Artificial Assembly of Myoglobin and Flavodoxin Reductase Using Designed Coiled-coil Peptides

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A unique and artificial assembly of myoglobin and flavodoxin reductase was constructed using a heterodimeric coiled-coil formation of de novo designed polypeptides in order to demonstrate a novel method for the artitecture of the active-site directed fusion protein.

Myoglobin (Mb) is a relatively small monomeric hemoprotein containing a noncovalently bound iron-protoporphyrin IX (heme).[1] Although in nature the essential role of Mb is oxygen storage, semiartificial myoglobins possessing nonnatural functions and/or artificial molecular binding sites have been prepared by a reconstitutional method that is the incorporation of an artificial heme having multiple functional groups into apomyoglobin (apo-Mb).[2–4] The reconstitution using a chemically modified cofactor is valuable for the active-site directed incorporation of various functional groups, but the previously reported functionalities were relatively small molecules.[2–4] The introduction of biopolymers such as polypeptide, protein, RNA, and DNA may expand the utility of the reconstitutional method. In this regard, we designed a semiartificial Mb having an amphiphilic peptide and achieved the introduction of flavin molecule via the heterostranded coiled-coil formation of the polypeptides.[5] In this study, we used the above strategy to construct an artificial assembly of the Mb and an electron transfer (ET) protein in solution. Additionally, we evaluated the ET ability from NADPH to the heme of the Mb via the introduced ET protein.

The design and preparation of semiartificial Mb, Mb-1αK, was described in the previous paper.[5] Briefly, the Mb-1αK was constructed through the reconstitution of apo-Mb with an artificial heme–peptide conjugate, Heme-1αK, in which a cationic 28-residue peptide chain (1αK) was coupled on its heme propionate group. Two peptides, 1αK and/or 1αE, contained a hydrophobic core composed of Leu and Val residues, basic (Lys) and acidic (Glu) residues to direct a heterodimeric two-α-helix structure, and also incorporated a buried Asn residue, which plays an important role to specify the formation of the parallel dimeric coiled-coil (Figure 1).[6,7] As an ET protein, *Escherichia coli* flavodoxin reductase (FDR) was chosen to demonstrate the utility of the specific peptide interaction for the introduction of a large biomolecule near by the heme active site. The FDR contains an FAD as a cofactor and transports electron from NADPH to flavodoxin.[8] The designed helix 1αE was fused to the *C*-terminal of FDR via a 6-residue linker to generate the construct, FDR-1αE. On the basis of the information from three-dimensional structure of FDR,[8] the *C*-terminal end of FDR places nearby the FAD in the active site of a protein. Accordingly, we expected that the heme and FAD cofactors of both proteins would be ar-

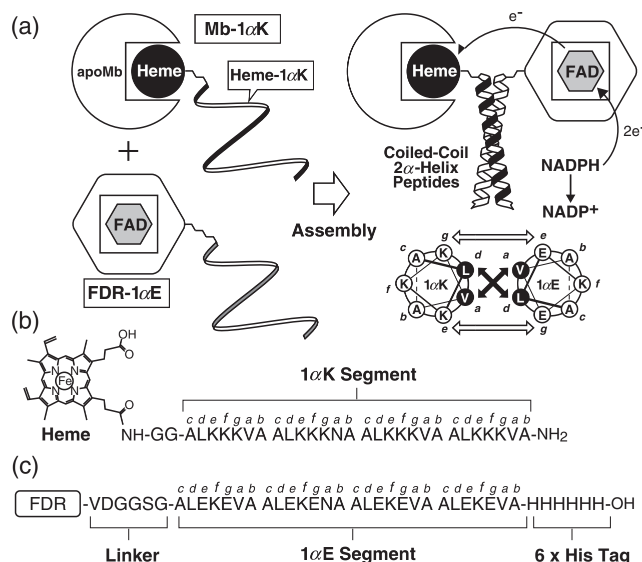

Figure 1. (a) Schematic illustration of an artificial protein assembly using designed coiled-coil peptides; (b) Chemical structure of a designed peptide, Heme-1αK; (c) Structure of a designed protein, FDR-1αE.

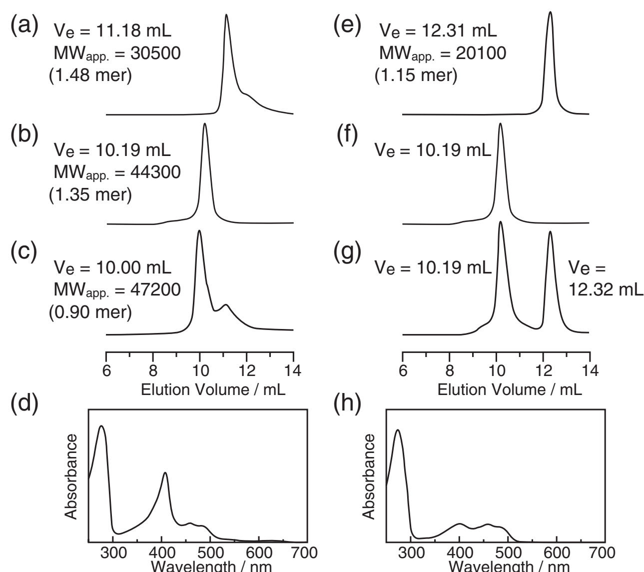

Figure 2. (a–c, e–g) Size-exclusion chromatograms of Mb-1αK alone (a), FDR-1αE alone (b,f), Mb-1αK/FDR-1αE mixture (c), native Mb alone (e), and native Mb/FDR-1αE mixture (g), detection at 280 nm; [protein] = 5 μM; Column, Superdex 75HR 10/30 (1 × 30 cm), 20 mM Tris HCl/150 mM NaCl, pH 7.4 at 25 °C; flow rate, 0.4 mL min^{-1}; Ve denotes elution volume of protein. (d,h) UV–vis Spectra of the first eluting peak from Figure 2c (d) and Figure 2g (h). The spectra were measured using a Shimadzu SPD10AVP photodiode array UV–vis detector.

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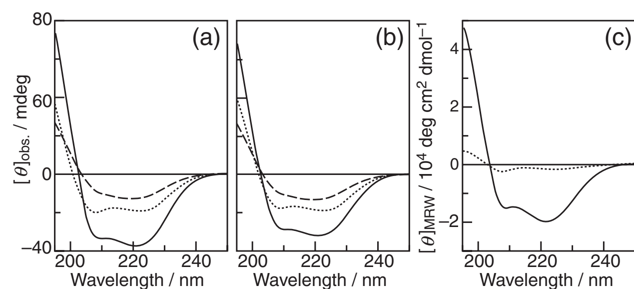

Figure 3. (a) CD Spectra of Mb-1αK (······), FDR-1αE (– –), and Mb-1αK/FDR-1αE mixture (——); (b) CD Spectra of native Mb (······), FDR-1αE (– –), and native Mb/FDR-1αE mixture (——); (c) Difference CD spectra of Mb-1αK/FDR-1αE mixture (——) and native Mb/FDR-1αE mixture (······) obtained by the subtraction of the sum of each protein's spectrum from the spectra of protein mixture. Spectra were measured in 20 mM Tris HCl/150 mM NaCl buffer (pH 7.4) at 25 °C. [Protein] = 5 μM.

ranged in close proximity in the designed assembly. In order to simplify the protein purification, we also appended the 6 × His tag to the *C*-terminal end of the construct. The plasmid containing the gene of the FDR-1αE sequence was prepared using standard methods.[9] The protein was overexpressed in *E. coli* BL21(DE3) and purified with Ni–NTA column.[10]

In order to examine molecular association states of the designed proteins in aqueous solution, the protein samples were passed through a size-exclusion column. Each protein showed a sharp single peak on its chromatogram (Figure 2). The designed proteins were likely to exist as monomeric forms, since the apparent molecular weight (MW_{app}) of each protein, estimated using natural globular proteins as standards, was almost coincident with theoretical one. When the Mb-1αK solution containing the FDR-1αE was chromatographed, a new peak appeared at a higher molecular weight (Figure 2c). The estimated MW_{app} and the UV–vis spectrum of the new peak were identical to those of the Mb-1αK and FDR-1αE complex ($MW_{app.}$ = 47200, 0.9 mer of 1/1 assembly) (Figures 2c, 2d). These results suggested that the Mb-1αK and FDR-1αE bound each other with 1/1 stoichiometry. In contrast, mixing of native Mb and FDR-1αE did not produce a new peak on the chromatogram and each protein was eluted separately (Figure 2g). This indicates that the coiled-coil peptides conjugated to proteins play an essential role to make an assembly in solution.

Circular dichroism (CD) study also revealed the assembly of Mb-1αK and FDR-1αE via the coiled-coil formation (Figure 3). The Mb-1αK showed a CD spectrum typical for α-helix-dominant proteins and is similar to that of native Mb. The CD spectrum of FDR-1αE was almost identical to that of wild-type FDR.[7] On the basis of the difference CD spectrum obtained by subtraction of the sum of each protein's spectrum from the spectrum of Mb-1αK/FDR-1αE mixture, the ellipticity changes at 222 nm was estimated as $-20100\,\mathrm{deg\,cm^2\,dmol^{-1}}$. Taking into consideration that the addition of FDR-1αE to native Mb did not affect their CD spectra (Figures 3b and 3c), it can be reasonably concluded that the α-helix improvement can be ascribed to the coiled-coil formation on the protein.

The ET reaction to the heme center via FDR-1αE was carried out using NADPH as an electron donor under an aerobic condition. Upon addition of NADPH, the absorption maxima at 406 and 630 nm due to met-Mb-1αK decreased with the si-

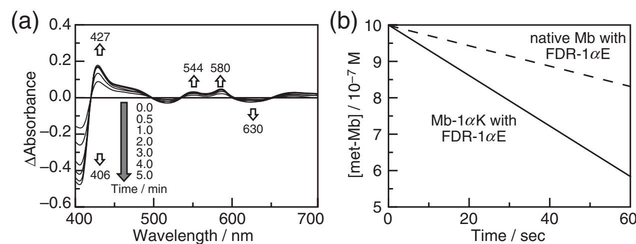

Figure 4. (a) UV–vis difference spectral change of Mb-1αK in the presence of FDR-1αE under aerobic conditions in a buffer at 25 °C. [Mb-1αK] = [FDR-1αE] = 5 μM, [NADPH] = 250 μM; (b) Time courses of met-Mb-1αK and met-Mb reduction by NADPH under aerobic conditions in a buffer (pH 7.4) at 25 °C. [Mb-1αK] = [FDR-1αE] = 1 μM, [NADPH] = 250 μM. The reactions were initiated by the addition of NADPH into the solution and followed by monitoring the absorbance change at 406 nm.

multaneous increase in the absorptions at 427, 544, and 580 nm, which are characteristic of absorptions of oxy-Mb (Figure 4a).[5] The initial rate ($v_{init.}$) for met-Mb-1αK reduction in the presence of FDR-1αE (1.0 equiv.) was $0.42\,\mu\mathrm{M\,min^{-1}}$. On the other hand, native Mb was reduced more slowly under the conditions in the presence of FDR-1αE ($v_{init.} = 0.17\,\mu\mathrm{M\,min^{-1}}$). This indicates that Mb-1αK and FDR-1αE were bound to each other through 1αK–1αE interaction, leading to the higher met-Mb-1αK reduction rate by a factor of about 2.5 compared to the case of native Mb.

In conclusion, we have achieved to create an artificial assembly of proteins. The acceleration of ET rate by the protein assembly via coiled-coil formation will be improved through the optimization of the mutual distance, the relative orientation, and reorganization energy between two active sites in the design. Although detailed study on the structure of the molecular assembly is needed, further systematic screening of the functional units and/or the amino acids in the peptide, Mb, and FDR will lead to the design of artificial protein assembly on the basis of the de novo reconstitutional method.

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References and Notes

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9 The FDR gene was amplified by PCR using chromosomal DNA of *E. coli* XL1-Blue strain and cloned into pET22b(+) plasmid vector (Novagen) to produce a pET22b-FDR plasmid. The gene for 1αE segment was chemically synthesized and ligated to pET22b-FDR to give a pET22b-FDR-1αE plasmid.
10 The purified FDR-1αE showed a single band at the proper molecular weight on SDS-polyacrylamide gel. A UV–vis spectrum of FDR-1αE was essentially identical to that of wild-type FDR in a buffer.

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