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Ribozymes that use redox cofactors*

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Abstract: This review summarizes the history and most recent advances in aptamers and ribozymes that bind and use redox(reduction–oxidation) cofactors. Redox reactions, catalyzed by protein enzymes in an extant world, play a central role in the metabolism of numerous biological molecules in the living organisms. The burden of catalyzing these reactions in a pre-protein-based world (i.e., the hypothesized RNA World) could have been borne by RNA molecules. To this end, we raise a fundamental question: can RNA accelerate the redox chemical transformation? We hope that this article will be able to shed light on this intriguing question.

INTRODUCTION

The discovery of RNA molecules with endonuclease and self-splicing activities in the early 1980s [1,2] has given rise to a hypothesis that primitive life-like forms could be RNA-based. This notion, the so-called "RNA World", postulates that RNA molecules could have played prominent roles in both heredity and catalysis, which are essential functions to evolve primitive life [3,4]. It is obvious that RNA can fulfill the function of heredity since it is functionally and structurally nearly identical to DNA. On the other hand, the catalytic function is not so clear. Even though we have witnessed the endonuclease activity in more than a half dozen naturally occurring ribozymes, such activities are, of course, insufficient to claim that RNA is capable of evolving ancient life.

The technique of in vitro selection, developed in the late 1980s [5–7], has aided researchers in exploring the catalytic potentials of RNA. This technology involves selection of active RNA populations based on a function or functions from diverse combinatorial sequences of RNA (generally involving more than 10^{13} unique sequences), amplification of the selected sequences using RT/PCR (reverse transcription/polymerase chain reaction), followed by transcription to generate enriched RNA populations. Repeating this process allows researchers to isolate rare active RNA sequences exhibiting desired functions. Such efforts have yielded both aptamers and ribozymes. The former bind a variety of targets from small organic molecules to large proteins, whereas the latter catalyze a variety of chemical reactions with remarkable ability.

The catalytic prowess of such ribozymes has been extensively discussed in a number of excellent reviews [8,9]. Thus, in this review, we would like to focus on the history and most recent advances in aptamers and ribozymes that bind and use *redox* (reduction–oxidation) cofactors. Redox reactions, cat-

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alyzed by protein enzymes in the extant world, play a central role in the metabolism of numerous biological molecules in living organisms. The burden of catalyzing these reactions in a pre-protein-based world (i.e., the hypothesized RNA World) could have been borne by RNA molecules [10–12]. To this end, we raise a fundamental question: can RNA accelerate the redox chemical transformation? We hope that this article would be able to shed light on this intriguing question.

NECESSITY OF REDOX RIBOZYMES IN THE RNA WORLD

Protein-based metabolic enzymes are responsible for catalyzing the redox chemistry in modern biological systems [13]. Although some modern metabolic enzymes catalyze redox reactions via sophisticated mechanisms involving transition-metal ions, or by using organic cofactors, such as nicotinamide adenine dinucleotide (NAD^+/NADH), flavin adenine dinucleotide (FAD/$FADH_2$), and cyanocobalamin (vitamin B_{12}) (Fig. 1), it should be noted that all these organic redox cofactors contain a ribonucleotide moiety. Therefore, even some protein enzymes, which are the current end products of billions of years of evolution, still fundamentally rely on these RNA-based cofactors for the redox function.

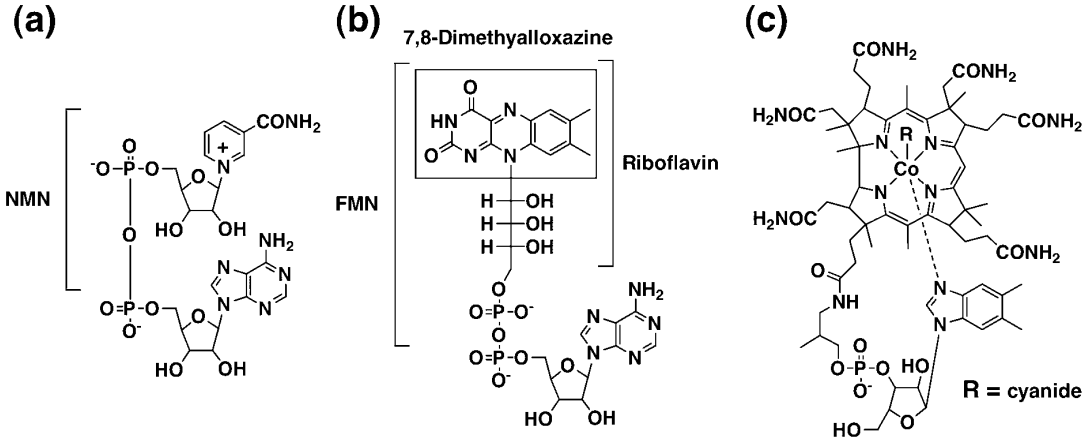

Fig. 1 Chemical structures of redox cofactors: (a) nicotinamide adenine dinucleotide (NAD^+); (b) flavin adenine dinucleotide (FAD); (c) cyanocobalamin.

From the view of the RNA world, the above fact postulates that these cofactors could be molecular remnants of the ancient life-like form [10,11]. Notably, Miller et al. have experimentally demonstrated potential prebiotic pathways for the synthesis of NAD^+, and thus NAD^+ could have been already available before the RNA world [14]. Then, are the redox cofactors alone able to oxidize or reduce substances? Although they are able to execute the chemistry, the rate is extremely slow [10]. This explains why the protein enzymes are necessary for the modern biological system. Thus, in the RNA world, redox ribozymes could have existed to accelerate redox chemistry in collaboration with the redox cofactors.

RNA APTAMERS THAT BIND TO REDOX COFACTORS

An obvious primary question to ask is whether RNA is able to specifically interact with redox cofactors. This question was answered by performing in vitro selection of RNA aptamers that show high affinity toward redox cofactors.

Burgstaller and Famulok screened RNA aptamers against flavin mononucleotide (FMN) and FAD from a pool consisting of 10^{15} members derived from a 74-nucleotide random sequence [15]. The affin-

ity selection of RNAs against resin-immobilized FMN or FAD yielded a consensus RNA motif composed of a secondary structure of stem-bulged loop-stem, where the 11-nucleotide internal bulge was made of six and five bases in the upper and lower strands, respectively (Fig. 2). Binding studies of the aptamer using a FMN-immobilized resin competitively eluting with the corresponding ligands revealed that the aptamer exclusively recognizes the flavin portion (7,8-dimethylalloxazine, see box in Fig. 1b) of the cofactor with a K_d of ~500 nM. The solution structure of the aptamer–FMN complex determined by NMR [16], revealed that the isoalloxazine ring intercalates into the helix consisting of a G9·G27 mismatch and a G10·U12·A25 base triple. The recognition specificity is dictated by the collaboration of the base stacking and hydrogen bonding of the uracil-like edge of the FMN ring to the Hoogsteen edge of A26 at the intercalation site. Thus, the three-dimensional (3D) structure of the RNA motif creates the pocket that binds to the flavin portion in a highly specific manner.

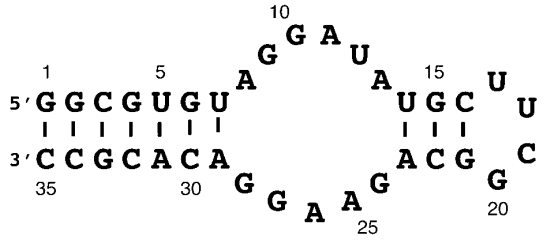

Fig. 2 Proposed secondary structure of the FMN-aptamer isolated by Burgstaller and Famulok [15].

Lauhon and Szostak performed similar selections of aptamers against riboflavin (Rb) independently from Famulok's study [17]. An interesting outcome from this study is that even though the structure of Rb is nearly identical to that of FMN, the selected RNA motif isolated in Szostak's study was not the same as that in Famulok's study. The anti-Rb aptamer consisted of a G-rich motif, presumably forming a G-quartet-like structure proposed based on chemical modification studies (Fig. 3a). This aptamer exhibits tight binding to Rb as well as FMN and FAD, with an observed K_d of 1–5 μM. It should be noted that the anti-Rb aptamer was unable to distinguish the structural difference between oxidized and reduced forms of 5-deazaRb, which is a structural analog of Rb. Because of the lack of 3D structural data of this aptamer, it is yet unknown how the G-quartet-like motif binds to the FMN ring. Clearly, the binding mode between two anti-FMN aptamers isolated by the Famulok and Szostak teams significantly differs from each other.

In the same article, the Szostak team also described the selection of anti-NMN^+ (nicotinamide mononucleotide) aptamers. A representative anti-NMN^+ aptamer was further characterized, showing that it is able to discriminate between NMN^+ and NMNH in solution by approximately 15-fold. To define the critical bases that interact with NMN^+, a dope-mutagenized pool of RNA sequences based on the anti-NMN^+ aptamer motif was prepared and active sequences specific to NMN^+ were reselected using the counter selection procedure with NMNH. This experiment led to the determination of the minimal NMN^+-binding motif consisting of two domains, a larger stem-bulge/stem-loop domain and a smaller stem-loop domain (Fig. 3b). Although it seems that the larger domain is primarily responsible for the binding activity, the smaller domain most likely enhances the stability of the tertiary structure of the aptamer. Although the exact interaction mode of the aptamer to the ligand is unknown, it is clear that the RNA molecule is able to form a specific binding pocket to NMN^+.

In addition to the above studies, anti-FAD aptamers [18] and anti-cyanocobalamin aptamers [19] have been generated by similar in vitro selection strategies. Remarkably, Breaker's group recently discovered that certain messenger RNAs contain the aptamer domain within the noncoding sequences, which bind specific metabolites with remarkably high affinity and specificity without the need of proteins [20–24]. The metabolite-binding motif, so called "riboswitch", uses allosteric mechanisms to regulate gene expressions of proteins involved in the biosynthesis, transport, or utilization of the target

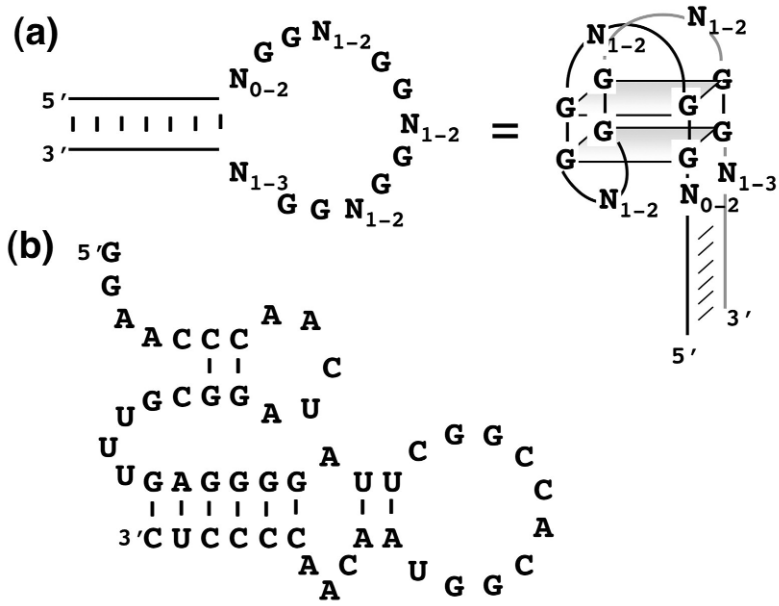

Fig. 3 Proposed secondary and tertiary structure of (a) riboflavin-aptamer (N with numbers indicate any numbers of nucleotides that can bridge the critical bases) and (b) NMN-aptamer isolated by Lauhon and Szostak [17].

metabolite. Riboswitches for various cofactors including coenzyme B_{12} [20], thiamine pyrophosphate [21], FMN [22], *S*-adenosylmethionine [24] have been identified so far. It is quite possible that there would be more examples of riboswitch-controlled gene regulations existing in the modern biological systems. If such mechanisms are preserved in a wide array of organisms, the natural aptamers may be another kind of molecular fossils, in addition to ribozymes, from the RNA world.

All results taken together, it is evident that the specific structures of RNA molecules are able to bind various redox cofactors. Although binding to the redox cofactors is necessary for RNA to perform catalysis, catalysis requires a more sophisticated mechanism. Thus, a question remaining to be answered is whether RNA molecules are capable of using such cofactors for redox chemistry.

SELF-INCORPORATION OF REDOX COFACTORS INTO RNA

To catalyze redox chemistry, redox cofactor-dependent enzyme needs to precisely place both substrate and redox cofactor at the active site. Therefore, the enzymatic event for redox reaction is inherently a tri-molecular reaction. Although such tri-molecular reactions are effectively catalyzed in the modern protein-based catalytic systems, such entropically unfavorable reactions might not be an easy task for primitive RNA-based catalytic systems. To overcome this entropical disadvantage, it has been proposed that redox cofactor could be covalently incorporated into the structure of ribozymes. In this way, the cofactor could become a prosthetic group of ribozyme, thus facilitating redox reaction of substrates. Simultaneously, this could have extended the limited repertoire of the functional groups available in RNA molecules.

To test whether cofactors can be self-incorporated into RNA sequences, Breaker and Joyce have used the *Tetrahymena thermophila* group I intron ribozyme [25]. This ribozyme normally promotes its own excision from a pre-rRNA molecule by catalyzing two successive phosphoester transfer reactions, resulting in self-splicing of intron and ligation of the flanking 5′- and 3′-exons [1]. In the initial step of transesterification reactions, the 5′-exon is released from the pre-rRNA while the exogenous guanosine becomes covalently attached to the 5′-terminus of the ribozyme via a 3′-5′-phosphodiester linkage.

They have exploited this chemistry to achieve the incorporation of alternative groups into its own 5′-end by replacing the guanosine nucleophile with appropriate cofactors or analogs that would likely be recognized by the guanosine binding site in the ribozyme (Fig. 4). A 3′-truncated version of the group I ribozyme was shown to mediate self-incorporation of nicotinamide guanine dinucleotide (NGD^+). Other related cofactors such as NAD^+ and dephosphorylated CoA-SH could also be incorporated but with only modest efficiencies.

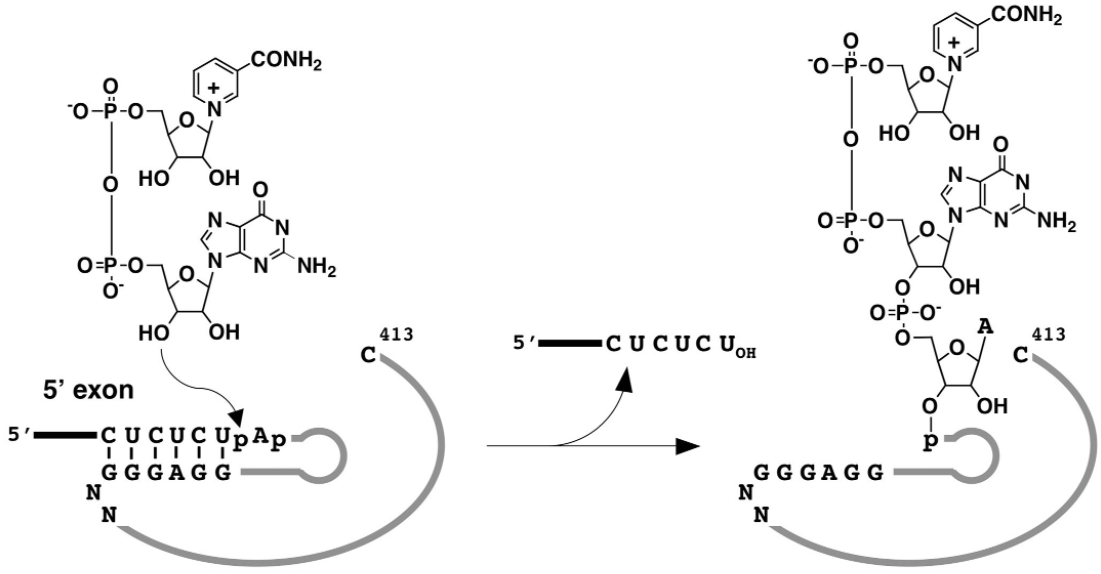

Fig. 4 Self-incorporation of nicotinamide guanine dinucleotide by a 3′-truncated version of the *Tetrahymena thermophila* group I ribozyme [25].

The Joyce team's demonstration showed that the manipulation of the catalytic mechanism of an existing splicing ribozyme could alter its function to the incorporation of redox and other type of cofactors into its own structure. Is this the only mechanism that is able to incorporate the cofactor into RNA molecules? The answer to this question came from an unexpected result from a selection designed by Huang and Yarus [26]. This team originally planned to select RNA species that can self-conjugate the amino acids to yield an aminoacyl-phosphate bond. Interestingly, the selection did not give the desired active RNA populations, but gave those exhibiting amino acid-independent pyrophosphatase activity. Moreover, the selected RNA species subsequently self-5′-labeled with radioactive pyrophosphate. Then, the strategy of the selection was modified to enrich the species that effectively self-conjugate the 5′-end with UTP-agarose. After 24 rounds of selection, RNA species with 5′-self-capping activity were successfully enriched. One of the cloned RNA sequences was characterized to show that self-capping activity is Ca^{2+}-dependent [27]. Further studies of this ribozyme revealed that it self-caps the 5′-end in the presence of GDP forming GpppRNA, but also decaps GPPi 10–50 times slower than the capping rate depending upon pH [27]. Notably, this self-capping ribozyme did not show selectivity toward the phosphate nucleophile, i.e., other cofactors bearing phosphate such as CoA (the 3′-phosphate is the nucleophile in this case) could be incorporated [28]. More recently, Jadhav and Yarus reevolved the above self-capping ribozyme into one that caps a given RNA with CoA at the 5′-end *in trans* (Fig. 5) [29]. Moreover, this CoA-RNA also has a domain that charges an AMP-activated acyl group on thiol of the 5′-CoA, thus acting as a self-*S*-acylating CoA-ribozyme. As a result, the combination of the CoA-capping ribozyme with the self-*S*-acylating CoA-ribozyme was able to yield an acyl-CoA–RNA starting from the corresponding 5′-triphosphate-RNA.

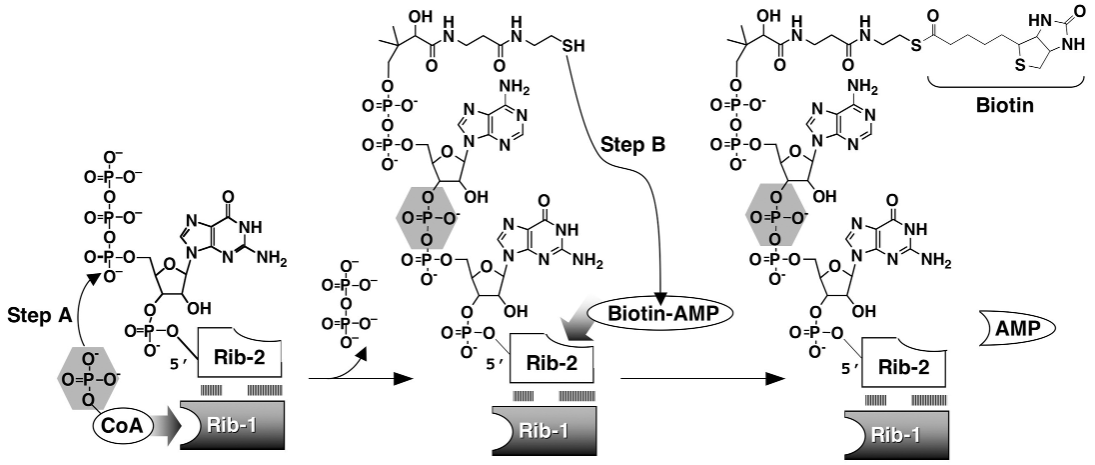

Fig. 5 Incorporation of CoA by the *trans*-acting capping-ribozyme (Rib-1) [26,27] and ribozyme-catalyzed acyl-CoA synthesis (Rib-2) [29]. The nucleophile of phosphate is shaded.

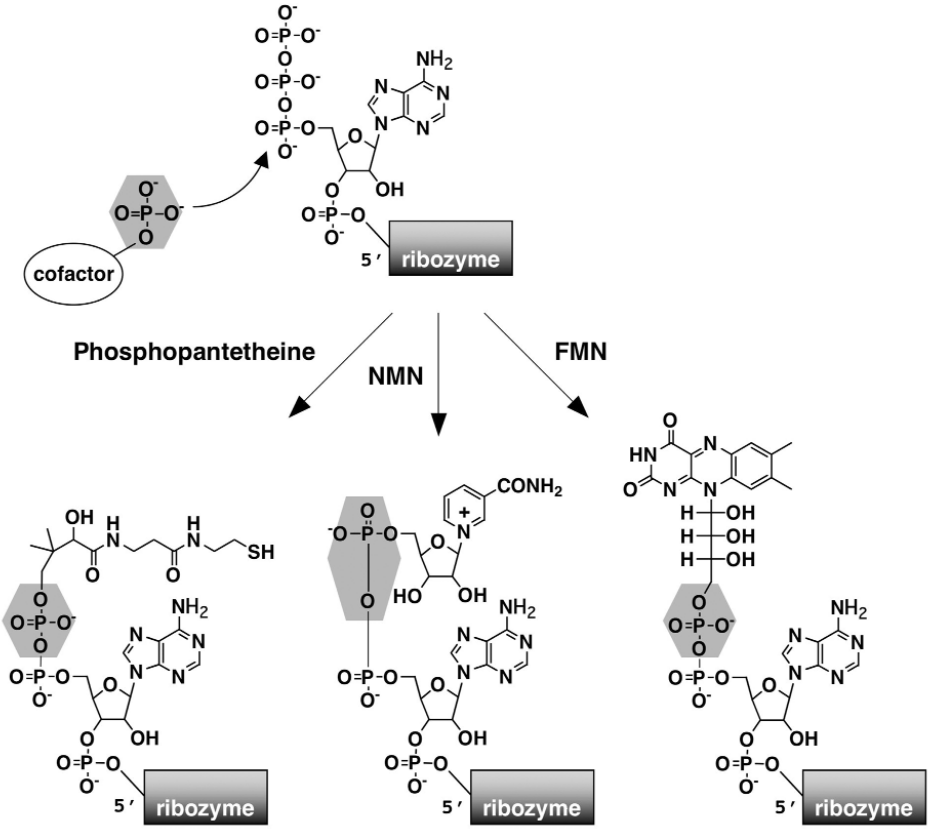

Fig. 6 RNA-catalyzed CoA, NAD^+, and FAD synthesis from phosphopantetheine, NMN, and FMN [30]. The nucleophile of phosphate is shaded.

The same team has made another significant progress in generating ribozymes that incorporate cofactors using the similar strategy as above. Huang and Yarus selected ribozymes that self-incorporate various cofactors at the 5′-end of RNA [30]. These ribozymes use the same mechanism as the above self-capping ribozyme for the incorporation of the cofactors; the α-phosphate of the ribozyme's 5′-triphosphate is attacked by the phosphate nucleophile on the cofactors, resulting in formation of the 5′-cofactor-ribozyme conjugate. Significantly, since the ribozymes were primed with adenosine triphosphate, the conjugation of their 5′-end with phosphopantetheine, NMN, and FMN yielded the linkage analogous to CoA, NAD, and FAD, respectively (Fig. 6).

In summary, the above experiments have demonstrated that cofactors including the redox ones can be incorporated into a specific site (the 5′-end) by ribozyme catalysis. It should be noted that the ribozymes thus far studied lack the specificity toward particular cofactors. However, such a broad activity toward various cofactors might be beneficial for developing various ribozyme systems that are able to use the cofactors as a prosthetic group for desired chemistries.

A RIBOZYME DISPLAYING ALCOHOL DEHYDROGENASE ACTIVITY

Despite this wealth of information about aptamers and ribozymes that interact or react with nucleotide cofactors, our initial question whether RNA is capable of catalyzing redox reaction still remains unanswered. The binding ability of RNA to the redox cofactor is necessary to perform redox catalysis, but to achieve the catalysis more sophisticated mechanism is most likely required; i.e., the proper juxtapositioning of the cofactor and the substrate in the active site and their collaboration with the catalytic residues to lower the activation energy. It seemed to us, however, that the reason why a redox ribozyme has not been isolated so far might be the lack of a suitable selection strategy, rather than the inability of RNA to catalyze a redox reaction. Thus, isolation of redox-active ribozymes could be achieved by utilizing a new in vitro selection strategy that enables direct enrichment of active sequences from the library of RNA sequences.

Two technical challenges must be overcome in order to design the suitable selection strategy. First, how can active species be separated from a large number of inactive members in the pool? Although this issue applies to any selection designed for new reactions, the selection of redox-active RNAs requires more in-depth considerations and planning since the change in structure or functional group before and after redox reaction is very small, i.e., a single hydride or electron-transfer process is involved in redox chemistry compared to other types of chemistry such as acylation, alkylation, or nucleotide addition, etc. Second, as discussed earlier, the cofactor-dependent redox chemistry is a trimolecular reaction, which is generally difficult for ribozyme selection particularly when neither of the substrates is an oligonucleotide. In order to facilitate the selection of active RNAs as self-modifying enzymes, one of the substrates, either the redox cofactor or the reacting substrate, has to be a part of RNA sequence. Then, which substrate should be covalently linked to the RNA pool in the context to facilitate the selection of active species? How can we incorporate either substrate into the RNA pool?

To this end, we made our decision to perform in vitro selection of ribozymes displaying NAD^+-dependent alcohol dehydrogenase (ADH) activity (Fig. 7) [31]. The protein enzymes of this kind catalyze the reversible dehydrogenation of primary and secondary alcohols to aldehydes and ketones, respectively. This chemistry was chosen because of a unique chemoselective reactivity of the product to hydrazine derivatives, yielding the hydrazone bond. When the primary alcohol, for instance, is covalently attached to an RNA pool, rare redox-active species capable of oxidizing the alcohol to aldehyde can be selectively labeled with biotin-hydrazide, hence allowing us to separate them from inactive RNA molecules on streptavidin-agarose. To incorporate the primary alcohol to the RNA pool, a pool of random RNA sequences was synthesized by priming with guanosine 5′-monophosphorothioate (GMPS), and then the 5′-phosphorothioate group was selectively reacted with a benzylalcohol derivative **1**. Each step of the selection processes, including selective incorporation of **1** into the RNA pool as well as iso-

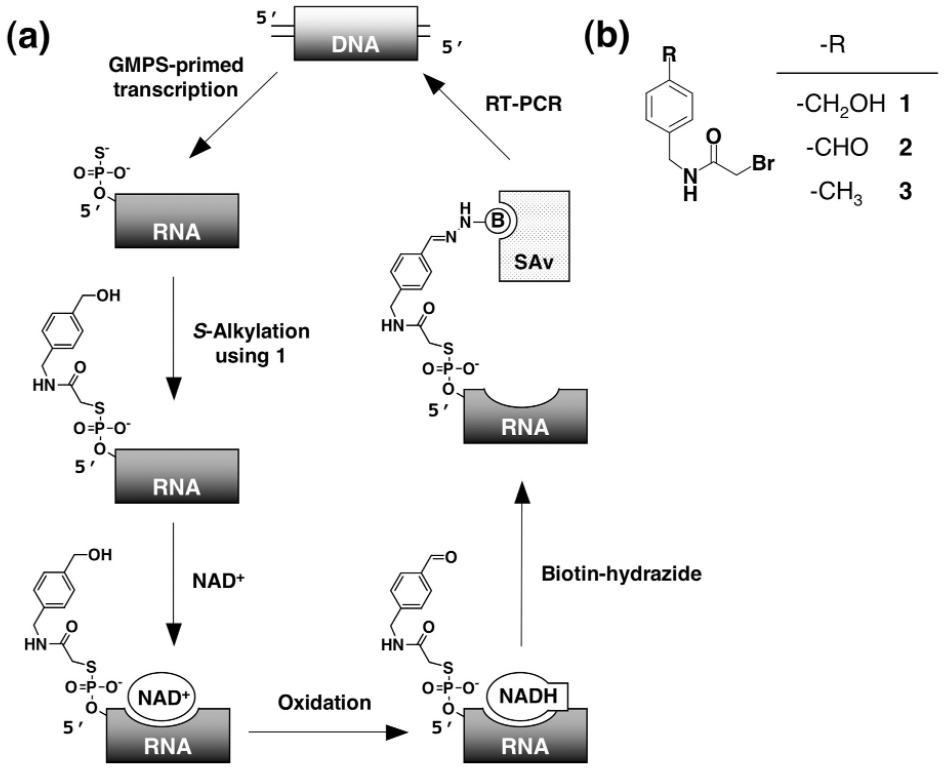

Fig. 7 In vitro selection of an alcohol dehydrogenase ribozyme: (a) selection strategy; (b) chemical structures of substrate derivatives [31]. Abbreviations: GMPS, guanosine 5′-monophosphorothioate; SAv, strepatavidin-agarose; B, biotin.

lation of the control sample of RNA bearing the benzaldehyde **2** instead of **1**, were confirmed prior to the selection. Thus, the selection strategy was set to go.

In retrospect, the conditions for the selection were very critical. We used two different conditions, where one included 100 mM Mg^{2+} as a divalent metal ion while the other involved 100 mM Mg^{2+} and 0.5 mM Zn^{2+}. The former condition is a standard selection protocol used in our laboratory for ribozyme selections, whereas the latter condition was chosen because some ADH protein enzymes actually use Zn^{2+} as an essential metal cofactor for the redox chemistry [32]. In fact, although we performed two selections independently under these conditions, only the latter selection with Mg^{2+} and Zn^{2+} successfully yielded the desired ribozyme.

The RNA pool conjugated with **1** was incubated in the presence of NAD^+, and active RNA sequences were selected according to the selection strategy. A total of 15 rounds of selection and evolution were carried out, and 10 clones were arbitrarily chosen for sequencing. Sequence alignment analysis of these clones revealed two classes of similar sequences and four independent sequences. We focused on a representative clone 02 (hereafter, referred to as ribox02, for a *ribo*zyme that catalyzes a red*ox* reaction) found in the major class I for further studies.

One of the most striking features of ribox02 is that the redox activity of ribox02 is strictly dependent upon the presence of Zn^{2+} and NAD^+. Removing these two cofactors completely wiped out the redox activity of ribox02. In contrast, removing Mg^{2+} resulted in only a partial reduction of the activity. Presumably Mg^{2+} plays an important role in stabilizing the tertiary structure of ribozyme but not in function. Another remarkable feature of the ribox02 system is the rate enhancement. Kinetic analysis of **1**-ribox02 converting to **2**-ribox02 with various concentrations of NAD^+ (0.1–10 mM) revealed the

single-turnover kinetic parameters of $k_{cat} = 0.71 \pm 0.02$ min^{-1} and $K_M = 5.33 \pm 0.31$ mM. Spontaneous oxidation of alcohols by NAD^+ is an energetically unfavorable process under the conditions used in the selection. Indeed, no detectable level of activity was observed using the initial random **1**-RNA pool after 24 h incubation with 0.1 M NAD^+. Given our minimum detection limit (0.01 %), we estimate the uncatalyzed reaction rate to be slower than 7×10^{-8} min^{-1}. This implies that the ribozyme achieves a rate enhancement of *at least* seven orders of magnitude.

To confirm the selective oxidation of the benzylalcohol, we prepared ribox02 in which substrate **1** was replaced with an oxidation-inert toluene derivative **3**, and this control construct was tested for redox activity. As expected, this replacement resulted in complete loss of activity, which is indicative of selective oxidation of the alcohol moiety [31]. To obtain direct evidence for the formation of the benzaldehyde, we used two analytical methods. Two-dimensional thin-layer chromatography was used to analyze the ^{32}P-labeled product, **2**spG*p (sp, thiophosphate; G, guanosine; *p, ^{32}P-labeled phosphate), generated by the RNase T2 digestion of the NAD^+-treated **1**-ribox01. Comparison of the above reaction product with authentic samples of ^{32}P-labeled **1**spG*p and **2**spG*p conclusively proved the formation of the benzaldehyde. The formation of benzaldehyde was also confirmed by a spectroscopic method. When 1,2-diamino-4,5-dimethoxybenzene (DDB) forms a conjugate with benzaldehyde, the resulting heterocyclic molecule enhances its characteristic fluorescent emission at near 400 nm [33]. In fact, the DDB treatment of the NAD^+-reacted **1**-ribox02 shows increase in the fluorescent intensity at 400 nm, and the intensity increase is consistent with the expected yield of the benzaldehyde formation determined on the streptavidin-dependent PAGE analysis.

The secondary structure of ribox02 was determined by a series of biochemical experiments with the aid of m-fold predictions. The data from nuclease, chemical structural mapping, and a series of compensatory mutations on stems P1 and P3 revealed a pseudo-knotted structure for ribox02 (Fig. 8a). This wild-type ribozyme was further truncated by deletion of the entire P5 domain, replacement of L2 loop with a stable UUCG tetra-loop, and shortening of P2b stem. The above truncation along with the shortening of the P4 stem yielded a compact ribox02 with a length of just 76-nucleotides (Fig. 8b).

Modern protein-based biology involves sophisticated metabolic and energy-production pathways that use multiple redox processes with various cofactors and/or protein-cofactor coupled systems [13]. The isolation of ribox02 prompted us to construct a multicomponent redox system that regenerates NAD^+ from NADH. To this end, we attempted to couple the NAD^+-dependent ribox02 redox system to a spontaneous electron-transfer process between NADH and FAD (Fig. 9). To assay the multistep redox system, we ran the reaction with **1**-ribox02 in the presence of NADH and an excess of FAD. After incubation for 2 and 6 h, **1**-ribox02 transformed to **2**-ribox02 in 4 and 12 % yields, respectively. No product was formed in the absence of NADH or FAD, clearly showing the necessity of the NADH–FAD electron-transfer process. Although the rate of conversion observed in this relay system is slower than that observed with NAD^+ alone, we suspect that the presence of NADH in the beginning of the redox relay results in reduction of **2**-ribox02 to **1**-ribox02. This is supported by our recent finding that **2**-ribox02 could be effectively reduced to **1**-ribox02 in the presence of NADH [34]. The reaction in the redox relay system is thus slower due to the competition between forward and reverse reactions catalyzed by ribox02. Nonetheless, this experiment clearly demonstrates that the ribozyme-dependent redox system can be coupled to a redox relay between two naturally abundant nucleotide cofactors.

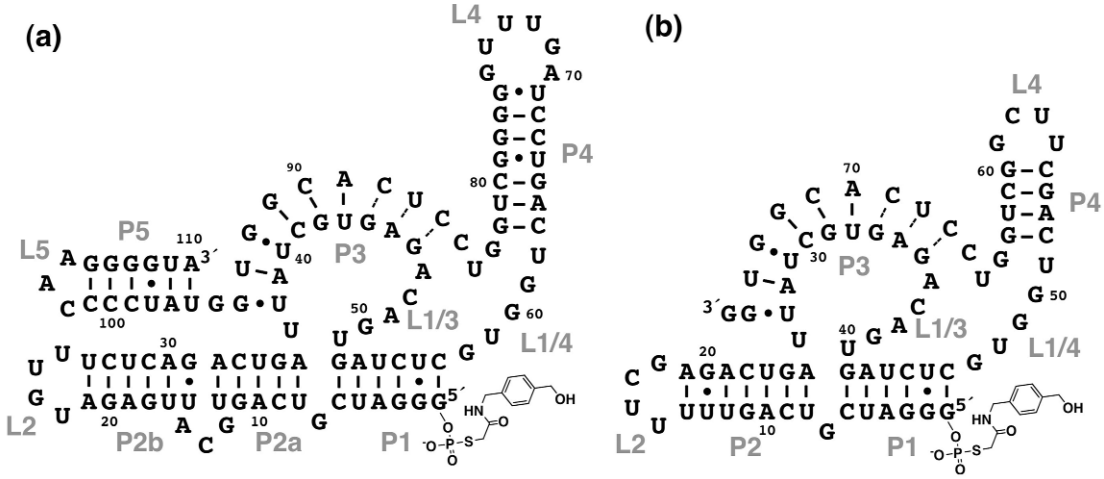

Fig. 8 Proposed secondary structure of (a) wild-type ribox02 and (b) a truncated 76 nt ADH ribozyme [31].

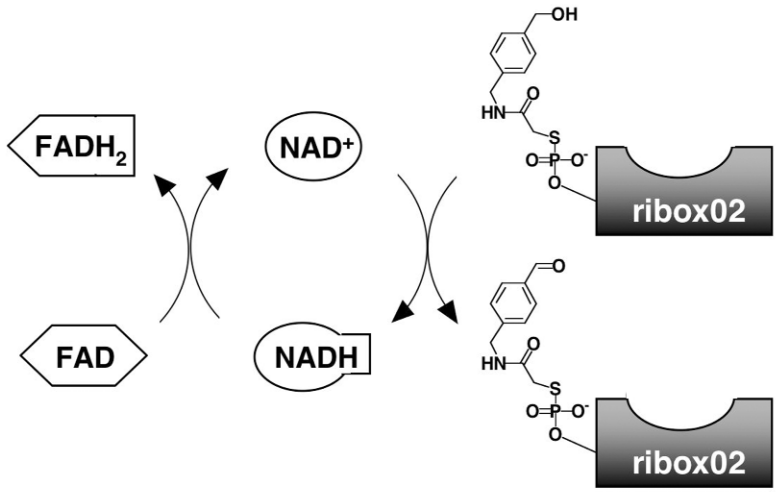

Fig. 9 A multicomponent redox system involving **1**-ribox02, NAD^+/NADH, and FAD/$FADH_2$ [31].

CONCLUSION AND PERSPECTIVES

The ADH ribozyme, ribox02 (Fig. 8a), is able to oxidize the benzylalcohol attached to the 5′-end (**1**-ribox02) in the presence of two cofactors, NAD^+ and Zn^{2+}. The rate acceleration is remarkably high (>10^7) even though the spontaneous alcohol oxidation by NAD^+ is an energetically unfavorable process. The truncated 76-nucleotide ribox02 folds into a compact pseudoknotted structure consisting of four stems and two single-stranded junctions (Fig. 8b). Biochemical studies suggest that both NMN^+ and AMP portions in NAD^+ cooperatively contribute to the binding to ribox02. Hence, the above structural elements somehow organize in the tertiary space to constitute the binding site for NAD^+. Presumably, like protein ADH enzymes, both cofactors, NAD^+ and Zn^{2+}, are precisely placed at the reaction center (the benzylic hydrogen) and their orchestration with catalytic residues in ribox02 facilitates the hydride (or electron) transfer. Although the cofactor-binding site is yet to be determined, we suspect that the GU-rich J1/4 is a potential site because it is physically located in close proximity to the 5′-benzylalcohol, upon the formation of P1 stem. In the future, it would be interesting to elucidate how

the pseudo-knotted structure of ribox02 governs the overall tertiary structure and constitutes the active site for the redox chemistry. These studies are particularly intriguing when the data are compared with those from other known natural and artificial "pseudo-knot" ribozymes.

The multicomponent redox relay between NAD^+/NADH and FAD/$FADH_2$ (Fig. 9) demonstrates the possibility of sophisticated RNA-based metabolic cycles. For instance, $FADH_2$ can be oxidized in the presence of oxygen or an inorganic oxidant to regenerate FAD for the catalytic cycle. Alternatively, ribozymes that use FAD/$FADH_2$ as a redox cofactor can be generated and coupled with the NAD^+/NADH ribox system.

It is now very clear that RNA molecules are not only capable of binding or reacting to redox cofactors but also using one of the redox cofactors for catalysis. We predict that appropriately designed selection strategies should enable us to generate ribozymes that use other redox cofactors such as FAD/$FADH_2$, cyanocobalamin or even transition-metal ions. Because redox reactions are involved in biosynthesis of a wide range of organic molecules, including sugars, amino acids, fatty acids, etc., we believe that the present study provides an entry to the discovery of large families of redox ribozymes. Therefore, it simply gave us an opportunity to open a new avenue of investigation toward RNA-originated biosynthetic pathways of such molecules. More will come in the future.

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