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Reconstitution of Myoglobin with a Heme Cofactor Bearing a Ruthenium Tris(2,2'-bipyridine) Pendant

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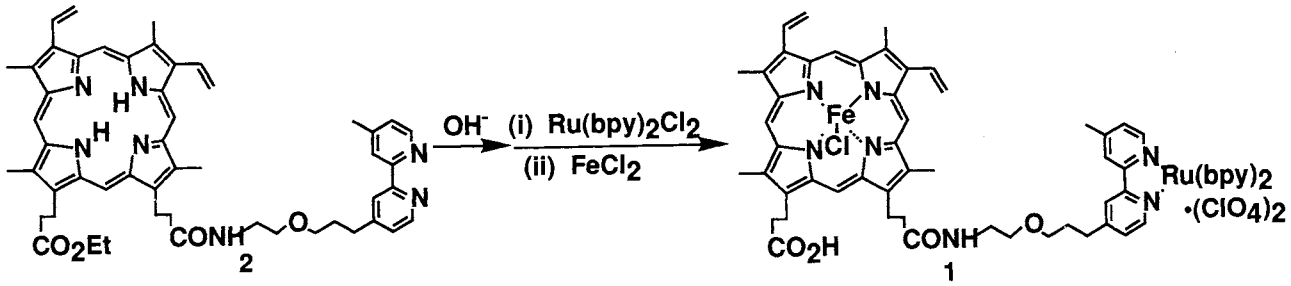
A protoheme derivative with covalently-bound tris(2,2'-bipyridine) ruthenium (II) $[Ru(bpy)_3]^{2+}$ was successfully incorporated into apo-myoglobin. In the reconstituted myoglobin emission of $^*[Ru(bpy)_3]^{2+}$ was quenched intramolecularly, the efficiency being greater by more than 30-fold than that of the corresponding intermolecular process.

The long range electron transfer reaction in the rigid protein matrices is one of the most active research areas because of their essential actions in many biological systems.[1] In order to elucidate their complicated reaction mechanisms, considerable effort has been devoted to prepare modified proteins with redox active groups at a specific position. Gray and coworkers, for example, used myoglobins with a ruthenium ammine complex on the protein surface.[2] Recently, they also synthesized cytochrome c in which the histidine residue was modified with ruthenium bis(2,2'-bipyridine) and observed the long range electron transfer with a large driving force.[3] Durham et al. prepared cytochrome c in which $[Ru(bpy)_3]^{2+}$ is covalently-bound at the lysine residue.[4] Since these modifications are not site-specific but random, the purification process becomes inevitably complex. As an active-site directed modification, we here demonstrate, on the basis of the cofactor reconstitution method, the synthesis and properties of the semi-artifical myoglobin modified with a $[Ru(bpy)_3]^{2+}$ pendant in the proximity of the active site.



Scheme 1.

Protoporphyrin derivative **2** was complexed with bis(2,2'-bipyridine) dichloro ruthenium (II) [$Ru(bpy)_2Cl_2$], followed by iron-insertion to yield the protohemin **1** as shown in Scheme 1.[5] The reconstituted myoglobin (Mb) from **1** was prepared according to the method reported in the literature.[6] The heme **1** (1.2 equiv.) in pyridine was added dropwise to the solution containing apo-myoglobin (from horse heart, 0.1 mM, 1 M = 1 mol dm^{-3}) in an ice-bath and incubated at 4 °C for 12 h. The mixture was centrifuged, dialyzed, and then purified through gel chromatography (Sephadex G-25) to afford the $Ru(bpy)_3$ pendant myoglobin ($Ru(bpy)_3$-Mb).

Figure 1 shows a UV-visible spectral change of **1** induced by the addition of apo-myoglobin (at 10 min intervals). The broad Soret-band at 390 nm due to aggregated heme was shifted to 409 nm and intensified with added apo-Mb. The spectral change was nearly saturated at the 1/1 ratio of the heme **1** over apo-Mb (See inset of Fig. 1), indicating that one equivalent of **1** was incorporated into one equiv. of apo-Mb.

The absorption spectrum of the purified $Ru(bpy)_3$-Mb in met-form (Fig. 2) gave a sharp Soret-band at 409 nm and a Q-band at 633 nm with a shoulder at 457 nm which is ascribed to a metal-to-ligand charge transfer (MLCT) band of the $[Ru(bpy)_3]^{2+}$ unit. This is similar to the spectrum made from the simple sum of the spectra of 1 equiv. of $[Ru(bpy)_3]^{2+}$ and of native met-Mb. It is clear that there is little interaction between $[Ru(bpy)_3]^{2+}$ and the heme of Mb in the ground state.

Figure 2 shows absorption spectra of the reduced and the dioxygen complex of $Ru(bpy)_3$-Mb as well as the oxidized form (met-form). Met-$Ru(bpy)_3$-Mb was rapidly reduced by addition of $Na_2S_2O_4$ powder to deoxy-form (ferrous-heme, λ_{max} = 435 and 557 nm). To this solution was bubbled dioxygen gas to afford dioxygen complex (oxy-form, λ_{max} = 418, 543, and 581 nm). The oxy-form of $Ru(bpy)_3$-Mb was slowly auto-oxidized to met-form at 30 °C through overnight. When the sixth ligand of the heme was exchanged from H_2O to azide or fluoride ion, spectral changes characteristic of these ligands occurred (azide form: 422, 542, and 576 nm, fluoride form: 408, 546, and 606 nm). These spectral changes in redox and ligand exchange reactions were very similar to those in native Mb.[7] These results reveal that heme **1** is certainly reconstituted in the active site of myoglobin.

Emission spectra of $[Ru(bpy)_3]^{2+}$ derivatives are compared in Fig. 3. The aqueous solution of $[Ru(bpy)_3]^{2+}$ gave a broad emission at 590 nm. The intensity was slightly lessened by intermolecular quenching when 1 equiv. of native Mb was added. At the same concentration, the emission intensity from $Ru(bpy)_3$-Mb was decreased to less than 4%. It may be suggested, therefore, that the intramolecular electron transfer quenching occurs much more efficiently in the present $Ru(bpy)_3$-Mb than the simple intermolecular process.

Reconstitution of hemoproteins has been widely conducted for the elucidation of interactions between heme and apo-protein.[8] In contranst, there are few examples which demonstrate that the reconstitution method is useful for introduction of artificial functional molecules in the proximity of the active site. We believe that the

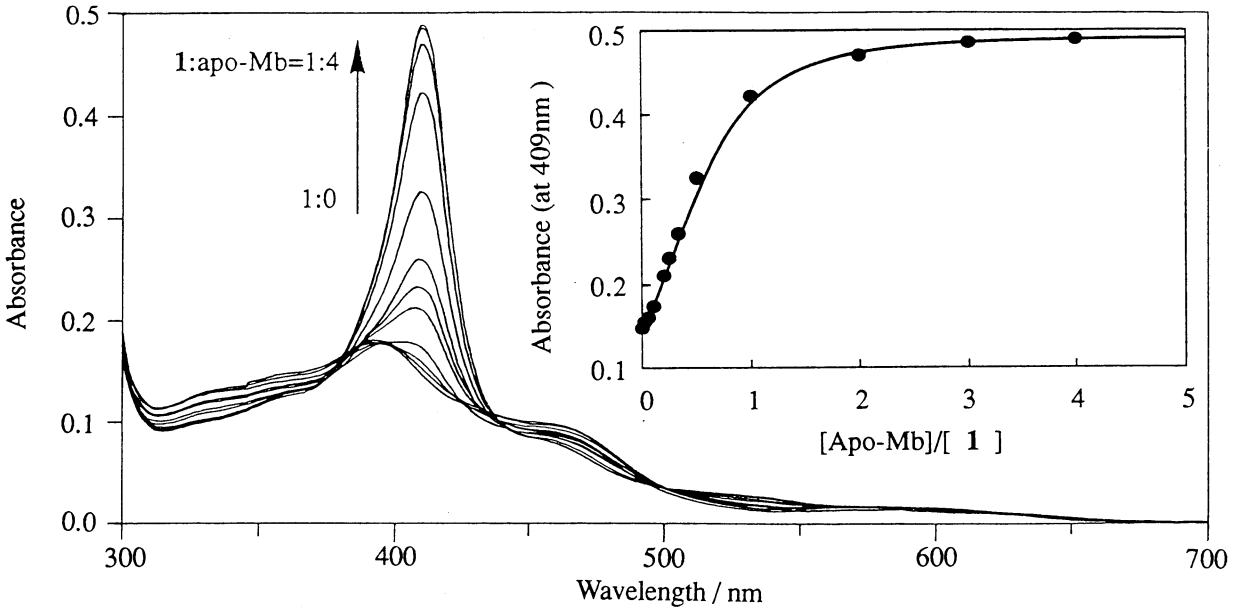


Fig. 1. Absorption spectral changes of the heme **1** by addition of apo-Mb (0 to 4.0 equivalents). **1** (3.3 μM) in 10 mM phosphate buffer, pH 7.5 at 25 °C. Inset: titration curve of **1** with apo-Mb monitored at 409 nm.

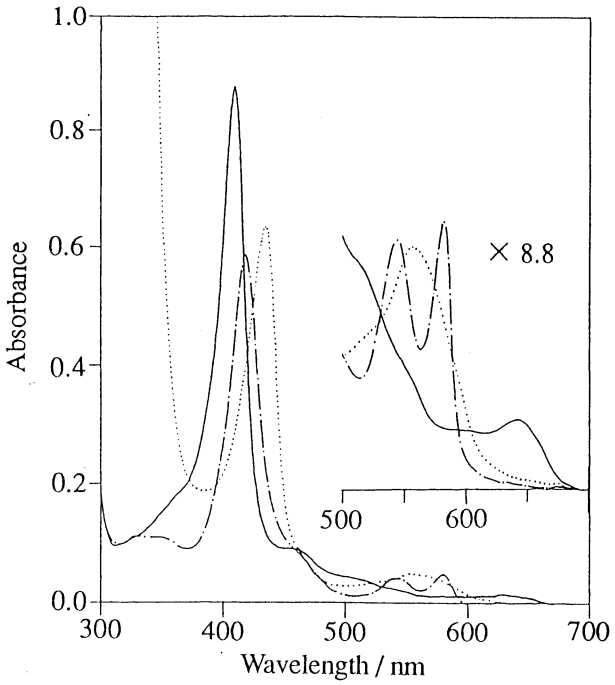


Fig. 2. UV-visible spectra of $Ru(bpy)_3$-Mb in met form (————), deoxy-form (·····), and oxy-form (—·—·—·). $Ru(bpy)_3$-Mb 5.4 μM in 10 mM phosphate buffer, pH 6.0.

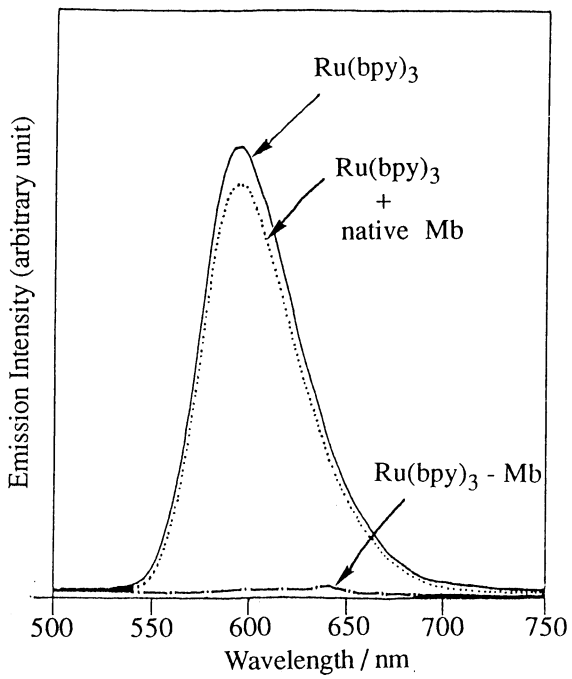


Fig. 3. Steady state emission spectra of $[Ru(bpy)_3]^{2+}$ derivatives 13 μM : $[Ru(bpy)_3]^{2+}$ only (————), equimolar mixture of $[Ru(bpy)_3]^{2+}$ and native Mb (·····), $Ru(bpy)_3$-Mb (—·—·—). excitation at 450 nm.

present method will be applicable more generally and fruitfully to other cofactor-bearing enzymes. Further studies on the photo-regulation of the catalytic activity of our $Ru(bpy)_3$-Mb are currently under progress.

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References

1) R. A. Marcus and N. Sutin, *Biochim. Biophys. Acta*, **811**, 265 (1985).
2) J. R. Winkler and H. B. Gray, *Chem. Rev.*, **92**, 369 (1992).
3) I.-J. Chang, H. B. Gray, and J. R. Winkler, *J. Am. Chem. Soc.*, **113**, 7056 (1991).
4) L. P. Pan, B. Durham, J. Wolinska, and F. Millet, *Biochemistry*, **27**, 7180 (1988); B. Durham, L. P. Pan, J. E. Long, and F. Millett, *Biochemistry*, **28**, 8659 (1989).
5) The protoporphyrin derivative **2** was synthesized by condensation of protoporphyrin mono-ethylester with 4-methyl-4'-(6''-amino-4''-oxahexyl)-2,2'-bipyridine in the presence of diethylcyano phosphate; Anal. Found: C, 72.85; H, 6.65; N, 11.25%. Calcd for $C_{52}H_{57}N_7O_4 \cdot 1/2H_2O$: C, 73.20; H, 6.87; N, 11.50%. The protohemin **1** was purified through gel column chromatography (Sephadex LH20, eluent = MeOH), **1**: Anal. Found: C, 54.46; H, 4.94; N, 9.37%. Calcd for $C_{70}H_{67}N_{11}O_{12}Cl_3FeRu \cdot 2H_2O$: C, 54.09; H, 4.61; N, 9.92%. Regio-isomers were not separated in this study.
6) T. Asakura, *Methods in Enzymology*, ed by S. Fleiser and L. Packer, Academic Press, New York (1978), Part C, p. 446 ; F. W. J. Teale, *Biochim. Biophys. Acta*, **35**, 543 (1959).
7) Q. H. Gibson and M. H. Smith, *J. Physiol.*, **136**, 27 (1957); C. K. Chang and T. G. Traylor, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2647 (1973); M. Tamura, T. Asakura, and T. Yonetani, *Biochim. Biophys. Acta*, **295**, 467 (1973).
8) For example: G. N. La Mar, U. Pande, J. B. Hauksson, R. K. Pandey, and K. M. Smith, *J. Am. Chem. Soc.*, **111**, 485 (1989).

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