Light-Driven Activation of Reconstituted Myoglobin with a Ruthenium Tris(2,2′-bipyridine) Pendant

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Received July 16, 1993

Development of a general methodology for the regulation of protein/enzyme activities by external stimuli is essential for the design of sophisticated biocatalysts which have potential applications to a wide area of biotechnology and medicine. Chemical modification of enzymes with nonnatural functional groups such as photochromic or redox-active molecules, polymers, and electrodes is gradually being proven to be one of the most promising approaches.[1] To establish a more general basis for such modifications, it is desirable to clarify how a site-specifically introduced nonnatural group acts in the semisynthetic protein.

We describe herein the light-driven activation of a semisynthetic myoglobin that is directly modified at the heme-cofactor active site. A reconstituted myoglobin with a heme bearing a photosensitizable tris(2,2′-bipyridyl)ruthenium(II) ($[Ru(bpy)_3]^{2+}$) pendant was effectively activated by visible light to function as a dioxygen storage protein.

Protoheme derivative **1** (Chart I) bound covalently to $[Ru(bpy)_3]^{2+}$ dissolved in pyridine[2] (1.2 equiv to apo-myoglobin (apo-Mb)) was added dropwise to an aqueous solution of apo-myoglobin[3] (0.1 mM, pH 7.0, phosphate buffer) at 4 °C and incubated for 12 h in the dark. The mixture was purified by centrifugation, dialysis, and gel chromatography (Sephadex G25) to afford a reconstituted myoglobin.[4]

Spectrophotometric titration of the heme **1** with apo-Mb clearly shows the formation of a 1:1 complex between **1** and apo-Mb. Purified Ru(bpy)$_3$-pendant myoglobin (oxidized form, met-Ru-Mb, λ_{max} = 409 and 633 nm) shows behavior similar to native Mb in the ligand-exchange reaction from H_2O to fluoride and azide.[5] Met-Ru-Mb is readily reduced by $Na_2S_2O_4$ to deoxy-Ru-Mb (λ_{max} = 435 and 557 nm) and subsequently oxygenated by dioxygen bubbled into this solution (oxy-Ru-Mb, λ_{max} = 418, 543, and 581 nm). These redox properties as detected by absorption spectroscopy are essentially the same as those in native Mb.[6] It is clear, therefore, that Ru-Mb is satisfactorily reconstituted as native Mb.

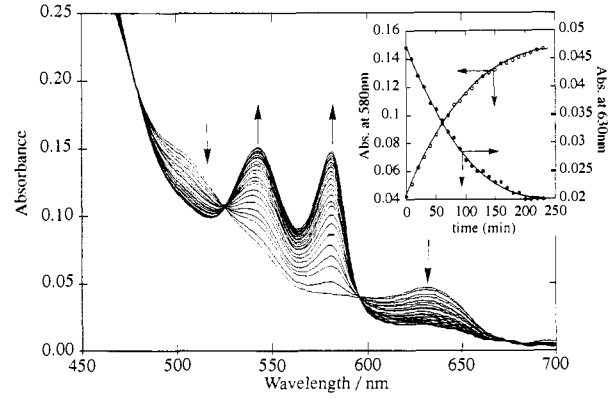
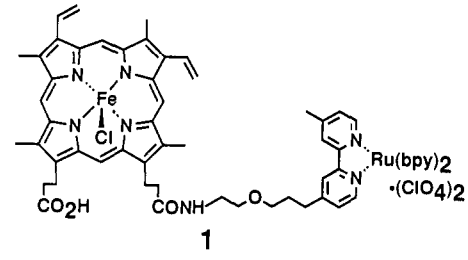


Figure 1. UV–visible spectral changes of Ru-Mb by light irradiation (wavelength cutoff below 450 nm, distance 5 cm) under aerobic condition: 1.2×10^{-5} M Ru-Mb and 1 mM EDTA in 10 mM phosphate buffer, pH 6.0, 25 °C. Measurements were made every 10 min. The arrows indicate directions of the intensity change. Inset: time course of the photoreaction which was monitored by the absorbance increase at 580 nm (oxy-Ru-Mb) and the decrease at 630 nm (met-Ru-Mb).

Chart I



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When the visible light (450-W high-pressure Hg lamp, wavelength cutoff below 450 nm) was applied to the met-Ru-Mb in the presence of EDTA (sacrificial donor, 1 mM) under N_2 atmosphere,[7] the absorbance due to deoxy-Ru-Mb (435 and 557 nm) increased with the decrease in the absorbance due to met-Ru-Mb (409 and 633 nm). The photoproduced deoxy-Ru-Mb quantitatively adsorbed dioxygen to form oxy-Ru-Mb (418, 543, and 581 nm) when dioxygen gas was bubbled into the solution. Photoirradiation of met-Ru-Mb under aerobic conditions gave the dioxygen complex of Ru-Mb directly, accompanying the decrease of met-Ru-Mb (Figure 1). First-order kinetics are observed for both the appearance of oxy-Ru-Mb and the disappearance of met-Ru-Mb, as shown in the inset of Figure 1. Oxy-Ru-Mb thus obtained was stable for a few days and slowly autooxidized to met-Ru-Mb with a half-life of 18 h in the dark at room temperature. No activation reaction of Ru-Mb occurred in the absence of EDTA and/or visible light.

The effect of light irradiation is clearly shown by the light on-and-off experiment (Figure 2a): Ru-Mb was activated to the dioxygen complex only when the visible light was on. These results reveal that the active center (protohemin) of Ru-Mb was reduced from the ferric to the ferrous state by photoinduced electron transfer, followed by the reaction with dioxygen gas. In native Mb, oxy-Mb cannot be formed by light irradiation.

It is of particular interest to compare the efficiency of light-driven activation of Ru-Mb with that of an intermolecular reaction (i.e., equivalent molecular mixture of $[Ru(bpy)_3]^{2+}$ and native Mb) by the rate of oxy-Mb formation. In sharp contrast to the intramolecular activation of Ru-Mb (Figure 2b), the intermolecular reaction stopped and leveled off at about 10% conversion (Figure 2c). The stopped reaction restarted when EDTA (10

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(7) Met-Ru-Mb gave an absorption spectrum similar to that made from the simple sum of $[Ru(bpy)_3]^{2+}$ and of native met-Mb. A metal-to ligand charge transfer (MLCT) band of the $[Ru(bpy)_3]^{2+}$ at 457 nm (shoulder) is observed.

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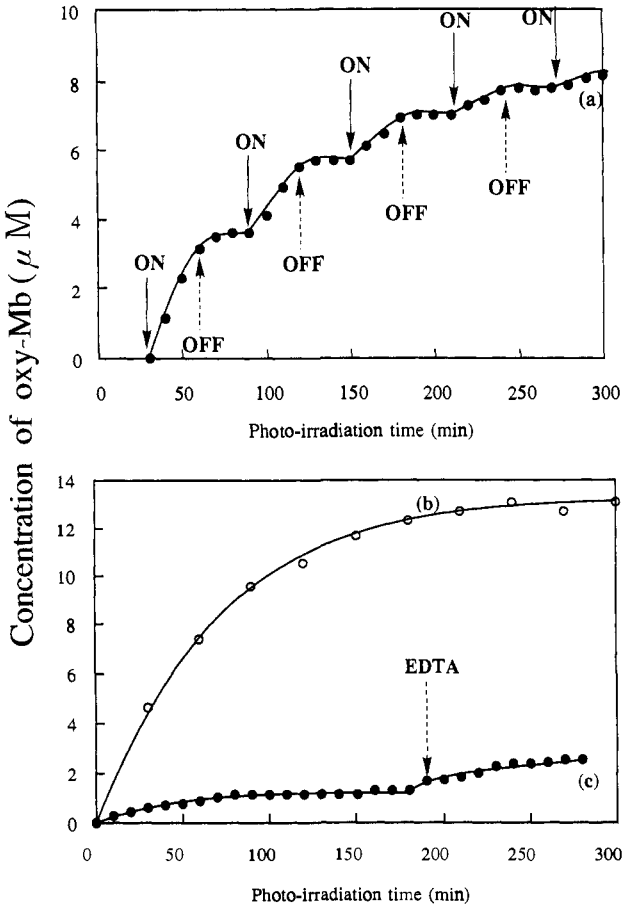

Figure 2. Time course of the photogeneration of oxy-Mb. (a) The light on-and-off experiment of Ru-Mb. The solid arrows and the broken arrows indicate that visible light is on and off, respectively. (b) The intramolecular (Ru-Mb) reaction (the reaction conditions are identical with those of Figure 1). (c) The intermolecular reaction (1.2×10^{-5} M $[Ru(bpy)_3]^{2+}$, 1.2×10^{-5} M native met-Mb, and 1 mM EDTA in 10 mM phosphate buffer, pH 6.0, 25 °C). The broken arrow indicates the point of addition of the extra amount of EDTA (10 mM).

mM) was added. The conversion of the intermolecular reaction strongly depend on the initial concentration of EDTA (1–100 mM). A concentration of 100 mM EDTA is needed to complete the formation of oxy-Mb.[8] Therefore, the efficiency of light activation of Ru-Mb is 100 times more enhanced than that of the simple mixed system.

(8) The reaction rate and the conversion in Ru-Mb are not dependent on the EDTA concentration in the range of 0.5–10 mM.

The steady-state emission spectrum shows that the emission intensity of Ru-Mb is lower by 30-fold than that of the intermolecular system (λ_{ex} = 480 nm, λ_{em} = 600 nm). The emission lifetime studies of Ru-Mb also indicate a single exponential decay in emission, with excited $[Ru(bpy)_3]^{2+}$ lifetime of 35 ns (λ_{ex} = 355 nm (Nd-Y laser excitation)) under Ar atmosphere, which is remarkably shorter than that of the corresponding intermolecular quenching (the lifetime of the excited state of $[Ru(bpy)_3]^{2+}$ was 717 ns (a single exponential)). Conceivably, such differences in electron-transfer rates from excited $[Ru(bpy)_3]^{2+}$ to the heme may affect the total efficiencies of the light activation process of Mb.[9]

In summary, it is concluded that the present protein Ru-Mb, which is synthesized by a cofactor reconstitution method, is photoactivatable through a long-range electron transfer. Considerable efforts have been devoted to elucidating the mechanism of long range electron transfer in proteins for the past several years.[10] Compared to previous approaches, the present approach is quite unique because (i) a cofactor reconstitution method is demonstrated to be applicable for the active-site directed introduction of nonnatural functional groups and (ii) a long-range electron-transfer rate can remarkably influence the net activity of the semisynthetic protein. The covalent bond between $[Ru(bpy)_3]^{2+}$ and the active site may facilitate electron-transfer communication as an electron wire.[11] Detailed studies of the mechanisms and kinetics of light activation of Ru-Mb are now underway in our laboratory.

Acknowledgment. We are grateful to Professors Taku Matsuo and Sunao Yamada and Mr. Mitsuhiro Kawazu for the measurement of emission lifetimes of Ru complexes. This research is partially supported by Izumi Science and Technology Foundation.

(9) There may be another mechanism in which EDTA acts as a reductive quencher of the excited $Ru(bpy)_3^{2+}$ to generate $Ru(bpy)_3^{1+}$, especially in the intermolecular activation of Mb. We are currently investigating the mechanism of the long range electron transfer in the light activation reaction.

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