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DNA-Binding Hemoproteins Tethering Polyamine Interface

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Artificial prosthetic groups tethering a polyamine moiety at the terminal of two peripheral heme–propionate side chains as a molecular interface were inserted into apocytochrome b_{562} to provide positively charged reconstituted hemoproteins, which exhibit a strong binding with double-stranded DNA.

Biological molecules are attractive building units for nanoscale assemblies based on bottom-up strategies. In particular, DNA molecules have been proven to serve as well-defined nanoscaffolds, where DNA–protein interaction generates fascinating conjugates.[1,2] The expanding repertoire of naturally occurring DNA–protein interaction using artificial surrogates is a promising target both in the fields of medicinal chemistry and nanobiotechnology. To this end, a strategy for converting ubiquitous proteins to DNA-binding ones is required. Especially, the conjugation of redox-active hemoproteins with DNA molecules has attracted increasing interest, because a series of hemoproteins function as an electron-transfer mediator, catalyst, and sensor. One intriguing approach to build a designed moiety on the hemoprotein surface is cofactor substitution, in which we can replace the native hemin with an artificial one having a DNA-binding interface. For example, myoglobin reconstituted with a modified hemin having an intercalator at the terminal of a heme–propionate side chain provides a stable DNA–myoglobin complex.[3] Furthermore, the covalent introduction of a single-stranded DNA or DNA-binding peptides to the heme–propionate side chain leads to DNA-conjugated hemoproteins.[4,5]

In contrast, we have recently reported an artificial protein–protein complexation using negatively or positively charged myoglobins;[6–10] e.g., myoglobin reconstituted with a modified hemin with a carboxylate cluster at the terminal of two propionate side chains forms a stable complex with a positively charged cytochrome *c*. Our next objective is to create unique nanobiomaterials involving a hemoprotein–DNA composite.

It is known that various polyamine structures might be effective as a strong binding interface for the DNA moiety.[11–14]

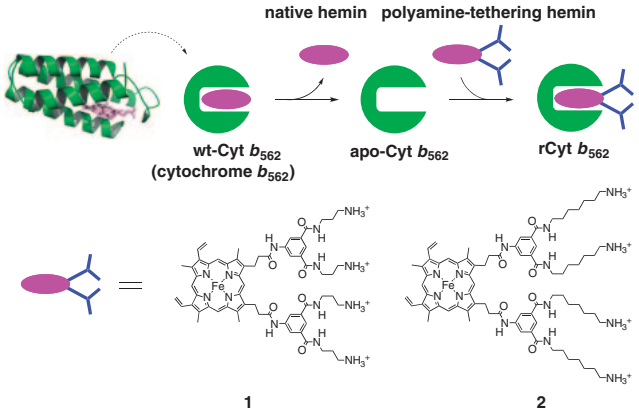

Figure 1. Schematic representation of reconstituted cytochrome b_{562} with polyamine moiety.

Therefore, we next prepared two kinds of cytochrome b_{562}s reconstituted with polyamine-tethering hemins, **1** and **2**, as a new DNA-binding hemoprotein (Figure 1). Here we report the characterization and the binding properties of positively charged hemoproteins.

The cofactors were prepared as follows: The terminal of two propionates of the protoporphyrin IX were coupled with 5-amino-1,3-bis(*t*-butoxycarbonylaminopropyl)carbamoylbenzene and the Boc groups were then deprotected by TFA/HCO_2H, yielding the positively charged free-base porphyrin. The corresponding propionate-modified porphyrins were treated with $FeCl_2$, and purification on a LH-20 column gave the final products, **1** and **2**. The products were characterized by FAB MS (**1**: calcd. 1166.5265; found 1166.5273, **2**: calcd. 1390.7769; found 1390.7767). From the characteristic absorption at 360, 396, and 608 nm, both amine-linked hemins were present as an equilibrium mixture of the Cl-bound hemin and its μ-oxo dimer in 10 mM HEPES buffer solution (pH 7.4).

The hemin derivatives were inserted into the apocytochrome b_{562} (apo-Cyt b_{562}) by a conventional method[15] followed by purification using a gel filtration column to produce the reconstituted cyt b_{562}s, rCyt b_{562}(**1**), and rCyt b_{562}(**2**). During the preparation, the reconstituted proteins were isolated as the ferrous (Fe^{2+}) form with His and Met ligations, providing well-characterized absorptions at 426, 531, and 562 nm, suggesting that each heme was located in the normal cyt b_{562} heme pocket. The ferric form was then prepared by oxidation with potassium hexacyanoferrate(III) exhibiting absorptions at 420, 537, and 566 nm.[16] Successful installation of the modified hemins was also determined by ESI-TOF MS experiments of the rCyt b_{562}(**1**) and rCyt b_{562}(**2**). The reconstituted ferric proteins were used for the following experiments. These reconstituted proteins were stable for several days at 4 °C.

The conformational characterization of the reconstituted proteins was performed by CD measurements (Figure 2). Characteristic 222- and 208-nm minima, and a crossover point at 201 nm indicate that the α-helical folding of both reconstituted ferric cyt b_{562}s are similar to that of the wild type cyt b_{562} (wt-Cyt b_{562}).[17] Although having the positively charged moieties, the reconstituted cyt b_{562}s retain mostly monomeric structure without interaction with the negatively charged region

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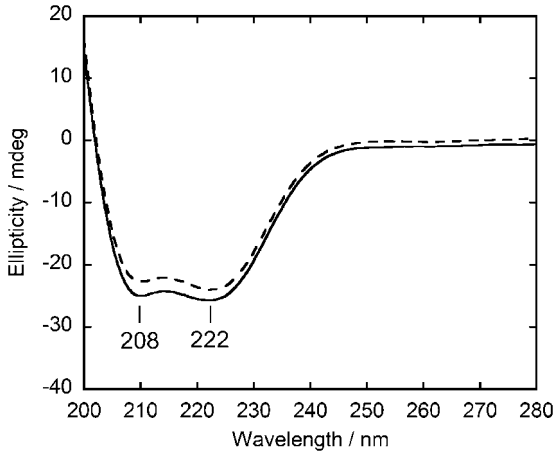

Figure 2. CD spectra of rCyt b_{562}(**1**) (solid line) and rCyt b_{562}(**2**) (dashed line). Conditions: 10 μM protein, 5 mM HEPES, 10 mM Tris-HCl, 1 mM EDTA (pH 8.2), 25 °C.

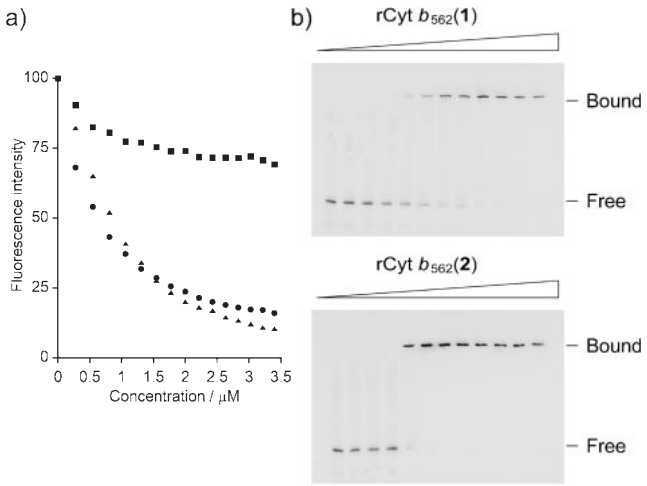

Figure 3. (a) Normalized fluorescent intensity (I_{max}) as a function of the cyt b_{562} concentration. rCyt b_{562}(**1**) (filled circle), rCyt b_{562}(**2**) (filled triangle), and wt-Cyt b_{562} (filled square). (b) EMSA analyses of DNA binding of rCyt b_{562}(**1**) and rCyt b_{562}(**2**). Protein concentrations: 0–10 μM.).

on the protein surface, supported by size exclusion chromatographic analyses.

We first investigated the qualitative binding ability of the reconstituted proteins to plasmid DNA by fluorescence assay using the fluorescent staining dye, GelStar, which exhibits a strong emission at 534 nm upon intercalation within the base pairs.[18] As shown in Figure 3a, the fluorescence of both polyamine-displaying cyt b_{562}, rCyt b_{562}(**1**), and rCyt b_{562}(**2**), decreased prominently. This effective quenching could be explained by two possible reasons. The binding of the reconstituted proteins expelled the intercalated dyes and/or the iron-containing protein bound to the double-stranded DNA (dsDNA) quenched the strong emission derived from the dyes within the DNA. While in the wt-Cyt b_{562} without the polyamine moiety, the fluorescence decreased slightly probably because only dynamic interaction between the DNA and the proteins are predominant. Therefore, the results clearly indicated that static binding of wt-Cyt b_{562} is negligible and that the tailored amine moiety on the reconstituted protein surface provides a strong DNA binding.

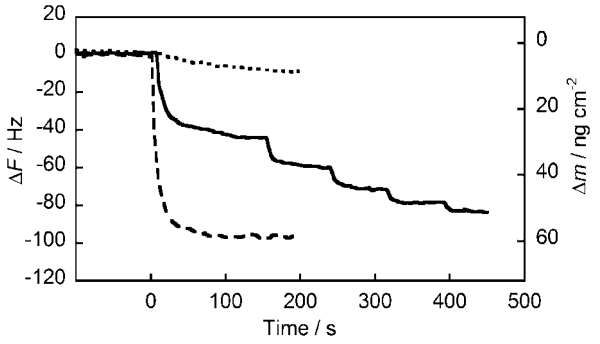

Figure 4. Frequency changes in the QCM in response to the sequential (solid line) and the single addition (dashed line) of rCyt b_{562}(**1**). The addition of wt-Cyt b_{562} is shown by the dotted line. Conditions: 10 mM HEPES, (pH 7.4), 25 °C.

Next, to investigate in detail the binding event between the reconstituted protein displaying the polyamine moiety and dsDNA, we performed an electrophoretic mobility shift assay (EMSA), in which we can obtain the exact ratio of the bound and free DNA to estimate the binding affinity. The EMSA analyses using the ^{32}P-labeled 50 bp DNA target showed that wt-Cyt b_{562} did not bind to the target at all. In contrast, rCyt b_{562}(**1**) and rCyt b_{562}(**2**) exhibited clearly shifted bands (Figure 3b), supporting the fact that both polyamine-tethering reconstituted proteins are potential species for strong DNA binding with the dissociation constants of 6.0×10^{-7} and 4.8×10^{-8} M, respectively, at 4 °C.[19] Positively charged amine groups with longer alkyl chain in rCyt b_{562}(**2**) might provide more effective multivalent electrostatic and/or hydrogen-bond interaction with the phosphate backbone and the base pair within the groove. Actually, the DNA binding here was interrupted in the high salt concentration.

The binding behavior of the reconstituted proteins on the dsDNA was also analyzed using a quartz crystal microbalance (QCM), which has proven to be a useful tool for detecting the DNA–protein interaction as mass increase on the QCM electrode.[20] The dsDNA with a thiol group on the 5′-terminus was immobilized together with 6-mercapto-1-hexanol on the Au electrode of the 27-MHz QCM. The frequency changes versus time of the dsDNA-immobilized QCM upon the additions of the reconstituted proteins revealed the apparent frequency decrease (−100 Hz), while wt-Cyt b_{562} did not exhibit a significant frequency change as shown in Figure 4. The sequential addition of rCyt b_{562}(**1**) exhibited a stepwise decrease in frequency, revealing that the observed frequency decay is due to the mass increase by the binding of the reconstituted protein on the DNA. The calculated K_d value, 1.6×10^{-7} M, for rCyt b_{562}(**1**) determined from the sequential addition decay of the QCM profile is in good accordance with that from the EMSA analyses. These results suggest that a total of approximately four positively charged proteins are attached to the target DNA, which seems to be in coincidence with the significantly shifted bands in the EMSA.

In conclusion, we have successfully prepared the reconstituted cyt b_{562} with the hemins tethering the polyamine moiety,

and our reconstituted proteins provide a strong binding affinity for dsDNA to afford a DNA complex with multiple hemoproteins. The present hemoprotein–DNA conjugate would serve as a way to create functional nanomaterials such as a protein-immobilized electrode. A study of the kinetic and thermodynamic aspects of these complexations is now in progress.

Experimental

Preparation of Cytochrome b_{562} Reconstituted with Polyamine-Tethering Hemins. To an apoprotein solution (10 mM HEPES, pH 7.4, 500 mM KCl) was slowly added dropwise an aqueous solution of **1** with gentle shaking at 4 °C and the mixture was incubated at 4 °C for 12 h. The protein solution was concentrated and potassium hexacyanoferrate(III) was added to obtain the ferric protein. The crude protein was purified by Sephadex G-25 gel filtration with elution of buffered solution (10 mM HEPES, pH 7.4) to give the reconstituted cyt b_{562} with **1** (rCyt b_{562}(**1**)). UV–vis (10 mM HEPES, pH 7.4) λ_{max}/nm 420, 537, and 566; ESI-TOF MS m/z calcd for $(M + H)^+$: 12947.8, found 12948.2. The reconstituted cytochrome b_{562} with **2** (rCyt b_{562}(**2**)) was obtained according to the same procedure. UV–vis (10 mM HEPES, pH 7.4) λ_{max}/nm 421, 536, and 562; ESI-TOF MS m/z calcd for $(M + H)^+$: 13172.0, found 13175.1.

Fluorometric Analyses. The fluorescence spectra were measured in a quartz microcell at 25 °C and recorded from 500 to 600 nm with 493 nm excitation. Plasmid DNA pBR322 (4361 bp) (5.0 μg mL^{-1} = 1.9 nM)) in 10 mM HEPES buffer (pH 7.4). Protein concentration was varied between 0 to 3.4 μM.

Electrophoretic Mobility Shift Assay (EMSA). The annealed 50 bp dsDNA (top strand: 5′-AGCTCTCATGCGGATGCACGTCAATAGTTGCTTCG-GAAGATCGACTAGATCCTG-3′, bottom strand: 3′-GAGTACGCCTACGTGCAGTTATCAACGAAGCCTTCTAGCTGATCTAGGACTCGA-5′) was labeled at the 5′ terminus with [γ-^{32}P]ATP by T4 polynucleotide kinase, and then the ^{32}P-labeled DNA fragment was purified by gel filtration using a buffered solution (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) as an eluent. The different concentrations of reconstituted/wild type protein (10 mM HEPES, pH 7.4) and the purified ^{32}P-labeled DNA fragment were incubated at 37 °C for 1 h. The DNA-bound proteins and free DNA were electrophoretically separated on a 12% polyacrylamide gel with Tris-borate-EDTA buffer (44.5 mM Tris-borate, 1.0 mM EDTA) at 230 V and 4 °C. The gel was preelectrophoresed for 30 min before running the samples. The gels were vacuum-dried and visualized by autoradiography using FLA-7000 (FUJIFILM Co., Ltd.).

Quartz Crystal Microbalance (QCM) Measurements. QCM measurement was performed by AFFINIX $Q_N\mu$ (INITIUM INC.). The cells were equipped with a 27 MHz QCM plate oscillating at fundamental frequency. To immobilize DNA on the Au electrode, the cleaned bare Au electrode of the QCM cell was immersed in 50 μL of a solution containing 0.5 μM 5′-thiol-modified 50 bp dsDNA, 10 mM Tris-HCl buffer (pH 8.0) at 25 °C for 30 min. After being washed with water, the cell was next immersed in 50 μL of a solution containing 200 μM of 6-mercapto-1-hexanol and 10 mM of Tris-HCl buffer (pH 8.0) at 25 °C for 30 min and washed again. The DNA-immobilized QCM cell was filled with 500 μL of a buffered solution (10 mM HEPES, pH 7.4) and the time course frequency changes were observed at different protein concentrations (99, 200, 290, 380, 480, and 500 nM). The proteins bound to the DNA-immobilized QCM cell were removed by washing with 1.0 M KCl solution for several times.

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Supporting Information

Synthesis of **1** and **2**, UV–vis spectra, ESI-TOF MS, and fluorometric DNA-binding analyses of reconstituted cyt b_{562}s. This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

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18 The characteristic absorptions of **1** and **2** did not remarkably change upon the addition of the dsDNA with the range of μM concentrations, indicating that hemis **1** and **2** are stably inserted into the apoprotein.
19 $R = I_b/(I_b + I_f)$, where I_b and I_f are the intensities of peptide-bound and free DNA bands, respectively. The K_d values were evaluated by fitting the experimentally obtained values of R to the binding isotherm equation ($R = [\text{protein}]/([\text{protein}] + K_d)$) assuming that the influence of cooperative binding is absent.
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