

Direct In Vitro Selection of Hemin-Binding DNA Aptamer with Peroxidase Activity

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A novel hemin-binding DNA was synthesized using in vitro selection (SELEX) method. The selected DNA not only bound to the hemin, but also had peroxidase activity when complexed with hemin. A pool of 104-nt single-stranded DNA (ssDNA) molecules containing a randomized sequence of 60 nt was synthesized. The DNA pool having random sequences was incubated with a hemin-immobilized affinity column. Bound DNAs were eluted with hemin solution and amplified by PCR with biotin-labeled primers. ssDNAs were isolated from the biotin-labeled double-stranded DNA (dsDNA) molecules after each selection process. After four rounds of selection process, the selected DNAs were cloned and sequenced. Four DNA aptamers were chemically synthesized from the sequenced clones. Two aptamers exhibited binding affinity to hemin and peroxidase activity. A 21-nt oligonucleotide was designed from the aptamer sequence that formed a complex with hemin and exhibited high peroxidase activity.

Heme is an essential molecule that plays critical roles in numerous biological phenomena. Free heme can act as an intracellular messenger by playing a role in regulation of gene expression or in ion channel signal transduction.[1,2] Moreover, heme is also a prosthetic group in certain enzymes and participates in various biological reactions, such as electron- and gas-transfer. Peroxidases are heme enzymes and utilize hemin as a cofactor to catalyze the oxidation of various substrates.[3,4] Peroxidases have been used as a biocatalyst for bioreactors and biosensors;[5–8] however, several intrinsic properties of these enzymes, such as thermostability and high cost, limit the applications of their function as natural catalytic copolymers. Therefore, chemists and biologists have conducted a sustained research effort to mimic peroxidases using combinatorial bioengineering methods as well as rational design methods. For example, Takahashi et al. prepared a poly(ethylene glycol)-modified hemin that exhibits peroxidase activity in several organic solvents.[9] Kamiya et al. demonstrated that a surfactant–histidine–hemin complex shows peroxidase activity in organic media.[10] Furthermore, Cochran and Schultz reported a catalytic antibody that forms a complex with hemin and exhibits peroxidase activity.[11] This antibody was obtained by binding to *N*-methylmesoporphyrin IX as a transition state analog (TSA).[12] Subsequently, several groups also succeeded in preparing catalytic antibodies with peroxidase activity.[13–15]

In 1990, a new method termed in vitro selection (SELEX) was developed.[16,17] This method aims at the development of aptamers and aptazymes, which are oligonucleotides (RNA or ssDNA) that bind to their target molecules or catalyze various reactions. Numerous aptamers and aptazymes have been developed that have been used in nanotechnology, diagnostics, and therapeutic applications.[18–23] Several aptamers that can bind hemin have also been reported,[24–26] of which DNA aptamers developed by Sen and colleagues exhibit high peroxidase activity when complexed with hemin.[27,28] We have previously reported the first successful synthesis of a non-natural RNA molecule with peroxidase activity.[25] However, all of the nucleic acid aptamers mentioned above were selected in an indirect manner, since the selection was carried out on a column immobilized with either *N*-methylmesoporphyrin IX (NMM) or mesoprotoporphyrin IX (MPIX) as a scaffold. In the present study, a hemin-binding DNA aptamer was in vitro selected by using a hemin-immobilized agarose column. The selected DNAs were characterized and their peroxidase activity was investigated.

Experimental

Materials. Hemin purchased from Sigma Co. (St. Louis, MO, USA) was used without further purification. Hemin-agarose beads and diammonium salt of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were obtained from Sigma Co. Dimethylsulfoxide (DMSO) and hydrogen peroxide were obtained from Junsei Chemical Co. (Tokyo, Japan). Toriton X-100 detergent was obtained from Calbiochem Co. (Darmstadt, Germany). Dynabeads MyOne Streptavidin C1 and magnet were obtained from Invitrogen Co. (Carlsbad, CA, USA). Deoxyribonucleotide triphosphate (dNTP) monomers and AmpliTaq DNA polymerase with reaction buffer were purchased from Roche Diagnostics Co. (Basel, Switzerland). All oligonucleotides containing ssDNA library were synthesized using an automated DNA synthesizer (H-8-SE model, Gene World Co., Tokyo, Japan) by typical phosphoramidite chemistry.

In Vitro Selection Process. The hemin-agarose columns (bed volume of 0.5 mL) were washed with decreasing concentrations of dimethylformamide (DMF): 100% DMF (10 mL), 66% DMF (10 mL), and 33% DMF (10 mL), followed by washing with Milli-Q water (20 mL). The hemin adsorbed on the resin was removed by rewashing with 25% pyridine until the wash was colorless, followed by a wash with Milli-Q water (20 mL), and a final preequilibration in 10 mL of binding buffer (20 mM Tris-AcOH,

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pH 8.0; 100 mM NaCl; 200 mM KCl; 5 mM $MgCl_2$; 0.5% Triton X-100; 5% DMSO: 1 M = 1 mol dm^{-3}).[29]

For the first round of selection, a synthetic single-stranded DNA library (8 μg) containing a random region of 60 nt (5′-TAGGGAATTCGTCGACGGATCC–N_{60}–CTGCAGGTCGACGCATGCGCCG-3′) was purified using denaturing polyacrylamide gel electrophoresis on an 8% gel and dissolved in 200 μL of annealing buffer (20 mM Tris-AcOH, pH 8.0; 100 mM NaCl; 200 mM KCl; and 5 mM $MgCl_2$). The DNA solution was heated at 90 °C for 5 min and allowed to cool to room temperature for 1 h, then topped to 1 mL with binding buffer. Affinity selection was performed as described previously.[30] The folded DNAs were then passed through a hemin-agarose column and the flow-through was recirculated through the column three times. The DNAs were incubated with hemin for 30 min at 15 °C. Unbound DNAs were eluted with 30 column volumes of the binding buffer. The DNAs bound to hemin in the column were eluted with six column volumes of hemin-saturated binding buffer, and then twice extracted with phenol–chloroform. The DNAs in the aqueous phase were precipitated twice with ethanol in the presence of glycogen (20 μg).

The resulting DNAs were amplified using nine PCR cycles (94 °C, 15 s; 55 °C, 15 s; 72 °C, 15 s) in 100 μL of reaction solution containing AmpliTaq DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs, 0.5 μM 5′ primer (FP) (5′-TAGGGAATTCGTCGACGGATCC-3′), and 0.5 μM 3′ primer labeled with biotin at the 5′ terminus (RBP) (5′-Biotin–CGGCGCATGCGTCGACCT-3′). After an additional nine PCR cycles in 800 μL of reaction solution performed using the same cycling conditions, the amplified double-stranded (ds) DNA was extracted with phenol–chloroform and subsequently precipitated with ethanol. The purified biotin-labeled dsDNA (16 μg in 100 μL) was added to 100 μL of 2 × B&W buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 2.0 M NaCl) containing 1 mg of Dynabeads MyOne Streptavidin C1. The mixture was incubated for 15 min at room temperature. The beads coated with the biotinylated dsDNA were separated by using a magnet. After washing two to three times with 1 × B&W buffer in the presence of the magnet, 20 μL of 0.2 M NaOH were added to the beads, which were then incubated for 5 min at room temperature. The supernatant was removed from the beads and mixed with 4 μL of 1.0 M acetic acid. This separation process was repeated three times. Subsequently, the isolated ssDNA was precipitated with ethanol for the next round of selection. The amount of ssDNA was measured by UV/VIS spectrophotometer (JASCO V-550).

Cloning and Sequencing. After four rounds of selection, the pool of DNA was amplified using the FP primer and RP primer (5′-CGGCGCATGCGTCGACCT-3′). The purified DNA was ligated into the pCR2.1 plasmid using the T/A cloning kit (Invitrogen). The plasmid was then transformed in INVαF′ competent *Escherichia coli* cells (Invitrogen). Seven clones were picked and DNA sequenced using the dideoxy method.

Measurement of Binding Property of DNA Aptamer to Hemin. The ability of four selected DNAs (4c15, 4c19, 4c20, and 4c21) to form a complex with hemin was investigated by UV–visible spectrometric analysis using 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), described by Travascio et al.[28] The DNAs were heated at 90 °C for 5 min in TE buffer, then allowed to slowly cool down to room temperature. The DNAs were placed in 40KT buffer to allow proper folding. Each solution was then made up to final concentration of hemin and incubated for 30 min at room temperature. UV–visible spectra were obtained using the UV/VIS spectrophotometer.

The dissociation constant (K_d) for the DNA–hemin complexes, in which the DNA is a tetramer of 4c15-s DNA [$(4c15\text{-}s)_4$], was determined by plotting the changes in absorbance of hemin at 404 nm against varying concentrations of $(4c15\text{-}s)_4$. The plot was fitted to the following equation, described by Wang et al.:[31]

$$[DNA]_0 = K_d(A - A_0)/(A_\infty - A) + [P_0](A - A_0)/(A_\infty - A_0) \quad (1)$$

where $[DNA]_0$ is the initial concentration of DNA [$(4c15\text{-}s)_4$]; $[P_0]$ is the initial concentration of hemin; A_∞ and A_0 are the absorbances of hemin in the presence of a saturated concentration of DNA and in the absence of DNA, respectively; and A is the absorbance of hemin measured in the presence of varying concentrations of DNA [$(4c15\text{-}s)_4$].

The circular dichroism (CD) spectra obtained using a JASCO J-720 spectropolarimeter were measured at 25 °C, after incubation of 4c15-s (2 μM final concentration) and hemin (5.0 μM final concentration) in 40KT buffer.

Measurement of Peroxidase Activity. Peroxidase activity of hemin and DNA–hemin complexes was evaluated by determining the apparent rates of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) oxidation. Increases in absorbance at 414 nm were measured as a function of time by using UV/VIS spectrophotometer, at room temperature. The $\Delta\varepsilon_{414\,nm}$ value used was 36000 M^{-1} cm^{-1}.[32] Reactions were initialized by the addition of a solution of H_2O_2 into an equilibrated mixture of ABTS plus hemin or DNA–hemin complex in 40KT buffer, where the final concentrations of H_2O_2 and ABTS were 0.75 and 2.5 mM, respectively.

Results and Discussion

In Vitro Selection. A DNA aptamer was selected from a DNA library, in which a 60-nt random region was flanked by constant primer regions. The DNA library was chemically synthesized by phosphoramidite chemistry. About 10^{13} ssDNAs were used in the first round of in vitro selection. Affinity elution was performed using hemin-saturated binding buffer to allow recovery of even the tightest binding aptamers. The absorbance at 260 nm of the DNA loaded in the column or of the eluted bound DNA was measured and was subsequently used for calculating the binding percentage of DNA in every selection round. A significant increase in the binding percentage of DNAs was observed after the fourth round, compared with the first round (first round, 15%; second round, 39%; third round, 59%; and fourth round, 70%). Therefore, we amplified and cloned the ssDNA collected from the fourth round. Seven clones were picked randomly then sequenced using the dideoxy method. Table 1 shows the random sequences of these clones. Both G-rich (e.g., 4-15 and 4-21) and G-poor (e.g., 4-2 and 4-20) sequences were obtained. Since it was previously reported that several G-quartet DNAs bind to porphyrin derivatives,[24,33–35] we selected three kinds of G-rich sequences (4-15, 4-19, and 4-21) and one kind of G-poor sequence to investigate their binding affinity. We synthesized four kinds of DNAs (4c15, 4c19, 4c20, and 4c21), which correspond to the random region of the four clones (4-15, 4-19, 4-20, and 4-21), respectively.

Table 1. Sequences of the Random Region of Selected DNAs

No.	Random sequence region	G
4-2	GTAGAAAGATCAGGTTGCTAGTTGGGCTGTAGCGTCGTTCAGCTCTTACGTGCCGCGTTC	19
4-11	CCGTTAGGCTAGTTTGGGGGTGGGCTGTTACGGACGGATTGAGTTAAGAGGGGGCAGTAA	26
4-15	AGGTGGGGAGGAGCGGGGTGCTAGGCTCTTATGGAGTCAGCGCAAAAGGGTTGTTGGAGC	28
4-19	TTGCTGCCCCTGCCCCAACGGGAGGTTGGCGGCGGAGTGCGGCTCTGGGGCTGGGGCTGT	27
4-20	CTGAGCGAGTCACAGCCATCGGCCTAGAAGCGCCCACCCTCATGAAACTATTAGGGTGCT	15
4-21	GCAGCCATGGTCCGTGGGGGGCTGGGAGCACGGTGTGGAATTGCGTTTCCGGGGGTGGT	29
4-27	CGGGCCGTAATAACTTGACGAAACGGCGCTTGTACTCCGAGGGGTCGCAGTGT	18

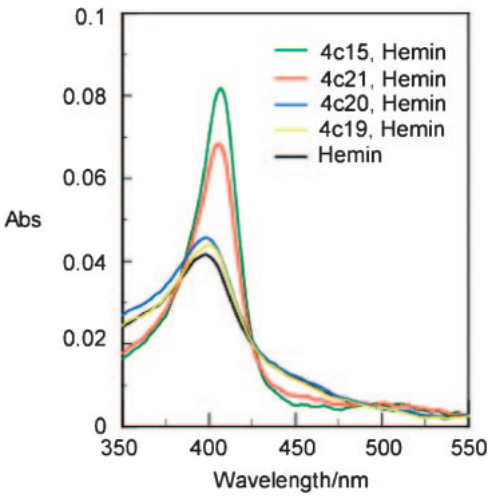

Figure 1. Spectra of hemin (0.5 μM) alone and in the presence of 4c15, 4c19, 4c20, and 4c21 (2 μM) in 40KT buffer. 4c15, green; 4c19, yellow; 4c20, blue; 4c21, red; and uncomplexed monomeric hemin, black.

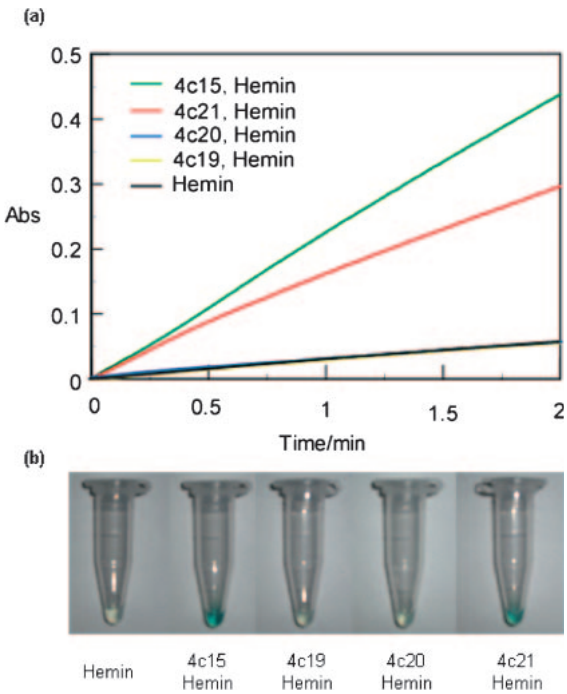

Figure 2. (a) Time-dependent absorbance (414 nm) changes upon analysis of peroxidase activity at different catalyst compositions. 4c15 and hemin, green; 4c19 and hemin, yellow; 4c20 and hemin, blue; 4c21 and hemin, red; hemin alone, black. (b) Visual detection of the peroxidase activity after 30 min of reaction at room temperature. In all experiments, the reactions consisted of the DNA, 1 μM; hemin, 0.25 μM; ABTS, 2.5 mM and H_2O_2, 0.75 mM in 40KT buffer.

Binding Affinity and Peroxidase Activity of Selected DNAs. First, the interaction of the various DNAs (4c15, 4c19, 4c20, and 4c21) with hemin was investigated by absorption spectroscopy (Figure 1). Travascio et al. reported that a hyperchromic effect is induced at the Soret band of hemin when a DNA aptamer binds to hemin.[28] Accordingly, a significant increase in the Soret absorption band was observed in response to titration with 4c15 and 4c21. In addition, the λ_{max} of absorbance at the Soret band exhibited a slight red shift in these two cases. These results seem to indicate that the 4c15 and 4c21 DNAs managed to bind hemin. In contrast, no significant changes were observed at the Soret band when titrating with either 4c19 or 4c20. This indicates that the 4c19 or 4c20 DNAs had no significant binding affinity for hemin.

The peroxidase activity of the four kinds of synthetic DNAs was investigated by the addition of ABTS and H_2O_2 to the solution used in the binding assay. The rate of ABTS oxidation by hemin in the presence or absence of the different DNAs was measured by monitoring the increase of absorbance at 414 nm. Comparing Figure 2a with Figure 1, the peroxidase activity results seem to be consistent with the binding affinities of the DNA molecules: an increased peroxidase activity was observed in the presence of hemin binding 4c15 or 4c21. The oxidation rate in the presence of 4c15–hemin was 7.5-fold faster than that of hemin alone. In contrast, no increase in peroxidase activity was observed in the presence of 4c19 or 4c20, which did not bind to hemin. The catalytic activity was also detectable by naked eye (Figure 2b): within thirty minutes, the reaction solution turned peacock-green in the presence of 4c15 or 4c21, but not in the presence of 4c19 or 4c20.

In natural peroxidase, heme is bound to a hydrophobic pocket of peroxidase, and the hydrophobicity of the environment around heme plays an important role in the enzyme reactivity.[3] Several groups have reported that peroxidase activity can be enhanced by increasing the hydrophobicity of the heme environment[9,36] Hyperchromicity of the Soret band has been considered an indicator of the hydrophobicity of the hemin environment. An increase of absorbance at the Soret band of hemin spectra indicates an increase in the hydrophobicity of the hemin environment, which is directly proportionate to the binding affinity.[37] The present study demon-

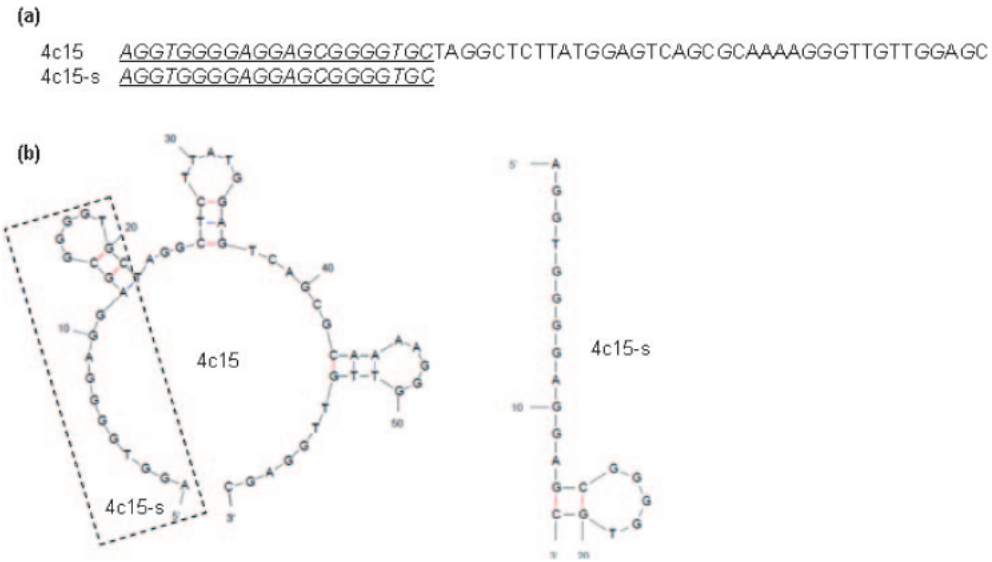

Figure 3. Sequence of 4c15 and its truncation (4c15-s) (a) and its predicted secondary structure (b).

strated that both binding affinity and peroxidase activity were observed in 4c15 and 4c21, but in neither 4c19 nor 4c20. Therefore, the differences in the peroxidase activity could be attributed to the differences in the hydrophobicity of hemin environment.

Since the 4c15 DNA molecule not only exhibited the highest binding affinity to hemin, but also possessed the highest peroxidase activity when complexed with hemin, when compared with the other oligonucleotides, the secondary structure of 4c15 was predicted using the software reported by Zuker (Figure 3).[38] It is notable that no featured secondary structure was found in 4c15. To investigate in detail the peroxidase activity as well as the hemin-binding property of this molecule, and considering that several G-quartet DNAs that bind porphyrin have been previously reported,[33–35] we therefore synthesized a G-rich motif (4c15-s) enclosed by a dashed frame.

Binding Affinity and Peroxidase Activity of Truncated Aptamer. A conformational analysis of the interaction between 4c15-s and hemin was performed by acquisition of CD spectra (Figure 4). The 4c15-s molecule exhibited a strong positive band at 260–265 nm and a negative band at 235–240 nm, indicating that 4c15-s adopts a parallel G-quartet structure, as described previously.[34,39,40] When hemin was added to the solution in large excess to 4c15-s, no significant change was observed in the spectra and the characteristic G-quartet signature was retained. This result suggests that the parallel G-quartet structure formed by 4c15-s remained, even after binding of hemin. Okazawa et al.[40] also reported that hematoporphyrin does not induce significant changes in the conformation of selected DNA aptamers.

The interaction of the truncated 4c15-aptamer (4c15-s) with hemin was investigated by UV–visible spectrometric analysis (Figure 5a). Absorbance at the Soret band of hemin increased with the increase of the concentration of 4c15-s. This result indicates that 4c15-s was capable of binding to hemin. Molar extinction coefficient of the 4c15-s–hemin complex at 404 nm ($\varepsilon = 165000\,M^{-1}\,cm^{-1}$) was determined. A Scatchard plot for

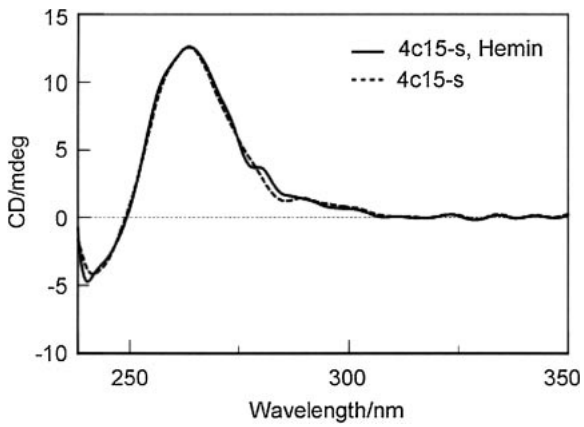

Figure 4. CD spectra of 4c15-s in the absence (dashed line) and presence (solid line) of hemin. 4c15-s and hemin concentrations were 2.0 and 5.0 μM, respectively.

the titration of hemin with a tetramer of 4c15-s was obtained by analyzing Figure 5a following a previous report (Figure 5b).[41] The dissociation constant was 45 nM at a stoichiometric ratio of 1:1 between hemin and the 4c15-s tetramer. This finding suggested that the 4c15-s tetramer formed a parallel G-quartet structure upon binding hemin. The absorbance changes at 404 nm depicted in Figure 5a was also plotted against the concentration of tetramer aptamer [$(4c15\text{-}s)_4$], as shown in Figure 6. The apparent dissociation constant (K_d) determined was 41 ± 3 nM, which gave close agreement with that determined from Scatchard plot, and the binding affinity was comparable to that of a PS2.M aptamer–hemin complex ($K_d = 27 \pm 3$ nM) described by Travascio et al., although they reported that the binding stoichiometry was 1:1.[28]

The peroxidase activity assay showed that the $(4c15\text{-}s)_4$–hemin catalysis rate was 8-fold higher than that for hemin catalysis alone (Figure 7). The observed catalysis rate for $(4c15\text{-}s)_4$–hemin was $8.6\,\mu M\,min^{-1}$, which was also comparable to the rate observed for PS2.M–hemin, using the same 40KT buffer conditions.[28] We investigated the mode of hemin

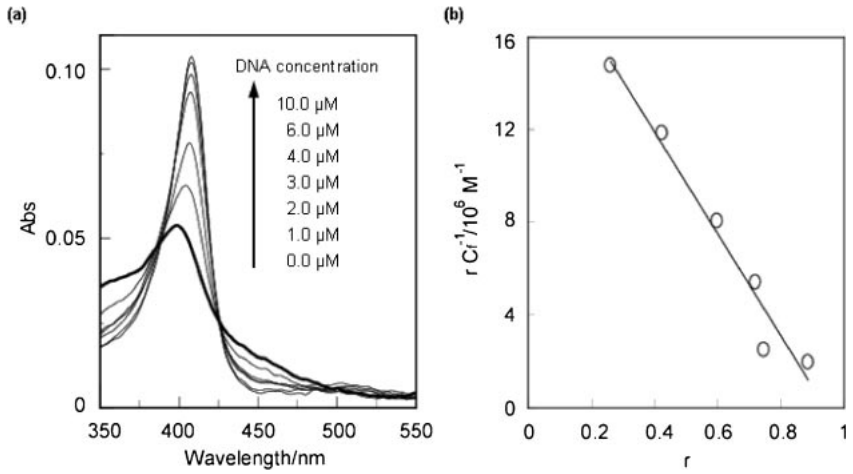

Figure 5. (a) UV–visible spectra around Soret band of hemin with increasing concentrations of 4c15-s. Concentrations of 4c15-s were 0, 1.0, 2.0, 3.0, 4.0, 6.0, and 10.0 μM. Titration of hemin with 4c15-s was carried out by incubating 4c15-s with hemin (0.67 μM) in 40KT buffer at room temperature. (b) Scatchard plot for the titration of hemin with 4c15-s. $r = C_b/[(4c15\text{-}s)_4]$; C_b is the concentration of bound hemin; C_f is the concentration of unbound hemin.

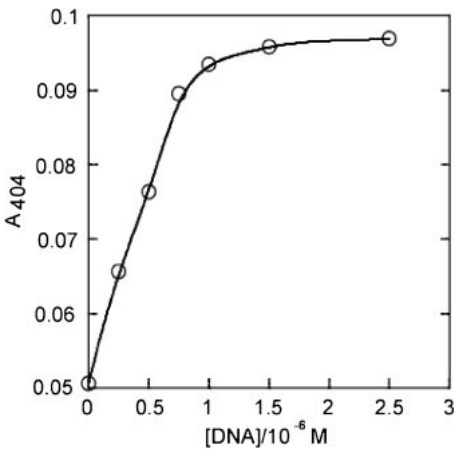

Figure 6. Absorbance at 404 nm versus varying concentrations of $(4c15\text{-}s)_4$. The data were from Figure 5a.

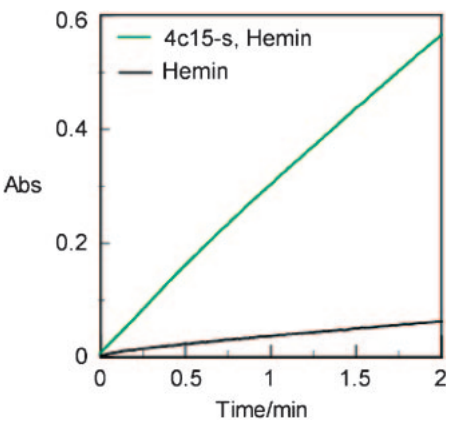

Figure 7. Time-dependent absorbance (414 nm) changes upon analysis of peroxidase activity of 4c15s–hemin (green) and hemin alone (black). In all experiments, the reactions consisted of the DNA, 2 μM; hemin, 0.33 μM; ABTS, 2.5 mM; and H_2O_2, 0.75 mM in 40KT buffer.

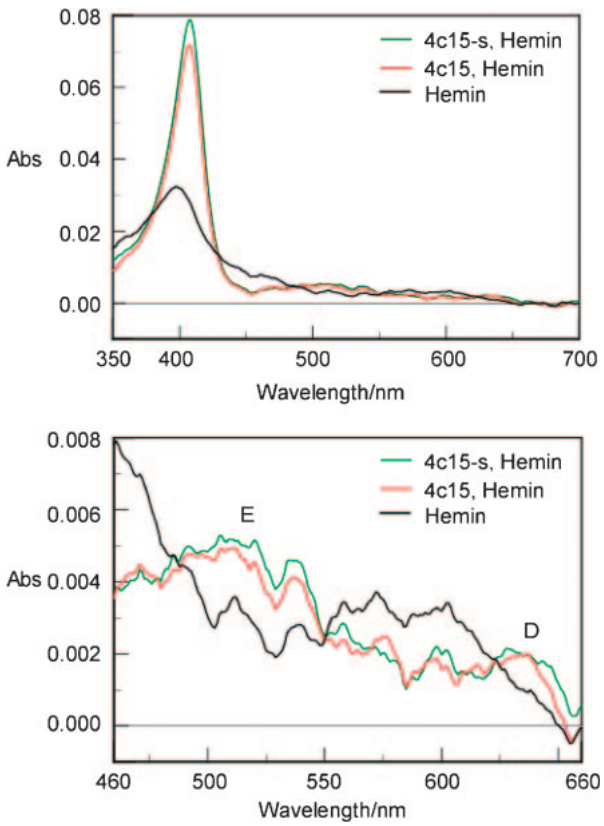

Figure 8. (a) UV–visible spectra of hemin (0.4 μM) alone and with 4c15 or 4c15-s (10 μM) in 40KT buffer: 4c15-s, green; 4c15, red; and uncomplexed monomeric hemin, black. (b) Visible region of the spectra shown in Figure 8a.

binding to 4c15 and 4c15-s (Figure 8). At the saturated concentration of aptamer (10 μM), all hemin (0.4 μM) formed complexes with 4c15 or 4c15-s. An almost equivalent hyperchromic effect was observed between 4c15–hemin and 4c15-s–hemin complexes in the Soret region. Similarly, in the visible region, when hemin formed a complex with 4c15 or 4c15-s,

new peak patterns, distinct from those of hemin alone, were observed. New bands, D and E, were considered to correspond to ligand to metal charge-transfer (LMCT) transitions usually observed in hemoproteins. The coincidence between 4c15 and 4c15-s indicated the same axial coordination for hemin. These results indicated that the hemin environment in 4c15 was very similar to that in 4c15-s and thus the sequence of 4c15 for binding affinity and for peroxidase activity was considered to be involved in 4c15-s.

Conclusion

In conclusion, a novel hemin-binding DNA aptamer was directly in vitro selected by using a hemin-immobilized agarose-column. The selected DNA aptamers not only bound to hemin, but also exhibited peroxidase activity by forming a complex with hemin. We also successfully designed a 21-nt oligonucleotide from one of the aptamers, and demonstrated that it not only bound to hemin but also exhibited high peroxidase activity. Although DNA aptamers that can bind hemin have been developed previously,[24] the binding mode was different from that of the aptamers described in the present study. The 21-nt DNA aptamer represents a promising biocatalyst and may also be widely used as a key component for the synthesis of nucleic acid-based biosensors.[42–46]

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