Poly(rA) binds poly(rG)·poly(rC) to form a triple helix

Michael Chastain and Ignacio Tinoco Jr

Department of Chemistry and Laboratory of Chemical Biodynamics, University of California, Berkeley, CA 94720, USA

Received September 3, 1991; Revised and Accepted November 15, 1991

ABSTRACT

Poly(rA) binds poly(rG)·poly(rC) to form a triple helix. Evidence for this structure includes ultraviolet absorbance mixing curves and melting curves, and circular dichroism spectroscopy. The formation of the triple helix depends on the length of the poly(rC) strand. Triple helix forms when the average length is around 100 nucleotides but does not form when the average length is about 500 nucleotides.

INTRODUCTION

Base triples have an important role in RNA structure and function. They occur or have been proposed to occur in tRNA (1), 5S rRNA (2), and in the *Tetrahymena* self-splicing intron (3). The guanosine binding site of the intron binds guanosine by the formation of a base triple (4). Base triples formed between a DNA duplex and a DNA third strand can regulate gene transcription (5–8). It is clearly important to be able to predict which base triples form, so the sequences that are capable of forming RNA triples need to be determined, and their structures and thermodynamic stabilities characterized.

In a systematic study which tested for triple helix formation between the two homopolymer Watson-Crick duplexes and each homopolymer, the following triple helices were shown to form (in 150 mM sodium chloride, 5 mM magnesium chloride, 10 mM sodium cacodylate, pH 7 at 4°C): rU·(rA·rU), rA·(rA·rU), rC⁺·(rG·rC), and rG·(rG·rC) (9). Several base triples not found in this study have recently been proposed to occur in the *Tetrahymena* intron on the basis of phylogenetic and mutational evidence (3,10). These include rA·(rG·rC) and rU·(rG·rC) base triples. In order to study the structure and thermodynamics of these base triples, we looked for their formation in polynucleotide triple helices. Here we present evidence that poly(rA) binds to poly(rG)·poly(rC) to form a triple helix at pH 7 in 0.10 M or higher concentrations of sodium chloride. Evidence for triple helix formation comes from ultraviolet absorption mixing curves, UV melting curves, and circular dichroism spectra. The formation of triple helix depends upon the length of the poly(rC) strand.

MATERIALS AND METHODS

Polynucleotides

All the polynucleotides used in this study were obtained from Pharmacia and were dialyzed for 24 hours against H₂O before use. The sedimentation coefficients were stated as 7.8, 7.8, 10.4,

5.7, and 5.1 for poly(rG), poly(rC), poly(rA), poly(rU), and poly(rI) respectively, which corresponds to average lengths of approximately 500, 500, 900, 300, and 200 nucleotides. Extinction coefficients used to determine concentration were: rA ϵ_{258} =9,800; rC ϵ_{269} =6,200; rG ϵ_{253} =10,400; rU ϵ_{260} =9,350; rI ϵ_{248} =10,200 (Pharmacia). Poly(rC) and poly(rG) were shortened by limited alkaline hydrolysis. Approximately 1 mg of polymer was dissolved in 1 ml of 0.01 M sodium hydroxide and incubated at 37°C for two hours. Hydrolysis was stopped by the addition of hydrochloric acid and sodium phosphate buffer, and the solution was then dialyzed against 10 mM sodium phosphate pH 7 for 24 hours. Denaturing gel electrophoresis showed that the size of the shortened poly(rC) and poly(rG) ranged from approximately 20 to 150 nucleotides with an average length of about 100 nucleotides.

Mixing curves

Although poly(rG) forms a very stable four-stranded structure, poly(rG) can be mixed with poly(rC) to form a poly(rG)·poly(rC) duplex (11,12). Stock solutions of poly(rA) and poly(rG)·poly(rC) were prepared at 60 μ M nucleotide concentrations. Solutions containing different ratios of poly(rG)·poly(rC) to poly(rA) were prepared by pipetting different amounts of stock solutions into eppendorf tubes. The tubes were weighed before and after the addition of the stock solutions to determine more accurately the ratio of poly(rA) to poly(rG)·poly(rC) in each tube. The samples (without added salt) were boiled for five minutes to denature the poly(rG) self-structure. The tubes were then placed in a heat block at 85°C for ten minutes during which time the correct amount of salt was added to bring the final salt concentration to 10 mM sodium phosphate pH 7, 0.10 M sodium chloride and the final sample concentration (the sum of the poly(rG)·poly(rC) and poly(rA) concentrations) to 48 μ M. The samples were then equilibrated overnight (or up to six weeks) at 4°C to make sure that the system had reached equilibrium. The absorbance of each sample was measured at 4°C between 220 nm and 320 nm on a Shimadzu UV-VIS Spectrophotometer UV-160 equipped with a temperature-controlled cell holder. Nitrogen was circulated through the cell-holding chamber to prevent condensation. Mixing curves were plotted at various wavelengths to determine that the absorbance at 260 nm is most sensitive to the interaction between poly(rA) and poly(rG)·poly(rC). No changes were seen in the mixing curves measured after overnight compared with further incubation, so the system was assumed to reach equilibrium overnight.

Melting Curves

Samples used to measure melting curves were diluted to 44 μM (in nucleotides) with water from 3.5 mM stock solutions. The samples were boiled for five minutes and then incubated at 85°C for ten minutes during which time salt was added to bring the samples to 10 mM sodium phosphate pH 7, 1 M sodium chloride. The final sample concentration was 35 μM in each nucleotide. Each sample was then dialyzed 24 hours against the buffer in which the melting curve was measured. The buffers all contained 10 mM sodium phosphate pH 7, and the sodium chloride concentrations were: 0, 0.1, 0.25, 0.5, 0.75, and 1 M. Melting curves were obtained on a single beam Gilford spectrophotometer Model 2530 interfaced to a Fountain 286 computer. The temperature was raised at a rate of 0.25°C per minute. The melting temperatures were estimated by fitting the curves with upper and lower baselines and then assigning the melting temperature to the midpoint of the transition.

Melting curves were also measured with different buffers and at different pH values. Experiments done in sodium cacodylate buffer did not show triplex formation, while experiments done in HEPES buffer or sodium phosphate buffer did show triplex formation. Melting curves were measured in sodium phosphate buffer at pH 6 and at pH 8 as well as pH 7. Both raising and lowering the pH slightly destabilized the triple helix.

Circular dichroism spectra

Samples used to measure CD spectra were prepared as the samples for mixing curves except that half of the samples contained only 10 mM sodium phosphate pH 7 while the other half also contained 0.10 M sodium chloride. The concentration of the samples was 24 μM in each nucleotide. CD spectra were recorded at room temperature on a JASCO J600 spectropolarimeter interfaced to an IBM XT computer.

Other sequences

To test the importance of the guanine amino group in forming the triple helix, poly(rI) was substituted for poly(rG). Assaying

for an interaction between poly(rA) and poly(rI)•poly(rC) was prevented by the alternate structures that poly(rI), unlike poly(rG), forms. Both $\text{rC}^+ \cdot (\text{rI} \cdot \text{rC})$ and $\text{rI} \cdot \text{rA} \cdot \text{rI}$ triple helices (13) form at pH 7 in 0.10 M sodium chloride.

We also looked for triple helix formation between poly(rU) and poly(rG)•poly(rC). Stock solutions at 80 μM nucleotide concentration were used for measurement of mixing curves and CD spectra as described above for poly(rA) with poly(rG)•poly(rC). Neither mixing curves nor CD spectra showed any sign of poly(rU) binding to poly(rG)•poly(rC) at 4°C or room temperature in 10 mM sodium phosphate pH 7, with the sodium chloride concentration either 0.10 M or 1.0 M. The failure of poly(rU) to bind to poly(rG)•poly(rC) was not changed by varying the length of the poly(rC) strands.

RESULTS

Ultraviolet absorption mixing curves (figure 1) measured at 260 nm show an interaction between poly(rG)•poly(rC) and poly(rA). A discontinuity in the curve is seen when poly(rG)•poly(rC) and poly(rA) are in a 1:1 mole ratio. The triple helix shows an increased absorbance relative to the poly(rG)•poly(rC) duplex and the poly(rA) single strand.

The stability of the triple helix is demonstrated in the UV melting curve shown in figure 2. The melting curve of the triple helix is characterized by a decrease in absorbance caused by the dissociation of the poly(rA) strand from the duplex, followed by an increase in absorbance due to unstacking of the poly(rA) single strand. The poly(rG)•poly(rC) duplex does not melt under these salt conditions. As shown by the melting curve, the polynucleotides do not form a triple helix in 10 mM sodium phosphate. Instead of seeing the decrease in absorbance caused by the poly(rA) strand dissociating from the duplex, the absorbance increases continuously due to the unstacking of the poly(rA) single strands.

Further evidence of triple helix formation comes from the dependence of the melting temperature on salt concentration. Theoretical and experimental studies on a variety of double and triple helices have shown that the melting temperature of a duplex changes by 17–19°C with a tenfold increase in salt concentration, whereas the melting temperature for the

Figure 1. The absorbance at 260 nm of different mixtures of poly(rA) and poly(rG)•poly(rC) is plotted versus fraction poly(rA). The experimental data follows the solid line. The dashed line is the absorbance expected if there is no interaction between the polynucleotides. The absorbance was measured at 4°C in 0.1 M sodium chloride, 10 mM sodium phosphate at pH 7.

Figure 2. The normalized absorbance at 260 nm is plotted versus temperature. The dark curve is data measured in 10 mM sodium phosphate, pH 7, 0.1 M sodium chloride. The light curve is data measured in 10 mM sodium phosphate, pH 7. The two curves were normalized at 85°C.

dissociation of a triple helix into a duplex and a single strand changes by a much greater amount, in the range of 30–60°C (14). Figure 3 shows the dependence of the $rA \cdot (rG \cdot rC)$ triple helix melting temperature upon the salt concentration when the salt concentration ranges from 0.10 M to 1 M sodium chloride. $dT_m/d\log[Na^+]$ is about 33°C which is characteristic of triple helix melting (14).

Circular dichroism (CD) spectra also show that $\text{poly}(rG) \cdot \text{poly}(rC)$ and $\text{poly}(rA)$ interact to form a triple helix. CD spectra of $\text{poly}(rG) \cdot \text{poly}(rC)$ and of $\text{poly}(rA)$ taken separately are shown in figure 4(a). The CD spectra of the triple helix and the sum of the $\text{poly}(rG) \cdot \text{poly}(rC)$ and $\text{poly}(rA)$ spectra are shown in figure 4(b). The triple helix shows an increase in CD intensity relative to the sum of the spectra taken separately. If there were no interaction between $\text{poly}(rA)$ and $\text{poly}(rG) \cdot \text{poly}(rC)$, the curves would be superimposable. When the spectra are taken in 10 mM sodium phosphate which is insufficient salt for triplex formation, the curves do become superimposable as shown in figure 4(c).

An unusual property of this triple helix is that formation of the triple helix depends on the length of the $\text{poly}(rC)$ strand. Initial attempts to repeat the melting curves with new polynucleotide samples failed. Varying the lengths of the polynucleotide strands showed that when the average length of the $\text{poly}(rC)$ strand is shortened to about 100 nucleotides the triple helix forms, whereas the purchased strand with a length of about 500 nucleotides does not form a triple helix. Changing the length of the $\text{poly}(rG)$ and $\text{poly}(rA)$ strands did not affect triple helix formation.

Triple helix formation dependent upon polynucleotide length was seen before in the $rA \cdot (rA \cdot rU)$ triple helix (15). In this case the length of the $\text{poly}(rA)$ strand was required to be between 28 and 150 nucleotides in order for triple helices to form. The dependence of triple helix formation on polynucleotide length could in principle be a kinetic or a thermodynamic effect. As suggested previously (15), the most likely explanation is that the effect is thermodynamic. Adding successive nucleotides to the triple helix may deform the helix backbone so that making long triple helices is unfavorable. Nicking the backbone of the double helix relieves the stress or bend induced by triple helix formation.

Figure 3. Melting temperatures ($\pm 3^\circ\text{C}$) are plotted versus the logarithm of the sodium chloride concentration. The slope of the line was determined by least squares fitting to be 33°C.

DISCUSSION

Two of the new base triples proposed to occur in the *Tetrahymena* intron are $rA \cdot (rG \cdot rC)$ and $rU \cdot (rG \cdot rC)$ (3,10). In the conditions we tried, only the $rA \cdot (rG \cdot rC)$ combination formed a polynucleotide triple helix. In the $rA \cdot (rG \cdot rC)$ base triple

Figure 4. Circular dichroism measured at room temperature is plotted versus wavelength. (a) The spectra of $\text{poly}(rA)$, $\text{poly}(rG) \cdot \text{poly}(rC)$, and $rA \cdot (rG \cdot rC)$ taken separately. (b) In 10 mM sodium phosphate, pH 7 and 0.1 M sodium chloride, the spectrum of the triple helix (dark line) differs from the sum of the $\text{poly}(rA)$ and $\text{poly}(rG) \cdot \text{poly}(rC)$ spectra taken separately (dashed line). (c) In 10 mM sodium phosphate the spectrum taken of the triple helix superimposes on the summation of the $\text{poly}(rA)$ and $\text{poly}(rG) \cdot \text{poly}(rC)$ spectra taken separately.

proposed in the *Tetrahymena* intron, A₁₀₅ in single-stranded region J3/4 binds in the minor groove to G₂₅₇•C₂₁₆ of helix P6 by forming two hydrogen bonds (3). This suggests the possibility that the poly(rA) strand binds in the minor groove of the poly(rG)•poly(rC) duplex. However, the structure of base triples can change depending on the context in which they are found. Two different structures for rA•(rG•rC) base triples formed in the major groove have been suggested by mutational evidence. A mutated form of the *Tetrahymena* intron which binds adenosine during the splicing reaction instead of guanosine forms an rA•(rG•rC) base triple in the major groove at the adenosine binding site (16). Mutational studies on tRNA^{Phe} also suggested that an rA•(rG•rC) base triple could form in the major groove (17).

In order to see if the adenosine is in the major or minor groove and to see if the dependence of triple helix formation on the length of the poly(rC) strand is a result of a distorted backbone conformation, NMR studies on oligonucleotides containing rA•(rG•rC) base triples are currently underway.

ACKNOWLEDGEMENTS

Michael Chastain is a Howard Hughes Medical Institute Doctoral Fellow. This work was supported in part by National Institutes of Health Grant GM 10840, and by the Department of Energy, Office of Energy Research, Office of Health and Environmental Research under Grant DE-FG03-86ER60406.

REFERENCES

1. Goddard, J.P. (1977) *Prog. Biophys. molec. Biol.* **32**, 233–308.
2. Westhof, E., Romby, P., Romaniuk, P.J., Ebel, J.-P., Ehresmann, C. and Ehresmann, B. (1989) *J. Mol. Biol.* **207**, 417–431.
3. Michel, F. and Westhof, E. (1990) *J. Mol. Biol.* **216**, 585–610.
4. Michel, F., Hanna, M., Green, R., Bartel, D.P. and Szostak, J.W. (1989) *Nature* **342**, 391–395.
5. Boles, T.C. and Hogan, M.E. (1987) *Biochemistry* **26**, 367–376.
6. Cooney, M., Czernuszewicz, G., Postel, E.H., Flint, S.J. and Hogan, M.E. (1988) *Science* **241**, 456–459.
7. Maher III, L.J., Wold, B. and Dervan, P.B. (1989) *Science* **245**, 725–730.
8. Orson, F.M., Thomas, D.W., McShan, W.M., Kessler, D.J. and Hogan, M.E. (1991) *Nucleic Acids Res.* **19**, 3435–3441.
9. Letai, A.G., Palladino, M.A., Fromm, E., Rizzo, V. and Fresco, J.R. (1988) *Biochemistry* **27**, 9108–9112.
10. Michel, F., Ellington, A.D., Couture, S. and Szostak, J.W. (1990) *Nature* **347**, 578–580.
11. Pochon, F. and Michelson, A.M. (1965) *Proc. Natl. Acad. Sci. USA* **53**, 1425–1430.
12. Thiele, D. and Guschlbauer, W. (1971) *Biopolymers* **10**, 143–157.
13. Howard, F.B. and Miles, H.T. (1977) *Biochemistry* **16**, 4647–4650.
14. Record, Jr., M.T. (1967) *Biopolymers* **5**, 975–992.
15. Broitman, S.L., Im, D.D. and Fresco, J.R. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5120–5124.
16. Been, M.D. and Perrotta, A.T. (1991) *Science* **252**, 434–437.
17. Sampson, J.R., DiRenzo, A.B., Behlen, L.S. and Uhlenbeck, O.C. (1990) *Biochemistry* **29**, 2523–2532.