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Reduction of an Aldehyde by a NADH/Zn^{2+}-Dependent Redox Active Ribozyme

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Redox chemistry is involved in various biological events, such as metabolism of biological molecules, detoxification, energy production, and regulation of protein functions.[1] Although only protein enzymes in modern biology are able to catalyze redox reactions in concert with organic/inorganic cofactors, potential functions of RNA molecules in redox chemistry had been speculated in the context of the RNA world hypothesis.[2−4] Specifically, the notion that RNA with cofactors might have been involved in ancient life before protein enzymes has been thus far supported by the following two facts: (1) circumstantial evidence that many organic cofactors such as nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD) consist of ribonucleotides[3] and (2) experimental evidence that in vitro selection had yielded several RNA aptamers[5−7] or RNA enzymes (ribozymes) that bind or react such nucleotide cofactors.[8−12] However, no redox-active ribozyme was known until recently.

Recently, we reported a ribozyme that exhibits activity analogous to alcohol dehydrogenase (ADH).[13] The ribozyme, called ribox02 (Figure 1A), was selected in vitro from a pool of random RNA sequences in the presence of NAD^+ and Zn^{2+}, based on the ability to convert a benzyl alcohol derivative **2** to the corresponding aldehyde **1** that was covalently attached to the 5′-end of the RNA pool (Figure 1B). In fact, ribox02 oxidizes the benzyl alcohol **2** to benzaldehyde **1** in NAD^+- and Zn^{2+}-dependent manner, with a rate acceleration of at least 7 orders of magnitude over the uncatalyzed rate. This successful demonstration of ADH-like function in ribox02 gives rise to a question whether it is able to catalyze the reverse reaction (Figure 1C), i.e., the reduction of the benzaldehyde in the presence of NADH, as protein ADHs generally carry out both chemistries using the microscopic reversibility of redox reactions.

To investigate such an activity of ribox02, we constructed the conjugate of ribox02 with 4-(bromoacetamidemethyl)-benzaldehyde **1**, **1**-ribox02, via the selective S-alkylation reaction of the 5′-GMPS (guanosine-5′-monophosphorothioate) on ribox02 (Figure 1A,B). When **1**-ribox02 was treated with biotin-hydrazide, the aldehyde group chemoselectively reacted with the hydrazide, and thus **1**-ribox02 was tagged with biotin. By means of polyacrylamide gel electrophoresis (PAGE) in the presence of streptavidin (SAv), the band of the biotinylated **1**-ribox02 was retarded and thus allowed us to determine the biotinylation efficiency of **1**-ribox02 in 82 ± 2% (lane 1 in Figure 2). On the basis of this result, we should be able to assess whether the reduction of **1**-ribox02 occurred in the presence of NADH and Zn^{2+}, since the product, **2**-ribox02, should not be retarded on SAv-dependent PAGE because the alcohol group could not be tagged with biotin.

Indeed, the incubation of **1**-ribox02 in the presence of 1 mM NADH and 0.5 mM Zn^{2+} increased the intensity of the faster migrating band from 17% (this value reflected the nonbiotinylated **1**-ribox02, lane 1 in Figure 2) to 70 and 77% after 2 and 30 min, respectively (lanes 4 and 5). Thus, the conversion yields are 53 and 60%, respectively. No change of 17% of the faster migration

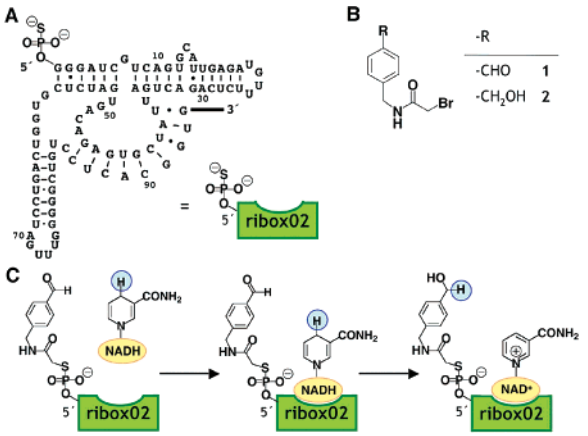

Figure 1. An alcohol dehydrogenase RNA-catalyzed reduction of benzaldehyde. (A) Proposed secondary structure of ribox02 (a total of 110 nucleotides in length). The thick line represents a mini-helix domain that can be deleted without affecting the ribozyme activity.[13] (B) Chemical structure of substrate derivatives used in this study for conjugation. (C) Schematic drawing of benzaldehyde reduction catalyzed by ribox02 in the presence of NADH.

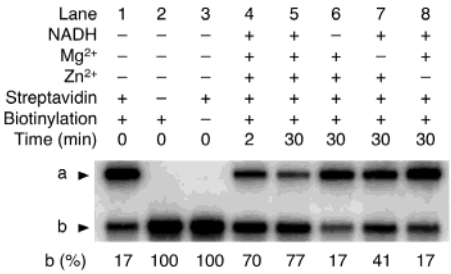

Figure 2. Reduction activity of ribox02 evaluated by SAv-dependent PAGE. Lanes 1−3, before the reaction with NADH (controls); lanes 4−5, reduction reaction catalyzed by ribox02 after 2 min (lane 4) and 30 min (lane 5) incubation; lanes 6−8, negative controls for lane 5 in the absence of NADH (lane 6), Mg^{2+} (lane 7), or Zn^{2+} (lane 8). Arrows: a, biotinylated-**1**-ribox02 complexed with SAv; b, **2**-ribox02 (reacted **1**-ribox02). Conditions: [**1**-ribox02] = 1 μM, [NADH] = 1 mM, [$MgCl_2$] = 100 mM, [$ZnCl_2$] = 0.5 mM in EK buffer ([EPPS] = 50 mM, [KCl] = 0.5 M, pH = 8.0).

band was observed in the absence of NADH or Zn^{2+}, (i.e., no reaction occurred), indicating that both NADH and Zn^{2+} are essential cofactors for reduction (lanes 6 and 8). In the absence of Mg^{2+}, activity was yet sustained with a substantially lower conversion yield (24%, lane 7), suggesting its role in stabilizing the tertiary structure rather than the catalytic role. All these results are consistent with our previous observations for the function of oxidation reaction of ribox02.

To obtain direct evidence for the formation of the benzyl alcohol, we performed two-dimensional (2D) thin-layer chromatography of the RNase T2-digested reaction sample (Figure 3A−C). This enzymatic digestion of ribox02 transcribed with α-[^{32}P]-GTP yields

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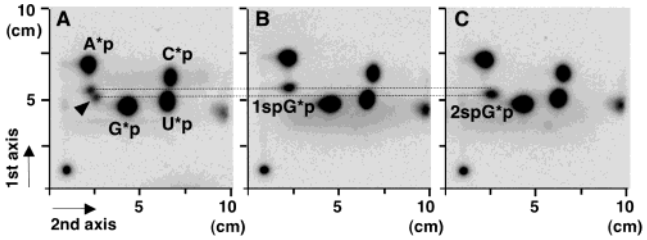

Figure 3. Product analyses of the ribozyme-catalyzed reduction of benzaldehyde by 2D TLC. (A) RNase T2 digests of NADH-treated **1**-ribox02. The arrow indicates the new migrating spot corresponding to the alcohol. (B) Digests of **1**-ribox02. (C) Digests of **2**-ribox02. Each digested nucleotide, except for **1**spG*p and **2**spG*p, was assigned on the basis of literature data.[14] sp, thiophosphate; G, guanosine; *p, [^{32}P]-labeled phosphate.

mononucleotide [^{32}P]-3′-monophosphate when its following nucleotide is guanosine, and these individual mononucleotides become visible on the 2D TLC as radioactive spots.[13] However, the **1**- or **2**-conjugated GMPS-[^{32}P]-3′-phosphate (**1**spG*p or **2**spG*p, asterisk indicates the radioisotope) should migrate differently from other mononucleotides since it has an additional negative charge along with the hydrophobic benzene ring of the aldehyde or alcohol. In fact, we have previously shown that all these mononucleotides, including **1**spG*p or **2**spG*p, could be separated on the 2D TLC, demonstrating that the method is a reliable means to determine the conversion. As expected, the analysis of the RNase-digested reaction product of **1**-ribox02 (Figure 3), referencing to the authentic samples prepared by the RNase-digested **1**-ribox02 and **2**-ribox02 (Figure 3B,C), clearly showed the conversion of **1**spG*p to **2**spG*p. Moreover, the yield of the conversion after 30 min determined by this TLC method was 55%, which was consistent with the estimated yield by the PAGE method. This suggests that both methods, the conventional gel-shift assay as well as the 2D TLC, are equally reliable to determine the conversion rate.

To determine the kinetic parameters, the conversion rate of **1**-ribox02 to **2**-ribox02 with various concentrations of NADH was determined by means of the PAGE method. The rate linearly increased up to 0.5 mM NADH, and over 1 mM accurate assessment of the rate was hampered because of fast kinetics beyond the limitation of our manual quenching method. Instead, we calculated the apparent second-order rate constant, k_{cat}/K_M, to be 1.1×10^4 M^{-1} min^{-1} (Figure 4). The rate constant of the uncatalyzed reaction was determined using the initial pool of **1**-RNA (round 0 pool) under the same experimental conditions except that 100 mM NADH was used to observe the conversion from **1**-RNA to **2**-RNA. The observed rate was 3.7×10^{-4} min^{-1}, and thus the apparent second-order rate constant for the spontaneous reaction was estimated to be 3.7×10^{-3} M^{-1} min^{-1}. This translates to a rate enhancement of 3×10^6-fold achieved by ribox02.

We have also explored whether ribox02 is able to reduce an acetophenone derivative that is structurally analogous to the benzaldehyde. In contrast to the aldehyde experiment, the ketone group was not reduced at all, as determined by both PAGE and TLC analyses. This may be attributed to the specific nature of substrate recognition of ribox02. However, more investigations are necessary to understand the specific contacts of the substrate and cofactors within the active site of ribox02, and such studies are currently underway in our laboratory.

In conclusion, we have demonstrated that the redox active ribozyme is able to reduce an aldehyde assisted by NADH/Zn^{2+}.

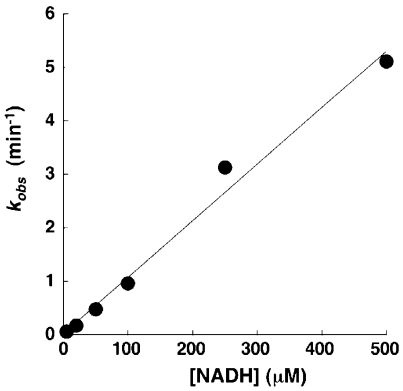

Figure 4. Kinetic plot of the reduction of benzaldehyde catalyzed by **1**-ribox02. The data were fit to the linear line of $y = 0.014946 + 0.010589x$, $R = 0.99349$.

Although the reversibility of phosphodiester[15] and acyl[16] transfer reactions catalyzed by ribozymes was known, that of other chemical reactions has not been well established. This study has demonstrated the reversibility of a hydride transfer chemistry, involving C−H bond formation/cleavage, catalyzed by the ADH ribozyme. Though its exact mechanism remains to be determined, the fact that the ribozyme shares many features with the protein ADHs, e.g., reversibility and NADH/Zn^{2+}-dependence, makes this RNA-based redox system intriguing. Most importantly, our results showing the abilities of RNA molecules for redox chemistry strengthen the view of the RNA world in the evolution of ancient life.

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Supporting Information Available: Experimental procedures for the preparation of **1**- and **2**-ribox02, 2D TLC analysis, and kinetic studies (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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