Cholesterol Esterase Activity by in Vitro Selection of RNA against a Phosphate Transition-State Analogue

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Received June 3, 1999

Creation of efficient catalysts with high specificities is one of the most challenging goals for chemists. Various attempts have been made to induce catalytic sites in natural and synthetic polymers by the use of transition-state analogues (TSA).[1] Antibodies have been raised against TSA and used as catalysts for chemical transformations. The rates of these antibody-catalyzed transformations are enhanced over background reactions.[2] On the basis of the discovery of catalytic antibodies, attempts have been made to extend the concepts involved in the antibody catalysis to the development of other biopolymeric catalysts.[3] RNA molecules are also potential catalytic biopolymers because of their unique conformations, molecular diversity, and relative ease of generation. However, most of the catalysts in this family, which have been successfully generated so far[4] have been developed by using direct selection of self-modified RNA.[5] While numerous attempts to select RNA catalysts via TS stabilization have been made, only a few successful results were reported for hydrophobic TSA,[6,7] owing to the hydrophobic nature of the RNA pocket.[8] These observations suggest that the hydrophobic interactions may be more precise and specific than hydrophilic interactions.[9]

We reason that the TSA of cholesterol esterase should be sufficiently hydrophobic to select specific RNA binders. An attempt has already been made to generate catalytic antibodies against a similar hapten as a phosphate ester **2**, a TSA in the cholesterol esterase hydrolysis of the carbonate ester **1** (Figure 1).[10] However, this failed, possibly as a result of poor immunogenicity.[11] The intrinsic difficulties associated with poor immunogenicity can be solved by use of in vitro selection of RNA, a process that takes a relatively short period of time. As part of our continuing interests in the design of TSA-induced catalysts, we for the first time have succeeded in generating RNA with a defined cholesterol esterase activity.

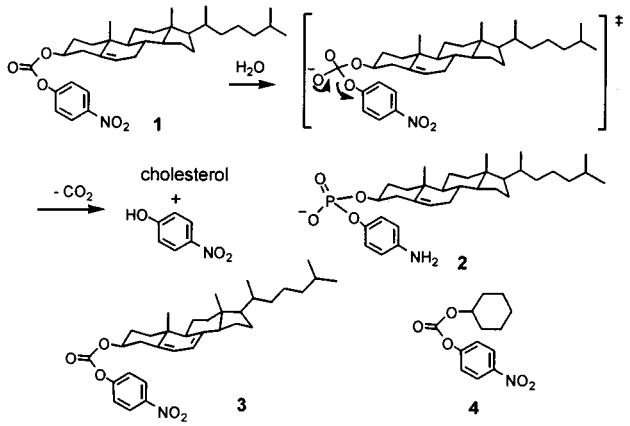

Figure 1. TS for cholesterol ester (**1**) hydrolysis and structures of the TSA analogue, **2** and other substrates.

The design and synthesis of the TSA **2** were carried out by the use of known procedures.[12] Immobilization of **2** on agarose was done under mildly basic conditions.[13] A 110-mer DNA library was designed to contain random nucleotides in 70 positions flanked by defined sequences at both ends for the purpose of PCR amplification and in vitro transcription.[14] The in vitro selection of RNA was made by the use of modification protocols.[15] Enrichment of the RNA was confirmed by means of affinity chromatography and elution procedures with ^{32}P-labeled RNA.[4(c),15] After six cycles of selection, enrichment was achieved as judged by the fact that 30% of the applied RNA was specifically eluted by **2**. The selected RNA was then cloned and sequenced by standard protocols.[16] Since the affinity elution was performed under less stringent conditions due to the poor solubility of **2** in the aqueous phase, no identical sequence was found among 11 cloned RNA molecules.

To measure the binding affinity of **2** to the cloned RNA, a surface plasmon resonance (SPR) technique was utilized. In the first process, the affinities between RNAs and **2** were measured by changing the concentration of RNA in solution with immobilized **2** in a flowcell.[17] One clone[18] showed the best binding ($K_D = 4.0 \times 10^{-8}$ M) as compared the original pool ($K_D = 1.0 \times 10^{-5}$ M). The K_D values of other cloned RNAs were similar (K_D

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(14) Twenty-four micrograms of 131-mer DNA (Midland Certified Company, Midland, TX) have 3.3×10^{14} of diversity. Usually, 1 μg was used to perform PCR for the initial RNA library, affording 1.4×10^{13} of diversity. The DNA template was as shown below:

5′-CC*TAATACGACTCACTATAGGG*GAGCTC**GGTACC**GAATTC- N70 -AAGCTTTGCAGAGGATCCTT-3′
T7 RNA polymerase SacI **KpnI** EcoRI HindIII BamHI

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(18) The random region of the sequence of this clone is as follows: 5′-GTGGGGTCGT CTTGGTTAAA CTCCTTGCGC GTCACGAGGT TAGC-CAGCTT GATACCTCAA GGTGGTGCCT-3′.

10.1021/ja991848u CCC: $18.00
Published on Web 11/04/1999

$= 1.1 \times 10^{-7}$ M) to those of the selected pool. Despite differences in the sequences of the cloned RNAs, the binding affinities were similar, suggesting that the binding might be mediated only by nonspecific stacking of RNA bases. To rule out this possibility, binding affinities to the cloned RNA by a variety of hydrophobic molecules were determined using the RNA clone as an immobilized ligand[19] in a SPR techniques. Compound **2** and hydrophobic analogues, such as ergosterol, vitamin D_3, β-estradiol, and *p*-aminophenyl phosphate in solution were injected to the RNA-immobilized flowcells. The same K_D value was observed for binding of **2** to the RNA by use of this process. However, cholesterol and other hydrophobic molecules, as well as *p*-aminophenyl phosphate, did not show binding affinity to this RNA clone. These data suggest that the hydrophobic binding site in this RNA has quite specific complementarity to **2** and that the structure which combines both hydrophobic cholesterol and the relatively hydrophilic *p*-aminophenyl phosphate moieties are necessary for the binding of the RNA.

The RNA that was selected for the TSA **2** had a 250-fold greater affinity of binding than original RNA. Thus, we anticipated that this RNA would significantly reduce the activation energy for the hydrolysis of carbonate ester **1**. All cloned RNA molecules were incubated with a 0.1 mM solution of the substrate **1** in a saline buffer at 37 °C and pH 7.5.[20] The *p*-nitrophenyl substrate was used in this process to elicit prompt and precise observation of catalytic activity. Two clones including the one displaying maximum binding to **2** showed catalytic activity. The reaction of **1** catalyzed by the maximum-binding RNA showed saturation kinetics. Enzymatic constants were determined to be $k_{cat} = 1.3 \times 10^{-3}$/h and $K_m = 29$ μM at pH 7.5 by use of a Lineweaver−Burk plot (Figure 2).[21] The background hydrolysis rate was measured under the same conditions ($k_{uncat} = 1.2 \times 10^{-5}$/h).[22] The rate enhancement (k_{cat}/k_{uncat}) was 110. The catalytic activity was thoroughly inhibited by the TSA, indicating that the binding pocket for **2** also is the catalytic site for hydrolysis. No rate enhancement was observed from the hydrolysis of a similar carbonate ester **3**[10] or of a small carbonate ester **4**[23] by the maximum-binding RNA.[21,22] Nuclease protection study with the maximum-binding RNA and TSA **2** yielded a protected fragment of approximately 30 nucleotides in 10% acryl amide gel, whereas the RNA without or with nonspecific compounds did not show any protected band.[12] Both studies of substrate specificity and nuclease protection resulted in the conclusion that hydrolysis of **1** by the maximum-binding RNA is only derived from the specific hydrophobic pocket for TSA **2**.

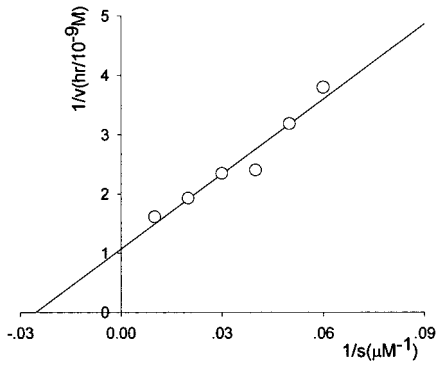

Figure 2. Lineweaver−Burk plot for cholesterol esterase activity by the maximum-binding RNA clone.

Although the rate enhancement is not as high as usual for catalytic antibodies, it appears to be a reasonable value on the binding energy of 12 kJ/mol measured by the affinity selection (K_D values of clone 8 versus original RNA). If all of this binding energy were fully converted to TS stabilization, the predicted rate acceleration (k_{cat}/k_{uncat}) would be 250 at 37 °C.[24] The observed rate enhancement was about half as much as this value. This suggests that a very significant fraction of binding energy of RNA to the TSA is reflected in the catalytic activity.

Our results demonstrate the first successful selection by TSA screening of an RNA that catalyzes a reaction that requires a nucleophilic or general base-activated nucleophilic substitution at the carbonyl functionality. Detailed molecular recognition studies are in progress to elucidate the mechanism of this catalytic process. In addition to the catalytic activity of ester hydrolysis, the submicromolar affinity of the selected RNA against a cholesterol derivative suggests that RNA molecules might possibly be used as antibody substitutes against haptenic hydrophobic molecules, such as cholesterol and the related steroid hormones.

Acknowledgment. Financially support for this work was provided by KIST (2V00363). S.J. is a beneficiary of Academic Research Fund of Ministry of Education, Korea (GE97-211). We thank Mr. Joon-Young Jun for help with the SPR studies. This contribution is dedicated to J.Y.'s late father Professor Byung-Sul Yu on the 10th anniversary of his passing, on December 15, 1999.

Supporting Information Available: Experimental procedures for preparation of **2**, for selection of RNA, for binding affinity studies, and for nuclease protection studies are available (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA991848U

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(20) Since the RNA was selected at pH 7.5, the catalytic activity was observed only at that pH. For the initial screening, concentrated solution of RNA (17 μM) in 100 μM of substrate **1** was incubated at 37 °C for 24 h in assay buffer (10 mM EPPS, 100 mM NaCl, 0.02% NaN_3 at pH 7.5). The product conversion was measured by injecting 10 μL aliquots of the reaction mixture onto an HPLC column and monitoring at 315 nm for *p*-nitrophenol. A C18 column (Microsorb; 4.6 mm × 15 cm, 5 μm) was used as the stationary phase and 50% aqueous acetonitrile as the mobile phase.

(21) To a 90 μL of RNA solution (0.67 μM) in assay buffer, 10 μL of 10 × substrate solution (variable from 0.15 mM to 1.0 mM in THF) was added. The resulting solution (0.6 μM of RNA, 15−100 μM of variable substrate concentration) was incubated at 37 °C for 3 days. The product conversion was measured by injecting 10 μL aliquots of the reaction mixture onto an HPLC column in every 24 h.

(22) In the absence and presence of original pool RNA (0.6 μM), background hydrolysis rate of the substrate was measured, affording the same number.

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(24) $k_{cat}/k_{uncat} = \exp(E_{uncat} - E_{cat}/RT)$