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Rational Design of a New IMP Aptamer Based on a TPP Riboswitch and a Hypoxanthine Aptamer

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We rationally designed a new inosine monophosphate (IMP) aptamer based on structural information from the natural thiamine pyrophosphate (TPP) riboswitch and an in vitro-selected hypoxanthine aptamer. The value of the binding affinity (K_a) for the newly designed IMP aptamer for IMP was $(2.6 \pm 3.2) \times 10^7\,M^{-1}$, which was 10-times larger than that for TPP. These results demonstrated that structure-based design of aptamers can be a useful scheme to develop artificial aptamers.

Aptamers are a class of functional RNAs that specifically interact with target molecules.[1] Although aptamers have been developed by random chemistry such as in vitro selection[1] (SELEX),[2] higher affinity, and specificity for a target molecule are still required for practical applications. An alternative strategy for development of aptamers, which can be rational design, is of interest for many research fields. However, de novo design of an aptamer is a very challenging task. Therefore, we here propose a combination of sequence information of the randomly-selected aptamer and naturally-observed riboswitch as a structural scaffold toward rational design of a new aptamer.

A riboswitch is found in an mRNA that can directly bind a target metabolite, resulting in its alteration of gene expression.[3] Previously, we discovered a TPP (Figure 1a) dependent riboswitch (Figure 1b) in the 5′-untranslated region (UTR) of *thiA* in *Aspergillus oryzae* and studied its function.[4–6] The 173-mer TPP riboswitch is composed of two stems (P2–P3 and P4–P5) connected by a three way junction (L1).[5] Notably, the P2–P3 and P4–P5 stems bind the pyrimidine group and phosphate group, respectively, of TPP in an independent manner.[7,8] That the pyrimidine-binding region in the P2–P3 stem binds to TPP via stacking interactions with the pyrimidine, whereas the P4–P5 stem binds the pyrophosphate group in TPP via Mg^{2+}, which reduces electrostatic repulsion between the pyrophosphate groups of TPP and the riboswitch.[7,8] Since the TPP riboswitch has an independent binding mechanism to recognize TPP, we expected that we could rationally design a new aptamer by introducing an in vitro selected aptamer having a stem-loop structure as a binding site instead of the original stem.

In order to prove this concept, we chose 5′-inosine monophosphate (IMP) as the target metabolite. IMP is known as the umami taste, and includes a hypoxanthine and phosphate groups (Figure 1a). It is possible that the phosphate group of IMP can be recognized by the P4–P5 stem because the stem binds the pyrophosphate group via Mg^{2+} coordination. Previously, Kiga et al. developed a hypoxanthine aptamer by in vitro selection.[9] The hypoxanthine aptamer folded into a stem-loop structure with a bulge that is similar to the secondary structure of the P2–P3 stem of the TPP riboswitch (Figure 2). Based on this structural information, we rationally designed a new aptamer by combin-

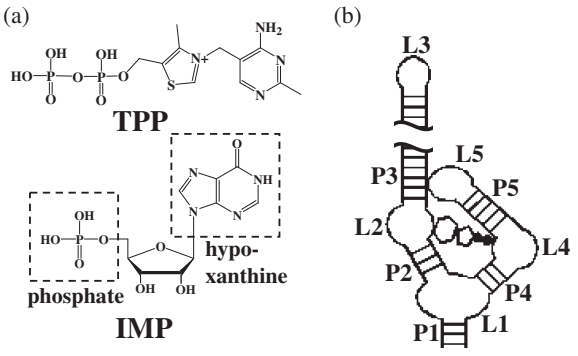

Figure 1. (a) Structure of TPP and IMP. (b) The model of the TPP riboswitch wrapping TPP. TPP is in the central position of the riboswitch. Hexagon: pyrimidine; pentagon: thiazole; closed circles: phosphates. The L2 loop binds to the pyrimidine group of TPP. The L4 loop binds to the phosphate group of TPP through an Mg^{2+} ion. The L5 loop binds to the P3 region and, as a result, TPP is wrapped in the riboswitch.

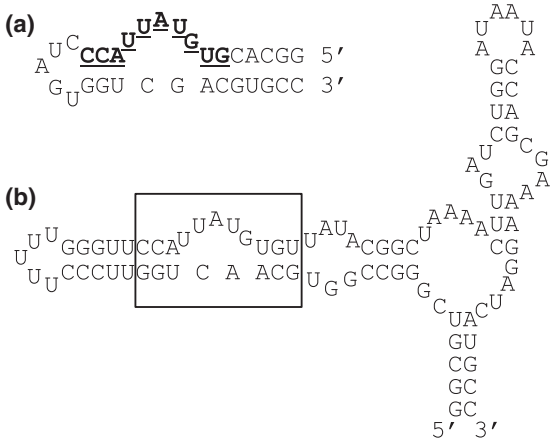

Figure 2. (a) The structure of the hypoxanthine aptamer. The consensus sequence of the aptamer is underlined. (b) The structure of the designed aptamer. The mutated region is enclosed within the square.

ing the TPP riboswitch and the hypoxanthine aptamer. We introduced the hypoxanthine aptamer, which contains critical bases for the hypoxanthine binding, in place of the pyrimidine-binding region of the TPP riboswitch P2–P3 stem (Figure 2b). By use of mfold,[10] we confirmed that the whole secondary structure of the designed aptamer was similar to that of the TPP riboswitch. Previously, we found that a truncated TPP riboswitch with a shorter P3 stem (mP3TPP riboswitch) was able to maintain its function (data not shown). This allowed us to make the P3 region shorter in the designed aptamer from 108 to 24 bases (Figure 2b).

In order to confirm the binding of the designed aptamer for IMP, we evaluated its binding affinity (K_a) for IMP. Samples of RNA were prepared by in vitro transcription and purified as described previously.[5] RNA samples were incubated at 70 °C for 3 min, cooled, and incubated at 20 °C for 30 min. Titration experiments using circular dichroism (CD) spectrometry (Jasco J-820 Hachioji) were carried out at 20 °C with a 1 cm path length at 265 nm. IMP was titrated against 0.2 μM RNA in a buffer containing 10 mM NaCl, 1 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.0). CD intensities decreased with an increase in IMP concentration. It was found that the CD intensity changes were relatively small. This is partly because the TPP riboswitch even with or without the target molecule forms A-form duplex and maintains its global structure during the titration experiments. In addition, the degrees of the CD intensity changes of the TPP riboswitch upon binding with TPP and Mg^{2+}, confirmed by X-ray crystallography,[8] were comparable with that observed here for the designed aptamer upon the binding with IMP.[5,6] Thus, it is possible to conclude that the small change in CD intensity observed here is reasonable.

The data were fitted to Eq. (1) based on a model that postulates one binding site to estimate the equilibrium parameters:[11]

$$\theta = \Delta\theta \cdot K_a[\text{metabolite}]/(K_a[\text{metabolite}] + 1) + \theta_0 \quad (1)$$

where $\Delta\theta$ is the change in the CD intensity, θ_0 is the initial CD value, and K_a is the apparent binding constant. The value of K_a for the designed aptamer for IMP was evaluated to be $(2.6 \pm 3.2) \times 10^7\,M^{-1}$ (Figure 3a). Of note, the K_a for the oridinal hypoxanthine aptamer for hypoxanthine was $4.8 \times 10^5\,M^{-1}$ at 25 °C.[9] This value is two-orders smaller than that of the designed aptamer for IMP, indicating that the P4–P5 stem of the aptamer participates in the binding with IMP as we expected. In the same way, the K_a of the designed aptamer for TPP was evaluated to be $(3.5 \pm 4.4) \times 10^6\,M^{-1}$ (Figure 3b), which is one-order smaller than that of the designed aptamer for IMP. Furthermore, the K_a of the mP3TPP riboswitch to TPP was $(1.3 \pm 0.2) \times 10^7\,M^{-1}$ (Figure 3c) under the same condition. On the other hand, a significant difference in the CD intensity for the mP3TPP riboswitch upon the IMP titration was not observed (Figure 3d). Therefore, binding affinity and specificity of the mP3TPP riboswitch were almost maintained in the designed aptamer with the different target molecule. In addition, we quantified the designed aptamer binding to adenosine diphosphate (ADP); however, no CD intensity change was observed (data not shown), indicating no binding with ADP. These results further support the belief that the designed aptamer can act as the IMP aptamer with a high specificity.

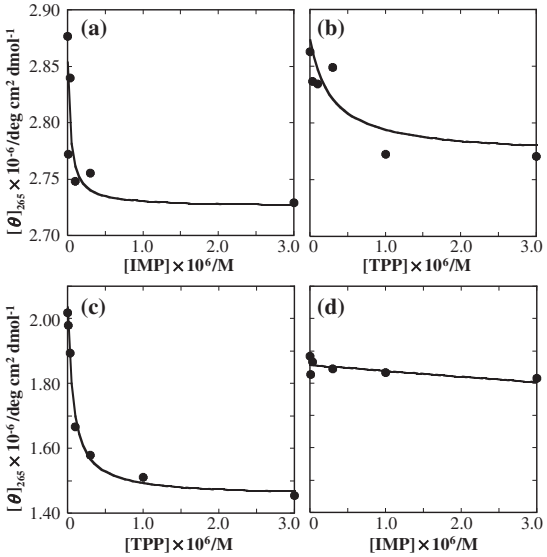

Figure 3. CD intensity of 0.2 μM of the designed aptamer (a and b) and the P3 truncated *A. oryzae* TPP riboswitch (c and d) at 265 nm in 50 mM Tris-HCl (pH 7.0) containing 10 mM NaCl, 1 mM $MgCl_2$, and various concentrations of IMP (a and d) or TPP (b and c) at 20 °C.

In conclusion, we rationally designed an IMP aptamer based on structural information from the TPP riboswitch and the hypoxanthine aptamer. The designed IMP aptamer has high affinity toward its target metabolite but not toward off-target metabolites, indicating specificity. Importantly, this aptamer is based on a riboswitch so it may function as the IMP riboswitch. Thus, this method developed could be useful for design of various riboswitches, although further studies are required to confirm a gene regulational function. Natural riboswitches are generally regulated by natural metabolites that are critical to cell growth. We hope that this method will allow us to utilize any ligands that are useful as an external stimulus.

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