Re-engineering an *in-vitro* evolved ribozyme.

Swetansu B Pattnaik\(^1\), Hiroaki Suga\(^1,2\)

\(^1\)Department of Chemistry, SUNY Buffalo, NY 14260, USA and \(^2\)Research Center for Advanced Science and Technology, The University of Tokyo, 113-8656 Tokyo, Japan.

ABSTRACT

Ribox2 is an *in vitro* evolved RNA molecule that exhibits the activity analogous to that of alcohol dehydrogenase, catalyzing redox reactions in a cofactor dependent manner. It forms a compact pseudoknotted structure and oxidizes an alcohol seven orders of magnitude faster than the estimated spontaneous rate. To gain insights into the structure-function relationship of Ribox2, we firstly performed mutational studies to identify the critical nucleotide bases for catalysis and then engineered the ribozyme structure to improve its overall fold. The re-engineered Ribox2, named Robust, was able to catalyze the reaction approximately 25% more efficiently, leading us to generate a trans-acting system.

INTRODUCTION

Redox ribozymes could have been an integral component of the RNA-based life forms given the central role played by redox reactions in the metabolism of various biological molecules in living organisms. Ribox2 is the first *in vitro* evolved ribozyme utilizing external cofactors (NAD*⁺*/NADH, Zn*⁺*) for catalysis (1, 2). The efficiency of the oxidation reaction is limited to 43% presumably due to the inherent alternative folding property of RNA (3, 4). This makes it very challenging to develop an efficient trans-acting ribozyme especially when the cis-precursor is only partly efficient.

![Diagram of Ribox2 and Robust structures](https://academic.oup.com/nass/article-abstract/51/1/381/1023995)

Our attempt to improve the efficiency of ribox2 by adjusting the metal ions and cofactor concentration was met with limited success. Computer aided secondary structure prediction algorithms (5, 6) suggested the presence of several alternative folds which may be interfering with the assembly of the prerequisite pseudoknot (Fig.1A). Based on these considerations, mutational studies were carried out to improve the stability of the overall fold. The re-engineered clone, Robust, showed a boost in efficiency of the reaction by at least 25%.

Also, single base mutational studies were carried out on the J1/3, J1/4 and J1/5 region formed by the pseudoknot structure. These mutations revealed a stringent conservation of the nucleotide bases pointing towards the significance of their disposition in the formation of the catalytic core structure.

RESULTS AND DISCUSSION

P1 mutant that had U45C mutation showed a marginal, but distinct increase in the yield. This mutation, by tethering the 5'-end of the ribozyme through a more stable Watson-Crick base pair, might have helped the more complicated pseudoknot structure to populate. Similarly, in P3 mutant, a substantial increase in yield was observed, when the 3'-end was secured more tightly by G75U74G73 \(\rightarrow\)C75G74C73 and U27A28U29 \(\rightarrow\)G27C28G29 substitutions. The P4 mutant also showed a distinct increase in yield when the stem was extended by addition of extra GC pairs. Computer algorithms suggested the extension would stabilize the P4 stem to prevent the imminent strand slippage leading to the formation of alternative folds.

These mutations were incorporated into a single clone, Robust, which was capable of catalyzing the redox reaction approximately 25% more efficiently than its precursor (Fig.1B).

![Diagram of putative tripartite active site of ribox2](https://academic.oup.com/nass/article-abstract/51/1/381/1023995)

Fig. 2. The putative tripartite active site of ribox2 comprised of the single stranded J1/3, J1/4 and J1/5 regions.

Preliminary kinetic studies suggest that the engineered
Robust folds at a much faster rate than ribox02 (data not shown). The accurate kinetic parameters of Robust will be determined by future studies.

Mutational analysis was carried out to understand the nature of interactions in the single stranded junction region comprised of the J1/3, J1/4 and J1/5 (Fig. 2). The yields of the different mutants relative to ribox02 are plotted in Fig. 3.

![Figure 3](image-url)  The yields of the mutants plotted relative to ribox02. The mutations at the J1/3, J1/4 and J1/5 regions are intolerable.

Since all the mutations in the J1/3, J1/4 and J1/5 region severely compromised the yields, the conserved nature of these residues is essential for catalysing the reaction. These observations highlight the existence of a very specific order accompanying the complex redox reaction.

![Figure 4](image-url)  Strategy for designing a trans-acting model. (A) The tolerance of insertion mutations at the G-bulge in ribox and robust. (B) Proposed trans-acting model with engineered Robust.

Lastly, we investigated the importance of single G-bulge in the ribox02. The tolerance of insertion mutations at the G-bulge region of ribox02 was surprising. The clones M1 and M2, wherein the G-bulge is extended by one and two adenine bases showed wild-type activity. Interestingly, we were able to extend the G-bulge further to a 5 base-paired stem loop in the clone M3, although incurring a slight loss of efficiency. This model is likely to retain the native fold better in a trans-acting set up as compared to the earlier construct. The tolerance of insertions at the G-bulge was also observed in the clone M4 designed from the engineered Robust clone. Fig. 4A summarises the tolerance of insertion mutation in ribox02 and Robust based clones. Based on the above considerations, the in G-bulge region of Robust will be engineered and tested in a trans-acting model as shown in Fig. 4B.

**CONCLUSION**

In conclusion, it is observed that the stability of the pseudoknot and the integrity of the active site residues are among the significant factors governing the efficiency of ribozyme mediated catalysis. Most importantly, the results so far have demonstrated that our attempt to engineer a more stable and efficient version of an in-vitro evolved ribozyme has succeeded. These results would allow us to develop an efficient trans-acting system.

**REFERENCES**


*Corresponding Author. E-mail: hsuga@rcast.u-tokyo.ac.jp*