De Novo Design, Synthesis, and Function of Semiartificial Myoglobin Conjugated with Coiled-Coil Two-α-Helix Peptides

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Abstract: The introduction of a flavin chromophore on the myoglobin (Mb) surface and an effective electron-transfer (ET) reaction through the flavin were successfully achieved by utilizing the self-assembly of heterostranded coiled-coil peptides. We have prepared a semiartificial Mb, named Mb-1αK, in which an amphiphilic and cationic α-helix peptide is conjugated at the heme propionate (Heme-1αK). Heme-1αK has a covalently bound iron–protoporphyrin IX (heme) at the N terminus of a 1αK peptide sequence. This sequence was designed to form a heterostranded coiled-coil in the presence of a counterpart amphiphilic and anionic 1αE peptide sequence in a parallel orientation. Two peptides, Fla[1]-1αE and Fla[31]-1αE, both incorporating a 10-methylisoalloxazine moiety as an artificial flavin molecule, were also prepared (Fla = 2-[7-(10-methyl)isoalloxazinyl]-2-oxoethyl). Heme-1αK was successfully inserted into apomyoglobin to give Mb-1αK. Mb-1αK recognized the flavin-modified peptides and a two-α-helix structure was formed. In addition, an efficient ET from reduced nicotinamide adenine dinucleotide to the heme center through the flavin unit was observed. The ET rate was faster in the presence of Fla[1]-1αE than in the presence of Fla[31]-1αE or the equivalent molecule that has no peptide chain. These results demonstrate that the introduction of a functional chromophore on the Mb surface can be achieved by using specific peptide–peptide interactions. Moreover, the dependence of the ET rate on the position of the flavin indicated that the distance between the heme active site and the flavin chromophore was regulated by the three-dimensional structure of the designed polypeptide.

Keywords: chromophores · electron transfer · helical structures · heme proteins · myoglobin

Introduction

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 involves incorporation of an artificial heme with multiple functional groups into apomyoglobin (apo-Mb).[2–4] For example, Hamachi and co-workers have synthesized artificial heme molecules modified with various functional chromophores, such as catenane,[2a–c] a ruthenium–tris(bipyridine) complex,[2d–g] iminodiacetic acid,[2h] riboflavin,[2i] C_{60},[2j] lipids,[2k] β-cyclodextrin,[2l–n] and boronic acids.[2o–q] By using these synthetic heme molecules, they achieved the functional conversion and regulation of Mb. As another example, Hayashi and co-workers applied the reconstitution strategy to create a new molecular recognition site near to the heme center.[3] They introduced multiple charged groups or hydrophobic units at the two heme propionates and demonstrated the recognition of small substrates and cytochrome *c* on the protein surface. Willner and co-workers conducted a vectorial photoinduced electron transfer (ET) with Zn^{II}–protoporphyrin IX modified with bipyridinium groups and introduced by using the reconstitution method.[4]

Although the incorporation of a chemically modified cofactor into the apoprotein is valuable for active-site-directed incorporation of various functional groups, the previously reported functionalities were relatively small organic and/or inorganic molecules. In addition, there are few examples in which the distance and orientation between the heme and the introduced functionalities were considered. In order to create an artificial protein that satisfies tailor-made functions and/or substrate specificity, it will be necessary to control the mutual arrangement of the heme active site and the functional groups. As well as considering these aspects, we

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decided to attempt the introduction of a polypeptide segment into the heme propionate group by the replacement of natural heme with a heme-conjugated peptide (Figure 1). By utilizing the rigid three-dimensional structure of the polypeptide, it should be possible to control the relative disposition of the heme active site, the introduced functionalities, and the amino acid side chains in a defined 3D space. In addition, because the peptide may provide various interactions, including charge–charge interactions, hydrophobic interactions, van der Waals contacts, and hydrogen bonding, it was expected that specific molecular recognition, comparable to that in natural systems, could be achieved.

In this study, we have used a designed two-α-helix structure for the introduction of a flavin derivative onto the surface of Mb. A heterostranded coiled-coil was chosen to demonstrate the specific peptide interaction on the Mb surface, because the coiled-coil structure is one of the motifs that have been well studied and established in the field of de novo design of polypeptides.[5–11] One of the heterostranded coiled-coil chains (1αK) was conjugated at the heme propionate of Mb. The flavin chromophore was attached at different positions in the other strand (1αE) in order to control the mutual distance between the heme active site and the flavin chromophore. By utilizing the noncovalent interaction between two peptides, we tried to introduce the flavin on the Mb surface and to estimate the interaction by using UV/Vis, circular dichroism, and fluorescence spectroscopy measurements. Additionally, we observed the ET reaction from NADH to the heme center with the flavin as an electron mediator. We report here the utility of the designed 3D polypeptide structure for the regulation of the distance between the heme center and the introduced functional group on the semiartificial protein surface.

Results and Discussion

Design and synthesis of peptides: Two peptide sequences, 1αK and 1αE, were designed to give a heterodimeric two-α-helix structure by mutual interactions (Figure 2). The design for the helices was based on the sequences reported by Hodges and co-workers and Kim and co-workers.[5b,6c–e] Both 28-residue segments were designed to take an amphiphilic α-helix structure, with the Leu and Val residues deployed on the α-helix segment in the same manner as hydrophobic amino acids in coiled-coil proteins. Coiled-coil sequences in proteins consist of heptad repeats containing two characteristic hydrophobic positions, that is, $(abcdefg)_n$ in which the *a* and *d* residues are hydrophobic ones, such as Leu and Val. Each of the two designed peptide sequences consists of a heptad in which *a* is Val, *d* is Leu, *b* and *c* are Ala, and *f* is Lys. Instead of Val, a single Asn residue was placed at a buried *a* position. This modification was based on the sequence of the native GCN4 Leu-zipper where the Asn plays an important role in specifying the parallel dimer formation of the coiled-coil structure.[6a,b,d,8b,12] The 1αK peptide contains positively charged Lys residues and the 1αE contains negatively charged Glu residues at both the *e* and *g* positions. Both 1αK and 1αE would exhibit interhelical ionic repulsions in a homostranded coiled-coil and the two-α-helix structure would thereby be destabilized. By contrast, the formation of a heterostranded coiled-coil consisting of 1αK and 1αE, which would be stabilized by interhelical E–K ionic attractions, is expected. The Fe–protoporphyrin IX was introduced at the N terminus of the 1αK peptide by an amide bond between the heme propionyl group and the α-amino group of the first Gly residue (Heme-1αK). The 10-methylisoalloxazine moiety was attached to the side chain of a Cys residue linked at the first or thirty-first posi-

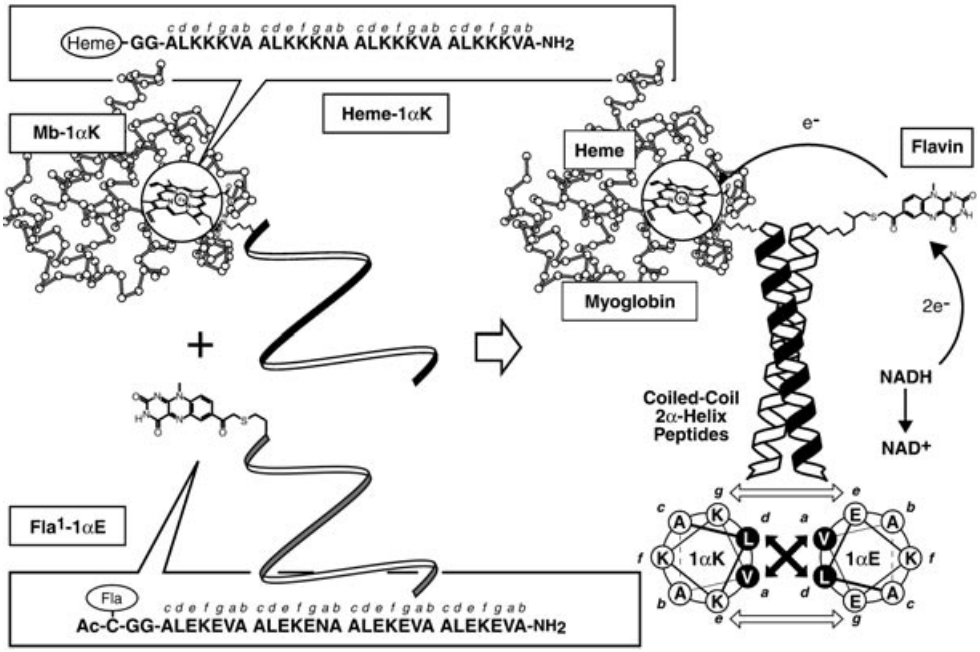

Figure 1. Schematic illustration of a semiartificial Mb conjugated with designed coiled-coil peptides. Fla = 2-[7-(10-methyl)isoalloxazinyl]-2-oxoethyl, NADH = reduced nicotinamide adenine dinucleotide, NAD^+ = oxidized nicotinamide adenine dinucleotide.

tion of the 1αE peptide (Fla[1]-1αE and Fla[31]-1αE, respectively) through a flexible Gly–Gly spacer. In addition to these heme- and flavin-conjugated peptides, we prepared W[1]-1αK, Dns[1]-1αE, and Dns[30]-1αE, in order to easily characterize the interaction between the 1αK and 1αE sequences (Figure 2). The peptide W[1]-1αK has a Trp residue at the N terminus of the 1αK peptide. In Dns[1]-1αE and Dns[30]-1αE, a dansyl group was introduced at the α-amino group of the Gly residue at position 1 and at the ε-amino group on the side chain of the Lys residue at position 30, respectively. By utilizing Trp and Dns groups as fluorescent probes, we intended to estimate the relative orientation and oligomerization state of the originally designed coiled-coil polypeptides.

The peptides were synthesized by means of a solid-phase method with the Fmoc strategy (Fmoc = 9-fluorenylmethoxycarbonyl).[13] The *N*-hydroxysuccinimide monoester of protoporphyrin IX was allowed to react with the Gly[1] α-amino group of the 1αK peptide on a resin.[14] After cleavage from the resin with trifluoroacetic acid, iron(III) was inserted into the protoporphyrin IX in the peptide by adding $Fe(OAc)_2$ in 50% AcOH/trifluoroethanol (TFE) to the mixture at 50 °C under nitrogen. For the preparation of flavin-conjugated peptides, 7α-bromoacetyl-10-methylisoalloxazine was allowed to react with the Cys side chains of purified 1αE peptides in 50% TFE/0.1 M tris(hydroxymethyl)aminomethane·HCl (Tris·HCl) buffer (pH 8.5).[15,16] The ε-amino group of Lys30 in Dns[30]-1αE was specifically protected with a 4-methyltrityl (Mtt) group to introduce the Dns group at the later stage. The Mtt group was removed by treating the peptide resin with dichloromethane/TFA/triisopropylsilane (94:1:5, 5×2 min) without cleavage of the other protecting groups and dansyl chloride was allowed to react with the ε-amino group of Lys30 on the resin. All the peptides were purified with reversed-phase HPLC (RP-HPLC), and identified by amino acid analysis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The amino acid analysis was also utilized for determination of the peptide concentration of the stock solutions.

CD and fluorescence spectroscopy studies of W[1]-1αK, Dns[1]-1αE, and Dns[30]-1αE: The conformation of the designed 1αK and 1αE peptides in solution was determined by CD measurements on W[1]-1αK, Dns[1]-1αE, and Dns[30]-1αE. The three peptides exhibited CD spectra characteristic of a random coil in 20 mM Tris·HCl buffer (pH 7.4), whereas they showed an α-helical pattern in TFE, which is known to be a helix-stabilizing solvent.[5b] Table 1 summarizes the molecular ellipticities at 222 nm ($[\theta]_{222}$) and the α-helix contents estimated from the $[\theta]_{222}$ values for the peptides.[17] We did not consider the potential contribution of the added aromatic chromophores (Dns and Trp) to the far-UV CD spectra of the polypeptides since the peptides showed weak CD intensity in the absorption region of the Dns and Trp groups. Neither the 1αK nor the 1αE sequence took a homostranded coiled-coil form in the buffer, possibly due to interhelical ionic repulsion.[5b] On the other hand, an equimolar mixture of peptides W[1]-1αK and Dns[1]-1αE showed a typical α-helical CD pattern with two negative maxima at 208 and 222 nm in buffer (Figure 3). From the $[\theta]_{222}$ value, the α helicity was estimated as 96%. As the ratio of the two peptides was changed, the peptide mixture showed a maximum α helicity at a [W[1]-1αK]/[Dns[1]-1αE] ratio of 1:1, a result suggesting that the 1αK and 1αE peptides formed a heterostranded coiled-coil with 1:1 stoichiometry (Figure 3b). Similar CD results were observed in the case of the W[1]-1αK and Dns[30]-1αE peptide mixture (Figure 3c,d). This result suggested that the position of the Dns group did not largely affect the coiled-coil structure composed of 1αK and 1αE sequences.

Table 1. Molecular ellipticities and α-helix contents of the designed peptides.

Peptide	In buffer (pH 7.4)		In TFE	
	$[\theta]_{222}$[a]	α helicity[b]	$[\theta]_{222}$[a]	α helicity[b]
W[1]-1αK	−3460	10	−24160	71
Dns[1]-1αE	−4680	13	−21750	66
Dns[30]-1αE	−5010	15	−23830	70
W[1]-1αK+Dns[1]-1αE	−32700	96	–	–
W[1]-1αK+Dns[30]-1αE	−30600	90	–	–

[a] [θ] values are expressed as mean residue ellipticities [deg cm^2 dmol^{-1}]. [b] α Helicities are estimated from $[\theta]_{222}$ values by using the equation described by of Scholtz et al.[17]

Fluorescence titration of W[1]-1αK with Dns[1]-1αE or Dns[30]-1αE was carried out in buffer (pH 7.4) in order to estimate the relative helix orientation of the heterostranded 1αK–1αE coiled-coil (Figure 4). Upon increasing the concentration of Dns[1]-1αE, a remarkable decrease in fluores-

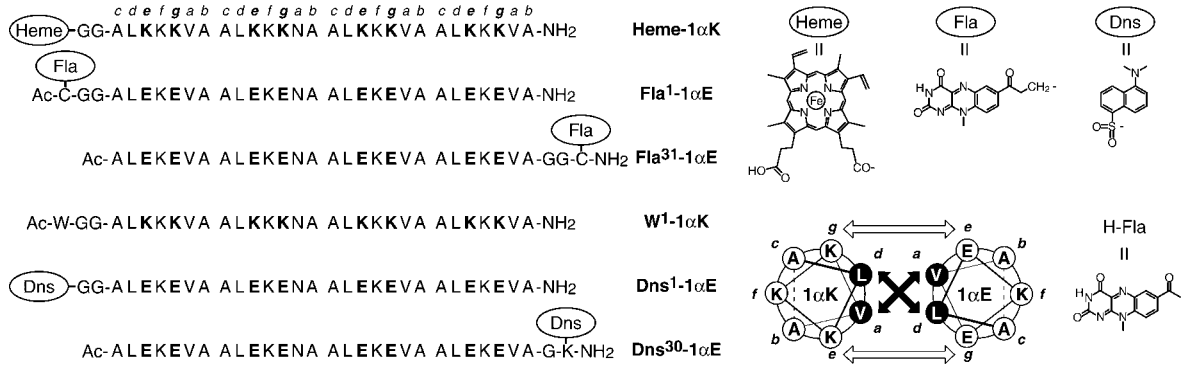

Figure 2. Chemical structures of the designed peptides, Heme-1αK, Fla[1]-1αE, Fla[31]-1αE, W[1]-1αK, Dns[1]-1αE, Dns[30]-1αE, and H-Fla. Dns = dansyl.

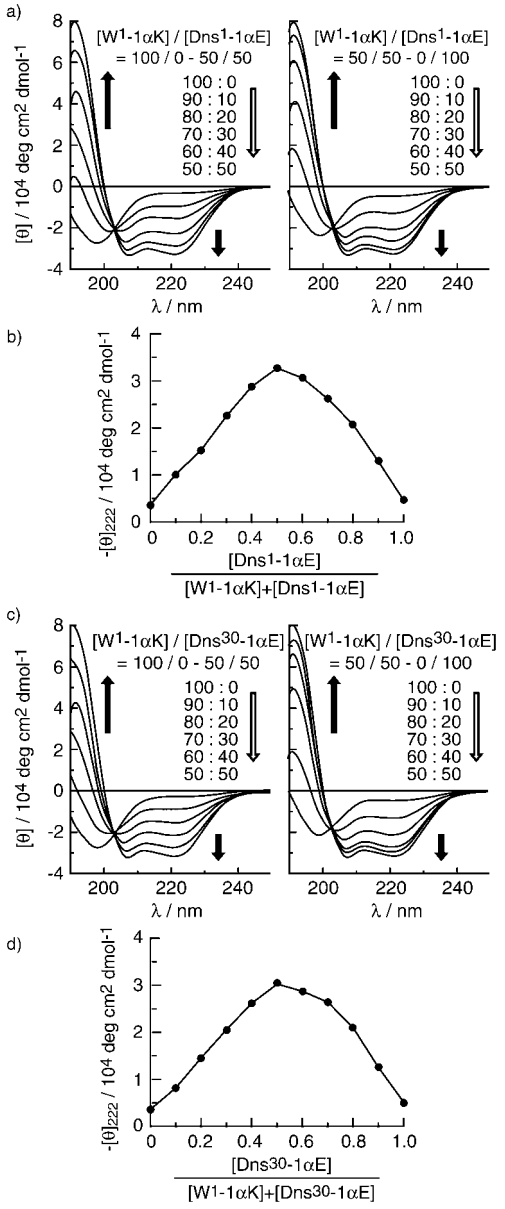

Figure 3. a) CD spectra of peptide mixture W^1-1αK and Dns^1-1αE at W^1-1αK/Dns^1-1αE ratios of 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, and 0:100 in buffer (pH 7.4) at 25 °C. b) Plot of the mean residue ellipticity at 222 nm versus the W^1-1αK/Dns^1-1αE ratio. c) CD spectra of peptide mixture W^1-1αK and Dns^{30}-1αE at W^1-1αK/Dns^{30}-1αE ratios of 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, and 0:100 in buffer (pH 7.4) at 25 °C. d) Plot of the mean residue ellipticity at 222 nm versus W^1-1αK:Dns^{30}-1αE ratio. [total peptide] = 20 μM.

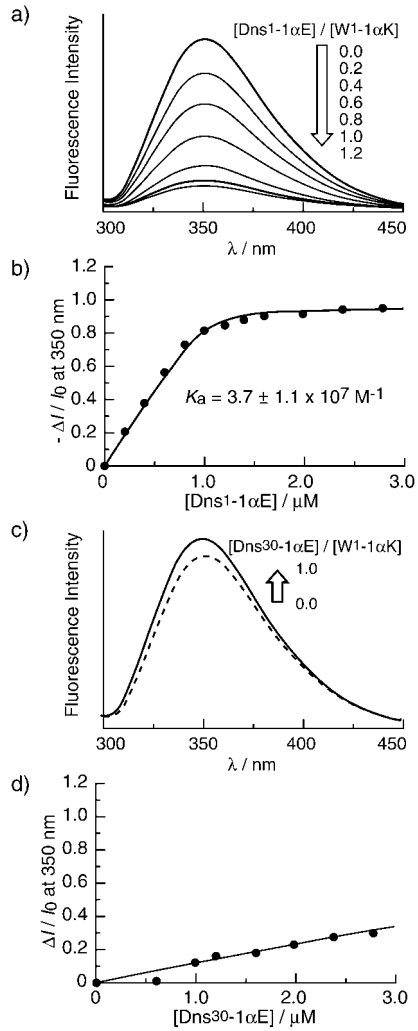

Figure 4. a) Fluorescence spectra of W^1-1αK with increasing concentrations of Dns^1-1αE in buffer (pH 7.4) at 25 °C. b) Plot of the fluorescence intensity at 350 nm as a function of Dns^1-1αE concentration in buffer (pH 7.4) at 25 °C. c) Fluorescence spectra of W^1-1αK in the presence (——) and absence (-----) of Dns^{30}-1αE (1.0 equiv) in buffer (pH 7.4) at 25 °C. d) Plot of the fluorescence intensity at 350 nm as a function of Dns^{30}-1αE concentration in buffer (pH 7.4) at 25 °C. [W^1-1αK] = 1.0 μM, λ_{ex} = 292 nm.

cence intensity of Trp1 at 350 nm was observed (Figure 4a). In contrast, addition of Dns^{30}-1αE had little effect on the fluorescence spectrum of W^1-1αK. This result suggests that the Trp1 residue and the Dns moiety at the N terminus of Dns^1-1αE exist in close proximity in the heterostranded coiled-coil and effective fluorescence resonance energy transfer (FRET) therefore occurs between the two chromophores. Meanwhile, the distance between Trp1 and the Dns group on the side chain of Lys30 is too long to induce FRET between the two chromophores. Accordingly, we propose that the designed 1αK and 1αE sequences form a coiled-coil structure in a parallel orientation. The plot of the change in Trp fluorescence as a function of Dns^1-1αE concentration showed the beginning of a plateau at a [W^1-1αK]/[Dns^1-1αE] ratio of 1:1, a fact supporting the formation of a 1:1 heterostranded coiled-coil of 1αK and 1αE peptides. The binding constant (K_a) determined based on the change of fluorescence intensity at 350 nm by using a 1:1 binding equation[18] was $3.7 \times 10^7\ M^{-1}$. This value is sufficient to obtain the dimeric coiled-coil structure in the concentration range used in this study.

Reconstitution of Heme-1αK with apo-Mb: The reconstitution of Heme-1αK with apo-Mb was carried out according to the methods reported by Hamachi and co-workers with minor modifications.[2] Heme-1αK in 50% TFE/20 mM Tris·HCl buffer (pH 7.4) was mixed with apo-Mb in the same buffer in an ice-bath. The reaction mixture was incu-

bated at 4 °C overnight. After centrifugation at 4 °C, the reaction solution was purified by using a Sephadex G-50 size-exclusion column and 20 mM Tris·HCl (pH 7.4).

The reconstitution was characterized by the UV/Vis titration of Heme-1αK with apo-Mb in buffer (pH 7.4). Heme-1αK itself showed a Soret band at 392 nm and a prominent broad band near 350 nm. Similar spectra have been reported for some heme–peptide adducts[19,20] and can be attributed to intermolecular heme–heme association. Simple porphyrins are known to aggregate at micromolar concentrations in aqueous solution[21] and similar behavior would be expected for Heme-1αK since the heme was introduced at the N terminus of the peptide and it will be exposed to the solvent. Upon increasing the concentration of apo-Mb, an increase of the Soret band at 409 nm and a decrease of the broad band at around 350 nm were observed with an isosbestic point at 390 nm (Figure 5a). The plot of the absorbance

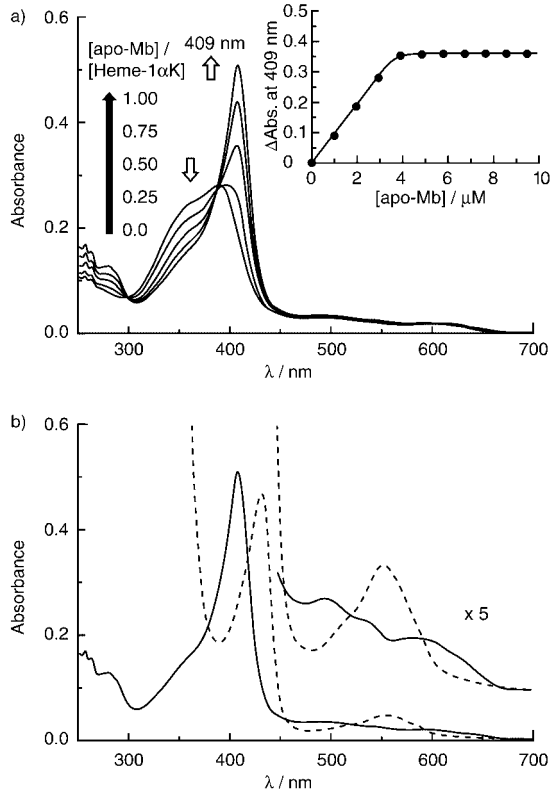

Figure 5. a) UV/Vis spectra of Heme-1αK with increasing apo-Mb concentrations in buffer (pH 7.4) at 25 °C. [Heme-1αK] = 4 μM. Inset: Plot of absorbance change at 409 nm as a function of apo-Mb concentration. b) UV/Vis spectra of Mb-1αK in the met-Mb form (——) and deoxy-Mb form (-----) in buffer (pH 7.4) at 25 °C. [Mb-1αK] = 4 μM.

change at the Soret band as a function of apo-Mb concentration showed the beginning of a plateau at the [apo-Mb]/[Heme-1αK] ratio of 1:1, a result suggesting that apo-Mb bound the Heme-1αK with 1:1 stoichiometry to give a semiartificial Mb. The K_a value of apo-Mb toward Heme-1αK was too large to be estimated correctly under the conditions utilized. Aggregation of Heme-1αK apparently did not affect the titration curve in the micromolar concentration range because of the large K_a value.

Figure 5b shows the UV/Vis spectra of the reconstituted Mb (Mb-1αK). The absorption maxima at 409 nm (sharp Soret band), 501 nm (β-band (Q_v)), and 540 nm (α-band (Q_o)) are almost coincident with the values of the native Mb under the experimental conditions (Table 2). The most significant difference between the spectra of Mb-1αK and native Mb is the π–iron charge-transfer band. While the native Mb showed the π–iron charge-transfer band at 630 nm, only a shoulder was observed near 625 nm in the spectrum of Mb-1αK because of the enhanced absorbance near 600 nm. By the addition of an azide or fluoride anion, the sixth ligand of the heme iron center in met-Mb is readily exchanged from water to the anion and the spectroscopic properties with the ligand binding are useful for probing the heme environment of Mb. When the sixth ligand was changed, Mb-1αK showed spectra similar to those of native Mb (λ_{max} (fluoride form) = 406, 488, and 603 nm; λ_{max} (azide form) = 419, 541, and 574 nm; Table 2). Additionally, a deoxy-Mb-1αK (ferrous heme state) was also obtained by adding a minimal amount of sodium dithionite to a solution of met-Mb-1αK in buffer (Soret band at 434 nm and Q-band at 556 nm; Figure 5b). It is suggested that the heme environment of the Mb-1αK is almost identical to that of native Mb.

Table 2. Wavelengths of the peak maxima in the UV/Vis spectra of Mb-1αK and native Mb in buffer.

Conditions	Protein	Soret band [nm^{-1}]	Q-bands [nm^{-1}]
met form	Mb-1αK	409	501, 540, 625[a]
	native Mb	409	504, 540, 634
F^- form[b]	Mb-1αK	406	488, 603
	native Mb	408	495, 606
N_3^- form[b]	Mb-1αK	419	541, 574
	native Mb	421	541, 574
deoxy form	Mb-1αK	433	556
	native Mb	434	556

[a] Shoulder. [b] The UV/Vis spectra of the F^- form and the N_3^- form were measured by addition of 30 mM KF and 20 mM NaN_3, respectively.

Fluorescence study of Fla[1]-1αE and Fla[31]-1αE in the presence and absence of Mb-1αK: The fluorescence spectra of the flavin-conjugated peptides Fla[1]-1αE and Fla[31]-1αE were measured in order to evaluate the interaction between the 1αK and 1αE segments on the Mb surface (Figure 6). Peptides Fla[1]-1αE and Fla[31]-1αE showed a fluorescence emission peak at 505 nm in buffer. The fluorescence intensity of the flavin-conjugated peptides was much weaker than that of the 7-acetyl-10-methylisoalloxazine (H-Fla) molecule that has no peptide chain. This is attributed to the flavin linked to the sulfur atom of the Cys side chain as reported previously.[16c] On addition of Mb-1αK (1.0 equiv), the fluorescence intensities of the flavin-conjugated peptides were decreased to approximately 70% of those observed in the absence of Mb-1αK. On the other hand, the addition of native Mb or apo-Mb had little effect on the fluorescence intensity of the flavin in the peptides. Furthermore, there was no change in the fluorescence spectrum of the H-Fla molecule on addition of Mb-1αK or Mb. These results indicate that

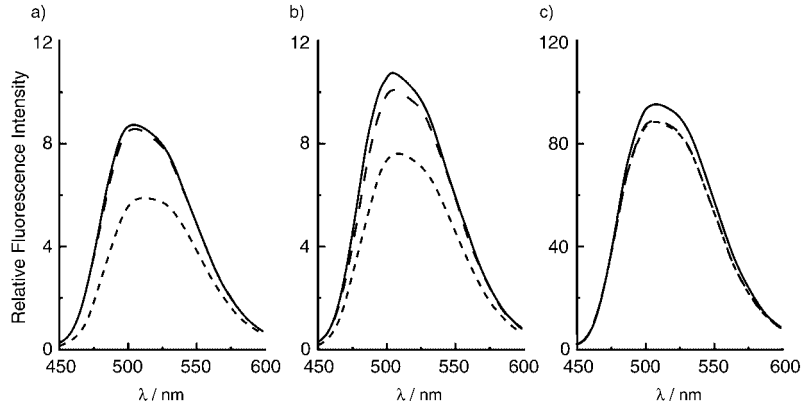

Figure 6. Fluorescence spectra of a) Fla[1]-1αE, b) Fla[31]-1αE, and c) H-Fla in the absence (——) and presence of Mb-1αK (-----) and the presence of native Mb (– – –) (1.0 equiv) in buffer (pH 7.4) at 25 °C. [Fla-1αE] = 2 μM, λ_{ex} = 430 nm.

the 1αE sequence was successfully recognized by the 1αK segment conjugated with the heme propionate.

CD spectra of Mb-1αK in the presence and absence of Fla-1αE peptides: Mb-1αK showed a CD spectrum with two negative maxima at 208 and 222 nm in buffer (Figure 7). This CD pattern is typical for α-helix-dominant proteins and is similar to those of native Mb and apo-Mb.[22] However, the CD intensity of Mb-1αK was lower than that of the native Mb, which might indicate that the 1αK segment introduced at the heme propionate group partially perturbs the 3D structure of Mb. On the other hand, the α-helix content of Mb-1αK was dramatically increased by the addition of Fla[1]-1αE or Fla[31]-1αE in a buffer. Taking into consideration that the addition of Fla-1αE peptides to native Mb and apo-Mb did not affect their CD spectra (data not shown), it can be reasonably concluded that the α-helix improvement can be ascribed mainly to the two-α-helix coiled-coil formation of Fla-1αE and the 1αK segment on the Mb surface. On the presumption that the observed changes of the CD spectra were due only to the conformational change of the 1αK and 1αE segments, the ellipticity changes at 222 nm were estimated as −22900 (67%) and −25900 deg cm^2 dmol^{-1} (76%) for Fla[1]-1αE and Fla[31]-1αE, respectively, based on the differential CD spectra (Figure 7b,c, insets).

Denaturation experiments by using guanidine hydrochloride: Denaturation experiments with guanidine hydrochloride (GuHCl) were conducted for the peptide-conjugated Mb in the presence and absence of Fla[1]-1αE peptide in buffer (pH 7.4; Figure 8). Increasing amounts of the denaturant destroyed the 3D structure of Mb, so as to release the heme cofactor or Heme-1αK from the heme crevice. The denaturation process was spectrophotometrically monitored by the broadening of the Soret band of the heme.[23] The stabilities of Mb-1αK with and without Fla-1αE were expressed as the GuHCl concentration at which half of the Mb-1αK was unfolded ($[GuHCl]_{1/2}$). Both in the absence and presence of Fla-1αE peptides, Mb-1αK exhibited a cooperative denaturation curve in buffer, a result suggesting that the Mb portion was retaining its native-like properties. However, Mb-1αK was less resistant to GuHCl denaturation ($[GuHCl]_{1/2}$ = 2.0 M) than the native Mb was ($[GuHCl]_{1/2}$ = 2.5 M) under the experimental conditions. By the linear extrapolation method, the free energy changes ($-\Delta G_{H_2O}$) of Mb-1αK and native Mb were evaluated as −5.3 and −8.6 kcal mol^{-1}, respectively. The 1αK segment conjugated at the heme propionate group seemed to destabilize the 3D structure of Mb. The addition of Fla[1]-1αE peptide caused further destabilization of the Mb domain ($[GuHCl]_{1/2}$ = 1.8 M, $-\Delta G_{H_2O} = -4.5$ kcal mol^{-1}). Possibly, the formation of a large coiled-coil structure near to the heme crevice is sterically unfavorable for the 3D structure of Mb. However, the change in the stability of Mb-1αK on addition of Fla-1αE

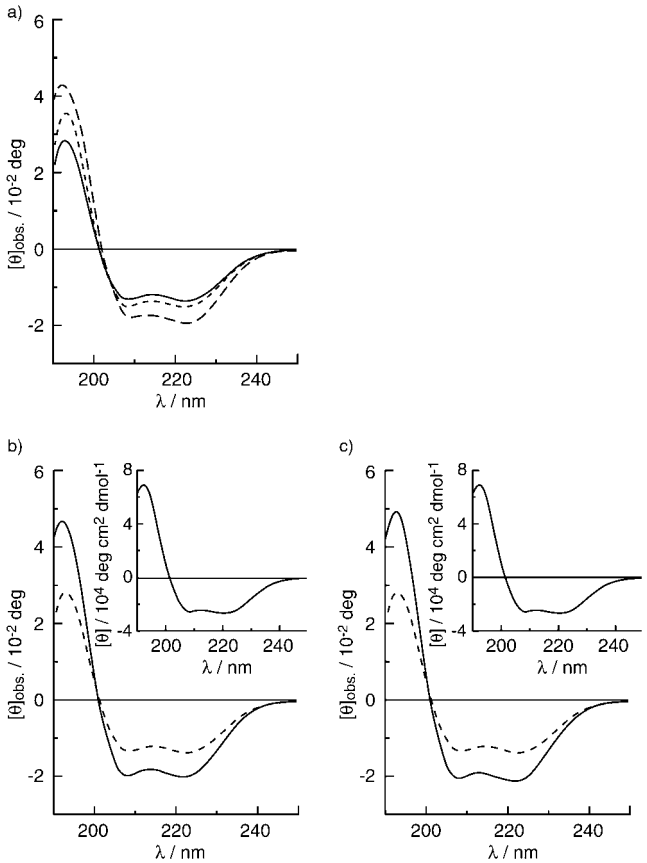

Figure 7. a) CD spectra of Mb-1αK (——), Mb (– – –), and apo-Mb (-----). b) CD spectra of Mb-1αK in the presence (——) and absence (-----) of Fla[1]-1αE (1.0 equiv). c) CD spectra of Mb-1αK in the presence (——) and absence (-----) of Fla[31]-1αE (1.0 equiv). Inset: Difference CD spectrum obtained by subtraction of the spectrum of Mb-1αK alone from the spectrum of the mixture of Mb-1αK and Fla-1αE. Spectra were measured in buffer (pH 7.4) at 25 °C by using a cell with a 1-mm path length. [protein] = 5 μM.

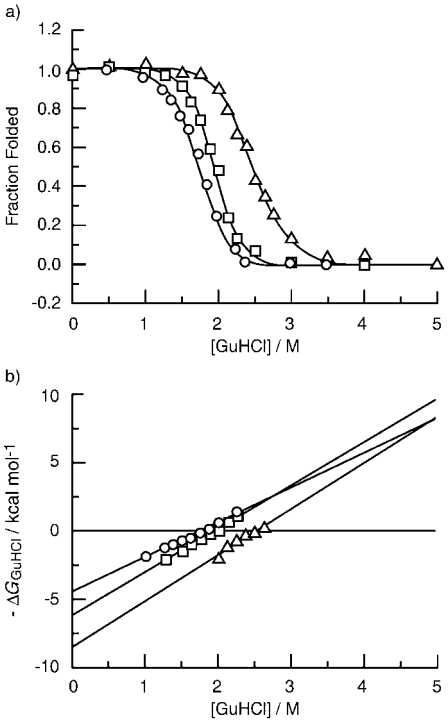

Figure 8. a) GuHCl denaturation profiles of Mb and Mb-1αK. b) Profiles of $-\Delta G_{GuHCl}$ at different GuHCl concentrations. Measurements were carried out in a buffer (pH 7.4) at 25 °C. Native Mb (△), Mb-1αK in the absence of Fla^1-1αE (□), and Mb-1αK in the presence of Fla^1-1αE (1.0 equiv; ○). [Protein] = 5 μM.

peptide is evidence for specific interaction between the 1αK and 1αE peptides on the Mb, since no change in the GuHCl resistance of the native Mb was observed on addition of Fla-1αE peptide.

The ET reaction from NADH to heme through the flavin unit: The ET reaction to the heme center through the flavin moiety was examined by using NADH as an electron donor under aerobic conditions. On addition of NADH, the reduction of the heme iron center and the production of oxy-Mb-1αK occurred simultaneously, as monitored with the UV/Vis spectrum (Figure 9). The pseudo-first-order rate constants, k_{obs}, calculated from the initial rates were proportional to the concentration of NADH (Figure 10). The apparent second-order rate constant, k_{2nd}, for oxy-Mb-1αK production in the presence of Fla^1-1αE (1.0 equiv) was 161 $M^{-1}s^{-1}$. On the other hand, both in the presence and absence of H-Fla lacking the 1αE peptide, the Mb-1αK was slowly reduced by NADH (k_{2nd} = 16.3 and 10.6 $M^{-1}s^{-1}$, respectively; Figure 11). These results indicate that Fla^1-1αE and Mb-1αK are bound to each other through the 1αK-1αE interaction, an interaction leading to an oxy-Mb-1αK production rate that is faster by a factor of about 10 than that observed in the case with peptide-free H-Fla. The Fla^{31}-1αK also accelerated the electron transfer from NADH to the heme, although the rate constant was about one-fifth of that observed with Fla^1-1αE (k_{2nd} = 32.3 $M^{-1}s^{-1}$). The flavin unit introduced on the Cys1 side chain of 1αE seems to be positioned near to the heme center and acts more effectively as an electron mediator

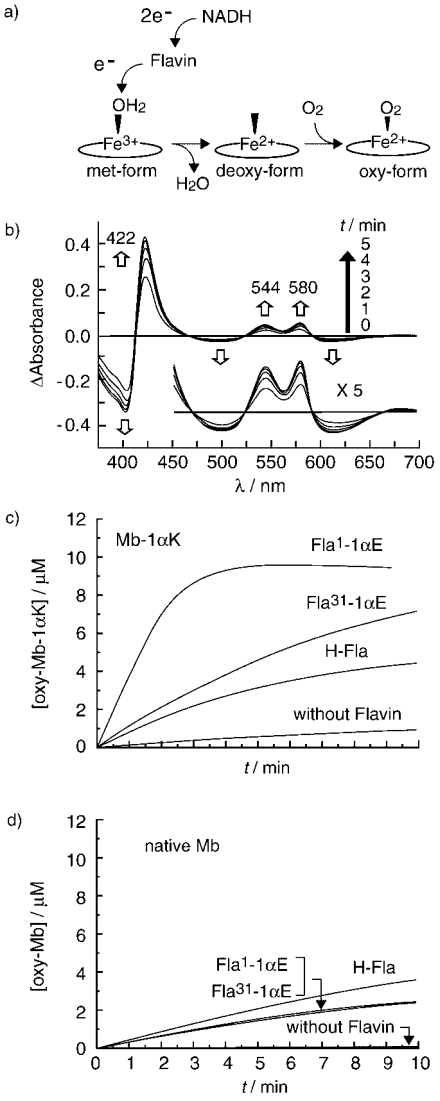

Figure 9. a) Possible reaction scheme of oxy-Mb-1αK formation. b) Difference spectra of Mb-1αK in the presence of Fla^1-1αE under aerobic conditions in buffer at 25 °C. [Mb-1αK] = 10 μM, [Fla^1-1αE] = 10 μM, [NADH] = 25 μM. c) Time-course plots of oxy-Mb-1αK formation by NADH under aerobic conditions in buffer (pH 7.4) at 25 °C. [Mb-1αK] = 10 μM, [Flavin] = 10 μM, [NADH] = 50 μM. c) Time-course plots of oxy-Mb formation by NADH under aerobic conditions in buffer (pH 7.4) at 25 °C. [Mb] = 10 μM, [Flavin] = 10 μM, [NADH] = 50 μM. The reactions were initiated by addition of NADH into the Mb-containing aqueous solution and followed by monitoring the absorbance change at 580 nm.

than that on the Cys31 side chain. These results suggest that the 1αK segment conjugated with the Fla-1αE peptide bound to the heme propionate, thereby forming a parallel two-α-helix structure on the surface of Mb.

In contrast to the above results, the addition of Fla^1-1αE and Fla^{31}-1αE into the native Mb system decreased the rate of oxy-Mb formation by a factor of 2.0 relative to that in the presence of H-Fla. As there was no significant difference in the reaction rate between native Mb and Mb-1αK in the presence of H-Fla (k_{2nd} = 17.5 and 16.3 $M^{-1}s^{-1}$, respectively), it seems that the 1αE sequence conjugated to the flavin molecule interrupts the ET process from NADH to the heme through the flavin molecule. One of the reasons for this

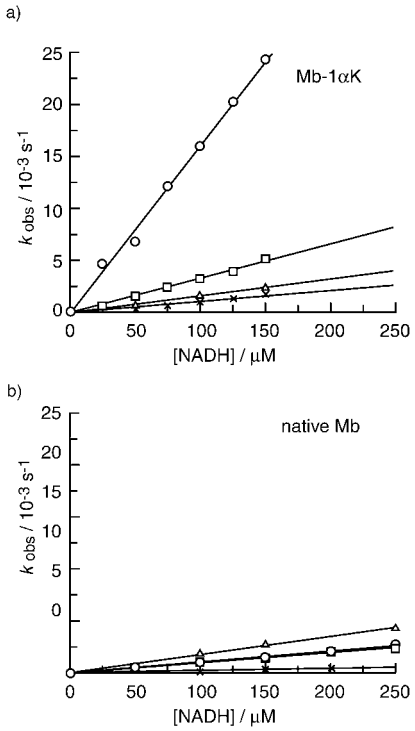

Figure 10. Profiles of the pseudo-first-order rate constants (k_{obs}) of a) oxy-Mb-1αK and b) oxy-Mb formation in the absence (×) and presence of Fla^{1}-1αE (○), Fla^{31}-1αE (□), or H-Fla (△) as a function of NADH concentration under aerobic conditions in buffer (pH 7.4) at 25 °C. [Mb-1αK] and [Mb] = 10 μM, [Flavin] = 10 μM.

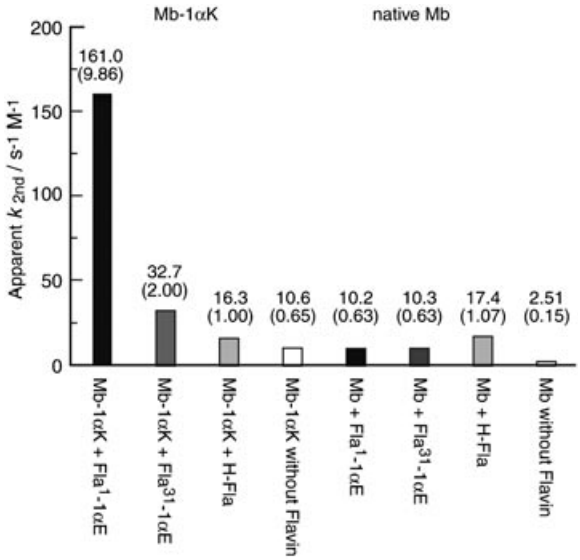

Figure 11. Apparent second-order rate constants (k_{2nd}) for oxy-Mb-1αK and oxy-Mb formation by NADH in the presence or absence of various peptides.

might be electrostatic repulsion between NADH and the Fla-1αE peptide because the net charge of the 1αE sequence is −4 (Glu×8 and Lys×4). This observation also confirms that the interaction between the peptide segments is responsible for the effective ET function in the present system.

Conclusion

Heme- and flavin-conjugated peptides, Heme-1αK and Fla-1αE, which formed a heterodimeric coiled-coil structure, were successfully designed and synthesized. By the incorporation of Heme-1αK into apo-Mb, we have produced a de novo semiartificial Mb which possesses an artificial peptide segment at the heme propionate group. Although the covalently introduced peptide segment on the heme propionate partially perturbed the 3D structure and caused the destabilization of Mb, the Mb-1αK showed cooperative denaturation curves, as seen in natural small proteins. This result implies that the Mb-1αK maintains most of its native-like properties. Spectroscopic measurements including CD, UV/Vis, and fluorescence spectroscopy studies showed that the attached 1αK segment recognized counterpart Fla-1αE peptides and together they take the form of a two-α-helix coiled-coil structure on the Mb surface. The flavin unit introduced close to the heme center through the specific 1αK–1αE interaction functioned as an efficient electron mediator and enhanced the ET rate by a factor of ≈10, relative to the H-Fla molecule lacking the 1αE peptide. The ET rate was different depending on the position of the flavin unit on the 1αE sequence, which can be regulated by the peptide design. The different ET rates could be due to the distance between the heme and the flavin mediator that arises from the parallel coiled-coil structure. This demonstrates that the designed peptide is useful for controlling of the three-dimentional arrangement of the heme active site and other functional units and for modulating the ET reaction on the protein. The novel strategy for the preparation of semiartificial Mb described in the present paper may permit the systematic study of the ET reaction on the protein surface. Since the flavin chromophore attached on the 1αE sequence can be replaced with other functional groups, one can easily construct new redox systems on the Mb surface. In addition, this strategy will be applicable to the exploration of a new type of fusion protein, in which the arrangement of active sites are regulated by the designed polypeptides, if we can introduce a natural cofactor, such as flavin adenine dinucleotide or flavin mononucleotide, instead of the artificial flavin unit. Although detailed examination of the 3D structure of Mb–peptide conjugates will be needed, further systematic substitution of functional units and/or amino acids in the peptide and Mb will lead to the design of artificial heme proteins by using the de novo reconstitution method.

Experimental Section

Materials and methods: All chemicals and solvents were of reagent or HPLC grade. Protoporphyrin IX was purchased from Aldrich. 7α-Bromoacetyl-10-methylisoalloxazine was synthesized according to the method of Levine and Kaiser.[15a] Amino acid derivatives and reagents for peptide synthesis were purchased from Novabiochem (Darmstadt, Germany). All peptides were synthesized manually according to a standard solid-phase method by using Fmoc strategy.[13] The peptides were purified and analyzed with RP-HPLC on a Cosmosil 5C18-AR-II column (Nacalai Tesque, Kyoto, Japan; 10×250 mm) or a Cosmosil 5C18-AR-II analytical column (4.6×150 mm) by employing a Shimadzu LC-10ACvp

pump equipped with a Shimadzu SPD10A UV/Vis detector and a Shimadzu CTO-10Avp column oven. MALDI-TOF MS was measured on a Shimadzu MALDI III mass spectrometer by using 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix. Amino acid analysis was carried out after hydrolysis in 6.0 M HCl at 110 °C for 24 h in a sealed tube and subsequent labeling with phenyl isothiocyanate. Each peptide concentration was determined by quantitative amino acid analysis by using Phe as an internal standard.

Synthesis of W^1-1αK, Dns^1-1αE, and Dns^{30}-1αE: The peptides were synthesized by the stepwise elongation of Fmoc-protected amino acids on Novasyn TGR resin (Novabiochem) according to a reported procedure with Fmoc-AA-OH (Fmoc-Ala-OH·H_2O, Fmoc-Asn(Trt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Trp-OH, Fmoc-Val-OH) by using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxy-benzotriazole hydrate (HOBt·H_2O) and diisopropylethylamine (DIEA) as coupling reagents.[16] The Lys30 residue of Dns^{30}-1αE was protected with the Mtt group and the other Lys residues were protected with Boc groups. Each coupling efficiency was checked by the Kaiser test.[24] For the acetylation of the N-terminus amino group of the W^1-1αK or Dns^{30}-1αE peptides, Fmoc-deprotected peptide resin was treated with acetic anhydride (10 equiv) and DIEA (5 equiv) in 1-methyl-2-pyrrolidone (NMP). To introduce the Dns unit at the N-terminus amino group of Dns^1-1αE, Fmoc-deprotected peptide resin was treated with dansyl chloride (5 equiv) and DIEA (2 equiv) in NMP for 5 h at 25 °C. In the case of Dns^{30}-1αE, the Mtt group protecting the side chain of Lys30 was selectively cleaved in CH_2Cl_2/TFA/triisopropylsilane (94:1:5, 5×2 min). Then, dansyl chloride (5 equiv) was allowed to react with the liberated ε-amino group on the Lys30 side chain in the presence of DIEA (2 equiv) in NMP for 5 h at 25 °C. The protecting groups and the resin were removed by stirring the dried resin for 2 h at 25 °C with TFA/*m*-cresol/ethanedithiol/thioanisole (85:6:6:2). The solvent was evaporated and the residues were solidified with diethyl ether in an ice-bath to give the crude peptides. The crude peptides were purified with RP-HPLC on a Cosmosil 5C18-AR-II column (10×250 mm) by using a linear gradient of MeCN/0.1% TFA (1.0% min^{-1}) to give the product with a single peak on analytical HPLC (Cosmosil 5C18-AR-II column, 4.6×150 mm, linear gradient of MeCN/0.1% TFA at 1.0% min^{-1}). The peptides were identified by MALDI-TOF MS and amino acid analysis; total yields: W^1-1αK 14%; Dns^1-1αE 26%; Dns^{30}-1αE 10%; MALDI-TOF MS: W^1-1αK *m*/*z*: 3334.2 $[M+H]^+$ (calcd for: 3332.9); Dns^1-1αE *m*/*z*: 3346.0 $[M+H]^+$ (calcd for: 3345.2); Dns^{30}-1αE *m*/*z*: 3457.8 $[M+H]^+$ (calcd for: 3457.3); amino acid analyses: W^1-1αK found (calcd): Ala 7.92 (8), Asn 0.85 (1), Gly 2.16 (2), Leu 4.04 (4), Lys 11.9 (12), Val 3.00 (3); Dns^1-1αE found (calcd): Ala 7.71 (8), Asn 0.88 (1), Glu 8.08 (8), Gly 1.57 (2), Leu 4.06 (4), Lys 3.93 (4), Val 3.00 (3); Dns^{30}-1αE found (calcd): Ala 7.70 (8), Asn 0.86 (1), Glu 7.84 (8), Gly 1.04 (1), Leu 4.08 (4), Lys 4.09 (4), Val 3.00 (3).

Synthesis of PPIX-1αK, Cys^1-1αE, and Cys^{31}-1αE: Peptide PPIX-1αK was a precursor of Heme-1αK with a free base protoporphyrin IX at the N-terminus amino group of the 1αK sequence. Peptides Cys^1-1αE and Cys^{31}-1αE were designed as intermediates to prepare Fla^1-1αE and Fla^{31}-1αE, respectively. The peptides were synthesized by the solid-phase method on Novasyn TGR resin with Fmoc-AA-OH by using HBTU, HOBt·H_2O, and DIEA as coupling reagents. The Fmoc-deprotected 1αK peptide resin was treated with the mono-*N*-hydroxysuccinimide ester of protoporphyrin IX (4 equiv) in the presence of DIEA (3 equiv) in NMP for 5 h at 25 °C to give a PPIX-1αK peptide resin.[14] The protecting groups and the resin were removed by stirring the dried resin for 2 h at 25 °C with TFA/*m*-cresol/ethanedithiol/thioanisole (85:6:6:2). The solvent was evaporated and the residues were solidified with diethyl ether in an ice-bath to give the crude peptides. The crude peptides were purified with RP-HPLC on a Cosmosil 5C18-AR-II column (10×250 mm) by using a linear gradient of MeCN/0.1% TFA (1.0% min^{-1}) to give the product with a single peak on analytical HPLC. The peptides were identified by MALDI-TOF MS; total yields: PPIX-1αK 31%; Cys^1-1αE 33%; Cys^{31}-1αE 24%; MALDI-TOF MS: PPIX-1αK *m*/*z*: 3648.5 $[M+H]^+$ (calcd for: 3649.4); Cys^1-1αE *m*/*z*: 3256.5 $[M+H]^+$ (calcd for: 3257.0); Cys^{31}-1αE *m*/*z*: 3256.1 $[M+H]^+$ (calcd for: 3257.0).

Heme-1αK, Fla^1-1αE, and Fla^{31}-1αE: The iron(III) ion was inserted into the protoporphyrin IX group of peptide PPIX-1αK by mixing the peptide with Fe^{II} acetate (10 equiv) in AcOH/TFE (5:5) under nitrogen for 6 h at 50 °C. The flavin was covalently attached on the Cys side chain of Cys^1-1αE and Cys^{31}-1αE by allowing 7α-bromoacetyl-10-methylisoalloxazine (5 equiv) to react with the Cys-1αE peptide in TFE/0.1 M Tris·HCl (pH 8.5; 1:1) for 2 h.[15,16] Crude peptides were purified with RP-HPLC on a Cosmosil 5C18-AR-II column (10×250 mm) by using a linear gradient of MeCN–0.1% TFA (1.0% min^{-1}) to give the peptide with a single peak on analytical HPLC. The peptides were identified by MALDI-TOF MS and amino acid analysis. A molar extinction coefficient of each peptide was estimated from the data obtained by quantitative amino acid analysis; yield of Heme-1αK from PPIX-1αK 28%; yield of Fla^1-1αE from Cys^1-1αE 52%; yield of Fla^{31}-1αE from Cys^{31}-1αE 41%; MALDI-TOF MS: Heme-1αK *m*/*z*: 3704.3 $[M+H]^+$ (calcd for: 3705.2); Fla^1-1αE *m*/*z*: 3525.6 $[M+H]^+$ (calcd for: 3525.3); Fla^{31}-1αE *m*/*z*: 3526.0 $[M+H]^+$ (calcd for: 3525.3); amino acid analyses: Heme-1αK found (calcd): Ala 7.68 (8), Asn 0.96 (1), Gly 2.06 (2), Leu 4.04 (4), Lys 11.2 (12), Val 3.00 (3); Fla^1-1αE found (calcd): Ala 7.71 (8), Asn 0.95 (1), Glu 7.94 (8), Gly 2.03 (2), Leu 4.05 (4), Lys 3.91 (4), Val 3.00 (3); Fla^{31}-1αE found (calcd): Ala 7.70 (8), Asn 0.93 (1), Glu 8.06 (8), Gly 2.03 (2), Leu 4.05 (4), Lys 3.90 (4), Val 3.00 (3); molar extinction coefficients in mol^{-1} cm^{-1} L (λ_{max} in nm^{-1}): Heme-1αK (5 μM in 50% pyridine/0.1 M NaOH with sodium dithionite): 29900 (556); Fla^1-1αE (10 μM in 20 mM Tris·HCl, pH 7.4): 10600 (430), 30100 (289); Fla^{31}-1αE (10 μM in 20 mM Tris·HCl, pH 7.4): 11500 (430), 32700 (289).

Reconstitution of semiartificial myoglobin modified with peptide: Apomyoglobin was prepared from horse heart myoglobin (Sigma) by the standard method.[25] The peptide–heme conjugate Heme-1αK was incorporated into the heme binding site of apomyoglobin by a slightly modified version of the procedure reported by Hamachi and co-workers.[2] The peptide Heme-1αK (1.0 equiv) dissolved in 50% TFE/20 mM Tris·HCl buffer (pH 7.4; 100 μL) was mixed with a solution of apomyoglobin in the same buffer (pH 7.4; 400 μL) in an ice-bath. The resulting mixture was incubated at 4 °C overnight. The solution was centrifuged at 4 °C and 10000 rpm for 30 min. The supernatant was passed through a Sephadex G-50 size-exclusion column (1.0×10 cm) and the fraction containing Mb-1αK was collected and used for analyses without further purification.

CD measurements: CD spectra were recorded on a JASCO J-720 spectropolarimeter by using a quartz cell with 1.0-mm pathlength at 25 °C. Peptides were dissolved in 20 mM Tris·HCl buffer (pH 7.4) at a peptide concentration of 20 μM. For the measurements of Mb, apo-Mb, and Mb-1αK, proteins were dissolved in 20 mM Tris·HCl buffer (pH 7.4) at a protein concentration of 5 μM.

Fluorescence measurements: Fluorescence spectra were recorded on a Hitachi 850 fluorescence spectrophotometer with a 1.0×1.0 cm quartz cell. Peptides were dissolved in 20 mM Tris·HCl buffer (pH 7.4) at a peptide concentration of 1.0 μM for the W^1-1αK peptide and 2.0 μM for the Fla-1αE peptides. For the fluorescence titration, W^1-1αK in buffer was titrated with Dns^1-1αE or Dns^{30}-1αE in increments of about 0.2 equivalents. After each addition of Dns-1αE peptide, samples were equilibrated for 15 min at 25 °C, then fluorescence spectra (300–450 nm), excited at 292 nm, were measured at 25 °C. The change in fluorescence intensity at the maximum emission wavelength of the W^1-1αK peptide with increasing Dns-1αE concentration was corrected for dilution and fitted by a single-site binding equation [Eq. (1)],[18] by using Kaleida Graph software (Synergy Software), where W_0 and D_0 represent the initial concentrations of the W^1-1αK and Dns-1αE peptides, respectively. ΔI denotes the difference in the fluorescence intensity of W^1-1αK in the absence (I_0) and presence of Dns-1αE at each concentration (I). When all the W^1-1αK forms the coiled-coil structure with Dns-1αE, ΔI is equal to ΔI_{max}.

$$\Delta I/I_0 = \{(\Delta I_{max}/I_0)/2D_0\}[(W_0+D_0+1/K_a)-\{(W_0+D_0+1/K_a)^2-4W_0D_0\}^{1/2}] \quad (1)$$

UV/Vis measurements: UV/Vis spectra were recorded on a Shimadzu UV-2200 spectrophotometer by using a quartz cell with 1.0 cm pathlength. Heme-1αK in buffer was titrated with apo-Mb in increments of about 0.25 equivalents. The increase in absorbance at the maximum of the Soret band with increasing apo-Mb concentration was corrected for dilution and plotted as a function of apo-Mb concentration. For the UV/

Vis spectrum of deoxy-Mb-1αK, iron(III) in the heme was reduced by the addition of an excess amount of solid sodium dithionite.

Assay for reaction rate of oxy-Mb-1αK formation: The ET rate from NADH to the heme center in the presence or absence of the H-Fla or Fla-1αE peptides was assayed by measuring the amount of the produced oxy-Mb.[2i] The reaction was initiated by the addition of NADH (final concentration of 25–250 μM) to mixtures of Mb derivative (10 μM) and flavin (10 μM) in 20 mM Tris·HCl buffer (pH 7.4) at 25 °C. The reaction was followed by monitoring the increase in absorbance of the Q-bands at 540 and 580 nm, as they are characteristic of the absorption of a typical dioxygen complex of Mb (oxy-Mb).

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