Incorporation of a Phebox Rhodium Complex into apo-Myoglobin Affords a Stable Organometallic Protein Showing Unprecedented Arrangement of the Complex in the Cavity

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Novel organometallic proteins have been synthesized as a 1:1 composite of rhodium 2,6-bis(2-oxazolinyl)phenyl (Rh•Phebox) complexes with the apo-form of myoglobin, which is an oxygen-transport protein having *b*-type heme (iron porphyrin IX) as a natural prosthetic group. The X-ray structural analysis reveals that the Rh•Phebox complex with phenyl substituents is included in the cavity with an almost perpendicular arrangement to that of the heme. The unique arrangement is supported by hydrogen bonding and a number of hydrophobic interactions, especially $\pi-\pi$ interactions between the phenyl rings of the substituents and the imidazole moiety of His93, which is a ligand of the rhodium atom. Semiquantitative analysis of the composite stability by ESI mass spectroscopy clearly indicates that the stability of the composites depends on an extent of the interaction between the substituents of the Rh•Phebox complexes and His93. Enantioselectivity for the chiral (*S,S*)- and (*R,R*)-Rh•Phebox complexes with the phenyl substituents is also observed in terms of the difference in the stability of the composites. According to the arrangement of the amino acid residues around the Phebox ligand, the (*S,S*)-form has a suitable configuration to fit one of its phenyl rings into the hollow formed by Leu89, His93, and Ile99, while structural distortion will result if the (*R,R*)-isomer adopts an arrangement similar to that of the (*S,S*)-isomer, which is confirmed with the enhancement of the maximal and minimal ellipticities of the circular dichroic spectrum in comparison with that of the intact (*R,R*)-isomer. Such structural strain would reduce the efficient $\pi-\pi$ interaction between the ligand substituents and His93, resulting in less stability of the composite containing the (*R,R*)-isomer. The results obtained here demonstrate that the myoglobin cavity is capable of accommodating organometallic compounds totally different from the heme in its molecular shape and the arrangement in the cavity. The extension and application of the present method will allow us to produce artificial organometallic proteins bearing functions that are difficult to achieve with natural prosthetic groups.

Introduction

Incorporation of non-native metal cofactors in well-characterized stable proteins has been regarded as an attractive strategy to prepare artificial metalloenzymes having non-natural functions.[1−10] There are several reports that apply this strategy such as reconstitution of heme proteins with chemically modified heme analogues,[11−15] covalent attachment of metal complexes to proteins through modification of specific amino acids,[1,16−20]

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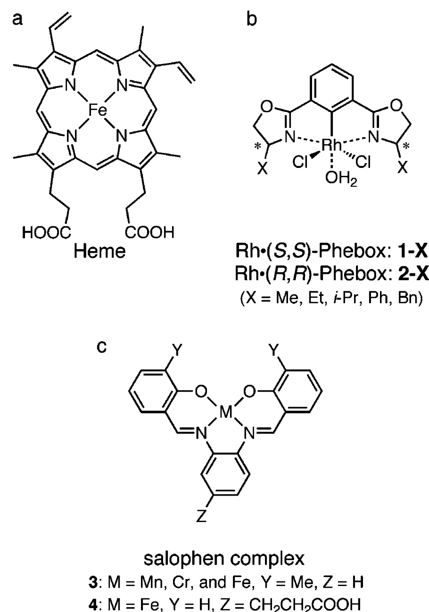

Figure 1. *b*-Type heme (a) and synthetic prosthetic groups employed in the present (b) and previous (c) studies.

and encapsulation of biotinylated metal complexes into an avidin or streptavidin cavity through specific interaction between the proteins.[21-23] On the other hand, we have developed a novel method to introduce artificial metal cofactors into apo-hemoproteins such as apo-myoglobin (apo-Mb) and heme oxygenase (HO), in which the natural prosthetic group, *b*-type heme (Figure 1a), is replaced with a series of salophen-metal complexes (**3** and **4** in Figure 1c).[7-10] The crystal structures of apo-Mb reconstituted with **3** clearly show that the coordination of His93, the original heme ligand of Mb, to the salophen complexes is retained to fix the salophen complexes in the cavity with assistance of hydrophobic interactions between amino acid residues and the complexes. Close inspection of the crystal structure allows us to design the salophen derivatives that regulate the asymmetric sulfoxidation of thioanisole from the *R*- to the *S*-isomers. Moreover, we have achieved reduction of the salophen complexes included in HO with the physiological reductant, cytochrome P450 reductase, by introducing a hydrogen-bonding site to the salophen ligands (**4**).[10] The reduced salophen complexes can activate dioxygen without oxidative degradation of the salophen ligands. The crystal structure and DFT (density functional theory) calculation of HO containing **4** have proved that the hydrogen bonding between an amino acid residue and the salophen ligands serves as the route for the electron flow from the interface between the reductase and HO to the iron ion of **4**. Thus, we have developed unique enzymatic and redox activities of the artificial metalloprotein by applying the salophen complexes as an artificial cofactor. In these studies, however, arrangements of the salophen complexes in the Mb and HO cavities are essentially the same as those of the heme. We, therefore, were concerned about the limitation of our method with artificial cofactors having structures similar to the heme.

In the present report, we describe successful reconstitution of apo-Mb with five analogues of rhodium 2,6-bis(2-oxazolinyl)-phenyl (Rh•Phebox, Figure 1b)[24] complexes that are totally different in molecular shape from the heme. The Rh•Phebox complexes are known to be a good agent to catalyze hydrosilylation of monosubstituted and α,β-disubstituted alkenes with high stereo- and enantioselectivity.[25] X-ray structural analysis of one of the obtained composites reveals that the Rh•Phebox complex is fixed in the Mb cavity with an unprecedented arrangement that is almost perpendicular to the heme. Subsequent ESI mass spectroscopy allows discussing the stability of the unique arrangement of the Rh•Phebox complex on the basis of interactions between the Phebox ligands and amino acid residues in the cavity.

Results and Discussion

Preparation of Composites and X-ray Structural Analysis. We have prepared five chiral Rh•(*S*,*S*)-Phebox complexes with different alkyl substituents on the oxazoline rings (**1-X**, **X** = Me, Et, *i*-Pr, Ph, and Bn, Figure 1b) according to previous reports.[25,26] apo-Mb prepared by the acid extraction method was used for the reconstitution. In order to prepare the composites, we have applied a method developed for modified hemes.[27] A slightly excess amount of a Rh•Phebox complex in a methanol solution was added to an apo-Mb solution followed by gentle stirring at 4 °C. After 30 min stirring, the solution was applied to desalting columns of Sephadex G25 and G75 in turn to separate the composite from nonreacted complexes. Through these simple processes, all the Rh•(*S*,*S*)-Phebox complexes can be reacted with the apo-forms of the wild-type (apo-Mb) and variant (apo-A71GMb) in which Ala71 is replaced with Gly, while only the composite consisting of **1-Ph** and A71GMb (**1-Ph**•A71GMb) afforded single crystals suitable for X-ray structural analysis among the composites. Figure 2a show the whole structure of **1-Ph**•A71GMb determined at a 1.8 Å resolution. The crystallographic data can be seen in the Supporting Information. The crystal structure clearly shows that **1-Ph** forms a 1:1 composite with A71G and occupies a unique position in the cavity in place of the heme. A comparison of arrangements in the cavity between **1-Ph** and the heme reveals that **1-Ph** takes an unprecedented orientation, which is almost perpendicular to the heme plane (Figure 2b). Histidine93, the axial ligand of the heme and the salophen complexes in the Mb cavity, still coordinates to the rhodium atom at a Rh−N distance of 2.31 Å. However, unlike the cases of the heme and salophen complexes in which His93 occupies axial coordination sites of the complexes, His93 ligates to the equatorial coordination site of **1-Ph** that was originally occupied by a water molecule before the composite formation. This unique arrangement of **1-Ph** is supported by a number of interactions between amino acid residues in the cavity and the Phebox ligand including the phenyl substituents on the oxazoline rings (Figure 2c,d,e). In the crystal

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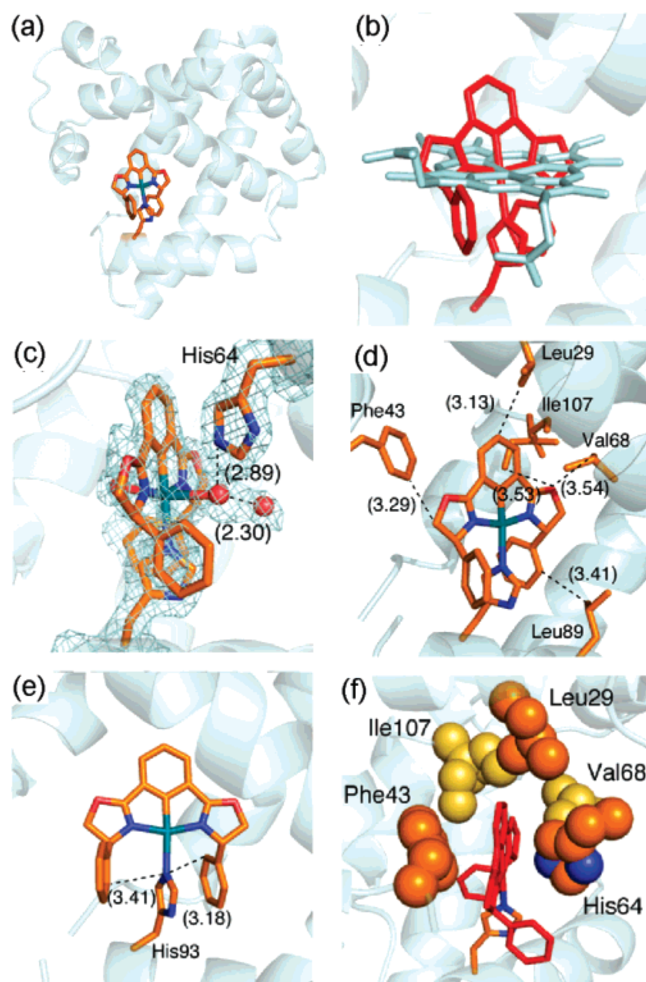

Figure 2. Crystal structure of **1-Ph**·A71GMb. (a) The whole structure; (b) Comparison of arrangement between **1-Ph** (red) and heme (white). (c) Hydrogen bondings over His64, the aqua ligand, and the water molecule. The $2|F_o| - |F_c|$ electron density (1σ contours) around the **1-Ph** is also shown. (d, e) Hydrophobic interaction between the amino acid residues and **1-Ph**. (f) Hydrophobic residues shown in (d) form a pocket suitable to hold the Phebox ligand plane. The values in parentheses show distances between two atoms (Å).

structure, both axial ligands of **1-Ph** have been replaced from the original chlorines with water molecules (Figure 2c), and one of the water ligands participates in hydrogen bondings with the Nϵ atom of His64 and a water molecule in the cavity. Although ESI mass data for **1-Ph**·A71GMb suggest the conservation of two chlorine ligands even in the composite (anal. 17,857.6; calc 17,857.3 with two chlorine ligands), we prefer to put water molecules in place of them in the crystal structure since the replacement rationalizes the electron density around the Phebox ligand and the close contacts observed for one of the axial ligands with the Nϵ atom of His64 (2.89 Å) or the water molecule in the cavity (2.30 Å). The analogous contradiction between ESI mass and crystallographic data has been reported in the reactions of cisplatin with proteins,[28,29] in which rationality of *B* factors of the platinum ligands has been argued to address whether ammonia or chlorine remains on the platinum atom in the crystal structures. Application of the argument to

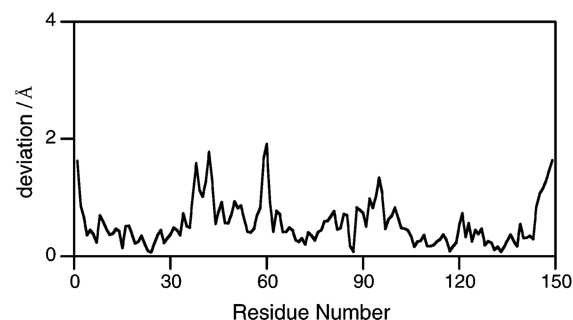

Figure 3. Deviations of **1-Ph**·A71GMb at C^{α}-positions from those of native Mb (PDB ID: 4MBN).

our crystallographic data supports the validity of water molecules rather than chlorines for the axial ligands. The refinement of the crystallographic data with the chlorine ligands on the axial coordination sites gives much higher *B* factor values for the chlorine atoms (∼45) than the rhodium atom (27.51; see Supporting Information), which would be unlikely in the crystal structure. To the contrary, when the crystallographic data are refined on the assumption that the water molecules occupy the axial coordination sites, the *B* factor values of the oxygen atoms decrease to a consistent value (∼25) with that of the rhodium atom in both sites. According to the UV−vis spectra, no significant change is caused in an electronic state of **1-Ph** by the incorporation process (see Supporting Information).

Hydrophobic interactions of **1-Ph** with five amino acid residues, Leu29, Phe43, Val68, Leu89, and Ile107, are observed with closest contacts of 3.29, 3.13, 3.54, 3.41, and 3.53 Å, respectively (Figure 2d). These interactions have induced shifts of the amino acid residues from their original positions in native Mb[30] to provide a pocket that fits in the ligand plane of **1-Ph** (Figure 2d,f). By contrast, the root-mean-square deviation (rmsd) of C^{α}-positions of **1-Ph**·A71GMb from native Mb is only 0.66 Å (Figure 3), comparable to those for Fe^{III}porphin·apo-Mb (0.77 Å) and biliverdin·apo-Mb (0.66 Å).[31,32] This indicates that the whole structure of Mb is virtually conserved for **1-Ph**·A71GMb regardless of the significantly different arrangement of the complex from the original. The flexible motion of the side chains to settle the complex and little deviation of the whole structure from native Mb suggest that the cavity of apo-Mb could be a versatile space to accommodate artificial prosthetic molecules without serious perturbation and denaturation of the protein structure. In this context, the replacement of Ala71 with Gly does not appear to serve any specific role in apo-Mb to form the stable composite with **1-Ph**. The closest contact, 7.4 Å, between Gly71 and **1-Ph** in the crystal structure suggests no direct interaction between them. The role of Gly71 in the facile crystallization of **1-Ph**·A71G remains to be solved in further investigations.

Another distinctive interaction is observed between His93 and the phenyl substituents of **1-Ph** (Figure 2e). The phenyl rings hold the imidazole moiety of His93 through the $\pi-\pi$ interaction with distances of 3.41 and 3.18 Å. This reminds us of the hydrophobic interaction found in the composite of apo-Mb with the salophen complexes, **3**, in which 3 and 3′-methyl substituents of **3** hold the alkyl chain of Ile107 in apo-Mb to stabilize the axial coordination of His93 to the metal centers.[9] Since the hydrophobic interaction has been considered to dominate the

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Table 1. Peak Intensity Ratios of the Composite to the Sum of the Composite and apo-A71GMb in ESI-MS[a]

1-Me	1-Et	1-*i*-Pr	1-Ph	1-Bn	2-Ph
23(1)	17(3)	14(3)	93(3)	53(10)	57(4)

[a] All the composites are dissolved in 5 mM ammonium acetate buffer, pH 6.7, for the measurement. Cone voltage and temperature were set to 40 V and 60 °C, respectively. Relative peak intensity ratios of the composite (%) are average values of four independent measurements with standard deviations in parentheses.

stability of the composites consisting of apo-Mb and **3**, one may imagine that holding His93 by the substituents of the Phebox complexes also plays a crucial role to afford the stable composites. We, therefore, assessed the effects of holding His93 by the substituents of the Rh·Phebox complexes on the stability of the composites by means of ESI mass spectroscopy.

Semiquantitative Analysis of Stability for the Composites. It has been demonstrated that ESI-MS is useful to estimate the relative stability of protein−DNA complexes formed through noncovalent bondings.[33] The stability of the complexes can be evaluated semiquantitatively by comparison of peak intensity ratios of the complex to the whole proteins, i.e., the sum of the complexed and uncomplexed proteins. We have applied this method to the present study. Table 1 shows peak intensity ratios of the each composite (**1-X**·A71GMb) to the sum of the composite and apo-A71GMb in the ESI mass spectra. The data are indicative of a certain trend in the stability. The phenyl substituent serves to form a more stable composite than the benzyl substituent (**1-Ph** > **1-Bn**). The substituents having a phenyl ring are more efficient than those consisting of alkyl chains (**1-Bn**, **1-Ph** > **1-Me**, **1-Et**, **1-*i*-Pr**) in the stability of the composites. A smaller alkyl group is likely to stabilize the composites (**1-Me** > **1-Et** ∼ **1-*i*-Pr**). These results and the obtained crystal structure allow the interpretation that the extent of the hydrophobic interaction between His93 and the substituents of the Phebox ligands accounts for the stability of the composites. As shown in Figure 2e, two phenyl rings of **1-Ph** are placed at optimum positions to interact with His93 through the $\pi-\pi$ interaction. Insertion of methylene linkers into the phenyl rings (**1-Bn**) will cause a deviation of the phenyl ring from the optimum position, resulting in a less efficient interaction. The alkyl chain substituents of **1-Me**, **1-Et**, and **1-*i*-Pr** would also cause hydrophobic interactions with His93, while this interaction would not be as effective as the $\pi-\pi$ interaction in **1-Ph**·A71G and **1-Bn**·A71G to stabilize the composites. The lower stability observed for the larger alkyl substituents is due to steric constraint of the methylene groups. Taking these considerations and the findings in the study of the salophen composites[7−9] into account, we propose the crucial role of effective holdings of amino acid residues through hydrophobic interactions to stabilize the composites, while we should mention that the composite stability estimated via ESI-MS does not represent the thermal stability of the proteins. In fact, melting points of all the composites, which are general indicators of the thermal stability, converge to 68−69 °C without any trends corresponding to the ESI-MS data. Therefore, the composite stability estimated here indicates affinity of the Rh·Phebox complexes to the Mb cavity. The significance of the hydrophobic interaction between the substituents of the Phebox ligands and His93 also leads to the difference in the stability of the composites containing (*S*,*S*)- and (*R*,*R*)-Rh·Phebox with the phenyl substituents.

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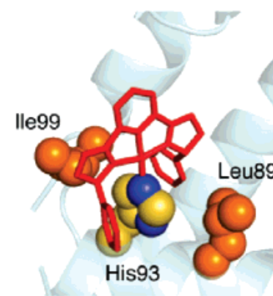

Figure 4. Side chains of Leu89, Ile99, and His93 (depicted with the sphere model) form a hollow suitable to accept the phenyl ring of **1-Ph**.

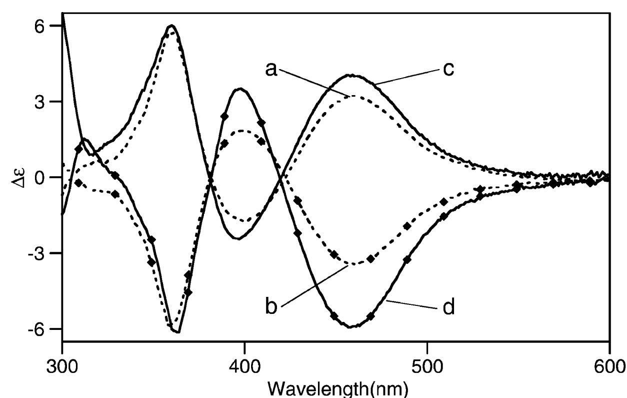

Figure 5. CD spectra of (a) **1-Ph**, (b) **2-Ph**, (c) **1-Ph**·A71GMb, and (d) **2-Ph**·A71GMb recorded at room temperature. The solvents of (a) and (b) are MeOH, and those of (c) and (d) are 10 mM Tris/HCl buffer (pH 7.0).

Enantioselectivity in (*S*,*S*)- and (*R*,*R*)-Isomers of the Rh·Phebox Complexes by apo-Mb. The ESI-MS data reveal another feature of the stability of the composites with respect to the (*R*,*R*)- and (*S*,*S*)-isomers of the Rh·Phebox complexes. Although the (*R*,*R*)-isomer of the Rh·Phebox complex with the phenyl substituents (**2-Ph**) forms a composite with apo-A71G (**2-Ph**·A71G), the stability of **2-Ph**·A71G is estimated to be lower than **1-Ph**·A71G based on the ESI-MS data (Table 1), suggesting that **1-Ph** is more preferable than **2-Ph** to form the composite with A71G. The crystal structure focusing on the vicinity of the phenyl substituents of **1-Ph** gives insight into the preference in light of the precise fitting of the substituents into the cavity (Figure 4). The side chains of Leu89, His93, and Ile99 form a hollow that appears quite suitable to fit one of the phenyl rings of **1-Ph**, while significant distortion would be induced on **2-Ph** in adapting the phenyl ring to the hollow if the arrangement of **2-Ph** in apo-A71GMb is similar to that of **1-Ph**. Circular dichroic data for **1-Ph**·A71GMb and **2-Ph**·A71GMb are consistent with this deduction (Figure 5). The spectrum of **1-Ph** in solution shows maximal and minimal ellipticities ($\Delta\epsilon$) at 360, 398, and 461 nm (line a), which are due to the chiralitic configuration of the phenyl substituents in the (*S*,*S*)-isomer. The exactly reversed spectrum of **1-Ph** is, therefore, observed for **2-Ph** (line b). Upon the incorporation into apo-A71GMb, however, **1-Ph** (line c) and **2-Ph** (line d) show distinctive changes in $\Delta\epsilon$. The spectrum of **1-Ph**·A71GMb is rather similar to that of **1-Ph** except for some enhancement in $\Delta\epsilon$ at 398 and 461 nm, indicating that **1-Ph** nearly retains the original structure in apo-A71GMb. By contrast, the formation of **2-Ph**·A71GMb accompanies apparent enhancement in $\Delta\epsilon$, which implies that significant structural distortion is induced on **2-Ph** by the incorporation into the cavity of apo-A71GMb, in agreement with the deduction from the crystal structure. Such

structural strain would reduce the efficiency of the $\pi-\pi$ interaction between **2-Ph** and His93, and consequently, the composite with **2-Ph** is less stable than that with **1-Ph**. According to these observations, we conclude that the cavity of apo-A71GMb is competent to recognize the asymmetric configuration of the Rh·Phebox complexes, which is reflected in the stability of the composites.

Conclusion

In the present study, we have prepared novel organometallic proteins consisting of apo-Mb and Rh·Phebox complexes. The X-ray structural analysis of **1-Ph**·A71G demonstrates that the cavity of apo-Mb is capable of forming a 1:1 composite with an organometallic compound that is different in molecular shape and the arrangement in the cavity from the heme. The unique arrangement of **1-Ph** in the cavity is due to the equatorial coordination of His93 to **1-Ph** with assistance of hydrogen bonding and a number of hydrophobic interactions between the amino acid residues and the Phebox ligands. The ESI-MS analysis reveals that the Rh·Phebox complexes bearing the more suitable substituents to hold His93 form the more stable composites, indicating that the interaction between the substituents of the Phebox ligands and His93 is crucial to the composite stabilities. The enantioselectivity observed for the (*S,S*)- and (*R,R*)-isomers of Rh·Phebox complexes with phenyl substituents could be explained along the same line. Recently, we have found other novel structures in composites of apo-Mbs with Cu(II) complexes,[34] which also evidence the capability of the heme cavity to contain various types of artificial prosthetic groups besides the heme. The results obtained here could be a guideline to produce stable organometallic proteins and regulate arrangements of artificial prosthetic groups in the cavity, which are fundamentals to bring about characteristic reaction activities of organometallic proteins. An investigation on the reactivity of **1-X**·A71GMb is currently in progress.

Experimental Section

The expression and purification of both the wild type and variant Mb (A71G) were performed as reported previously.[35] Phebox ligands and their rhodium complexes were prepared by literature methods.[36,37]

Preparation of the Composites of Rh·Phebox Complexes with the Wild-Type and A71G apo-Mb (1-X·Mb, 1-X·A71G). All the reactions were carried out at 4 °C. The methanol solution of a Rh·Phebox complex (1.3 mL, 1.2 mM) was slowly added to an apo-Mb solution (13 mL, 100 μM in 10 mM Tris/HCl, pH 7.0), and then the mixture was stirred for 30 min at 4 °C, followed by dialysis against 10 mM Tris/HCl buffer (pH 7.0) at 4 °C for 6 h. Then, the reaction mixture was passed through Sephadex G25 and G75 equilibrated with 10 mM Tris/HCl buffer (pH 7.0) for final purification of composites from excess complexes. The purified composites were concentrated to 1 mM and stored at 4 °C until use.

Mass Spectrometry. ESI-MS analyses were performed on a Micromass LCT instrument. Typical parameters are as follows: capillary voltage, 3 kV; cone voltage, 40 V; source temperature, 60 °C; flow rate, 3 μL min^{-1}. CsI (1 mg/mL) in 50% H_2O/MeCN was used for mass scale calibration. All samples were dialyzed against 5 mM ammonium acetate buffer (pH 6.7) at 4 °C for 8 h and diluted to 10 μM by the same buffer solution. The stability of the composites was evaluated by comparison of peak intensity ratios of the composite (Y) to the whole proteins (X + Y, where X represents the peak intensity of the apo-Mb). See Supporting Information for more details about X and Y.

Circular Dichroic Spectroscopy. Circular dichroism spectra were recorded on a JASCO model J-720 spectropolarimeter. Protein samples (2.5 μM in 10 mM Tris/HCl buffer, pH 7.0) were placed into a quartz cell (10 mm path length), and ellipticity was recorded from 200 to 600 nm at 25 °C.

Crystallization of 1-Ph·A71GMb. 1-Ph·A71GMb was purified with Sephadex G75 and crystallized at 4 °C with the hanging-drop vapor diffusion method. Drops of 4 μL containing approximately 5.4 mg/mL protein and sodium/potassium phosphate buffer (NaH_2PO_4/K_2HPO_4, pH 6.8, 1.35 M) were equilibrated against 1 mL of reservoir solution containing the same buffer in 2-fold higher concentration (2.7 M). The colorless crystal grew within 5 days. Before data collection, the crystal of **1-Ph**·A71GMb was briefly immersed in a cryoprotectant solution that consisted of 15% (w/v) glycerol and 2.7 M sodium/potassium phosphate buffer (NaH_2PO_4/K_2HPO_4, pH 6.8).

X-ray Data Collection and Crystallographic Refinement. X-ray diffraction data were collected at 100 K using a Rigaku FR-E X-ray generator (wavelength: 1.5418 Å, Cu Kα) and a R-AXIS VII detector at the High-Intensity X-ray Diffraction Laboratory, Nagoya University. The data were processed with the programs *DENZO* and *SCALEPACK*. The Mn^{III}(3,3′-Me_2-salophen)·apo-A71GMb structure (PDB code: 1V9Q) with deletion Mn^{III}(3,3′-Me_2-salophen) moiety was used as an initial model for the refinement of **1-Ph**·A71GMb. The refinement was carried out with the programs *REFMAC5*[38] and *CNS*[39] and manual model building with the program *COOT*.[40] Ramachandran plot parameters were calculated using *PROCHECK*.[41] The coordination data have been deposited in the Protein Data Bank under the accession code 2EF2.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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