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In vitro selection of hemin-binding catalytic RNA

Mingzhe Liu, Takuma Kagahara, Hiroshi Abe, Yoshihiro Ito *

Nano Medical Engineering Laboratory, RIKEN Advanced Science Institute, 2-1 Hirosawa, Wako-Shi, Saitama 351-0198, Japan

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ABSTRACT

Catalytic RNAs with peroxidase activity were obtained by the in vitro selection of RNA aptamer-binding hemin. One of the RNA aptamers selected showed binding affinity to hemin with a dissociation constant of 0.8 μM and exhibited high peroxidase activity by forming a complex with hemin. The catalytic efficiency of the RNA–hemin complex was 10-fold higher than that of hemin alone.

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Peroxidases are ubiquitous oxireductases. The horseradish peroxidase (HRP) has been used widely as a biocatalyst in organic synthesis and biosensing.^{1–4} All HRP isoenzymes contain iron (III) protoporphyrin IX, called simply a 'heme group'. Many groups have tried to synthesize mimics of this peroxidase using a rational design method.^{5–10} For example, Takahashi et al., prepared a polyethylene glycol-modified hemin that exhibits peroxidase activity in several organic solvents.⁵ Hayashi et al. reported that horse heart myoglobin (Mb) reconstituted with a modified heme enhances the peroxidase activity of the Mb.⁹ Several groups have reported catalytic antibodies with peroxidase activity.^{11–14} For example, Cochran et al., reported a catalytic antibody that forms a complex with hemin and exhibits peroxidase activity.¹¹

Nucleic acid catalysts with peroxidase activity have also been developed on the basis of the in vitro selection (SELEX) method.^{15–17} This method was developed in 1990 and is used widely to develop new catalysts and aptamers that bind the desired molecules.^{18–24} Travascio et al., reported on a DNA aptamer–hemin complex that exhibits peroxidase activity,¹⁶ which was the first example of a nucleic acid catalyst with peroxidase activity. Our earlier study reported for the first time the development of nonnatural ribozyme-carrying 2'-amino groups that exhibit peroxidase activity in the presence of hemin.¹⁷ However, each of the nucleic acid catalysts were selected using an indirect method in which selection proceeded on a column immobilized with *N*-methyl mesoporphyrin IX (NMM). It is unknown whether natural ribozyme,

whose sequence can be selected by the SELEX method, exhibits peroxidase activity.

Recently, direct in vitro selection of RNA–aptamer binding to hemin has been attempted using a hemin-immobilized agarose column.²⁵ The purpose of our present study was to use the SELEX method to find natural ribozymes that bind hemin and exhibit peroxidase activity. We investigated in detail the binding affinity and peroxidase activity of the RNAs selected using this method.

The hemin–agarose columns were washed²⁶ and preequilibrated in binding buffer. In vitro selection was performed as described previously.²⁷ The library of single-strand (ss) DNA (103 nt) containing a random region of 59 bases (5'-TAG-GGA-ATT-CGT-CGA-CGG-ATC-C-N59-CTG-CAG-GTC-GAC-GCA-TGC-GCC-G-3') was synthesized and amplified through PCR using the 5'-primer containing the T7 promoter sequence (5'-A-TAA-TAC-GAC-TCA-CTA-TAG-GGA-ATT-CGT-CGA-CGG-AT-3') and 3'-primer (5'-CGG-CGC-ATG-CGT-CGA-CCT-G-3'). The amplified DNA was transcribed with T7 RNA polymerase. The product of the transcription reaction was purified by denaturing polyacrylamide gel electrophoresis (PAGE). For the first round of selection, a random-sequence RNA pool ($\sim 10^{11}$ molecules) was annealed and then loaded on the hemin-immobilized agarose column. Unbound RNAs were washed away. The bound RNAs were eluted with the binding buffer containing hemin. The collected RNAs were quantified by UV absorption at 260 nm and then amplified by RT-PCR. The amplified DNA was transcribed and applied to the hemin-immobilized column again for the next round selection. After the fourth round of selection, the ability of the RNAs to bind to immobilized hemin increased significantly, and we cloned the fourth and sixth rounds of the DNA

* Corresponding author.

E-mail address: y-ito@riken.jp (Y. Ito).

pool and sequenced them. The random regions of 21 isolated RNAs are shown in Table 1. The same sequences were observed in the 4c20/4c26 pair, 4c23/4c25 pair, and 6c4/6c5 pair.

We chose three RNAs (4c23, 4c26, and 6c5) from the clones having the same sequences and two RNAs (4c1 and 6c20) from the others, and their binding affinities to hemin were investigated as follows. These RNAs were incubated with hemin in 40KT buffer,¹⁶ and the absorption at the Soret band of hemin was measured by UV–vis spectrometry. As shown in Figure 1, a significant increase in the Soret absorption was observed in the presence of 4c26 or 6c5 RNA, whereas almost no change in Soret absorption was observed in the presence of 4c1, 4c23, or 6c20. Travascio et al., reported that a hyperchromic effect is induced at the Soret band of hemin when a DNA aptamer binds to hemin.¹⁶ We concluded that the former RNAs exhibit binding affinity to hemin, but the latter do not. Generally, the increase in selection rounds leads to increases in the population of the same sequences, which causes convergence of sequence for high binding activity. We considered that either 4c1 or 6c20 was not the convergent product for the high binding activity, because neither had the same sequences as the clones. On the other hand, we considered the convergent sequences were more likely to have high binding activity (i.e., 4c26 and 6c5 over 4c23, 4c26, and 6c5).

The peroxidase activity was also investigated for the five kinds of RNAs mentioned above by adding 2,2'-azino-bis(3-ethylbenzo-

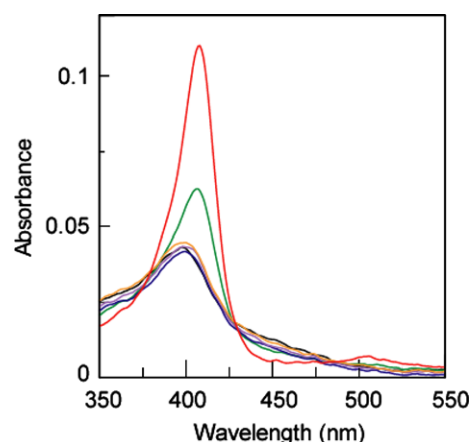


Figure 1. UV-visible spectra of hemin (0.5 μ M) alone and in the presence of selected RNA (4.0 μ M) in 40KT buffer (50 mM 2-morpholinoethanesulfonic acid hydrate (MES) pH 6.5; 100 mM Tris acetate; 40 mM potassium acetate; 1% DMSO; 0.05% Triton X-100). 6c5, red; 4c26, green; 6c20, orange; 4c23, purple; 4c1, blue; and uncomplexed monomeric hemin, black.

thiazoline-6-sulfonic acid) (ABTS) and H_2O_2 to the solution used in the binding assay. The oxidation rate of ABTS by hemin in the presence or absence of the RNA was measured by monitoring the increase in absorbance at 414 nm. The $\Delta\epsilon_{414}$ nm value, $36,000\text{ M}^{-1}\text{ cm}^{-1}$ reported previously,²⁸ was employed. A comparison of Figures 1 and 2 shows that the peroxidase activities correlated with the binding affinities of the RNAs: a significantly enhanced peroxidase activity was observed in the presence of hemin binding to 4c26 or 6c5 RNA. The observed oxidation rate was 25-fold faster in the presence of 6c5 RNA than with hemin alone. In the same reaction condition, 6c5 RNA caused a greater enhancement of oxidation rate than did the PS2.M-DNA aptamer¹⁶ ($V_{\text{obs}}(\text{PS2.M-hemin})/V_{\text{obs}}(\text{hemin}) = 20$). In contrast, almost no enhancement of peroxidase activity was observed in the presence of 4c1, 4c23, or 6c20, which did not bind to hemin. In a natural peroxidase, the heme group is bound in a hydrophobic pocket of peroxidase.²⁹ Several groups have reported that the increased hydrophobicity around hemin enhances the peroxidase activity.^{5,9} On the other hand, an increase in the absorption of the Soret band of hemin is thought to be caused by an increase in the hydrophobicity of the heme environment.³⁰ The relationship between the

Table 1
Sequences of the random region of selected RNAs

RNA clone	Sequence of the random region
4c1	AAGUACGCGGGUUGAUGGAGUCAUCGGCACUGGAGGAU UGUUCUGGAUGAGUGGACGA
4c4	GCACCCUACAUAUAGUCGGCCAGGUCUGCGUGUGUGA UGUUCUGGAGGAUUUGGGG
4c6	GGGAUACGCCAUACAUCAGAGGUGCUUUGAGCUGCAUUGU CAUAGUUGCCCAACUA
4c7	AGGGGCCUGUACACCAUAGCUGACGGGUUCCGAGAUGUGUAAU GGGAGAGGCGUCAUA
4c8	GCGAGUGCGGCGAUAAGCUGUUUGAGCAGGGAGGUUAGA GGGUACGGAUCAUGAGGCU
4c11	UCGGUCUUAACCGCUAGGGAUUGGCAGAAUACAUAACCGA CAGAUCCAAGGGUCCGACU
4c14	UUUAGCAGUUGAGCUGAUGAGGAUGCGCUGUGUCCAGG AAGGAUUUCGGGGCGACAG
4c15	GGGGCUGGAUCUCCAUUGGUUAGGAGACGGAGUGCCUA AUGGUUUGAAACUUAUGCG
4c19	AUUGCGUGUAGACCCAGGGUAUUCUACUGGUUACGCCAA GGCUAGGUGGGCUAGCACU
4c20	AUGGUAACAGGAAAGUCUGUUUGGGGUAUAAGGACAAC ACGCCGCGCCGAAGGUGA
4c23	UGUUGGCGUGGAUCCAUCGCGGGUUGAAGUUAUUGGUG GCUGCUAUACAUACAG
4c25	UGUUGGCGUGGAUCCAUCGCGGGUUGAAGUUAUUGGUG GGCUGCUAUACAUACAG
4c26	AUGGUAACAGGAAAGUCUGUUUGGGGUAUAAGGAC AACACGCCGCGCCGAAGGUGA
4c27	AGAACCAGUGACGAUACAUAACGAACCUUCUGAGAUC CGGUCAGGAGGUUUGAACA
4c28	AUGCGGUCAGCUGAUAUCUGCUACUGGCGUGAGCACACC GACUUAGGGAGGACGUA
4c35	ACGAGUGUGCGUGGCAUUAUCUGCUAUUACCUAUUGUG CGGCUAUCCAGUACUGGU
4c36	AGCAAAGAGCAGGUGCAUGGUUGGUGCUCCUGACACC AGUGAGAUUAUGUACUGCG
4c40	ACAGCGAGGCAACCAUUGAUCAUUGCGACACGUGGUGAC CGACGCAUAGACUAGGU
6c4	AGCUGAUGUGGACGUCAGUUUAAGGAAUGUGGAGGU UGGAAGUUAUGGCUUGCAUCAG
6c5	AGCUGAUGUGGACGUCAGUUUAAGGAAUGUGGAGGU GGAAGUUAUGGCUUGCAUCAG
6c20	AGUGCCAGGGGAGAUUAAGCCGAUUUGUGAUAGUGU CUCUUAACAGGCGUAUCAU

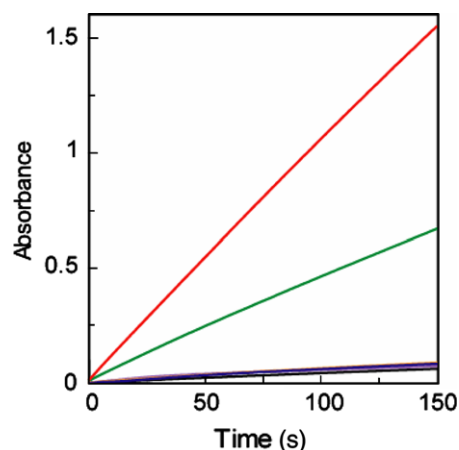


Figure 2. Time-dependent ABTS oxidation upon analysis of peroxidase activity at different catalyst compositions. 6c5 and hemin, red; 4c26 and hemin, green; 6c20 and hemin, orange; 4c23 and hemin, purple; 4c1 and hemin, blue; and hemin alone, black. The reaction was carried out in 40 KT buffer. RNA, 2.0 μ M; hemin, 0.25 μ M; ABTS, 2.5 mM; H_2O_2 , 0.75 mM.

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