Kinetics and Mechanism of RNA Binding by Triplex Tethered Oligonucleotide Probes

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Abstract: We have described a series of tethered oligonucleotide probes (triplex TOPs) that recognize one single-stranded and one double-stranded region of an RNA simultaneously through the formation of Watson−Crick and Hoogsteen base pairs, respectively. Here we describe studies on the kinetics and mechanism of triplex TOP-RREAU association and dissociation. Because triplex TOP-RREAU complexes cannot be observed by direct electrophoretic methods, kinetics was monitored by use of a competitive electrophoretic mobility shift assay that quantified the effect of a triplex TOP on the association and dissociation rates of an electrophoretically stable TOP-RREAU complex. Association and dissociation rate constants of triplex TOP-RREAU complexes were extracted from the experimental data by numerical integration. Triplex TOP-RREAU association reactions at 25 °C were characterized by rate constants between (7.8 ± 2.0) × 10^3 and (16 ± 3) × 10^3 M^−1 s^−1, while dissociation reactions were characterized by rate constants between (3.3 ± 1.0) × 10^−4 and (5.4 ± 2.0) × 10^−2 s^−1. Rate constants for association of triplex TOP-RREAU complexes were insensitive to the length and sequence of the 3′-oligonucleotide that mediates triple helix formation. Rate constants for dissociation of triplex TOP-RREAU complexes were sensitive to changes in tether length as well as the length and composition of the 3′-oligonucleotide. Taken together, these data suggest that triplex TOPs follow a kinetic pathway for binding RREAU in which duplex formation is rate-limiting and precedes triple helix formation. The implication of our data with regard to the kinetics of triple helix association within the context of a highly structured RNA is discussed.

Large, structured RNA molecules play critical roles in cellular and viral life cycles.1−3 A considerable body of biochemical structure-mapping data,4−6 along with several high-resolution structures,7−15 indicate that many large RNAs are composed of irregular arrangements of short single- and double-stranded regions joined by structures such as loops, bulges, and pseudoknots.16−19 These irregular architectures present complex functional group arrays that are recognized in nature by other nucleic acids,20–23 small organic molecules,24 and proteins,26−33 other nucleic acids,24,26 and small organic molecules.25 There is considerable current interest in the design of molecules that mimic the properties of these natural ligands, not only to inhibit the translation of messenger RNAs but also to interrupt the functions of catalytic and regulatory RNAs.26−32 Several strategies have been presented for the recognition of large, structured RNA molecules. Ribozymes and single-stranded oligonucleotides33−35 recognize RNA on the basis of primary sequence.36−40 Although a strategy based on primary sequence recognition is broadly applicable in theory, it requires a target sequence that is unique, accessible, and single-stranded.41−47 These requirements can be difficult to fulfill in practice.

(22) Burd, C. G.; Dreyfuss, G. Science 1994, 265, 615.
(24) Ribosomal RNA and Group I Introns; R. G. Landes: Austin, TX, 1996; Vol. 1.
Organometallic complexes and small organic molecules recognize more irregular elements of structure. For example, various rhodium complexes recognize expanded RNA major grooves or G-U mismatches, and the organometallic complexes methidiumpropyl-EDTA and bis(phenanthroline)copper(II) show some preference for junctions between duplexes and single-stranded loops or bulges. Our strategy is based on a class of molecules called tethered oligonucleotide probes (TOPs). A TOP consists of two short oligonucleotides joined by a tether whose length and composition may be varied using chemical synthesis. Each of the oligonucleotides within a TOP recognizes a single, accessible sequence within the target RNA, and the tether traverses the distance between the two sequences. In contrast to traditional oligonucleotides which recognize a single, contiguous RNA sequence, TOPs recognize two short, noncontiguous sequences that are proximal in the folded RNA. Because TOPs bind simultaneously to two accessible sequences, rather than one long sequence that may not be fully accessible, TOPs exhibit very high affinities for structured RNA targets and bind these targets more rapidly than molecules that must disrupt structure in order to bind.

Initially, we evaluated TOPs that recognized two noncontiguous single-stranded sequences within a target RNA through the formation of standard Watson–Crick base pairs. More recently, we evaluated TOPs that recognized one single-stranded and one double-stranded region simultaneously through the formation of Watson–Crick and Hoogsteen base pairs, respectively. These molecules were termed triplex TOPs. The RNA target for our triplex TOPs was a modified version of the HIV-1 Rev response element (RRE) (Figure 2A) in which twelve contiguous A-U base pairs replaced a portion of stem–loop IIB (RREAU, Figure 2B). The 5′-oligonucleotide within each triplex TOP recognized the accessible, single-stranded region between positions 69 and 76 of RREAU (site 1) to form a duplex and the 3′-oligonucleotide recognized the AU-rich duplex (site 4) to form a triple helix. Triplex TOPs designed to recognize RREAU sites 1 and 4 formed complexes with nanomolar dissociation constants and were effective inhibitors of Rev-RREAU complexation at equilibrium in vitro.

Here we characterize the kinetics and mechanism of triplex TOP-RREAU association and dissociation. Because triplex TOP-RREAU complexes cannot be observed by direct electrophoretic methods, kinetics were monitored by use of a competitive electrophoretic mobility shift assay that quanti-
fied the effect of a triplex TOP on the association and dissociation rates of a photoelectrophoretically stable TOP-RREAU complex. Association and dissociation rate constants were extracted from the experimental data by numerical integration. Triplex TOP-RREAU association reactions were characterized by rate constants between \((7.8 \pm 2.0) \times 10^3\) and \((16 \pm 3) \times 10^3\) M\(^{-1}\) s\(^{-1}\) at 25 °C, while dissociation reactions were characterized by rate constants between \((3 \pm 1) \times 10^{-4}\) and \((400 \pm 40) \times 10^{-4}\) s\(^{-1}\). Association rate constants were insensitive to changes in the length and sequence of the \(3'\)-oligonucleotide that mediates triple-helix formation, whereas dissociation rate constants were sensitive to changes in \(3'\)-oligonucleotide sequence and length as well as changes in tether length. Taken together, these data indicate that triplex TOPs bind RREAU through a kinetic pathway in which duplex formation is rate-limiting and precedes triple-helix formation.

**Experimental Section**

**Synthesis of Tethered Oligonucleotide Probes.** TOPs and oligonucleotides were synthesized on a 1 μmol scale\(^{66}\) by use of the solid-phase phosphoramidite method\(^{66}\) on a Perseptive Biosystems model 8909 nucleic acid synthesizer. Oligonucleotides S1 and SIEXT and TOP S1-5-S2 were cleaved from the solid support and deprotected upon treatment with 12 M \(\text{NH}_3\) in 55 °C for 10 h.\(^{67}\) All TOPs were deprotected with ammonia-saturated ethanol at 55 °C for 10 h.\(^{40}\) TOPs and oligonucleotides were purified by use of denaturing polyacrylamide gel electrophoresis (20% acrylamide, 19:1 acrylamide: \(\text{N}_{2}\) polymerase) and a chemically synthesized DNA template. Our experimental protocol was as follows: RREAU (250 nM or 1 mM final concentration) was chosen such that the fraction of \([32\text{P}]\)S1-5-S2 bound to RREAU was determined by use of the competitive method to determine kinetic constants for triplex TOP-RREAU complexes.

**Preparation of RREAU.** RREAU was prepared by use of T7 RNA polymerase\(^{23}\) (Promega) and a chemically synthesized DNA template. RREAU was purified by preparative denaturing gel electrophoresis (20% acrylamide, 19:1 acrylamide: \(\text{N}_{2}\)-methylenebis(acrylamide), 7 M urea).\(^{70}\) TOPs were stored in diethyl pyrocarbonate-treated (DEPC) water at -20 °C and thawed prior to use. S1-5-S2 was labeled on the 5'-end by use of \([\gamma-32\text{P}]\)ATP and T4 polynucleotide kinase.\(^{70}\)

**Kinetic Analysis: General Procedures.** All reactions were performed at 25 °C in binding buffer (10 mM 1,4-piperazinethanesulfonate (ethanesulfonate) (Research Organics, Inc.) (pH 7.0), 300 mM NaCl, 2 mM Hepes (pH 7.5), and 10% glycerol). RREAU (125 nM-1.25 μM) in RRE buffer (5 mM Hepes (pH 7.5), 25 mM NaCl) was renatured before each reaction by heating at 85 °C for 2 min and cooling at 25 °C for 10 min. Binding reactions were analyzed with nondenaturing gels (6% acrylamide, 79:1 acrylamide: \(\text{N}_{2}\)-methylenebis(acrylamide), 7 M urea) and desalted prior to use in kinetic experiments. The sequence of the 5’-32 residues of RREAU was confirmed by enzymatic methods\(^{64,65}\) using protocols recommended by the supplier (Pharmacia). Aliquotted RNA stock solutions were stored in DEPC water at -20 °C and thawed prior to use.

**Table 1. Kinetic and Equilibrium Constants for Triplex TOP-RREAU Complexes at 25 °C**

<table>
<thead>
<tr>
<th>TOP or oligonucleotide</th>
<th>(10^{-3} \times k_a) (M(^{-1}) s(^{-1}))</th>
<th>(10^4 \times k_d) (s(^{-1}))</th>
<th>(K_d) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1-S2</td>
<td>7.8 ± 2.0</td>
<td>4 ± 0.5</td>
<td>51 ± 5</td>
</tr>
<tr>
<td>S1</td>
<td>7.8 ± 2.0</td>
<td>500 ± 200</td>
<td>7000 ± 300</td>
</tr>
<tr>
<td>S1-1-S4</td>
<td>16 ± 3</td>
<td>3 ± 1</td>
<td>21 ± 5</td>
</tr>
<tr>
<td>S1-3-S4</td>
<td>9.5 ± 0.8</td>
<td>29 ± 9</td>
<td>300 ± 150</td>
</tr>
<tr>
<td>S1-5-S4</td>
<td>7.8 ± 2.0</td>
<td>28 ± 10</td>
<td>363 ± 100</td>
</tr>
<tr>
<td>S1-7-S4</td>
<td>7.8 ± 2.0</td>
<td>120 ± 40</td>
<td>1540 ± 500</td>
</tr>
<tr>
<td>S1-1-S4(^{6})</td>
<td>7.8 ± 2.0</td>
<td>19 ± 10</td>
<td>240 ± 100</td>
</tr>
<tr>
<td>S1-1-S4(^{6,7})</td>
<td>7.8 ± 2.0</td>
<td>60 ± 30</td>
<td>810 ± 400</td>
</tr>
<tr>
<td>S1-1-S4(^{UC})</td>
<td>7.8 ± 2.0</td>
<td>400 ± 40</td>
<td>5130 ± 500</td>
</tr>
<tr>
<td>S1-1-S4(^{C})</td>
<td>9.5 ± 2.0</td>
<td>64 ± 30</td>
<td>400 ± 170</td>
</tr>
<tr>
<td>SIEXT</td>
<td>7.8 ± 2.0</td>
<td>240 ± 70</td>
<td>4200 ± 800</td>
</tr>
</tbody>
</table>

\(^{a}\) Experimental protocols described in text. \(^{b}\) This error represents the range of values that provided a good fit (residual less than 5%) to the experimental data. \(^{c}\) Calculated from the ratio \(k_d/k_a\). \(^{d}\) Determined by competitive equilibrium mobility shift analysis.\(^{61,62}\)

**Association and Dissociation Kinetics of \([^{32}\text{P}]\)S1-5-S2-RREAU**

**Direct Method.** In order to verify the accuracy of the competitive kinetic analysis, we measured the rate constants for association and dissociation of the \([^{32}\text{P}]\)S1-5-S2-RREAU complex by direct electrophoretic method\(^{60,75,76}\) and compared these values to those obtained by competition and numerical analysis (Table 1). For association reactions, 0.01 nM \([^{32}\text{P}]\)S1-5-S2 was added to 30–500 nM RREAU in binding buffer at 25 °C and aliquots were applied at 2 min intervals to running gels. For dissociation reactions, 50–400 nM RREAU and 0.01 nM \([^{32}\text{P}]\)S1-5-S2 were equilibrated in binding buffer for 90 min at 25 °C. Dissociation was initiated by addition of 10 nM unlabeled S1-5-S2, and aliquots were applied at 30 s intervals to running gels. Data were analyzed as described previously.\(^{60}\) Association and dissociation reactions were performed at least twice at each RREAU concentration. Figure 3A illustrates the fraction of \([^{32}\text{P}]\)S1-5-S2 bound to RREAU as a function of time at RREAU concentrations of 30, 50, 100, 150, 200, and 500 nM. Figure 3B shows the first-order plots that were used to obtain \(k_{obs}\). A value for \(k_1\) (7.8 ± 2.0) × 10\(^{-5}\) M\(^{-1}\) s\(^{-1}\) (\(R = 0.98\)) was obtained from the slope of the plot of \(k_{obs}\) versus [RREAU] in Figure 3C. This value was identical to the value determined by the competitive method.

**Association and Dissociation kinetics of TOP-RREAU Complexes: Competitive Method.** Rate constants describing the association and dissociation of TOP-RREAU complexes and the S1-RREAU and SIEXT-RREAU complexes were determined by use of a competitive electrophoretic mobility shift assay\(^{65,66}\) in which the fraction of \([^{32}\text{P}]\)S1-5-S2 bound to RREAU in the presence of varying concentrations of competitor (TOP, S1, or SIEXT) was monitored as a function of time. Our experimental protocol was as follows: RREAU (250 nM or 1 mM in RRE buffer) was renatured as described above and equilibrated with \([^{32}\text{P}]\)S1-5-S2 in binding buffer for 1 h at 25 °C. The concentration of RREAU (100 or 400 nM final concentration) was chosen such that the fraction of \([^{32}\text{P}]\)S1-5-S2 bound to RREAU was between 0.7 and 0.9 prior to addition of competitor. Dissociation of the \([^{32}\text{P}]\)S1-5-S2-RREAU complex was initiated upon addition of between 500 nM and 15 mM competitor TOP or oligonucleotide, and aliquots of the reaction mixture were applied at 5 min intervals to running gels. Final concentrations were as follows: 0.01 nM \([^{32}\text{P}]\)S1-5-S2, 100 or 400 nM RREAU, 500


Determination of the rate constant for association of the \([^{32}P]S1-5-S2-RRE_{AU}\) complex by direct and competitive electrophoretic mobility shift analyses. (A) Plot of \((1 - F_t)\), the fraction \([^{32}P]S1-5-S2\) bound as a function of time, \(t\), in the presence of \([RRE_{AU}]=\Delta.30\ nM; \odot.50\ nM; \bigcirc.100\ nM; \bigtriangledown.150\ nM; \blacktriangledown.200\ nM; \triangle.500\ nM. (B) Determination of \(k_{obs}\). Plot of \(\ln[(F_t - F_{eq})(F_0 - F_{eq})]\) versus time for \([RRE_{AU}]=\Delta.30\ nM; \odot.50\ nM; \bigcirc.100\ nM; \bigtriangledown.150\ nM; \blacktriangledown.200\ nM; \triangle.500\ nM. \(F_{eq}\) was calculated from the concentration of \(RRE_{AU}\) and the \(K_d\) of the \(S1-5-S2-RRE_{AU}\) complex (68 nM). Each solid line represents the best linear least-squares fit to the data. (C) Determination of \(k_1\) and \(10^5x\). The open point at \([RRE_{AU}] = 0\) is from an independent measurement of \(k_1\). The solid line represents the best linear least-squares fit to the data. (D) Competitive analysis. Plot of the effect of \(S1-5-S2\) on the fraction of \([^{32}P]S1-5-S2\) bound to \(RRE_{AU}\) as a function of time. \(\theta = \text{fraction of } [^{32}P]S1-5-S2 \text{ bound to } RRE_{AU}.\) The solid line represents the simulated time course where \(k_1 = 7.8 \times 10^3\ M^{-1}s^{-1}\) and \(k_2 = 4 \times 10^4\ s^{-1}.\) This simulation fit the experimental data with the smallest average residual (2%). The hashed line represents the simulated time course where \(k_1 = 9.0 \times 10^{-7}\ s^{-1}\) (derived from independent measurement of \(k_1\) as described in part C) and \(k_2 = 7.8 \times 10^3\ M^{-1}s^{-1}\) (derived from direct association kinetic analysis, part C). This simulation fit the experimental data with an average residual of 28%. The dotted line represents the simulated time course where \(k_1 = 3 \times 10^{-3} s^{-1}\) and \(k_2 = 7.8 \times 10^3\ M^{-1}s^{-1}.\) This simulation fit the experimental data with an average residual of 10%. Residuals for each trial are shown above the corresponding plot.

nM–15 nM competitor in binding buffer. The fraction of \([^{32}P]S1-5-S2\) bound to \(RRE_{AU}\) in the presence of competitor TOP did not change once equilibrium was reached, indicating the absence of slow changes in \(RRE_{AU}\) conformation that altered the position of equilibrium.

**Rate Constant Determination.** Simulated time courses illustrating the time-dependent change in the fraction of \([^{32}P]S1-5-S2\) bound to \(RRE_{AU}\) as a function of competitor concentration were generated by numerical integration of differential equations 2–4 using a Runge–Kutta algorithm.77 Although the association and dissociation of triplex \(TOP\cdot RRE_{AU}\) complexes likely proceed through several discrete steps and intermediates, detection of many of these intermediates is difficult; our experimental assay detects only \([^{32}P]S1-5-S2\) and \([^{32}P]S1-5-S2-RRE_{AU}\). We therefore utilized a simple binding model in which the \([^{32}P]S1-5-S2-RRE_{AU}\) and triplex \(TOP\cdot RRE_{AU}\) association and dissociation reactions were represented by single second-order and first-order rate constants, respectively. This choice was validated by the observation that the rate constants for \([^{32}P]S1-5-S2-RRE_{AU}\) association and dissociation obtained from direct kinetic experiments were within a factor of 2 of those obtained from the competitive analysis. Although triplex \(TOP\cdot RRE_{AU}\) rate constants calculated from \(k_2\) and \(k^{-}\) were consistently 2.5–6.5-fold lower than those measured by equilibrium methods (Table 1), the former values are more accurate than the latter values, especially for weakly bound TOPs that are not well quantified by gel methods. A 2.5–6.5-fold difference in the values of \(k_2\) and \(k^{-}\) would not alter our mechanistic conclusions.

**Results**

A series of triplex TOPs (Figure 1) designed to recognize \(RRE_{AU}\) (Figure 2B) were synthesized by solid-phase methods. Triplex TOPs \(S1-1-S4, S1-3-S4, S1-5-S4,\) and \(S1-7-S4\) contained 5′- and 3′-oligonucleotides designed to interact with discrete regions of \(RRE_{AU}\): the 5′-oligonucleotide complements 8 bases spanning C69–G76 (site 1) to form a duplex, whereas the 3′-oligonucleotide complements the 12 base pairs within stem-loop IIB (site 4) to form a triple helix. Triplex TOPs \(S1-1-S4\) and \(S1-1-S4\) contained truncated 3′-oligonucleotide sequences, whereas triplex TOPs \(S1-1-S4\) and \(S1-1-S4\) contained mutated 3′-oligonucleotide sequences. Oligonucleotides S1 and S1EXT were complementary to only site 1.

**Association and Dissociation Kinetics of Triplex \(TOP\cdot RRE_{AU}\) Complexes.** We used a competitive electrophoretic mobility shift assay63–65 and a Runge–Kutta algorithm77 to determine the rate constants for association and dissociation of triplex \(TOP\cdot RRE_{AU}\) complexes. This assay quantified the effect of a triplex TOP on the association and dissociation rates of a \(TOP\cdot RRE_{AU}\) complex that was stable during gel electrophoresis and whose association and dissociation kinetics were established by direct methods. The \(TOP\cdot RRE_{AU}\) complex chosen for this task was \(S1-5-S2-RRE_{AU},\) which is stabilized by Watson–Crick interactions at both sites 1 and 2.

The reversible binding reactions monitored in our assay are represented by the following linked equilibria:

\[
R+C \xrightleftharpoons[k_{RC}^{-}\atop k_{RC}]{k_{RC}^{+}} R \cdot C \atop R \cdot T
\]

where \(K_{RC}^{+} = k_{RC}^{-}/k_{RC}, \) \(K_{RC}^{-} = k_{RC}/k_{RC}^{-},\) and \(R, C, T, R \cdot T,\) and \(R \cdot C\) represent \(RRE_{AU}\) competitor, \([^{32}P]S1-5-S2, [^{32}P]S1-5-S2-RRE_{AU},\) and competitor-\(RRE_{AU}\), respectively. \(k_1\) and \(k_1\) represent the second-order association and first-order dissociation rate constants of the \([^{32}P]S1-5-S2-RRE_{AU}\) complex; \(k_2\) and \(k_2\) represent the second-order association and first-order dissociation constants of the competitor-\(RRE_{AU}\) complex.

The kinetics of our competition assay are represented by the following differential rate equations:

\[
\frac{d[R]}{dt} = k_{RC}^{-}[R\cdot T] + k_{RC}[R\cdot C] - k_1[R][R] - k_2[C][R]
\]

\[
\frac{d[R\cdot C]}{dt} = k_{RC}^{-}[R\cdot C] - k_2[C][R] = -\frac{d[C]}{dt}
\]

\[
\frac{d[R\cdot T]}{dt} = k_{RC}^{-}[R\cdot T] - k_1[R][T] = -\frac{d[T]}{dt}
\]

These equations and a Runge–Kutta algorithm77 were used to simulate the time-dependent change in the concentration of \([^{32}P]-S1-5-S2-RRE_{AU}\) as a function of \(k_2\) and \(k^{-}\). Values for \(k_1\) and \(k^{-}\) were determined independently and fixed at \(7.8 \times 10^3\ M^{-1}s^{-1}\) and \(4 \times 10^{-4}\ s^{-1},\) respectively. These simulated time courses were compared to the experimental time courses, and \(k_2\) and \(k^{-}\) were varied to optimize the fit. Residuals for trials simulated in this manner ranged between 1.0% and 4.6%.

**Association Rate Constants (\(k_2\)).** The time-dependent effects of S1, S1EXT, and triplex TOPs on the fraction of \([^{32}P]-\)

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Kinetics and Mechanism of RNA Binding by Triplex TOPs

S1-5-S2 bound to RRE\textsuperscript{AU} are shown in Figure 4. Superimposed on each plot is the simulated trial that exhibited the best fit to the experimental data. Averaged rate constants for all triplex TOP-RRE\textsuperscript{AU} complexes are shown in Table 1. The data show that all triplex TOPs bound RRE\textsuperscript{AU} with similar association kinetics, with $k_2$ values between $(7.8 \pm 2.0) \times 10^3$ and $(16 \pm 3) \times 10^3$ M$^{-1}$ s$^{-1}$. For example, the association rate constant was insensitive to the length of the tether separating the 3'- and 5'-oligonucleotides. S1-1-S4, with a single abasic phosphodiester linkage between the 3'- and 5'-oligonucleotides, exhibited an association rate constant that was comparable to those for molecules containing three, five, and seven abasic phosphodiester units. Association rate constants were also insensitive to the length and sequence composition of the 3'-oligonucleotide. S1-1-S4, S1-1-S4\textsuperscript{G}, S1-1-S4\textsuperscript{C}, and S1-1-S4\textsuperscript{UC}, all of which contained a different 3'-oligonucleotide sequences and formed RRE\textsuperscript{AU} complexes with different stabilities, exhibited nearly identical association rate constants. Even an oligonucleotide in which an additional 10 nucleotides were appended to the 3'-end of S1 (S1\textsuperscript{EXT}) exhibited an equivalent association rate constant of $(7.8 \pm 2) \times 10^3$ M$^{-1}$ s$^{-1}$. Not only were all triplex TOP association rate constants of all triplex TOPs nearly identical to one another, they were also nearly identical to that measured for the association of S1 with RRE\textsuperscript{AU}.

Dissociation Rate Constants ($k_{-2}$). The rate constants for dissociation of triplex TOP-RRE\textsuperscript{AU} complexes varied by more than 2 orders of magnitude, between $(3 \pm 1) \times 10^{-4}$ and $(540 \pm 200) \times 10^{-4}$ s$^{-1}$. In general, triplex TOP-RRE\textsuperscript{AU} complexes with the most stable RRE\textsuperscript{AU} displayed the lowest dissociation rate constants. S1-1-S4, which formed the most stable RRE\textsuperscript{AU} complex ($K_d = 93 \pm 15$ nM),\textsuperscript{62} was characterized by the lowest $k_{-2}$ value ($(3 \pm 1) \times 10^{-4}$ s$^{-1}$), whereas S1-1-S4\textsuperscript{UC}, which formed the least stable RRE\textsuperscript{AU} complex ($K_d = (30 \pm 3700$ nM) was characterized by the highest $k_{-2}$ value ($(400 \pm 40) \times 10^{-4}$ s$^{-1}$). The rate constants for dissociation of the S1-5-S4 RRE\textsuperscript{AU} complexes varied with tether length over a 40-fold range with the following order: S1-1-S4 < S1-3-S4 = S1-5-S4 < S1-7-S4. Dissociation rate constants were also sensitive to the length and sequence of the 3'-oligonucleotide; sequence changes that resulted in mismatched RRE\textsuperscript{AU} complexes significantly increased the dissociation rates of triplex TOP-RRE\textsuperscript{AU} complexes. For example, the dissociation rate of the S1-1-S4\textsuperscript{UC}-RRE\textsuperscript{AU} complex ($k_{-2} = (400 \pm 40) \times 10^{-4}$ s$^{-1}$), which contained three mismatched Hoogsteen base pairs, was more than 130-fold greater than that of the cognate S1-1-S4-RRE\textsuperscript{AU} complex and approximately the same as that of the S1-RRE\textsuperscript{AU} complex ($k_{-2} = (500 \pm 200) \times 10^{-4}$ s$^{-1}$). Shortening the 3'-oligonucleotide by three or six nucleotides resulted in 6- or 20-fold increases in $k_{-2}$\textsuperscript{62}. The lifetime of the S1-1-S4-RRE\textsuperscript{AU} complex (3300 s) is comparable to the lifetime of the complex between RRE\textsuperscript{AU} and S1-5-S2 (2500 s), a TOP that binds RRE\textsuperscript{AU} sites 1 and 2 via duplex formation.

Discussion

Each triplex TOP shown in Figure 1 is comprised of two short oligonucleotides joined together with a flexible tether of variable length. We showed previously that triplex TOPs bind RRE\textsuperscript{AU} in a highly specific manner and that both the 3' and 5'-oligonucleotides hybridize to their prescribed binding sites in the complex.\textsuperscript{61,62} Here we used competitive kinetic experiments to investigate the stepwise pathway by which the triplex TOP-RRE\textsuperscript{AU} complex forms. Because there was no simple integrated solution to the differential equations describing the competitive binding reactions, we used numerical integration to simulate the kinetic data. Association rate constants of triplex TOP-RRE\textsuperscript{AU} complexes were comparable to that measured for the S1-5-S2-RRE\textsuperscript{AU} complex and were insensitive to the length and sequence of the 3'-oligonucleotide that mediates triple helix formation. By contrast, dissociation rate constants were sensitive to changes in tether length and the length and sequence of the 3'-oligonucleotide.

Two Pathways for Formation of Triplex TOP-RRE\textsuperscript{AU} Complexes. The assembly of a TOP-RRE\textsuperscript{AU} complex may proceed through two pathways (Figure 5, top). Pathway I is characterized by a first step in which a duplex forms between the 5'-oligonucleotide within each triplex TOP and site 1 of RRE\textsuperscript{AU} and a second step in which a triple helix forms.

Figure 4. Plots illustrating the effect of triplex TOP or oligonucleotide (○) on $\theta$, the fraction of [32P]S1-5-S2 bound to RRE\textsuperscript{AU} as a function of time. The solid line illustrates the simulated best fit to the experimental data. Above each plot is shown the residuals in percent (%). Residuals at each data point were calculated from the equation $(\nu_s - \nu_s)/\nu_s$, where $\nu_s$ and $\nu_s$ are the values obtained from experiment and simulation, respectively.\textsuperscript{91,92}
between the 3′-oligonucleotide and site 4. Pathway II is characterized by a first step in which a triple helix forms between the 3′-oligonucleotide of the triplex TOP and site 4 of RRE<sub>AU</sub> and a second step in which a duplex forms between the 5′-oligonucleotide and site 1.

Available data on the kinetics of duplex and triple-helix hybridization indicate that neither pathway I or II is unlikely for the assembly of TOP-RRE<sub>AU</sub> complexes. The association of short, unstructured oligonucleotides into duplexes is rapid ($k_{on} = 10^6$–$10^7$ M$^{-1}$ s$^{-1}$)<sup>19</sup> relative to rate constants measured.
for triple-helix association \( k_{\text{on}} = 10^3 \text{M}^{-1} \text{s}^{-1} \). However, when one of the oligonucleotide partners is structured, duplex association rate constants can decrease by several orders of magnitude into the range observed for triple-helix formation. \( 42,60,83 \) S1-5-S2, which disrupts intramolecular Watson—Crick and non-Watson—Crick base pairs within the RRE \( 84–86 \) to interact with sites 1 and 2, exhibits an association rate constant of approximately \( 10^7 \text{M}^{-1} \text{s}^{-1} \). Triplex TOPs also interact with site 1 and therefore must also disrupt these intramolecular base pairs. Since there is little or no information on the effect of RNA structure on triple-helix formation, it is possible that formation of the site 1 duplex could be slower than formation of the site 4 triple helix.

**Kinetic Data Provide Evidence for Pathway I.** Pathways I and II can each be represented by two energy diagrams that differ in terms of whether duplex formation or triple helix formation is rate-limiting (Figure 5, bottom). In pathways Ia and IIa, formation of a duplex at site 1 is rate-limiting, whereas in pathways Ib and Iib, formation of a triple helix at site 4 is rate-limiting. Our data indicate that all triplex TOPs bind RRE \( \text{AU} \) with similar second-order rate constants, with values between 7.8 \( \times \) \( 10^3 \) and 16 \( \times \) \( 10^3 \) \text{M}^{-1} \text{s}^{-1}. \) Not only are all triplex TOP association rate constants similar to one another, they are also similar to the rate constant measured for S1, an oligonucleotide that binds RRE \( \text{AU} \) at site 1 only. These data indicate that triplex TOP association kinetics are dominated by the rate with which the 5'-oligonucleotide binds site 1 and imply that formation of the site 1 duplex is the rate-limiting step in the binding reaction. This correlation rules out pathways Ib and Iib, in which triple-helix formation is rate-limiting.

Further examination of the kinetic data rules out pathway Iia. The principle of microscopic reversibility demands that a reaction follow the same pathway in the forward and reverse directions. \( 87 \) The first dissociative step along pathway Iia involves rate-limiting dissociation of the 5'-oligonucleotide within each TOP from site 1 of RRE \( \text{AU} \). Dissociation through this pathway would result in values of \( k_2 \) that correlated only with sequence within the 5'-oligonucleotide. Instead, triplex TOP dissociation rate constants correlate with sequence within the 3'-oligonucleotide. This analysis rules out pathway Iia and argues in favor of a binding mechanism in which duplex formation at site 1 is rate-limiting and precedes triple-helix formation (pathway Ia).

**Association Rate Constants.** Because triplex TOP-RRE \( \text{AU} \) association reactions follow a binding pathway in which duplex formation precedes triple-helix formation and is rate-limiting, values for \( k_2 \) reflect the rate with which the 5'-oligonucleotide binds site 1 of RRE \( \text{AU} \). These rate constants \( (k_2 \sim 10^3 \text{M}^{-1} \text{s}^{-1}) \) are approximately 2 orders of magnitude smaller than those measured for the association of unstructured oligonucleotides into short duplexes \( (k_{\text{on}} = 10^5–10^6 \text{M}^{-1} \text{s}^{-1}) \). It is well known that oligonucleotides can bind structured RNAs with rate constants that are considerably smaller than those found for binding unstructured RNAs. \( 88–90 \) The decreases in rate constant are attributable to static or dynamic secondary or tertiary interactions within the RNAs that must be disrupted for binding to occur. \( 41–43,83 \) There is considerable evidence that such interactions exist in RRE \( \text{AU} \); NMR studies on stem–loop IIB suggest that residues (within site 1) may be stably or transiently base paired. \( 84–86 \) Thus, the (relatively) slow association kinetics exhibited by triplex TOPs is most likely the result of structure within RRE \( \text{AU} \) that must be disrupted for binding to occur.

**Dissociation Rate Constants.** Because triplex TOPs bind RRE \( \text{AU} \) through a mechanism in which duplex formation is rate-limiting and precedes triple-helix formation, the values measured for \( k_2 \) reflect contributions from both duplex and triple-helix dissociation. The dissociation rate constant of the S1-1-S4-RRE \( \text{AU} \) complex, containing 12 triple-helical base pairs in addition to the site 1 duplex, was comparable to that of a DNA triple helix containing 22 triple-helical base pairs. \( 79,80 \) The dissociation rate constants of the S1-1-S4-RRE \( \text{AU} \) and S1-1-S4-RRE \( \text{AU} \) complexes, containing nine and six triple-helical base pairs, respectively, in addition to the site 1 duplex, were comparable to those of a DNA triple helix containing 11 or 13 triple-helical base pairs. \( 82 \) Although triplex TOP-RRE \( \text{AU} \) complexes containing three mismatched Hoogsteen base pairs dissociated more than 100 times faster than analogous complexes lacking mismatches, introduction of a mismatch into a DNA triple helix increases the dissociation rate constant by more than 1000-fold. \( 80 \) Presumably, the dissociation rate constants of triplex TOP-RRE \( \text{AU} \) complexes are less sensitive to mismatches because dissociation of both the duplex and the triple helix contributes to the dissociation rate constant \( k_2 \).

**Conclusions.**

Here we employed kinetic methods to analyze the interactions between triplex tethered oligonucleotide probes and a modified version of the Rev response element, RRE \( \text{AU} \). We find that the rate constants for association of triplex TOP-RRE \( \text{AU} \) complexes are independent of the structure and stability of the triple helix, whereas the rate constants for dissociation are not. These findings allow us to propose a binding pathway in which duplex formation is rate-limiting and precedes triple-helix formation (pathway 1a in Figure 5). This conclusion implies that the bimolecular reaction between a triplex TOP and RRE \( \text{AU} \) site 4 (the first step of pathway Iia) must proceed more slowly than the observed association rate constant, \( 10^4 \text{M}^{-1} \text{s}^{-1} \). Since this rate constant also represents that observed for forming model triple helices, our results imply that triple-helix association reactions are hindered in the context of the structured RREV \( \text{AU} \) target. Triplex TOPs are unique in that they appear to benefit from the thermodynamic stability of the triple helix as well as the (comparatively) rapid rate constants for duplex formation.

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\( (79) \) Maher, L. J.; Dervan, P. B.; Wold, B. *Biochemistry* 1990, 29, 8820.


\( (83) \) Lima, W. F.; Monia, B. P.; Ecker, D. J.; Freier, S. M. *Biochemistry* 1992, 31, 12055.


\( (88) \) Studier, F. W. *J. Mol. Biol.* 1969, 41, 199.


