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Photoreduction of cytochrome *c* with zinc protoporphyrin reconstituted myoglobin (ZnPP–Mb)

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Abstract

Photoreduction of cytchrome *c* (cyt. *c*) proceeded by irradiation of the visible light in the reaction system consisting of EDTA, ZnPP–Mb and cyt. *c*. About 90% of cyt. *c* was reduced in 20 min of irradiation of the light. Photoinduced electron transfer occurs between ZnPP–Mb and cyt. *c* in the above system. Laser flash photolysis showed that the oxidative quenching of ^{3}ZnPP–Mb* by the oxidized cyt. *c* took place and the quenching rate constant was $8.5 \times 10^5\ M^{-1}s^{-1}$.

Keywords: Cytochrome *c*; Electron transfer; Flash photolysis; Laser flash photolysis; Myoglobin; Photoinduced electron transfer; Protoporphyrin; Zinc

1. Introduction

Electron transfer between proteins is one of the critical reactions in the living organisms. In photosynthesis, for example, photoinduced electron transfer takes place in the reaction center to accomplish charge separation and then the stepwise electron transfer proceeds in the dark. Intra- and intermolecular electron transfer proceeds in the respiration chain coupled with the oxidative phosphoriration to form ATP. In these electron transfer systems, prosthetic groups such as heme, flavins, quinones, metal ions and metal clusters act as the active site for electron transfer to be oxidized or reduced. On one hand, polypeptide chain controls the reaction in the substrate specificity and the reactivity. The substrate specificity is caused by the molecular recognition of polypeptide chain. The redox potential of the prosthetic groups is tuned by the microenvironment around them in the protein molecules. Therefore, the same prosthetic groups sometimes show the different redox potential in the different proteins.

To study electron transfer between proteins, laser flash photolysis can be a useful tool if the prosthetic groups in the protein molecules have a long enough lifetime at the photoexcited state to occur electron transfer. As iron porphyrins have a short lifetime even at the excited triplet state, the native heme proteins containing iron porphyrins as the prosthetic groups cannot be used in laser flash photolysis. On the other hand, zinc porphyrins have longer lifetimes of the excited triplet state compared with other metalloporphyrins. Therefore, the excited triplet state of zinc porphyrins are active as a photosensitizer, and are useful

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as a photochemical probe to study photoinduced electron transfer [1,2].

As the substitution of metal ion or heme group can take place in heme proteins, zinc porphyrin reconstituted heme proteins can be prepared. [3–7] We have previously reported that the photoinduced electron transfer proceeds between zinc protoporphyrin reconstituted myoglobin (ZnPP–Mb) and methyl viologen [7]. When ZnPP–Mb is photo-excited at 532 nm by a Nd–YAG laser pulse, zinc protoporphyrin in ZnPP–Mb is excited to form the excited triplet state, which has a lifetime of 15 ms. Methyl viologen quenches the excited triplet state of ZnPP–Mb (^{3}ZnPP–Mb*) to form separated ion pair showing that photoinduced electron transfer takes place between ZnPP–Mb and methylviologen and that zinc porphyrin is a useful photochemical probe to study electron transfer in the biological system [7].

In this work, we will report photoinduced electron transfer between ZnPP–Mb and cyt. *c*, and show that the reduction of cyt. *c* occurs under steady irradiation of the visible light in the reaction system containing ZnPP–Mb as a photosensitizer and EDTA as an electron donor. And the rate constant has been determined by laser flash photolysis for photoinduced electron transfer between ZnPP–Mb and cyt. *c*.

2. Materials and methods

Myoglobin from horse skeletal muscle and cyt. *c* from horse heart (Type VI) were obtained from Sigma Chem. Co. Zinc protoporphyrin IX was purchased from Porphyrin Products, Inc. ZnPP–Mb was prepared as reported previously [7]. Cyt. *c* was oxidized before the experiment as described below. About 10 mg of cyt. *c* was dissolved in 5 ml of 10 mM phosphate buffer (pH 7.0), and 10^3 equivalent of potassium ferricyanide was added to be stirred for 10 min. The reaction mixture was loaded on a Sephadex G-25 column (1.5 × 30 cm) previously equilibrated with 10 mM phosphate buffer (pH 7.0). The column was run at 0.5 ml min^{-1} using 10 mM phosphate buffer (pH 7.0) as an eluent. The concentration of the purified cyt. *c* was determined by the absorbance at 360, 410, and 526 nm.

The steady irradiation of the visible light was carried out at 25°C using a 200 W tungsten lamp as a light source. The reaction mixture consisting of EDTA, ZnPP–Mb and cyt. *c* in a Pyrex tube equipped with an optical cell was degassed by several freeze–pump–thaw cycles before irradiation of the light. The concentration of the reduced cyt. *c* was determined by the change in the absorbance at 550 nm using ϵ(cyt. c_{red}) − ϵ(cyt. c_{ox}) = 18,500 M^{-1}cm^{-1} [8].

A Nd–YAG laser (Quanta Ray GCR-3) was used for laser flash photolysis. The wavelength of the laser pulse was 532 nm. The set-up of the system was the same as that reported previously [7]. The sample solution was degassed by freeze–pump–thaw cycles before the measurement. The measurement was carried out at 25°C.

3. Results and discussion

When the reaction mixture containing EDTA, ZnPP–Mb and cyt. *c* was irradiated by a tungsten lamp, a change was observed in the absorption spectra with the isosbestic points at 502, 527, 541 and 557 nm as shown in Fig. 1. The new absorption band at 520 and 550 nm increased in the intensity by the irradiation of the visible light. The

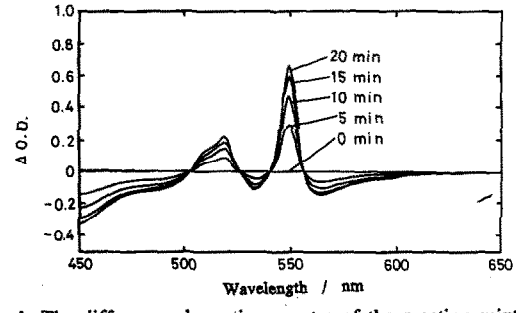


Fig. 1. The difference absorption spectra of the reaction mixture containing EDTA, ZnPP–Mb and cyt. *c* under steady irradiation of the visible light. The numbers in the figure show the irradiation time. EDTA (3.8×10^{-2} M), ZnPP–Mb (1.8×10^{-5}M) and cyt. *c* (3.5×10^{-5} M) were dissolved in 10 mM phosphate buffer (pH 7.0).

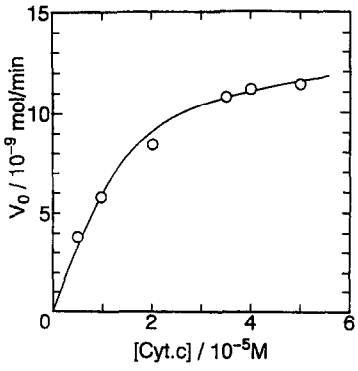
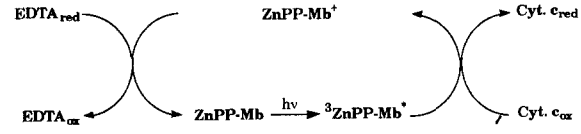
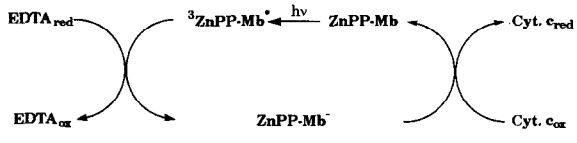


Fig. 2. The dependence of the initial reduction rate on the concentration of cyt. *c*. EDTA (3.8×10^{-2} M), ZnPP–Mb (1.8×10^{-5} M) and cyt. *c* were dissolved in 10 mM phosphate buffer (pH 7.0).



Scheme 1.



Scheme 2.

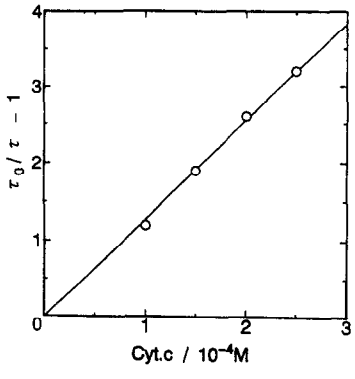


Fig. 3. The Stern–Volmer plot for the quenching of ^{3}ZnPP–Mb* by cyt. *c*. ZnPP–Mb (2.0×10^{-5} M) and cyt. *c* was dissolved in 10 mM phosphate buffer (pH 7.0).

absorption bands appeared by the irradiation of light are characteristic of the reduced cyt. *c* showing that the photoreduction of cyt. *c* took place in the reaction system consisting of EDTA, ZnPP–Mb and cyt. *c*. About 90% of cyt. *c* was reduced after 20 min of irradiation under the experimental conditions shown in Fig. 1.

The initial reduction rate was proportional to the concentration of ZnPP–Mb under the conditions that the concentration of EDTA and cyt. *c* were 3.8×10^{-2} and 3.5×10^{-5} M, respectively, in 10 mM phosphate buffer (pH 7.0). Fig. 2 shows the dependence of the initial reduction rate on the concentration of cyt. *c*. The initial reduction rate increased with increasing the concentration of cyt. *c* and then approached to the constant value. This is probably caused by the inner filter effect of cyt. *c*., i.e., as cyt. *c* has strong absorption bands in the visible region overlapped with those of ZnPP–Mb, the photoexcitation of ZnPP–Mb is inhibited by increasing cyt. *c*. When EDTA or ZnPP–Mb was omitted from the reaction mixture, no reduction of cyt. *c* was observed.

Two possible mechanisms can be thought for the photoreduction of cyt. *c* in the above system as shown in Schemes 1 and 2. ZnPP–Mb acts as a photosensitizer in both mechanisms. In the oxidative quenching mechanism, ^{3}ZnPP–Mb* is quenched by the oxidized cyt. *c* to form ZnPP–Mb$^+$ and the reduced cyt. *c* as shown in Scheme 1. ZnPP–Mb$^+$ is reduced to ZnPP–Mb by EDTA. In the reductive quenching mechanism, ^{3}ZnPP–Mb* is quenched EDTA to form ZnPP–Mb$^-$ and then the formed ZnPP–Mb$^-$ reduce cyt. *c* as shown in Scheme 2. The laser flash photolysis was carried out to establish which mechanism was correct in the above system.

The lifetime of ^{3}ZnPP–Mb* is determined to be 15 ms by the analysis of the single exponential decay curve of the transient absorption at 470 nm. [7] The lifetime of ^{3}ZnPP–Mb* decreased to 13 ms when 1.7×10^{-4} M of EDTA was added. This shows that the quenching of ^{3}ZnPP–Mb* by EDTA does not take place effectively. On the other hand, the lifetime of ^{3}ZnPP–Mb* was 5 ms when 1.5×10^{-4} M of cyt. *c* was added to the sample solution showing that cyt. *c* quenched ^{3}ZnPP–Mb* effectively.

The Stern–Volmer plot is shown in Fig. 3 in the case of cyt. *c* used as the quencher. The Stern–Volmer plot showed good linear relationship and the rate constant was calculated to be 8.5×10^5 $M^{-1}s^{-1}$ for the quenching of ^{3}ZnPP–Mb* by the oxidized cyt. *c*. The above results show that the reduction of cyt. *c* under steady irradiation of the visible light proceeds by the oxidative quenching mechanism as shown in Scheme 1.

The transient absorption of the reduced cyt. *c* or ZnPP–Mb^+ was not observed in the experiments of laser flash photolysis probably because the back electron transfer between the reduced cyt. *c* and ZnPP–Mb^+ was fast. The reduced cyt. *c* can be accumulated in the reaction system consisting of EDTA, ZnPP–Mb and cyt. *c* under steady irradiation because EDTA reacts with ZnPP–Mb^+ effectively to compete with the back electron transfer.

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