Dimeric Proteins

DOI: 10.1002/ange.201409267

Rational Design of Heterodimeric Protein using Domain Swapping for Myoglobin**

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Abstract: *Protein design is a useful method to create novel artificial proteins. A rational approach to design a heterodimeric protein using domain swapping for horse myoglobin (Mb) was developed. As confirmed by X-ray crystallographic analysis, a heterodimeric Mb with two different active sites was produced efficiently from two surface mutants of Mb, in which the charges of two amino acids involved in the dimer salt bridges were reversed in each mutant individually, with the active site of one mutant modified. This study shows that the method of constructing heterodimeric Mb with domain swapping is useful for designing artificial multiheme proteins.*

Protein design is a powerful tool for creating novel artificial proteins by redesigning natural proteins or designing novel proteins.[1] Successful examples include artificial oxidases,[2] reductases,[3] and hydrolases.[4] To produce an artificial protein, it is crucial to control its folding, and may be achieved by controlling the interaction between secondary structures using metal ions or cofactors.[5] Protein dimerization may also be used to control the folding and biological functions. For example, human soluble guanylate cyclase for NO signal transduction is a heterodimer consisting of α and β subunits (αα and ββ homodimers also exist).[6] Recently, Roelfes and co-workers designed an artificial metalloenzyme catalyzing the Diels–Alder reaction based on a dimeric transcription factor (lactococcal multidrug resistance regulator).[7] Development of other methods to design dimeric or oligomeric proteins is indispensable for increasing the variety of artificial enzymes.

Domain swapping is a phenomenon in which a helix or a secondary structure is exchanged between protein molecules. It has been observed in many proteins and associated with protein folding and function.[8] These proteins include prion,[9] cystatin C,[10] β2-microglobulin,[11] and serpin,[12] which are involved in deposition diseases. Domain swapping has also been observed for heme proteins,[13] including cytochrome *c* (cyt *c*),[14] and myoglobin (Mb).[15] Recently, we found that intermolecular hydrophobic interactions in cyt *c* at the early stage of protein folding controls the formation of the oligomer by domain swapping.[16] The domain-swapped dimer of cyt *c* exhibited different properties from those of its monomer,[14b,d] although the domain-swapped Mb dimer exhibited a similar oxygen-binding character as that of its monomer.[15] In this study, we show that domain swapping could be used to design a heterodimeric protein of Mb, and this method may be a new approach for creating artificial proteins.

Mb has been favored as a scaffold protein for heme protein design, and has been converted successfully into various artificial enzymes by redesigning its heme active site.[1b,g,i,m, 2a,b,d,e, 3a–c,e, 5e] Although Mb functions in the monomeric form, dimeric Mb was detected more than forty years ago.[17] We have resolved the X-ray crystallographic structure of dimeric horse Mb (PDB code: 3VM9), which exhibited a unique domain-swapped structure with each active site constructed by two protomers (see Figure S1 in the Supporting Information).[15] Therefore, we chose horse Mb as a model protein, and explored a new approach for designing an artificial protein based on domain swapping.

The key initial step for protein design based on dimeric wild-type (WT) horse Mb was to precisely control the dimerization. By close inspection of the X-ray crystallographic structure of dimeric Mb, we found that aside from hydrophobic interactions, four new salt bridges were formed between protomers in the dimer: between Lys78 in one protomer and Glu85 in the other protomer ($Lys78_A/Glu85_B$), $Glu85_A/Lys78_B$, $Lys79_A/Asp141_B$, and $Asp141_A/Lys79_B$ (Figure 1 A and Figure S1). Since these residues play an important role in stabilizing the dimer, we envisaged that dimerization of two different mutant Mbs may be controlled by altering these interactions. We replaced the two positively charged residues Lys78 and Lys79 with negatively charged residues (Glu78 and Asp79) in one mutant (K78E/K79D Mb, ED Mb), and the two negatively charged residues Glu85 and Asp141 with positively charged residues (Lys85 and Lys141) in the

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[**] We thank Leigh McDowell for his advice during manuscript preparation. This work was partially supported by Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science (Category B, No. 26288080 (S.H.) and Young Scientists B, No. 24750163 (S.N.)). Y.-W.L. acknowledges the support from JSPS (Invitation Fellowship Program). This work was also supported partially by the Natural Science Foundation of China (No. 31370812; to Y.-W. L.).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201409267.

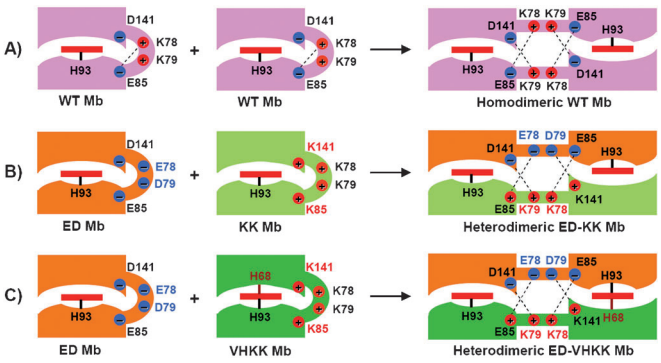


Figure 1. Design of heterodimeric Mb: A) Dimerization of WT Mb. B) Amino acid mutations to control the interaction between the protomers and obtain heterodimeric Mb. C) Amino acid mutations to obtain heterodimeric Mb with two different active sites.

other mutant (E85K/D141K Mb, KK Mb) (Figure S2 for locations of the mutated residues in monomeric Mb). We envisaged that the stability of homodimeric Mb of each mutant would decrease by removal of the salt bridges between the protomers because of the mutations (E78/E85 and D79/D141 or K78/K85 and K79/K141), whereas heterodimeric Mb created by ED Mb and KK Mb would be stabilized by the electrostatic interactions between the charge-reversed mutated residues (E78/K85 and D79/K141; Figure 1B).

ED Mb and KK Mb were purified using a procedure reported previously for WT Mb.[18] We prepared dimeric Mb using the modified procedure for preparation of dimeric WT Mb,[15] where 10 vol% 2-propanol was added to the monomeric Mb solution instead of 5 vol% ethanol to obtain a larger amount of dimer (see the experimental section in the Supporting Information). Only a small amount (ca. 8%) of the homodimers was observed by dimerization of ED Mb

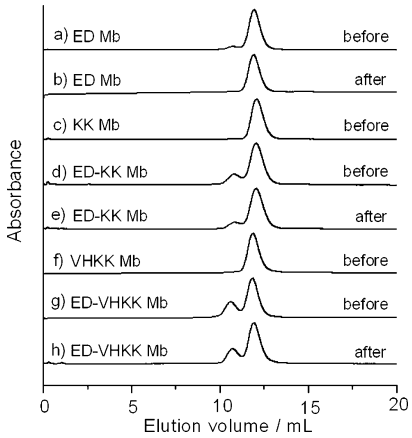


Figure 2. Elution curves of dimeric ED Mb (a,b), KK Mb (c), ED-KK Mb (d,e), VHKK Mb (f), and ED-VHKK Mb (g,h) treated with 10 vol% 2-propanol. The elution curves were taken before (a,c,d,f,g) and after (b,e,h) heating the protein solution at 45 °C for 30 min. Measurement conditions:Superdex 75 10/300 GL column, 50 mM potassium phosphate buffer (pH 7.0), monitoring wavelength: 409 nm, temperature: 4 °C.

with 2-propanol, according to size-exclusion chromatography (Figure 2a, and see Figure S3A). The homodimeric ED Mb dissociated completely after heating at 45 °C for 30 minutes (Figure 2b and Figure S3A). For KK Mb, only the monomer peak was detected in the chromatogram after treatment with 2-propanol, thus showing that KK Mb did not form homodimers (Figure 2c). When dimers were prepared from a mixture of ED Mb and KK Mb (1:1 ratio) under the same conditions, the number of dimers obtained increased (ca. 18%; Figure 2d and Figure S3B). The dimers obtained from the mixture of ED Mb and KK Mb may contain both homodimeric ED Mb, and the heterodimer of ED Mb and KK Mb (heterodimeric ED-KK Mb). However, after heating the obtained dimer solution (mixture of homodimeric ED Mb and heterodimeric ED-KK Mb) at 45 °C for 30 minutes, a certain amount of dimer (ca. 12%) was still observable (Figure 2e and Figure S3B). The dimer observed after heating should correspond to heterodimeric ED-KK Mb, since homodimeric ED Mb dissociated by the heating treatment. These results indicate that pure heterodimeric proteins were obtained by controlling the surface charges in domain swapping.

We further designed a heterodimeric Mb with two distinct heme coordination sites, which can be found in multiheme proteins in nature but are hard to achieve in a homodimeric protein.[19] According to the X-ray crystallographic structure of homodimeric WT Mb (PDB code: 3VM9), the two identical His/H_2O-coordinated heme sites in the dimer are very similar to that in the monomer.[15] We were interested in whether a bis(His)-coordinated heme site, as observed for native neuroglobin[20] and cytoglobin,[21] could be generated in one of the two heme centers in the heterodimer. It has been reported that by exchanging two distal residues, His64 and Val68, with Val and His, respectively (H64V/V68H Mb; Figure S2), His68 and His93 coordinate to the heme iron in both ferric and ferrous states.[22] Therefore, we referred to the H64V/V68H double mutation to generate a bis(His) coordination site at one of the heme sites in heterodimeric Mb, and designed a heterodimer with ED Mb and H64V/V68H/E85K/D141K Mb (VHKK Mb; Figure 1C). Purified VHKK Mb exhibited similar UV/Vis spectra (ferric, peak at $\lambda = 412$ nm; ferrous, peaks at $\lambda = 427$, 529, and 563 nm; see Figure S4C) as those of H64V/V68H Mb,[22] thus suggesting that the heme coordination did not change in either redox state by reversing the charges of two residues at the protein surface. Interestingly, the spectrum of VHKK Mb in the ferrous state resembled those of Mb crystals after X-ray irradiation ($\lambda =$ 529 and 568 nm).[23]

Heterodimeric ED-VHKK Mb was prepared using the same procedure as that used for heterodimeric ED-KK Mb. Similar to KK Mb, VHKK Mb did not form a homodimer by treatment with 2-propanol (Figure 2f), and indicated that the four Lys residues at the protomer interface destabilized the homodimer dramatically, although the bis(His) coordination tends to stabilize the dimer. Interestingly, a large amount of dimer (ca. 30%) was observed when constructing dimers from ED Mb and VHKK Mb (Figure 2g and Figure S3C), and about 24% of the protein still formed dimers after heating at 45 °C for 30 minutes (Figure 2h, and Figure S3C). The yield

of heterodimeric ED-VHKK Mb obtained was more than twice that of heterodimeric ED-KK Mb obtained by the same method, thus suggesting that the bis(His) coordination stabilizes the dimer. After heating the dimer solution at 45 °C for 30 minutes, we purified heterodimeric ED-VHKK Mb by using a Hi-Load Superdex 75 26/60 column, where it was separated from ED Mb and VHKK Mb (see Figure S5).

To elucidate the structure of heterodimeric ED-VHKK Mb, we performed X-ray crystallographic analysis (PDB code: 3WYO). The structure at 2.0 Å resolution showed that heterodimeric ED-VHKK Mb adopts a domain-swapped structure essentially identical to that of homodimeric WT Mb (Figure 3 A). Three strong salt bridges (E78/K85, 3.20 Å

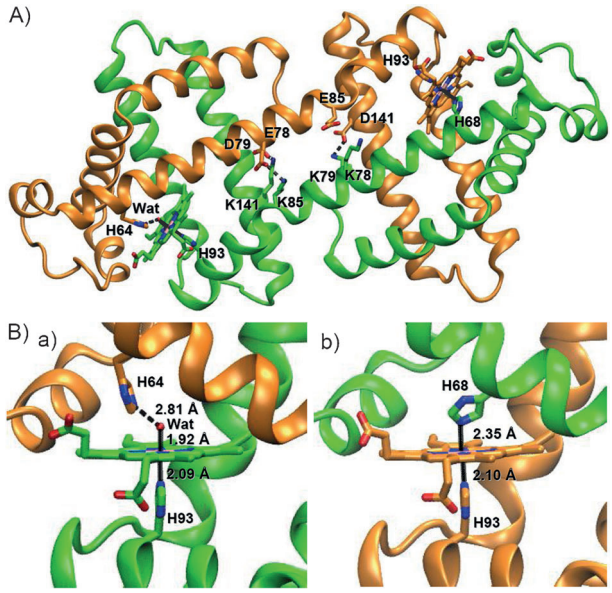


Figure 3. Crystal structure of heterodimeric ED-VHKK Mb (PDB code: 3WYO). A) Overall structure. B) Heme coordination structures: a) His/H_2O and b) bis(His) coordination. The coordination bonds and salt bridges are shown in solid and dashed lines, respectively. The hemes and His64, His68, and His93 are shown as stick models. The nitrogen and oxygen atoms of the hemes, His64, His68, His93, the key amino acids of the salt bridges, and coordinated water are shown in blue and red, respectively.

between heavy atoms; D79/K141, 2.77 Å; D141/K79, 2.82 Å) were formed in the ED-VHKK Mb heterodimer between the ED Mb and VHKK Mb protomers. However, one salt bridge which formed in the WT dimer was not detected in the crystal structure of the heterodimer (E85/K78, 4.83 Å), although it may form weakly in solution by their close arrangements. It was also revealed from the crystal structure that heterodimeric ED-VHKK Mb possesses two heme active sites with different coordination structures, that is, His/H_2O coordination (Figure 3 B a) and bis(His) coordination (Figure 3 B b). Distal His64 formed a hydrogen bond (2.81 Å) with the axial water molecule at the His/H_2O-coordinated heme site, similar to that of monomeric Mb.[18] At the bis(His)-coordinated heme site, His68 and His93 coordinated to the heme iron (Fe–His distances: His68: 2.35 Å; His93: 2.10 Å), similar to those in H64V/V68H Mb (His68: 2.31 Å; His93: 2.27 Å).[22] These observations demonstrate that two distinct heme sites can be designed in a single heterodimeric protein by domain swapping, without large structural alterations from the original site of the monomer.

We investigated the UV/Vis spectra of heterodimeric ED-VHKK Mb in different oxidation states, and compared them with those of the original monomers. At the ferric state, the absorption spectrum of ED-VHKK Mb exhibited a Soret band at $\lambda = 410$ nm (Figure 4 A, black), which was between

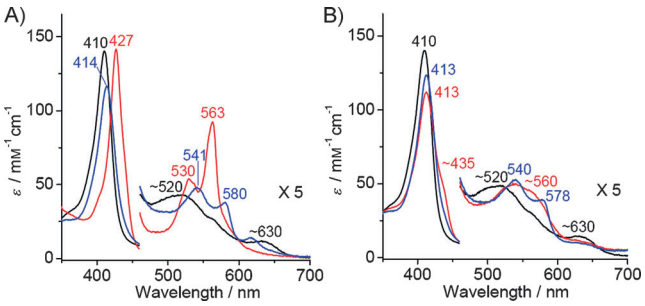
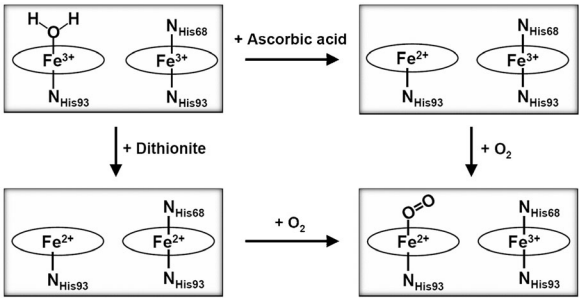


Figure 4. UV/Vis spectra of heterodimeric ED-VHKK Mb by reduction with A) dithionite (1 mM) and B) ascorbate (1 mM) in 50 mM potassium phosphate buffer (pH 7.0): before (black) and after reduction (red), followed by exposure to air (blue).

those of ED Mb ($\lambda = 408$ nm) and VHKK Mb ($\lambda = 412$ nm; Figures S4 A and S4 C). The spectrum of the visible bands of heterodimeric ED-VHKK overlapped well with the average spectrum of those of ED Mb and VHKK Mb monomers (see Figure S6 A). By reduction of both heme sites in ED-VHKK Mb with dithionite, the Soret band red-shifted to $\lambda = 427$ nm with two visible bands observed at $\lambda = 530$ and 563 nm in the spectrum (Figure 4 A, red). The spectrum after reduction also overlapped well with the average spectrum of those of ED Mb and VHKK Mb monomers reduced with dithionite (see Figure S6 B). By reduction with a mild reducing reagent, ascorbic acid, the Soret band of ED-VHKK Mb shifted to $\lambda =$ 413 nm with a shoulder peak at $\lambda = 435$ nm (Figure 4 B). The UV/Vis spectrum of heterodimeric ED-VHKK Mb in the presence of ascorbic acid was also similar to the average spectrum of those of ED Mb and VHKK Mb in the presence of ascorbic acid (see Figure S6 C). ED Mb was reduced to the ferrous deoxy form with ascorbic acid (see Figure S4 B), whereas VHKK Mb remained in the ferric state (see Figure S4 D). These results highlight that ED-VHKK Mb contains two distinct heme coordination sites resembling those in ED Mb and VHKK Mb monomers.

Scheme 1 illustrates the conversion of the oxidation and coordination states in heterodimeric ED-VHKK Mb by reduction with either dithionite or ascorbic acid, and subsequent exposure to air. O_2 bound to the ferrous deoxy heme and an oxygenated complex was formed when fully-reduced (by dithionite) ED-VHKK Mb was exposed to air, as indicated by generation of two visible bands at $\lambda = 541$ and 580 nm (Figure 4 A, blue). The spectrum obtained following reduction with dithionite and subsequent exposure to air was similar to that of half-reduced (by ascorbic acid) ED-VHKK



Scheme 1. Conversion of oxidation and coordination states in heterodimeric ED-VHKK Mb with two distinct heme centers.

Mb exposed to air (Figure 4B, blue). The final state obtained after exposure to air contained both a ferric bis(His)-coordinated heme and a ferrous oxy heme, since it exhibited a spectrum similar to the average spectra of ferric VHKK Mb and the oxy form of ED Mb (Figure S6D). These results show that the ferrous bis(His) heme center in ED-VHKK Mb was quickly oxidized to the ferric state with O_2, similar to the VHKK Mb monomer (Figure S4C), in agreement with the observation for H64V/V68H Mb reported previously.[22] Therefore, the two hemes may act as two independent sites.

In conclusion, we demonstrated that protein dimerization by domain swapping can be controlled efficiently through alteration of the salt bridges between the surfaces of two protomers in the dimer. This approach was successfully applied to design a heterodimeric protein of horse Mb with two different heme active sites, which exhibited different ligand binding properties. This study presents a new example for rational protein design with multiheme groups using domain swapping.

Received: September 19, 2014
Published online: November 4, 2014

Keywords: dimerization · heme coordination · heme proteins · salt bridges · X-ray crystallography

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