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On the ruthenium(II) polypyridine labeled cytochrome c_3

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Abstract

The ruthenium(II) polypyridine labeled cytochrome c_3 derivative, Ru(bpy)$_2$(dcbpy)-cytochrome c_3, was synthesized and characterized by using spectroscopic techniques and applied to hydrogen evolution. Ru(bpy)$_2$(dcbpy)-cytochrome c_3 can be used as a substrate on hydrogen evolution with hydrogenase. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Ruthenium(II) polypyridine labeled cytochrome c_3; Intramolecular electron transfer; Hydrogen evolution; Hydrogenase

1. Introduction

Photoinduced hydrogen evolution from water has been studied extensively using a system composed of four components: electron donor (D), photosensitizer (P), electron carrier (C) and catalyst as shown in Scheme 1 [1–4]. Among inorganic photosensitizers, ruthenium tris(2,2′-bipyridine) derivatives are suitable compounds for photoinduced hydrogen evolution system, because ruthenium complexes possess properties such as photostability in water, high extinction coefficients in the visible region, and relatively long-lived excited states.

In the four-component system, the yield of hydrogen evolution is low because of the diffusional electron transfer step from the photoexcited state of the photosensitizer to the catalyst. To improve the system, the rapid electron transfer between photosensitizer and catalyst is desired. As a catalyst, hydrogenase is used in this reaction. Hydrogenase from *Desulfovibrio vulgaris* (Miyazaki) catalyzes hydrogen evolution with reduced form of methylviologen as a substrate. On the other hand, in vivo the hydrogenase catalyzes hydrogen evolution with reduced cytochrome c_3. Therefore, cytochrome c_3 can be used as an electron carrier instead of viologen in the photoinduced hydrogen evolution, so that rapid electron transfer from the electron carrier to hydrogenase may occur. To achieve the rapid electron transfer from the photoexcited state of the photosensitizer to the catalyst in this system, cytochrome c_3 is used as an electron carrier and is connected to the photosensitizer.

In this paper, we hope to describe the preparation and the photochemical properties of Ru(bpy)$_2$(dcbpy)-cytochrome c_3. In addition, to investigate the substrate affinity for hydrogenase, hydrogen evolution with dithionite-reduced

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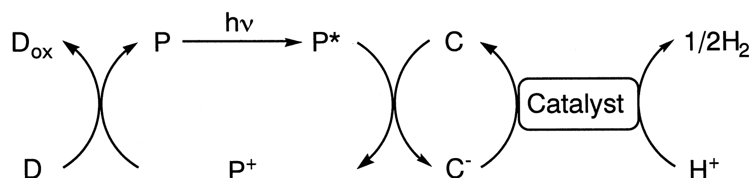

Scheme 1. Photoinduced hydrogen evolution system.

$Ru(bpy)_2(dcbpy)$-cytochrome c_3 as a substrate was carried out.

2. Experimental

2.1. Materials

All the reagents used were of analytical or of the highest grade available. 4,4′-dimethyl-2,2′-bipyridine (dmbpy), 4,4′-dicarboxy-2,2′-bipyridine (dcbpy) and $Ru(bpy)_2CO_3$ were prepared as previously described [5–7]. Cytochrome c_3 and hydrogenase were purified from *D. vulgaris* (Miyazaki) according to the literature [8,9].

2.1.1. Preparation of mono-N-hydroxysuccinimide ester of dcbpy

Mono-*N*-hydroxysuccinimide ester of dcbpy was prepared analogously to the literature [10]. Dcbpy (0.16 μmol) was dissolved in 3.0 ml of water and treated with 0.16 μmol of KOH to convert one of the two protonated carboxyl groups to K^+ salt. After removal of water under vacuum, the compound was dissolved in 1.2 ml of dry DMF. The compound was stirred with dicyclohexylcarbodiimide (0.16 μmol) and *N*-hydroxysuccinimide (0.16 μmol) at 30°C for 15 h. The white precipitate was removed by centrifugation and DMF was removed under vacuum. Mono-*N*-hydroxysuccinimide ester of dcbpy was dissolved in 1.5 ml of 10 mmol dm^{-3} EPPS buffer (pH 8.5). White di-*N*-hydroxysuccinimide precipitate was removed by centrifugation.

2.1.2. Preparation of dcbpy-cytochrome c_3 derivative

Cytochrome c_3 was treated with freshly prepared mono-*N*-hydroxysuccinimide ester of dcbpy (excess) in 10 mmol dm^{-3} EPPS buffer (pH 8.5) at 30°C for 3 h. The sample solution was applied to Sephadex G-25 gelfiltration column (Pharmacia) to remove the excess mono-*N*-hydroxy-succinimide ester of dcbpy. Dcbpy-labeled cytochrome c_3 was chromatographed on SP Sepharose Fast Flow HR10/10 column (Pharmacia) with 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4). The dcbpy-labeled cytochrome c_3 was eluted by using a linear gradient from 0 to 250 mmol dm^{-3} NaCl at 25 mmol dm^{-3} min^{-1}. After SP Sepharose Fast Flow column, Fr. 1 and 2 of dcbpy-labeled cytochrome c_3 were applied to Mono S HR5/5 column (Pharmacia) with 20 mmol dm^{-3} ammonium acetate buffer (pH 4.8) and then eluted by a linear gradient from 0 to 250 mmol dm^{-3} NaCl at 2.5 mmol dm^{-3} min^{-1}. Fr. 3 of dcbpy-labeled cytochrome c_3 after SP Sepharose Fast Flow was applied to Mono S column with 20 mmol dm^{-3} phosphate buffer (pH 6.0) and eluted with a linear gradient from 0 to 250 mmol dm^{-3} NaCl at 2.5 mmol dm^{-3} min^{-1}.

2.1.3. Preparation of $Ru(bpy)_2(dcbpy)$-cytochrome c_3 derivative

Dcbpy-cytochrome c_3 was treated with 10 equiv. of $Ru(bpy)_2CO_3$ in 100 mmol dm^{-3} sodium acetate buffer (pH 4.0) at 30°C for 24 h. The sample solution was applied to Sephadex G-25 column to remove the excess $Ru(bpy)_2CO_3$

and then chromatographed on CM Sepharose Fast Flow column with 20 mmol dm^{-3} phosphate buffer (pH 6.0). $Ru(bpy)_2$(dcbpy)-cytochrome c_3 was eluted with a linear gradient from 0 to 250 mmol dm^{-3} NaCl at 2.5 mmol dm^{-3} min^{-1}. The sample fractions were concentrated by using Centricon 10 (Pharmacia) and exchanged with 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4).

2.2. Spectroscopic measurements

UV–vis absorption spectra were measured in 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4) using a Shimadzu MultiSpec-1500 spectrometer.

The luminescence spectra were measured in 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4) at room temperature using Hitachi F-4000 spectrometer. The absorbance at the excitation wavelength was kept constant at 0.05 for all the sample solutions in these experiments.

2.3. Hydrogen evolution with $Ru(bpy)_2$(dcbpy)-cytochrome c_3 and hydrogenase

To investigate the substrate affinity of $Ru(bpy)_2$(dcbpy)-cytochrome c_3 for hydrogenase, hydrogen evolution with dithionite-reduced $Ru(bpy)_2$(dcbpy)-cytochrome c_3 as a substrate was carried out. The sample solutions containing dithionite (40 mmol dm^{-3}), $Ru(bpy)_2$(dcbpy)-cytochrome c_3 (2.0 μmol dm^{-3}) in 2.0 ml of 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4) under argon were incubated at 30°C for 10 min. The reaction was started by injection of 10 μl of hydrogenase solution (final conc.; 32 nmol dm^{-3}). Evolved hydrogen was detected by gas chromatography (Shimadzu GC-14B, detector: TCD, column: active carbon).

3. Results and discussion

3.1. Preparation of $Ru(bpy)_2$(dcbpy)-cytochrome c_3

$Ru(bpy)_2$(dcbpy)-cytochrome c_3 was prepared by two step procedures as shown in Fig. 1. The first step was the treatment of cytochrome c_3 with mono-*N*-hydroxysuccinimide ester of dcbpy to convert positively charged lysine amino groups to negatively charged dcbpy lysine groups. Singly labeled dcbpy-cytochrome c_3 was purified by SP Sepharose Fast Flow and fractionated in four fractions. MALDI TOF-mass measurements showed that each purified dcbpy-cytochrome c_3 (Fr. 1–3) contained a single equiv. of dcbpy and Fr. 4 was native-cytochrome c_3. Further purifications of dcbpy-cytochrome c_3 (Fr. 1–3) were carried out by Mono S column and fractionated in three fractions. The second step involved the treatment of the dcbpy-cytochrome c_3 (Fr. 1–3) with $Ru(bpy)_2$-CO_3 to form singly labeled $Ru(bpy)_2$(dcbpy)-cytochrome c_3 derivative. $Ru(bpy)_2$(dcbpy)-cytochrome c_3 was purified by using cation-exchange chromatography.

3.2. Photochemical properties of $Ru(bpy)_2$-(dcbpy)-cytochrome c_3

A typical absorption spectrum of Ru-$(bpy)_2$(dcbpy)-cytochrome c_3 (Fr. 1) is shown

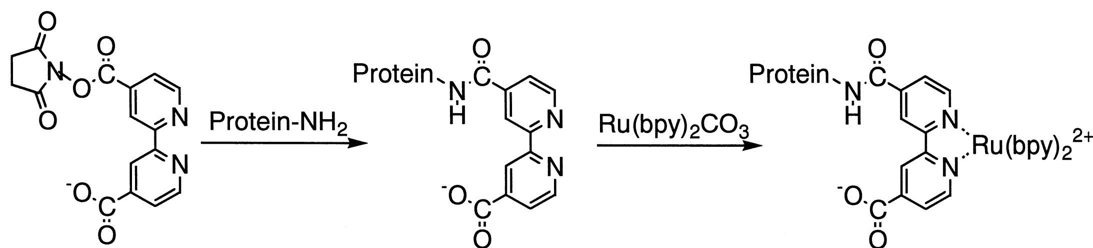

Fig. 1. Preparation of ruthenium(II) polypyridine labeled cytochrome c_3 derivative.

in Fig. 2. The absorption spectrum of $Ru(bpy)_2(dcbpy)$-cytochrome c_3 (Fr. 1–3) is almost the same as the spectrum of equimolar mixture of native cytochrome c_3 and $Ru(bpy)_2(dcbpy)$, indicating no electronic interaction between the $Ru(bpy)_2(dcbpy)$ site and the cytochrome c_3 in the ground state.

The photoexcited state of $Ru(bpy)_2(dcbpy)$-cytochrome c_3 was studied by luminescence emission spectra measurements. The luminescence spectra of $Ru(bpy)_2(dcbpy)$-cytochrome c_3 (Fr. 1–3) are shown in Fig. 3. For $Ru(bpy)_2(dcbpy)$-cytochrome c_3, the shapes of the luminescence spectra of $Ru(bpy)_2(dcbpy)$-cytochrome c_3 were the same as that of $Ru(bpy)_2(dcbpy)$ + cytochrome c_3 (1:1). However, the luminescence intensity of $Ru(bpy)_2(dcbpy)$-cytochrome c_3 was lower than that of $Ru(bpy)_2(dcbpy)$ + cytochrome c_3 (1:1). These results indicate that the photoexcited state of $Ru(bpy)_2(dcbpy)$ moiety is oxidatively quenched by the bound cytochrome c_3 and no electronic interaction occurs between the photoexcited state of $Ru(bpy)_2(dcbpy)$ moiety and bound cytochrome c_3.

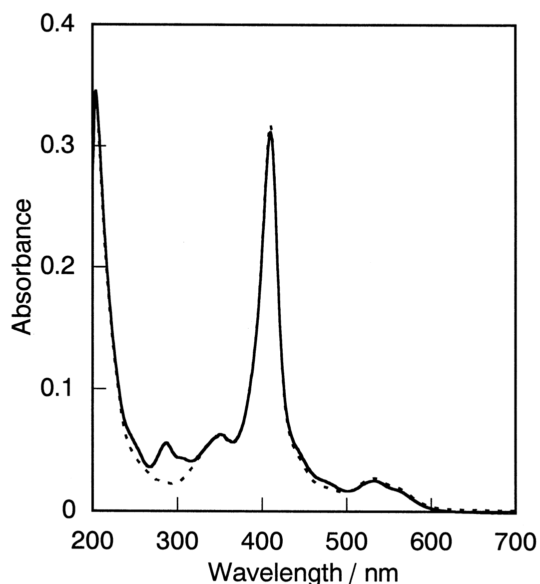

Fig. 2. Absorption spectra of $Ru(bpy)_2(dcbpy)$-cytochrome c_3 and native-cytochrome c_3 in 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4).

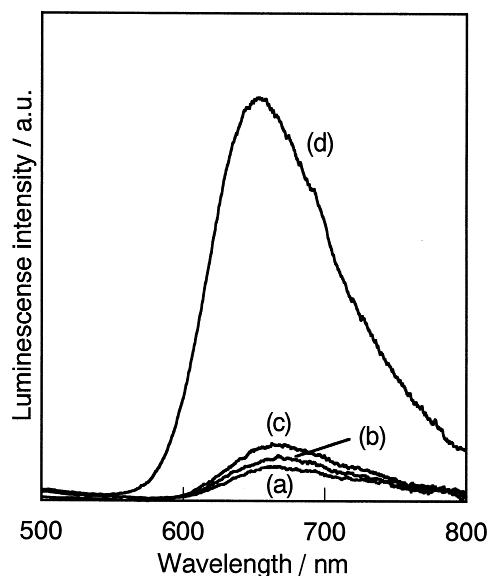

Fig. 3. Luminescence spectra of $Ru(bpy)_2(dcbpy)$-cytochrome c_3 (a: Fr. 1, b: Fr. 2, c: Fr.3) and $Ru(bpy)_2(dcbpy)$ (d) in 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4). The excitation wavelength was 287 nm.

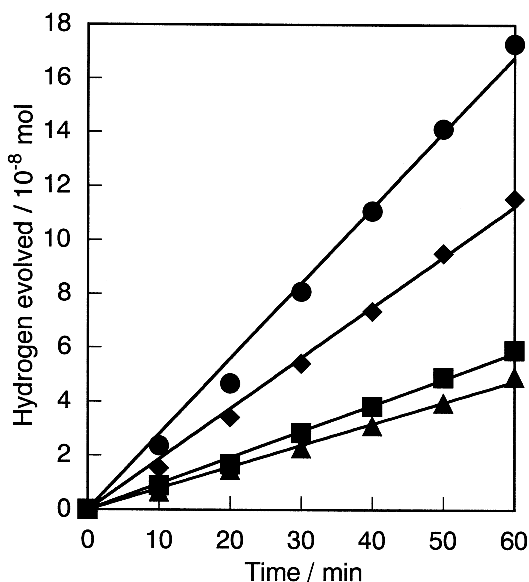

Fig. 4. Time dependence of hydrogen evolution with dithionite-reduced $Ru(bpy)_2(dcbpy)$-cytochrome c_3 and hydrogenase. The sample solution contained 2.0 μmol dm^{-3} of $Ru(bpy)_2(dcbpy)$-cytochrome c_3 (■: Fr. 1, ▲: Fr. 2, ◆: Fr. 3), 40 mmol dm^{-3} of dithionite and 32 nmol dm^{-3} of hydrogenase in 2.0 ml of 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4). For individual system, the solution contained 2.0 μmol dm^{-3} of cytochrome c_3 (●), 40 mmol dm^{-3} of dithionite and 32 nmol dm^{-3} of hydrogenase in 2.0 ml of 25 mmol dm^{-3} Tris–HCl buffer (pH 7.4).

3.3. Hydrogen evolution with Ru(bpy)$_2$(dcbpy)-cytochrome c_3 and hydrogenase

Fig. 4 shows the time dependence of hydrogen evolution with dithionite-reduced cytochrome c_3 and hydrogenase. In the case of the solution containing dithionite-reduced cytochrome c_3 and hydrogenase, hydrogen evolution was observed in proportion to the reaction time. For Ru(bpy)$_2$(dcbpy)-cytochrome c_3 hydrogen evolution was also observed, indicating that Ru(bpy)$_2$(dcbpy)-cytochrome c_3 can be used as a substrate on the photoinduced hydrogen evolution. However, the hydrogen evolution rate of Ru(bpy)$_2$(dcbpy)-cytochrome c_3 (Fr. 1–3) was lower than that of cytochrome c_3. One possible interpretation is that the attached ruthenium complex on the Ru(bpy)$_2$(dcbpy)-cytochrome c_3 may alter the geometry of the complex between cytochrome c_3 and hydrogenase, so that the hydrogen evolution rate decreases. In addition, the hydrogen evolution rates of Ru(bpy)$_2$(dcbpy)-cytochrome c_3 (Fr. 1–3) were between 0.66 and 1.5 nmol min^{-1}. From these results, it is suggested that Fr. 3 of Ru(bpy)$_2$(dcbpy)-cytochrome c_3 may be the optimized complex between cytochrome c_3 and hydrogenase for rapid electron transfer among all the Ru(bpy)$_2$(dcbpy)–cytochrome c_3 complexes.

Acknowledgements

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