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In vitro selection of hemin-binding catalytic RNA

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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 washed[26] 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

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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-ethylbenzothiazoline-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 $\Delta\varepsilon_{414\,nm}$ value, 36,000 M^{-1} 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 aptamer[16] (V_{obs}(PS2.M–hemin)/V_{obs}(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 hemin environment.[30] The relationship between the

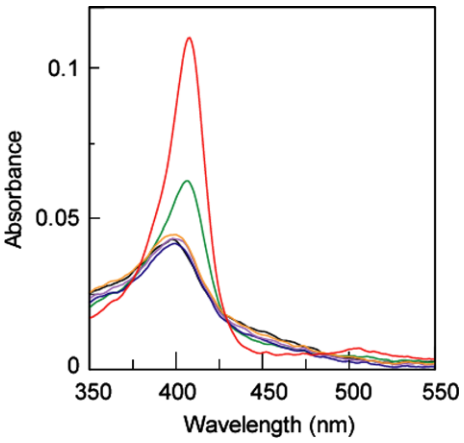

Figure 1. UV–visible spectra of hemin (0.5 μM) alone and in the presence of selected RNA (4.0 μM) in 40KT buffer (50 mM 2-morpholinoethanesulfonic acid hydrate (MES) pH 6.5; 100 mM Tris acetate; 40 mM potassium acetate; 1% DMSO; 0.05% Triton X-100). 6c5, red; 4c26, green; 6c20, orange; 4c23, purple;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

Table 1
Sequences of the random region of selected RNAs

RNA clone	Sequence of the random region
4c1	AAGUACGCGGGUUGAUGGGAGUCAUCGGCACUGGAGGAU UGUUCUGGAUGAGUGGACGA
4c4	GCACCCCUACAUAUAGUCGGCCAGGUCUGCGUGUGUGA UGUUCGUGGAGGAUUUGGGG
4c6	GGGAUACGCCAUACAUCCCAGAGGUCGUUUGAGCUGCAUUGU CAUAGUUGCCCAACUCA
4c7	AGGGGCCUGUACACCAUAGCUGACGGGUUCCGCAGAUGUGUAAU GGGAGAGGCGUCAUA
4c8	GCGAGUGCGGCGAUAAGCUGUUUGAGCAGGGAGGUUUAGA GGGUACGGAUCAUGAGGCU
4c11	UCGGUCUUACACGCUAGGGAUUGGCAGAAUACAUACCGA CAGAUCCAAAGGGUCCGACU
4c14	UUAUGCAGUUGUGAGCUAUGAGGAUGCGCUGUGUCCAGG AAGGAAUUCGGGGGCAGACG
4c15	GGGGCUGGAUCUCCAUUGGUUCAGGAGACGGAGUGCCUA AUGGUUUGAAACUCAAUGCG
4c19	AUUGGCUGUAGACCCAGGGUAUUCUACUGGUUUACGCCAA GGCUAGGUGGGCUAGCACU
4c20	**AUGGUAACAGGAAAGUCUGUUUGGGGUAAUAAGGGACAAC ACGCCGCGCCGAAGGGUGA**
4c23	*UGUUGGCUGGGAUCCAAUCGCGGGGUUGAAGUUAUUGGUGG GCUGCUAUACACUAUCAG*
4c25	*UGUUGGCUGGGAUCCAAUCGCGGGGUUGAAGUUAUUGGUG GGCUGCUAUACACUAUCAG*
4c26	**AUGGUAACAGGAAAGUCUGUUUGGGGUAAUAAGGGAC AACACGCCGCGCCGAAGGGUGA**
4c27	AGAACCAGUGCACGAUACAUGAACGAACCUUCUGAGAUC GCGUGCAGGAGGUUUGAACA
4c28	AUGCGGUCAGCUCAGUACUCUGCUACUGGCGUGAGCACACC GACUUAGGGAGGACGUCA
4c35	ACGAGUGUGCGUGGCAUUACUGUGCUAUUACCUAUUGUG CGGCUCAUCCAGUCAUCGUU
4c36	AGCAAAGAGCAGGGUGCAUGGUUGGUGCUCCCUGACACC AGUGAGAUAUAUGUCAUGCG
4c40	ACAGGCAGGCAACCAUUGAUCAAUGCGACACGUGGUGGAC CGACGCAAUAGACUAGGU
6c4	**AGCUGAUGUGGGACGUCAGUUAUAGGAAUGUGGAGGU UGGAAGUUAUGGCUUGCAUCAG**
6c5	**AGCUGAUGUGGGACGUCAGUUAUAGGAAUGUGGAGGUU GGAAGUUAUGGCUUGCAUCAG**
6c20	AGUGGCCAGGGGAGAUGUAAGCCGAUUUGUGAUAGUGU CUCUUACAGAGGCUGAUCAU

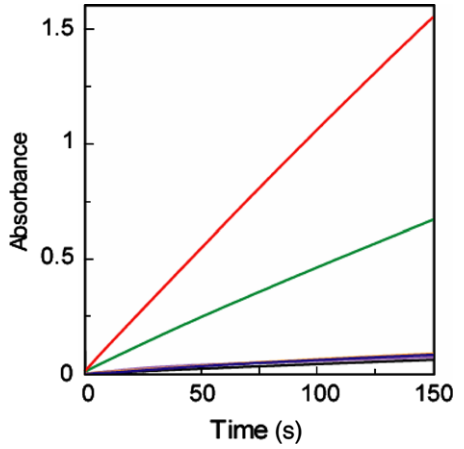

Figure 2. Time-dependent ABTS oxidation upon analysis of peroxidase activity at different catalyst compositions. 6c5 and hemin, red; 4c26 and hemin, green; 6c20 and hemin, orange; 4c23 and hemin, purple; 4c1 and hemin, blue; and hemin alone, black. The reaction was carried out in 40 KT buffer. RNA, 2.0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

binding affinity and peroxidase activity may be explained by the difference in the hydrophobicity of the hemin environment.

As described above, 6c5 RNA showed the highest binding affinity and the fastest ABTS oxidation rate. The interaction of 6c5 RNA with hemin was evaluated quantitatively by the titration of hemin with an increasing concentration of 6c5 RNA (Figure 3a). Absorption at the Soret band of hemin increased with increasing concentration of 6c5 RNA. The absorbance changes at 404 nm depicted in Figure 3a were plotted against the concentration of 6c5 RNA (Figure 3b). The apparent dissociation constant (K_d) determined was 0.8 μM. The K_d value of 6c5 RNA is similar to that of RNA aptamers reported by other groups, in which the K_d was in the submicromolar order.[16,17,31] Scatchard analysis gave a value of 1.3 moles of RNA bound per mole of hemin, suggesting that the binding stoichiometry was 1:1 (data not shown). The predicted secondary structure of either the 101-nt full-length or the random region of 6c5 RNA[32] suggested that the conserved domain enclosed by the dashed frame is the active domain that binds to hemin, as shown in Figure 4. It is notable that the sequence of 6c5 RNA did not contain a G-rich motif, which has been observed in other hemin-binding RNA aptamers.[17,31,33]

The catalytic reaction of the 6c5 RNA–hemin catalyst was investigated kinetically using Michaelis–Menten plots, which showed the ABTS oxidation rate as a function of the concentration of H_2O_2 (Figure 5). The peroxidase reaction with the 6c5 RNA–hemin catalyst gave a k_{cat} value of 650 min^{-1} and a K_m value of 9.0 mM.

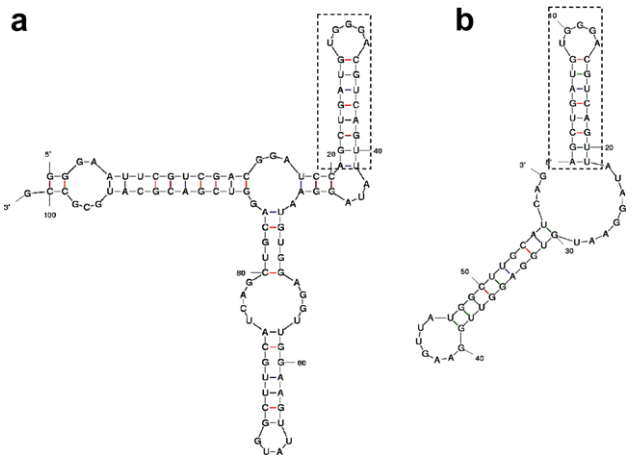

Figure 4. The predicted secondary structure of the 101-nt full-length (a) and the random region (b) of 6c5 RNA.

The value of catalytic efficiency (k_{cat}/K_m) of 6c5 RNA was 1203 M^{-1} s^{-1}. Our data contrast with those of Travascio et al., who found that the peroxidase reaction with hemin alone gave a k_{cat} value of 9 min^{-1}, a K_m value of 1.3 mM, and a k_{cat} / K_m value of 115 $M^{-1}s^{-1}$. The difference in K_m values suggests that the access of H_2O_2 to the reaction center of the 6c5 RNA–hemin complex may be more difficult than that to hemin. Considering that the buffer component and pH significantly affected the peroxidase activity[16] and the kinetics,[33,34] it is difficult to compare the activity directly. However, 6c5 RNA had considerably high catalytic efficiency. In addition, although this catalytic efficiency was considerably lower than that of HRP, it was higher than the efficiency of some hemoproteins that have peroxidase activity such as wild-type ferrimyoglobin (k_{cat}/K_m = 540 M^{-1} s^{-1}) and catalytic antibody (k_{cat}/K_m = 233 M^{-1} s^{-1}).[33] We achieved a 10-fold enhancement of the catalytic efficiency of the 6c5 RNA–hemin catalyst comparing with hemin alone. This enhancement by 6c5 RNA was twofold higher than that induced by unnatural ribozyme reported previously by our group, although the binding affinity of 6c5 RNA was a little lower than that of unnatural ribozyme.[17] This result suggests that the peroxidase activity depends on both the structure of the com-

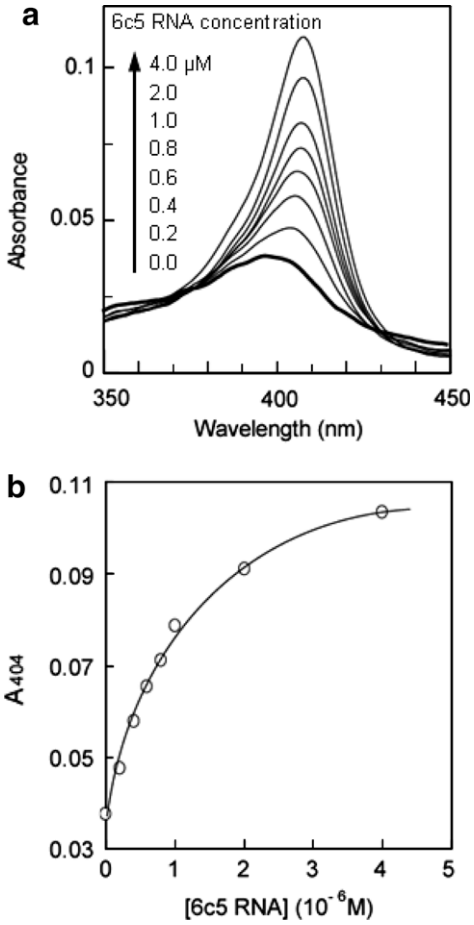

Figure. 3. (a) Absorption of Soret band of hemin with increasing concentrations of 6c5 RNA. The concentrations of 6c5 RNA were 0, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, and 4.0 μM. Titration of hemin with 6c5 RNA was carried out by incubating 6c5 RNA with hemin (0.5 μM) in 40KT buffer at room temperature. (b) Absorbance at 404 nm versus varying concentrations of 6c5 RNA. The data are from (a).

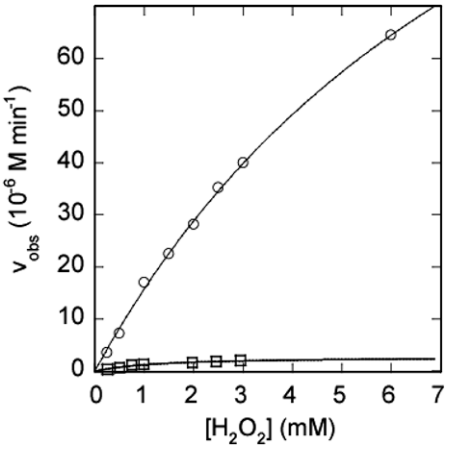

Figure 5. Michaelis–Menten plots of the peroxidation reaction in the presence of the 6c5 RNA–hemin complex (RNA, 2.0 μM; hemin, 0.25 μM) (○) and hemin alone (0.25 μM) (□). The reaction buffer comprised 50 mM MES pH 6.5, 100 mM Tris acetate, 40 mM potassium acetate, 1% DMSO, 0.05% Triton X-100, 2.5 mM ABTS, catalyst (6c5 RNA–hemin or hemin), and H_2O_2.

plex of RNA with hemin and the binding affinity between RNA and hemin.

In conclusion, a new catalytic RNA with peroxidase activity was developed by in vitro selection using a hemin-immobilized agarose column. We demonstrated for the first time that in vitro-selected natural RNA exhibits peroxidase activity. Although several DNA/RNA enzymes that exhibit peroxidase activity have been reported so far, they were in vitro selected against NMM and not hemin. We used direct in vitro selection against hemin in this study and found a high catalytic efficiency of the 6c5 RNA selected that was similar to that of other DNA/RNA enzymes reported previously. In this regard, a catalytic DNA with peroxidase activity has also been developed by in vitro selection against hemin.[35]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.01.016.

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