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Engineering of the Redox Ribozyme for the Determination of Its Architecture

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Here we report engineering efforts on an artificially evolved redox ribozyme, known as ribox02, and its more compact pseudoknot construct generated, combox02. The rigorous mutational works in the various regions suggested that the catalytically essential domain composed of three joining regions flanked by three stems. The architecture of combox02 can be an excellent framework for constructing libraries by randomizing sequences in this region for in vitro selection of novel ribozymes capable of catalyzing various chemical conversions of 5′-substrates.

An in vitro evolved ribozyme, referred to as ribox02,[1] catalyzes redox reaction of a benzyl alcohol (aldehyde) substrate reversibly in the presence of external cofactors NAD^+ (NADH) and Zn^{2+} (Figure 1). It achieves greater than a seven orders of magnitude rate enhancement over the background reaction, representing one of the prominent artificial ribozymes. The parental ribozyme of ribox02 was selected from a RNA pool that has the alcohol substrate at its 5′-end, and the further engineering effort reduced the size from 110 nucleotides (nt) to 76 nt ribox02. Our follow-up studies revealed that ribox02 did not accept various other alcohol as a substrate for redox reaction, suggesting that it is highly specific to the benzyl alcohol (Jin, Pattnaik, Futai, and Suga, unpublished data). We had also recently performed similar selection experiments to isolate novel redox ribozymes capable of catalyzing the conversion of an alkyl secondary alcohol to the corresponding ketone from a random RNA pool; unfortunately, this has thus far no success. We therefore needed to step back to investigate the essential catalytic domain(s) of ribox02, leading to its minimal structural scaffold. We hoped to see such a study would show us the meaningful direction in which the subsequent ribozyme selection might well to follow.

We have previously proposed the secondary structure of ribox02 consisting of a pseudoknot architecture based on the

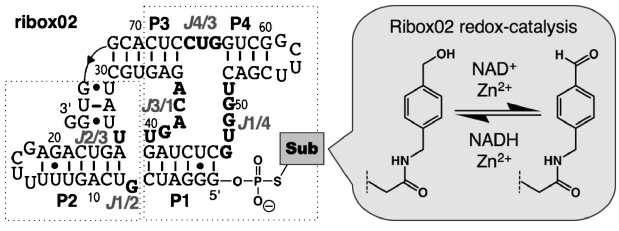

Figure 1. The secondary structure of ribox02. The dashed line boxes indicate two structural regions studied in this work. The bases involved in the joining regions are highlighted in bold. Ribox02 reversibly catalyzes redox reaction of the benzyl alcohol/aldehyde substrate attached to the 5′-end of ribox02.

results of compensatory mutations in the existing four stems, P1–P4 (Figure 1).[1] We here report intensive mutational analyses of the joining (*J*) regions between the stems, revealing that the catalytic core consists of three *J* regions, *J*1/4, *J*3/1, and *J*4/3. Based on this knowledge, the P2 stem can be entirely deleted by the substitution of an extended P3 (exP3), and a compact ribox02, referred to as combox02, is generated. The results show that the above *J* regions compose the essential catalytic core of the ribozyme embedded in a compact pseudoknot 3-dimensional architecture.

Ribox02 binds the benzyl alcohol substrate covalently ligated to its 5′-end via phosphothioate and simultaneously interact with the two cofactors. This termolecular interaction in the ribox02 active site is likely critical to achieve the efficient hydride transfer from the benzylic position of the substrate to the C4 carbon atom on the nicotiamide ring of NAD^+. Our previous attempt in employing chemical modifications of ribox02 in the absence and presence of substrate/cofactors has failed to identify potential bases that are responsible for the interaction with the substrate and cofactors (Tsukiji and Suga, unpublished results). Therefore, we decided to perform intensive mutational analyses in order to determine critical bases in ribox02 and locate the active site in the structure.

Our previous work suspected that the active site of ribox02 is presumably embedded in the GU-rich *J*1/4 and *J*3/1 regions because of their physically close proximity to the 5′-end to which the substrate is covalently attached.[1] Since G1 pairs with C46 by the formation of P1 (Figure 1), the downstream of C46 seems the most logical region for such an assumption. Careful sequence analysis in *J*1/4 suggested that G49–U51 in *J*1/4 could form base pairs with G64–C66 in *J*4/3 (Supporting Figure 1A).[21] We thus introduced mutations into G64–C66 that would maintain the pairing interaction. If these base pairings are required for the activity, mutants prepared should retain similar activities. To our disappointment, however, none of these mutants exhibited appreciable redox activity. An alternative hypothesis is the formation of the base pairs between A36–C37 in *J*3/1 and G64–U65 in *J*4/3 (Supporting Figure 1B).[21] Although the above interaction are limited to only two base pairs, the stacking of these two base pairs top on P3 with a C66 bulge may extend the P3 stem interaction. We therefore verified this pairing interaction by making mutants with compensatory base pairs that stabilize/retain the interaction. Again, none of these mutants showed appreciable activity, indicating that neither hypothesis was supported (Supporting Figure 1B).[21]

Since the rational approach seemed to us not working, we next performed arbitrary point mutations in each *J* region to identify the essential residues of ribox02 (Supporting Figure 2).[21] Although almost all the mutants showed a loss of activity, G7A mutation[1] as well as single/double adenosine

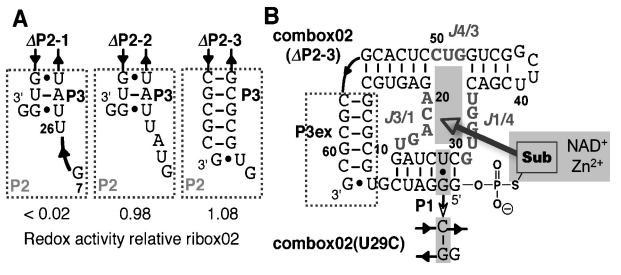

Figure 2. Structures of new ribox constructs. (A) Relative activities of ribox constructs involving the truncation of P2 along with the engineering of P3. (B) Secondary structures of combox02 (ΔP2-3) and combox02(G29C).

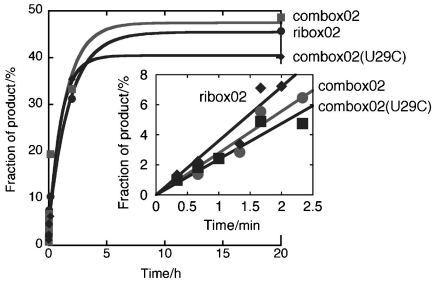

Figure 3. Initial rate and end-point of the conversion of 5′-benzyl alcohol to aldehyde under saturated NAD^+ concentration (15 mM).

insertion into *J*1/2 turned out to be as active as ribox02 (Supporting Figure 3).[21] Similarly, both U26C and U26G mutants in *J*2/3 exhibited near wild-type activities. Taken together, bases in the *J*1/2 and *J*2/3 regions are tolerated for mutations and insertions, giving us a new opportunity to engineer these regions.

By the encouragement of the above finding, we further engineered P2 and its *J* regions. To simplify the structure, we first prepared a construct, ΔP2-1, where P2 was deleted via a direct connection of G7 to U26 (Figure 2A, ΔP2-1); but this construct showed no activity. We then prepared another deletion construct containing a UA-insertion between G7 and U26 (Figure 2A, ΔP2-2). This minor change turned the parental inactive construct ΔP2-1 into an active one with a comparable activity to ribox02. Aiming at further stabilizing the P3 stem formation, ΔP2-3 containing an extended GC-rich P3 stem was designed, showing a minor improvement in the end-point activity, i.e., yield, from the parental ribox02 (Figure 2A, ΔP2-3). We referred to this construct as combox02 (**com**pact-ri**box**02, Figure 2B).

G2 near the 5′-end forms a G:U wobble pair with U45 in P1 (Figure 1). Since there are some known ribozyme examples that such a G:U wobble pair at or near the reaction site plays a critical role in ribozyme catalysis,[2–16] we wondered if the G2:U45 pair is essential for redox activity. A combox02 mutant containing U29C, referred to as combox02(U29C), was prepared for testing its activity (Figure 2B). To this end, we performed kinetic experiments to evaluate the redox rate of three constructs, ribox02, combox02, and combox02 (U29C) under a saturated NAD^+ concentration at 15 mM (it should be noted that K_M for ribox02 had been determined to be 5 mM[17]). The observed initial rates were all similar, and so were the end-point conversions (Figure 3). Thus we conclude that these constructs possess virtually the same catalytic properties, suggesting that the G2:U45 wobble pair is not essential for the redox activity but yet contributes to the optimal redox activity of ribozyme.

In conclusion, we have endeavored to elucidate the catalytic domain of ribox02 that has redox activity for the benzyl alcohol substrate in the presence of NAD^+ and Zn^{2+} cofactors and have shown here a new construct of ribozyme, combox02. The catalytic domain consists of three joining regions flanked by three stems, in which the essential bases compose the substrate/cofactors binding site via noncanonical interactions. Removing the catalytically redundant P2 stem region has simplified the pseudoknotted structure of ribox02, facilitating us to design a reliable library containing randomized regions embedded in the catalytic domain for new ribozyme selection. Moreover, the structure of combox02 would provide a suitable construct to implant an accessory domain into P4, e.g., U1A stem-loop,[18–20] that benefits to its crystallization as well as structural refinement.

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21 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

