Registry No. I, 108817-44-3; II, 108817-45-4; III, 108817-46-5; IV, 108817-47-6.

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Formation of a Left-Handed RNA Double Helix: Energetics of the A–Z Transition of Poly[r(G-C)] in Concentrated $NaClO_4$ Solutions†

Horst H. Klump and Thomas M. Jovin*

Institut für Physikalische Chemie, Universität Freiburg, D-7800 Freiburg, Federal Republic of Germany, and Abteilung Molekulare Biologie, Max-Planck-Institut für Biophysikalische Chemie, D-3400 Göttingen, Federal Republic of Germany

Received December 30, 1986; Revised Manuscript Received March 13, 1987

ABSTRACT: Ultraviolet spectroscopic and nuclear magnetic resonance (NMR) studies have shown that poly[r(G-C)] in a solution of 4 M $NaClO_4$ undergoes a transition to a left-handed Z-RNA helix upon raising the temperature to 60 °C [Hall, K., Cruz, P., Tinoco, I., Jr., Jovin, T. M., & van de Sande, J. H. (1984) *Nature (London) 311*, 584–586]. In the present report, the transition temperature of this particular order/order transition is shown to increase with decreasing $NaClO_4$ concentration to about 110 °C, above which only the helix-to-random coil transition is detectable. The reversibility and cooperativity of the helix/helix conversion has facilitated the quantitative evaluation of the transition enthalpy by means of differential scanning microcalorimetry. In 5 M $NaClO_4$, the transition temperature is 43 °C, the conversion enthalpy 4.2 kJ (1.0 kcal) per mole of base pair, and the corresponding entropy change 13 J (3.1 cal) deg^{-1}. The van't Hoff enthalpy for the same process, determined from the temperature dependence of the optical transition, is 0.26 MJ (62 kcal) per mole of cooperative unit. The ratio of the two enthalpy values yields an apparent cooperative length for the A–Z transition of poly[r(G-C)] of ~60 base pairs, indicative of a concerted all-or-none process.

Some of the early attempts to solve the three-dimensional structure of DNA single crystals involved oligonucleotides composed exclusively of alternating guanine and cytosine residues (Wang et al., 1981; Dickerson et al., 1981). These sequences provide very stable secondary structures in small oligomers as well as in polymers and lack the difficulties associated with the corresponding homopolymer pair. Unexpectedly, the first crystal structure of the duplex of the hexanucleotide $d(C\text{-}G)_3$ did not reveal the familiar right-handed helix of B-DNA but rather a left-handed conformation designated Z-DNA (Wang et al., 1981). It provided a structural basis for the previously reported cooperative optical transitions and anomalous circular dichroism spectra of poly[d(G-C)] at high salt concentrations (Pohl & Jovin, 1972). Numerous

† This investigation was supported by the Max Planck Society and the Deutsche Forschungsgemeinschaft (DFG, Schwerpunktprogramm Biophysik der Organisation der Zelle) and by the Fonds der Chemischen Industrie.

* Address correspondence to this author at Max-Planck-Institut für Biophysikalische Chemie.

0006-2960/87/0426-5186$01.50/0

factors have been shown to influence the equilibrium between the B- and Z-DNA conformations [reviewed in Rich et al. (1984) and Jovin et al. (1983, 1987)]. For example, Z-DNA is stabilized by organic cosolvents, by high concentrations of monovalent cations and low concentrations of polyvalent cations, and by chemical modification (methylation, halogenation) at the C5 position of the pyrimidine base.

A similar transition has been observed in the corresponding RNA polymer poly[r(G-C)], in this case between the A conformation at low salt concentration and a Z-DNA-like form at high salt concentrations and temperatures [Hall et al. (1984); for a recent review see Cruz et al. (1986)]. Thus, Z-RNA shares with Z-DNA (i) the alternation in phosphate conformation, (ii) the syn configuration about the purine glycosidic bond, (iii) the accessibility of the GH8 proton, and, most significantly, (iv) the left-handed sense of the double helix. Unfortunately, a crystallographic structure is not yet available, except for the oligomers methylated or brominated at C8 of G (Nakamura et al., 1985), a modification that stabilizes the syn conformation and, thereby, the global Z form. The A-to-Z RNA transition is much less favorable than the corresponding B-to-Z transition in DNA. Thus, high concentrations of chaotropic salts (e.g., $NaClO_4$) or mixed solvents are required as well as elevated temperatures, in contrast to the temperature-independent B–Z equilibrium of poly[d(G-C)] in uni-univalent salts (Pohl & Jovin, 1972; Soumpasis & Jovin, 1987). Calorimetric measurements of d(C-G) oligomers have yielded estimates for the very low enthalpic contribution to the B–Z transition of ≤ 0.3 kcal mol·bp^{-1} (see footnote 1) (Marky et al., 1982; Klump, 1986b). We report here studies by differential scanning microcalorimetry of the more energetic A–Z transition of poly[r(G-C)].

Materials and Methods

Materials. Poly[r(G-C)] was prepared according to minor modifications of the method of Hall et al. (1985). Chain-length analysis by polyacrylamide gel electrophoresis under nondenaturing conditions using RNA virus standards (Biebricher et al., 1982) yielded estimates for the bulk of the material produced in such syntheses of ca. 100–200 base pairs. The polynucleotide solution was thawed and exhaustively dialyzed against several changes of 5 M $NaClO_4$, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.2, and used as the stock solution throughout the following experiments. The concentrations of solutions were calculated by using $\epsilon_p = 6900$ M^{-1} cm^{-1} at 260 nm, a value we determined using phosphate analysis according to Vogel (1961). (Karstadt and Krakow (1970) have previously reported an ϵ_p of 6600 M^{-1} cm^{-1} at 257 nm for the A form of poly[r(G-C)].) All chemicals were of analytical reagent grade, and the water was doubly distilled. The stock solution was diluted with the standard buffer.

Spectroscopic Measurements. UV spectra and transition curves were recorded either on Hitachi Perkin Elmer Model 124 and Kontron Uvikon 820 spectrophotometers at atmospheric pressure or with a Pye-Unicam Model 1800 spectrophotometer equipped with a pressurable (10 bar) cell holder (Klump & Löffler, 1985). The latter instrument permitted the registration of transition temperatures above 100 °C without boiling the water solvent.

Differential Scanning Microcalorimetry. An adiabatic scanning microcalorimeter, Type DASM-1 from Mashpriborintorg (Moscow) based on a design of Privalov (1974),

[1] Abbreviations: mol·bp, mole base pairs of DNA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

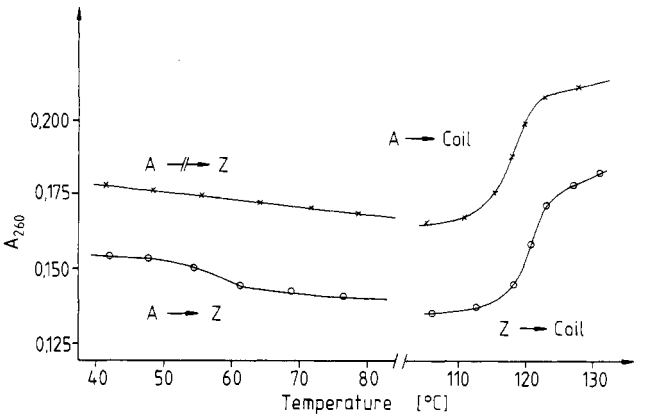

FIGURE 1: Absorbance of poly[r(G-C)] in $NaClO_4$ solutions as a function of temperature and under external pressure (10 bar): (○) 4 M $NaClO_4$; (×) 0.1 M $NaClO_4$.

was used. In a typical experiment, 1 mL of the concentrated RNA stock solution was filled into the sample cell and heated simultaneously with 1 mL of a solution of identical composition (except for the absence of the polynucleotide) filled into the reference cell. A differential measurement was then performed according to the published procedures. The enthalpy of the transition was calculated by comparison of the experimentally obtained peak area to an electrically induced calibration area of known enthalpy equivalents (Filimonov & Privalov, 1978).

Results

Absorption–temperature scans for two poly[r(G-C)] solutions differing only in the bulk $NaClO_4$ concentration are shown in Figure 1. At low counterion concentration (0.1 M Na^+) the absorbance recorded at 260 nm as a function of temperature remained unaffected up to almost 110 °C and then started to rise in a limited temperature interval due to the double-helix-coil transition of right-handed RNA. The scan was rerun with the same solution, yielding an entirely identical result. The second measurement shown in Figure 1 was performed in 4 M $NaClO_4$ and differed with respect to the behavior in the temperature range 50–60 °C. There was a small decrease of absorption with increasing temperature (hypochromic effect), characteristic of the order/order A–Z transition of the polynucleotide as reported by Hall et al. (1984). At higher temperatures, the absorbance showed a dramatic increase over a narrow temperature interval, indicative of the helix-coil transition of the Z-RNA. The latter process was rapidly reversible, whereas the low-temperature transition was comparatively slow and occurred at a distinctly lower temperature, i.e., demonstrated a hysteresis, upon reversal by cooling. We attribute this effect to the slow rate of the A–Z RNA interconversion and not to the population of metastable intermediates. For example, in other experiments the forward reaction initiated by introducing the RNA into 5 M $NaClO_4$ at 43 °C, i.e., the transition midpoint (Figure 2), exhibited a main phase (90% amplitude) with a $t_{1/2}$ of ~5 min and a somewhat slower final phase, possibly due to length heterogeneity. After the sample was cooled to 20 °C for 2 h, the A–Z RNA transition could be repeated as in the initial run, establishing that the order/order transition can be treated as a reversible process.

Figure 2 shows a number of A–Z transitions, measured by the characteristic hyperchromism at 290 nm, in which the initial sample (designated 1) was stepwise diluted by the appropriate buffer in order to lower the salt concentrations and thereby shift the transition temperature T_t to successively higher values. From the slopes of the symmetric transition

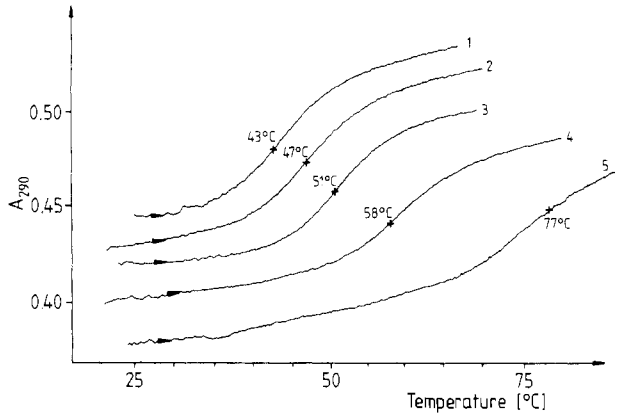

FIGURE 2: A–Z order/order transitions of poly[r(G-C)] solutions monitored by A_{290}. Different $NaClO_4$ concentrations were used: (1) 5 M; (2) 4.75 M; (3) 4.55 M; (4) 4.0 M; (5) 3.5 M. The ordinate refers to curve 3. The heating rate was 1 °C/min. The curves were analyzed by extrapolating the linear portions of the curves into the transition region, the T_t being defined at the intersection with a line halfway between the extrapolations (De Prisco et al., 1981).

curves at T_t, the van't Hoff enthalpies ΔH_{vH} were calculated according to standard thermodynamic relations applied to the equilibrium between two polymer conformations (Savoie et al., 1978; Soumpasis & Jovin, 1987)

$$\Delta H_{vH} = 4RT_t^2 \partial\theta/\partial T|_{\theta=0.5} \quad (1)$$

where R is the gas constant and θ is the degree of transition to the A form. Curves 1–4 of Figure 2 yielded ΔH_{vH} values of 0.25, 0.26, 0.26, and 0.23 MJ (~62 kcal) per mole of cooperative unit, respectively. T_t decreased linearly with the logarithm of the salt concentration (Figure 4). While the size of the cooperative unit in general cannot be derived from such spectroscopic measurements alone, the intrinsic (per mole of base pair) transition enthalpy ΔH can be extracted from the salt dependence of the transition temperature according to the expression (McIntosh and Jovin, submitted for publication; Soumpasis & Jovin, 1987)

$$d(1/T_t)/d \ln c = R\alpha/\Delta H \quad (2)$$

where α reflects the dependence of the intrinsic A–Z stability constant s on the salt concentration c; i.e., $s \propto c^{\alpha}$ (Pohl, 1983; Soumpasis & Jovin, 1987). From the above data, we derive the value $\Delta H/\alpha \sim 10$ kJ mol·bp^{-1}. Using $\alpha = 0.6$, obtained for d(G-C) oligonucleotides in NaCl (Pohl, 1983), ΔH equals ~6 kJ (or 1.4 kcal) mol·bp^{-1}.

The most direct estimation of the intrinsic thermodynamic parameters (ΔH, ΔS, $\Delta G°$), of course, is from calorimetric measurements. Scans of RNA solutions in 5 and 4.8 M $NaClO_4$ and the corresponding experimental base lines (buffer vs. buffer) are shown in Figure 3. The areas above the dotted-line segments provide the total enthalpy change which accompanies the inversion in the handedness of the helix (e.g., 0.579 mcal for the curve in Figure 3b).

The following information can be deduced from the calorimetric transition curves: (i) The A-DNA to Z-DNA transition is an endothermic reaction, favoring the left-handed conformation at elevated temperatures. (ii) The calorimetric transition coincides with the optical transition (Figure 1) but is slightly less symmetrical, partially due to distortion introduced by delays in instrument response. (iii) Comparison of the endothermic peak area to a calibration mark gives the exact transition enthalpy for the dissolved polynucleotide. Knowledge of the polymer concentration allows us to determine the molar transition enthalpy ΔH as 4.2 kJ (1.0 kcal) mol·bp^{-1}.

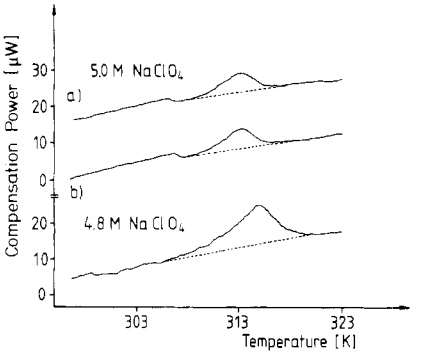

FIGURE 3: Calorimetric transition curve of the A–Z RNA transitions: (a) 5 M $NaClO_4$ (0.165 mg of poly[r(G-C)] in 1 mL); (b) 4.8 M $NaClO_4$ (0.393 mg of poly[r(G-C)] in 1 mL). A constant heating rate was used.

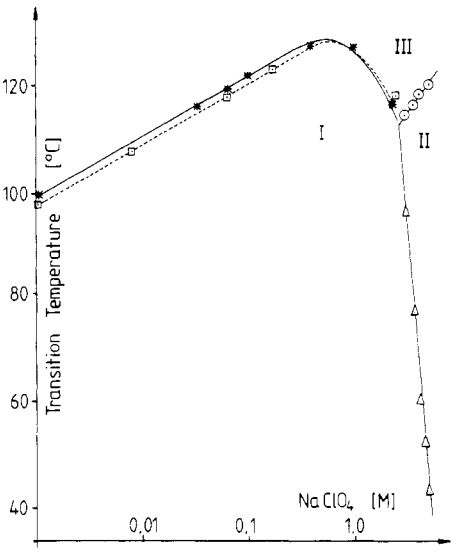

FIGURE 4: Phase diagram for poly[r(G-C)] as a function of $NaClO_4$ concentration and temperature. Area I gives the range of the right-handed A helix, area II the range of the left-handed Z helix, and area III the range of the random-coil form. The dotted lines and square symbols refer to poly(G)·poly(C). Other symbols: (*) A-coil transition; (Δ) A–Z transition; (O) Z-coil transition. The A–Z and Z-coil data were obtained in single experiments such as those shown in Figure 1.

The various determinations in Figure 3 yielded values within 3% of the mean. From the Gibbs–Helmholtz equation and assuming ΔH is temperature independent (as observed for ΔH_{vH}), the corresponding ΔS is 13 J (3.1 cal) deg^{-1} mol·bp^{-1} and $\Delta G°$ is 0.32 kJ (0.076 kcal) mol·bp^{-1} (25 °C). (iv) We can calculate the cooperative unit, i.e., the number of base pairs that undergo the A–Z RNA transition in a concerted manner, from the ratio $\Delta H_{vH}/\Delta H_{cal}$ and obtain the value of ca. 60 base pairs, indicative of an all-or-none transition for the rather short polymers constituting the poly[r(G-C)] preparations. (Population heterogeneity would increase the breadth of the optical transition and thus reduce the apparent ΔH_{vH}; thus, we do not consider the discrepancy with the estimated chain length significant.)

From these and the results of other experiments, we have constructed a state diagram that depicts the areas of stability for each of the conformations (Figure 4). Area I represents the conditions (temperature/counterion concentration) in which the right-handed A conformation is favored, area II shows the stability range for the left-handed Z conformation, and area III gives the conditions under which only the random coil exists. For comparison, the results of an investigation of the thermal stability of poly(G)·poly(C) are included; this

polymer has the same base composition as poly[r(G-C)], but the homopolymer pair structure does not undergo a transition to a left-handed form. In common to all other polynucleotides investigated so far, poly[r(G-C)] shows a linear increase of melting temperature T_m on log [Na^+], i.e., from 1 mM up to 0.3 M. Beyond this concentration, the T_m first levels off and then actually decreases as the sodium ion concentration surpasses 1 M. This drop is suddenly reversed as the concentration of $NaClO_4$ exceeds 2.7 M. As already discussed, the observation of a two-step process is consistent with an order/order transition (helix inversion) followed by a helix–random coil transition.

Discussion

In recent years there have been numerous studies based on microcalorimetry of the energetics of conformational changes in polynucleotides [Klump (1986a); Marky et al. (1982); Breslauer et al. (1986); Chaires and Sturtevant (1986) and references therein]. Most of these have treated deoxypolynucleotides including native heterogeneous DNA sequences. In particular, one of us (H.K.) has dealt with the energetics of formation of different secondary structures in poly[d(G-C)] and in poly[d(G-m^5C)], including order/order transitions as well as the order/disorder transitions (Klump & Löffler, 1985; Klump, 1986a,b). [Other data for the methylated polymer are available from Szu and Charney (1985) and Chaires and Sturtevant (1986).] The prerequisite for performing these experiments was the instrumental improvement of the UV spectrophotometer cell to withstand the 10 bar of external pressure required to shift the boiling point of the solvent water beyond the transition temperature of the most stable polynucleotides (140 °C). In a concurrent effort, the sophistication of adiabatic scanning microcalorimeters was improved so as to allow the scanning of small samples (0.5 mL) up to a temperature of 132 °C [as used in Klump (1986b)]. Thus enthalpy changes as low as 0.1 mcal were detectable, i.e., in the range characteristic of some B–Z DNA transitions.

There seems to be no fundamental difference in the formation of ordered secondary structures between DNAs and RNAs, although as a rule double-stranded RNAs are more stable than DNAs. The A–Z transition was demonstrated in poly[r(G-C)] by spectroscopic and NMR techniques (Hall et al., 1984; Cruz et al., 1986), but not with a continuous scan of the change of state. An appropriate method for demonstrating the helix inversion is the registration of the UV absorbance as a function of the temperature. From a comparison of the spectra of poly[r(G-C)] at low and high salt concentrations (Hall et al., 1984) or, more directly, from the corresponding difference spectra (Jovin, unpublished data), 290 nm is a favorable wavelength for scanning the A–Z RNA transition. The actual absorbance curves are symmetrical around the transition midpoint, suggesting that there is a single nucleation, i.e., inversion center, which probably originates at an end and propagates through the molecule.

The transition temperature decreases with increasing salt concentration (Figure 4), a property shared with members of the DNA (G-C) and (A-C)·(G-T) sequence families bearing C5 substitutions (methyl or halogen) and capable of assuming the left-handed Z-DNA conformation (Behe et al., 1985; Jovin et al., 1983; McIntosh and Jovin, submitted for publication; Soumpasis & Jovin, 1987). In this respect, the virtually temperature-independent B–Z equilibrium of the prototypic polynucleotide poly[d(G-C)] in NaCl solutions is a singular phenomenon. Indeed, in this case the salt-dependent free energy changes appear to be dominated by electrostatic and hard-sphere interactions (Soumpasis, 1986; Soumpasis et al., 1987; Soumpasis & Jovin, 1987). The equilibria between the B, A, and Z forms have also been treated by the same formalism (Soumpasis et al., 1987). However, in the case of poly[r(G-C)], the chaotropic effects of components such as the ClO_4^- anion are required in order to bring about a transition. [A relative stabilization of the Z-DNA structure also occurs in ClO_4^- solutions (Pohl & Jovin, 1972; Hamori & Jovin, 1987).] One can also surmise that alterations in the hydration state of the various conformations is an important factor (Westhof et al., 1985; Saenger et al., 1986; Soumpasis & Jovin, 1987). It is generally accepted that Z-DNA (Pohl, 1976; Soumpasis & Jovin, 1987) and probably Z-RNA (Cruz et al., 1986) are favored by dehydrating conditions.

The calorimetrically recorded heat capacity change, due to the inversion of the handedness of the ordered secondary structure, can be integrated in a limited temperature range so as to yield the true transition enthalpy ΔH. From the polymer concentration in terms of phosphate residues a transition enthalpy per base pair of 4.2 kJ (1.0 kcal) can be calculated, a value which is relatively small and remarkably similar to that determined for the B–Z order/order transitions of the pyrimidine-substituted deoxy polymers [0.9–1.2 kcal mol·bp^{-1}; summarized in Soumpasis and Jovin (1987)]. This finding is of particular interest in relation to the observed differences in the RNA and DNA systems alluded to above, as well as to the inherent structural differences between the corresponding right-handed (B-DNA, A-RNA) and possibly (Cruz et al., 1986) left-handed conformations. Similar ΔH values are also obtained from the phase boundary defined by temperature and salt variation (eq 2; Figure 4; McIntosh and Jovin, submitted for publication; Soumpasis & Jovin, 1987).

Standard thermodynamic equations can be utilized to calculate the other thermodynamic parameters from ΔH. The value for the transition entropy ΔS of 13 J (3.1 cal) mol·bp^{-1} deg^{-1} is much smaller than the entropies associated with helix-coil transitions. However, since an order/order transition is involved, there are no substantial contributions from the conformational entropies of the backbone. The small observed entropy change may reflect differences in (i) hydration of the two-handed helix configurations (Westhof et al., 1985; Saenger et al., 1986); (ii) hydrophobic interactions of the bases; (iii) surface charge densities (Soumpasis & Jovin, 1987); and (iv) vibrational entropies. The free energy change of only about 0.08 kcal mol·bp^{-1} at 25 °C reflects the small value of the transition enthalpy and the low transition temperature. In the case of the helix-coil transition, the cooperativity is calculated from the ratio of the van't Hoff enthalpy and the corresponding intrinsic enthalpy change determined directly from the calorimetric scan. However, we must express some caution in transferring this formalism to order/order transitions, for example, due to uncertainties related to the nucleation processes (Soumpasis & Jovin, 1987). The high cooperativity in our case implies that almost all the bases in the double-stranded helical structure change their disposition simultaneously, i.e., in a concerted all-or-none reaction.

The calorimetric measurements have been extended to poly[r(G-C)] analogues containing methyl and halogen substitutions in the C5 position of the pyrimidine base, and the results will be reported elsewhere. These modifications are interesting since they have been shown to have a relative destabilizing effect on the Z conformation of the RNA (Jovin, unpublished data) as opposed to the dramatic stabilization in the case of Z-DNA. This finding emphasizes the subtleties involved in determining the energetics of transitions between ordered states, and, furthermore, suggests that specific ligands

(small molecules, proteins) will exert a dominant effect on these processes in vivo.

ACKNOWLEDGMENTS

We thank G. Heim for synthesizing and characterizing the polymers, R. Lehmann for preparing the figures, and D. M. Soumpasis for critical reading of the manuscript.

Registry No. Poly[r(G-C)], 49846-05-1; $NaClO_4$, 7601-89-0.

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