base pairs but also increased pairing contributions for stabilities of internal base pairs. Both these effects (fixing vs fraying) vary the real pairing contributions of terminal base pairs in different directions. If they are of the same order of magnitude, they will compensate each other and the empirical pairing contributions will be a reliable estimation.

From these considerations we conclude that for terminal AU base pairs in symmetrical AU cooligomers base stacking contributes between 75 and 90% of the total excess stabilization. Therefore, for these systems with a high degree of fraying at the helix termini base pairing appears to play only a minor role.

Nature of the Transition in Double-Helical Nucleotides. By calculation of the size of the cooperative unit λ from DSC results by means of eq 4 it has been proved that intermolecular cooperation between double-helical complexes takes place. It is generally accepted that values of $\lambda > 1$ indicate a cooperative unit larger than the molecular unit in consequence of oligomerization or aggregation (Sturtevant, 1987). Aggregation between AU oligoribonucleotides is theoretically possible in consequence of fraying at the ends of the helix. A model of such equilibria is shown in Figure 8. Of course, this speculation should be confirmed in detail by means of other methods. Martin et al. (1971) had obtained sedimentation coefficients for double- and triple-helical complexes. For the double-helical molecules rA_6U_6 and rA_7U_5 sedimentation coefficients at the limit of measurability were obtained. Therefore, aggregation had been excluded for these systems. However, if the extent of aggregation is small, this procedure probably fails and more sensitive methods must be used to study these equilibria.

In consequence of these aggregation effects from calculating λ no decision is possible as to whether the helix to coil transition of the nucleotides under study proceeds in a two-state or a multistate manner. For this reason deconvolution of DSC data by means of the formalism developed by Freire and Biltonen (1978a–c) provides an interesting method to get more information about the nature of the transitions. From deconvolution of our DSC curves it can be stated that melting of all AU oligoribonucleotides under study occurs in a multistate manner. As a criterion for the degree of melting in a multistate manner we use the core duplex related maximum population of intermediate states $F_{\mathrm{I,max}}$.

From these values of $F_{\mathrm{I,max}}$ it becomes obvious that an increase in number of base pairs increases the population of intermediate states. Through fraying, the stability of base pairs becomes gradually lower from the middle to the end of the helix. If melting of the helix starts at its ends and proceeds to its center, intermediate states should contain intact base pairs in the middle and molten base pairs at the ends of the helix. For this reason it is clear that nucleotides with a higher number of base pairs provide more possible intermediate states. Furthermore, values of $F_{\mathrm{I,max}}$ for rA_5U_6 and rA_7U_5 are lower than the value for rA_5U_5. Dangling ends in these cases probably stabilize the terminal base pairs of the rA_5U_5 core helix and therefore lower the population in intermediate states.

Triple-Helical Structures. By analysis of DSC curves and IR spectra we have shown that triple-helical regions occur in AU oligoribonucleotides rA_xU_y if $y > x + 1$. For the corresponding polymeric complex poly(A)·2poly(U) it has been stated that antiparallel strands of poly(U) are realized by folding of poly(U) on poly(A) (Thrierr & Leng, 1972). If the oligomeric system under study behaves in a similar manner, the required U excess ($y > x + 1$) for the formation of triple base pairs can be understood as a consequence of U folding.

Calculation of the size of the cooperative unit λ and the maximum population of intermediate states $F_{\mathrm{I,max}}$ for systems with triple base pairs yields two remarkable results. First, $F_{\mathrm{I,max}}$ values for nucleotides of the same series rA_5U_y or rA_7U_y are equal within the limits of experimental uncertainty: on average 25% for rA_5U_y and 55% for rA_7U_y. These values are larger than those that have been obtained for the symmetrical core helices rA_5U_5 (21.2%) and rA_7U_7 (25.4%). From these facts we conclude that formation of triple-helical base pairs increases the degree of fraying at the ends of the core helix. Furthermore, this increase of fraying upon formation of triple-helical regions seems to be independent from the number of triple-helical base pairs. Second, the size of the cooperative unit λ for nucleotides of the series rA_7U_y is smaller than 1. In agreement with high values for $F_{\mathrm{I,max}}$ this result proves an extreme high degree of melting in a multistate manner in thermal denaturations for these nucleotides. From these results it is not clear if intermolecular cooperation takes place in these systems or not. However, from the behavior of nucleotides of the series rA_5U_y with triple-helical regions aggregation in these cases it is probable, too. Measurements of sedimentation coefficients could convey clearness. Martin et al. (1971) reported a value for the sedimentation coefficient for rA_5U_8 which clearly shows that in this case a multistranded aggregate is formed containing an average of about four to seven strands of rA_5U_8. Unfortunately, no corresponding result for an rA_7U_y nucleotide is available. In order to determine the reason for these differences in the melting behavior of rA_5U_y and rA_7U_y, more detailed studies on this field are neccessary.

ACKNOWLEDGMENTS

The expert technical assistance of W. Wangler and S. Küstermann is gratefully acknowledged.

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