Probing the Structural Determinants of a Catalytic RNA with Isomerase Activity

James R. Prudent, Joanna Staunton, and Peter G. Schultz*

Howard Hughes Medical Institute
Department of Chemistry, University of California
Berkeley, California 94720

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Recently, a number of strategies have been developed for isolating RNAs with novel catalytic activities from large libraries of random RNA sequences.[1] One such approach involves screening random pools of RNAs for molecules that bind an analogue of the transition state (TS‡) for the reaction of interest. By *in vitro* screening of RNAs for their ability to bind the planar TS‡ analogue **4**, a 165 nucleotide (nt) RNA (AA6) was isolated that catalyzes the isomerization reaction of the diastereomeric biphenyl **1** to its isomer **2**, with $k_{cat} = 2.8 \times 10^{-5}$ min^{-1} and $K_m = 542$ μM (Scheme 1); the reaction was competitively inhibited by **5**, with an inhibition constant (K_i) of 7 μM.[2] In an effort to gain greater insight into the nature of this RNA-catalyzed reaction, we have carried out a series of chemical and enzymatic modification experiments as well as a mutational analysis of this RNA.

To identify the minimum sequence capable of binding the TS‡ analogue, partial alkaline hydrolysis of 5′-^{32}P end-labeled AA6 was carried out.[3,4] The partially hydrolyzed RNA was heated to 90 °C, slowly cooled to room temperature, and loaded onto an affinity column derivatized with the TS‡ analogue **4**. The column was washed with 20 column volumes of binding buffer (200 mM NaCl, 6 mM $MgCl_2$, 5% dimethyl sulfoxide, 50 mM MES buffer, pH 5.75), and the bound RNA eluted with six volumes of 5 mM EDTA in water. Denaturing gel electrophoresis of the eluted RNAs indicated that a truncated 72 nt sequence (nts 1−72) was able to fold into a structure that binds **6** (Figure 1A). This 72 nt RNA makes up the first stem loop structure of the most stable predicted secondary structure of AA6[2] (Figure 2). Extension of this sequence into stem loops 2 and 3 of AA6 results in a loss of binding to **6** until each stem loop structure is largely formed. Similar experiments with the 3′-^{32}P end-labeled 72 nt RNA indicated that three nucleotides could be removed from the 5′ terminus (Figure 1A). The resulting 69 nt RNA was isolated by large-scale runoff transcription, followed by denaturing gel electrophoresis, electroelution, and ethanol precipitation.[2] The purified RNA was found to bind **6** and had roughly 50% the catalytic activity of full-length AA6.

To determine the sequence requirements for binding and catalysis, mutations and deletions were introduced into a sequence containing the first stem loop of AA6 to afford a library of ~10^{15} RNA variants.[5,6] The affinity selection procedure was then repeated,[2] and after three rounds of screening, with an amplification of ~200-fold per round,[2] ~50% of the RNA was retained on the affinity column. Sequence analysis of 10 clones revealed a 24 nt region (nts 37−60) in which the isolated clones differ from the original sequence by an average of 18% per sequence. Many of the positions covary in a fashion accordant with Watson−Crick base pairing (Figure 2). These 10 sequences, along with 40 others within this

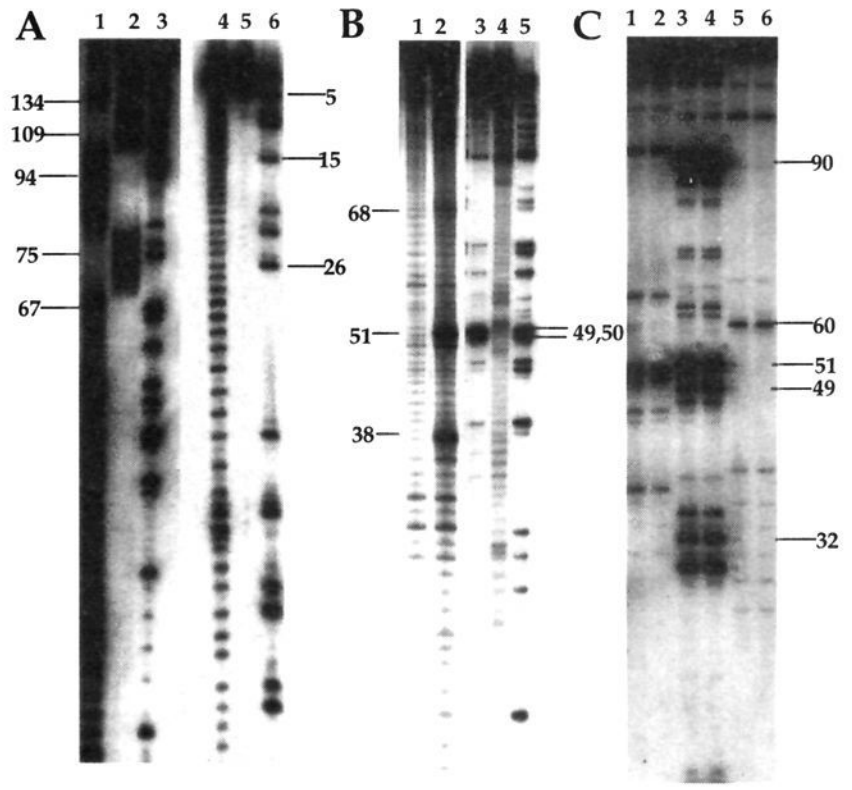
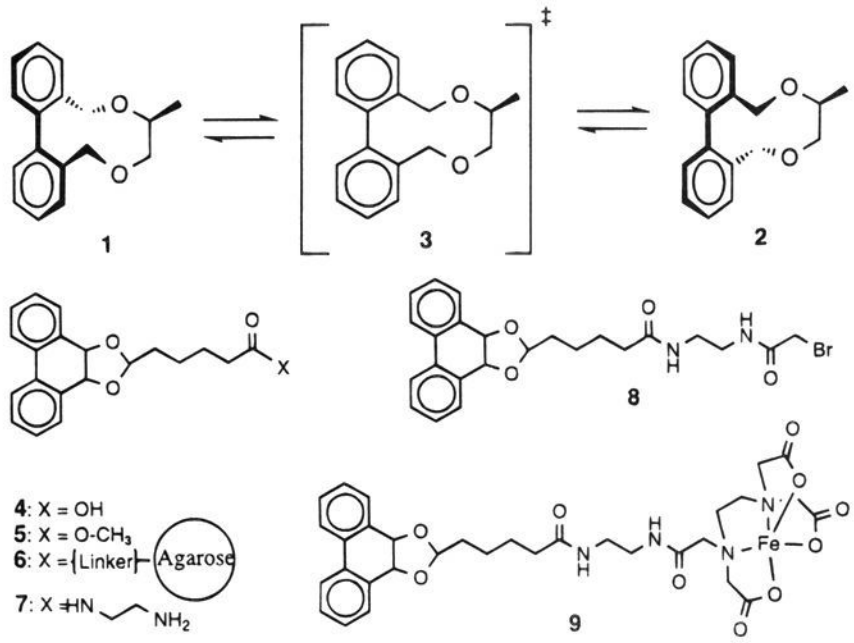


Figure 1. Autoradiogram of 11% denaturing polyacrylamide gels. Numbers to the right and left of gels correspond to base positions in Figure 2A. (A) Boundary experiments: lanes 1−3 contain 5′-^{32}P end-labeled truncated versions of AA6, and lanes 4−6 contain 3′-^{32}P end-labeled truncated versions of the 72 nt sequence. Lanes 1 and 4 correspond to sequences which do not bind to **6**, lanes 2 and 5 correspond to sequences that bind **6**, and lanes 3 and 6 are nuclease T1 digest. (B) Affinity labeling and cleavage experiments using 5-^{32}P end-labeled AA6. Lane 1 was treated with α-bromopropylacetamide; lane 2 was treated with affinity cleavage reagent **8**; lane 3 was treated with affinity cleavage reagent **9**; lane 4 was treated with EDTA·Fe(II); and lane 5 is a nuclease T1 digest. (C) Nuclease digestion of AA6 under nondenaturing conditions. Lanes 1 and 2 are nuclease T1 digests; lanes 3 and 4 are nuclease U2 digests; and lanes 5 and 6 are nuclease Cl3 digests. Lanes 1, 3, and 5 contain no ligand; lanes 2, 4, and 6 contain 200 μM **5**.

Scheme 1



* To whom correspondence should be addressed.

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(4) RNA (1 μM) was hydrolyzed by heating to 95 °C in 1 mM glycine, 0.4 mM $MgSO_4$, pH 9.5, for 5 min. The RNA mixture was immediately added to nine volumes of binding buffer, heated to 90 °C for 1 min, and slowly cooled back to room temperature.

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(6) A partially randomized 78 nt library was constructed as follows: a partially randomized synthetic DNA with the sequence 5′-GGGAGAGAGCT-CACGAATTCCGAGCCGCTCATATACGACCTCTGGACAGG-AGGACGGTCCAGCAAGACGGAATTCTCGCGA-3 and equally synthesized reactive mixtures of the other three phosphoramidites were used to dope the underlined sequence at a rate of 34% at each position. To introduce base deletions, acetic anhydride capping steps were removed during the synthesis. The library was deprotected in concentrated ammonium hydroxide at 90 °C for 1 h and carried on as previously described.[2]

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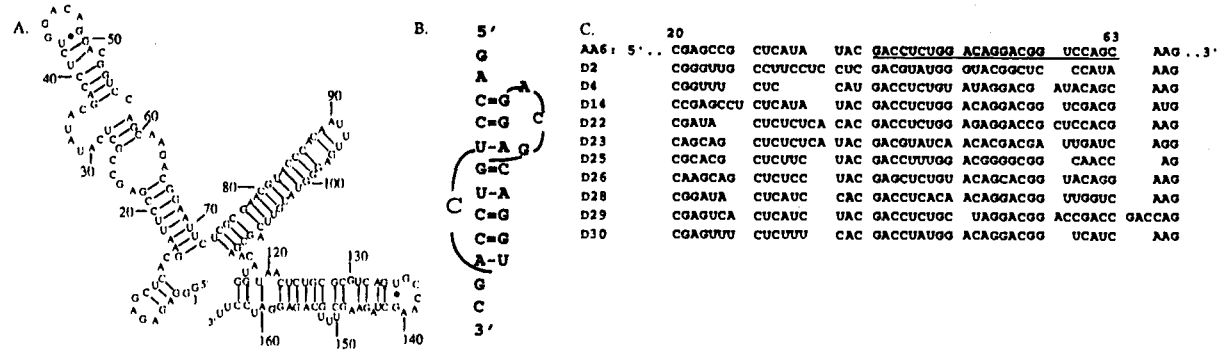

Figure 2. (A) The most stable predicted secondary structure of AA6.[2] (B) Proposed secondary structure of bases 37−60 of AA6. (C) Sequences of AA6 and 10 isolated clones from the mutagenized and selected 78 nt library. The first line gives the primer sequence; the underlined bases of AA6 correspond to the sequence in B.

selected pool, were then isolated and tested for binding and isomerase activity. Many of the transcribed 72 nt RNAs bound to **6** and were catalytically active, although the rates were again roughly half that of full-length AA6. Nondenaturing polyacrylamide gel analysis suggested that these truncated sequences adapt multiple conformations (in contrast to AA6, which runs as a single band), which may account for the decrease in observed catalytic activity. Several of the 24 nt variants were also transcribed and tested for binding and isomerase activity, but all were found to be inactive.

To locate the binding site for TS‡ analogue **4**, affinity cleavage experiments were carried out.[7] Reagents **8**[8] and **9**[9] consist of bromoacetic acid and ethylenediaminetetraacetic acid (EDTA) linked to the amino terminus of phenanthrene derivative **7**, respectively. The cleavage patterns[10,11] generated by treating 5′-^{32}P end-labeled AA6 with reagents **8** and **9** (Figure 1B) support the idea that the 24 nt sequence identified above is important for transition state binding and stabilization. Specific alkylation of bases A38, A51, and G50 by the *N*-bromoacetyl derivative **8** (lane 2, Figure 1B) indicates that these bases are in close proximity to each other. The EDTA·Fe(II)-derived affinity cleaving reagent **9** also cleaves within this region, specifically at G49 and G50 (lane 3, Figure 1B). In both cases, controls with α-bromopropylacetamide and EDTA·Fe(II) alone show that selective cleavage depends on the presence of the TS‡ analogue. Data from EDTA·Fe(II) footprinting experiments also indicate that the phosphate sugar backbones of A51 and C52 are partially protected in the presence of 400 μM **5** (data not shown).

Finally, nuclease digestions were carried out in the presence and absence of **5** to determine whether significant conformational changes occur in the RNA on ligand binding. Analysis of the cleavage patterns produced by nucleases T_1, U_2, and CL3 on 5′-^{32}P end-labeled AA6 revealed no differences between cleavage reactions with or without 200 μM ligand (lanes 1−6, Figure 1C). This is in contrast to the behavior of some other small molecule binding aptamers reported in the literature.[12] The experiments presented above are consistent with the formation of a folded structure involving nts 37−60, which creates a preorganized site capable of binding **3** and catalyzing the isomerization of **1**. It remains unclear why the structure of the 24 nt sequence is catalytically inactive, although nts 15−22 and 65−72 of the predicted secondary structure of AA6 (Figure 2) make up an 8-base-pair region of stem 1, which may stabilize the formation of the catalytic core and therefore be essential for catalysis. Additional experiments are presently underway to explore appropriate folding conditions and mutations of this structure in order to facilitate further structural analysis and enhance catalytic activity.

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(8) Affinity labeling reagent **8** was prepared by adding 2 equiv of triethylamine and 1 equiv of bromoacetyl bromide in chloroform to **7**[2] until all of **7** was consumed, as determined by thin-layer chromatography (10:1 chloroform/methanol). Reagent **8** was purified by silica gel chromatography (30:1 chloroform/methanol) and characterized by NMR and mass spectroscopy.

(9) To synthesize reagent **9**, phenanthrene derivative **7** was added to 1 equiv of EDTA triethyl ester,[8] *N*-hydroxysuccinimide, and 1,3-dicyclohexylcarbodiimide in dioxane, and the resulting triester was purified by silica gel chromatography (30:1 chloroform/methanol). Three equivalents of LiOH was added to the triester in a 1:1 mix of water/acetonitrile, and the mixture was stirred overnight. Compound **9** was purified by preparative HPLC with a C18 reverse-phase column and a mobile phase step gradient from 40 to 70% acetonitrile in a 50 mM triethylammonium acetate buffer, pH 5.5, over 30 min and characterized by NMR and mass spectroscopy.

(10) To a 50 μL solution of 10 μM 5′-^{32}P end-labeled AA6 in binding buffer was added 2 μL of a 10 mM stock solution of **8** in DMSO, and the mixture was incubated for 7 days at 29 °C. The reactions were ethanol precipitated, treated with 10 μL of 1.0 M Tris·HCl (pH 8.2) and 10 μL of a freshly prepared 0.2 M aqueous $NaBH_4$ solution, and incubated in the dark for 20 min. The reactions were again ethanol precipitated, treated with 20 μL of a freshly prepared aqueous solution of 1 M aniline, pH 4.5, and incubated for 20 min at 60 °C in the dark. The hydrolysis reactions were frozen, lyophilized, resuspended in 200 μL of water, and lyophilized again. Finally, the RNA was resuspended in a 50% aqueous mixture of formamide and analyzed on 11% polyacrylamide 8 M urea gels.

(11) All reactions were carried out with equimolar **9**·Fe(II) complexes made by combining a 40 mM aqueous solution of **9** with equimolar aqueous ferrous ammonium sulfate solution. The reactions were initiated by adding 2 μL of 20 mM **9**·Fe(II) solution to 196 μL of a solution containing 10 μg of 5′-^{32}P end-labeled AA6 in DMSO free binding buffer, followed by 2 μL of an aqueous 100 mM DTT solution. The reactions were vortexed and incubated at 22 °C for 15 min. The reactions were terminated by the addition of an equal volume of formamide, stored at −80 °C, and analyzed on 11% polyacrylamide 8 M urea gels.

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