Communication

Journal of Photopolymer Science and Technology
Volume10,Number2(1997) 271-274 ©1997TAPJ

Excited States of Iron-Free Cytochrome c-553 Extracted From *Desulfovibrio Vulgaris*, Miyazaki

Miriam Abe[1], Hiroshi Inoue[1], Toshihiko Nagamura[1,2], Tatsuhiko Yagi[1,3], and Hideyuki Itagaki[3]

[1] *Graduate School of Electronic Science and Technology, Shizuoka University 3-5-1 Johoku, Hamamatsu 432, JAPAN*

[2] *Crystalline Films Laboratory, Research Institute of Eletronics, Shizuoka University 3-5-1 Johoku, Hamamatsu 432, JAPAN*

[3] *Department of Chemistry, Faculty of Education, Shizuoka University 836 Ohya, Shizuoka 422, JAPAN*

Keywords: cytochrome c, porphyrin, fluorescence, transient absorption

Cytochrome c-553 extracted from *Desulfovibrio vulgaris*, Miyazaki is an electron carrier protein that has only one heme, bound to a single polypeptide chain of molecular weight 8,000 [1]. The purpose of this research is to clarify photophysical processes of the iron-free cytochrome c-553 so as to develop finally an opto-electronic protein device such as novel optical memory by means of photochemical hole burning (PHB).

Desulfovibrio vulgaris, Miyazaki, was cultured as described before [2]. Bacterial sonicate was prepared by disintegrating wet cells of *Desulfovibrio vulgaris* suspended in 5-6 volumes of H_2O with an ultrasonic disintegrator (UR-200P, Tomy Seiko Co., Tokyo) at 20 kHz, 180 W, for 12 min. QAE-Sephadex A-50 (Pharmacia) chromatographic column was used to concentrate c-553 from the bacterial sonicate treated with $(NH_4)_2SO_4$. Sephadex G-50 chromatographic column was used to separate high molecular weight cytochrome and cytochrome c-553 [1].

Preparation of iron-free cytochrome c-553, i.e., porphyrin cytochrome c-553, was carried out with minor modifications of the methods of Fisher et al. [3] and Vanderkooi and Erecinska [4]. Ten milligrams of cytochrome c-553 were cooled in an open Teflon beaker suspended in a Dewar flask containing liquid nitrogen. Anhydrous HF (pressure of 0.2 kg/cm^2) was passed into the beaker for four minutes and the cytochrome c-553 turned purple, indicating that a reaction had occurred. The beaker was transferred from the liquid nitrogen container to room temperature and HF was removed under a stream of nitrogen. The porphyrin cytochrome c-553 was dried under reduced pressure for 24 hours, and then was purified through

Received March 28, 1997
Accepted May 28, 1997

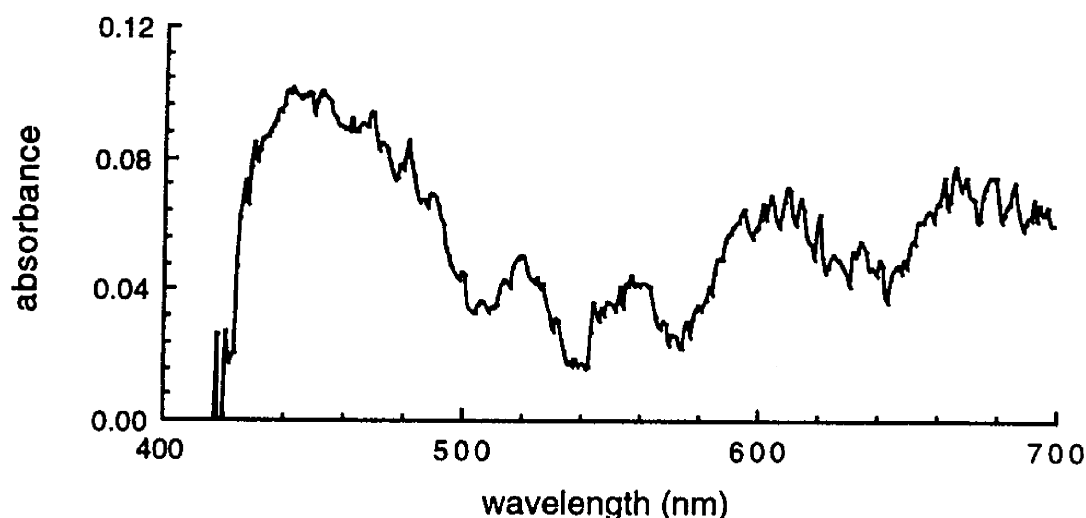

Figure 1 : Transient absorption spectrum of porphyrin cytochrome c-553 in tris-HCl solution at 26 ps after excitation with a fs laser at 400 nm.

Sephadex G-50 chromatographic columns using Tris-HCl buffer as eluent. The porphyrin cytochrome c-553 eluted a little earlier than normal ferricytochrome c-553, which indicated that the molecular sizes of the former are slightly larger than those of the latter due to a slight change in the structure of the protein. The fractions of the porphyrin cytochrome c-553 were pooled, dialyzed against distilled water, and applied to a CM-cellulose (NH_4^+) column.

The visible spectrum of the porphyrin cytochrome c-553 in Tris-HCl buffer at pH 7.2 showed four bands with absorption maxima at 503, 540, 568 and 620 nm in addition to strong absorption at 402 nm. These are bands characteristic for all free base porphyrins [5]. Although the primary structure of cytochrome c-553 is different from that of horse heart cytochrome c [4], the absorption peaks of iron free cytochrome c's were found to agree with one another.

In order to obtain the information on excited states, the time-dependence of transient absorption of porphyrin cytochrome c-553 was studied in a 1 cm cell upon excitation with a 400 nm femtosecond (fs) laser. Transient absorption measurements were performed using a pump-probe system with a regeneratively amplified, mode-locked fs Ti: sapphire laser [6,7]. The amplified Ti:sapphire laser delivered pulses with an FWHM of 200-250 fs, 10 Hz repetition rate and had maximum power of 6 mJ/pulse at 800 nm. A probe white light was obtained by focusing the residual 800 nm light into a cell containing a 2:1 v/v mixture of D_2O/H_2O after passing through a BBO crystal to obtain the second harmonics for pumping [6,7]. The transient absorption and decay dynamics were measured by a dual photodiode array system (Hamamatsu Photonics C6140-PMA) using an optical delay.

Fig. 1 shows the transient absorption spectrum of a Tris-HCl solution (pH 7.2) of porphyrin cytochrome c-553. The transient absorption bands extend to the blue from 700 nm and have a peak at 450 nm. The ground state bleaching is also observed at 510, 540, 570, and 620-640 nm, which correspond to the

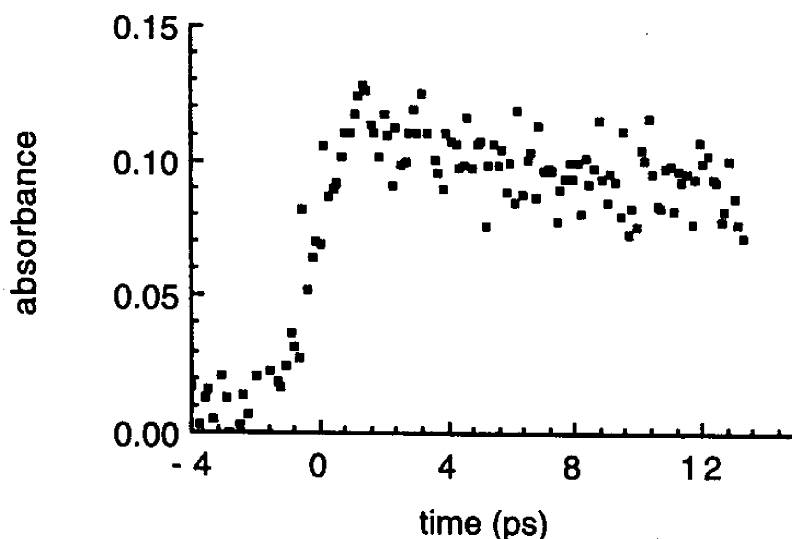

Figure 2 : Time profile of transient absorption at 450 nm for porphyrin cytochrome c-553 upon excitation with a fs laser at 400 nm.

absorption peaks of the visible spectrum of the porphyrin cytochrome c-553. The time profile of transient absorption at 450 nm is shown in Fig. 2, which demonstrated very fast formation and slow decay of a photoexcited state. Although the rise is slightly slower than the time resolution (< 1 ps) of our system probably due to larger optical length of the cell used to increase the transient absorption, the observed transient absorption could be attributed to the excited singlet state from following discussion. The transient absorption at 450 nm showed a double exponential decay with a minor fast component with a time constant ($\tau_{1/e}$) of 3.8 ps and a major slow one with $\tau_{1/e}$ of longer than a few nanoseconds (ns).

The fluorescence behavior of porphyrin cytochrome c-553 was also measured in degassed Tris-HCl solution and glycerol-water (5:1 v/v) solution. They exhibited relatively strong fluorescence of porphyrin moieties in the 600~700 nm region with peaks at 620 and 680 nm. There is no large difference between the two solutions. Fig. 3 showed temperature dependences of fluorescence spectra of porphyrin cytochrome c-553 in degassed glycerol-water (5:1 v/v) solution. The fluorescence peaks shift to the blue with a decrease in temperature. It appears to be induced by interaction among porphyrin moieties and solvents.

Fluorescence decay curves were obtained with a Horiba NAES-550 single photon counting machine. The emission of porphyrin cytochrome c-553 in degassed Tris-HCl solution (pH 7.2) was measured at 20℃ through an interference filter (630, 652, and 682 nm). The excitation light is separated through a B-390 filter with the transmittance peak at 390 nm. Time constants were calculated using the deconvolution method by fitting the data to equations consisting of the sum of exponentials. The decay curve with time (ns) at 630 nm was fitted very well to,

$$I_{630} = 0.22 \exp(-t / 13.0) + 0.78 \exp(-t / 3.2).$$

The time profiles of fluorescence at other wavelengths were also calculated to be the sum of two exponential functions with time

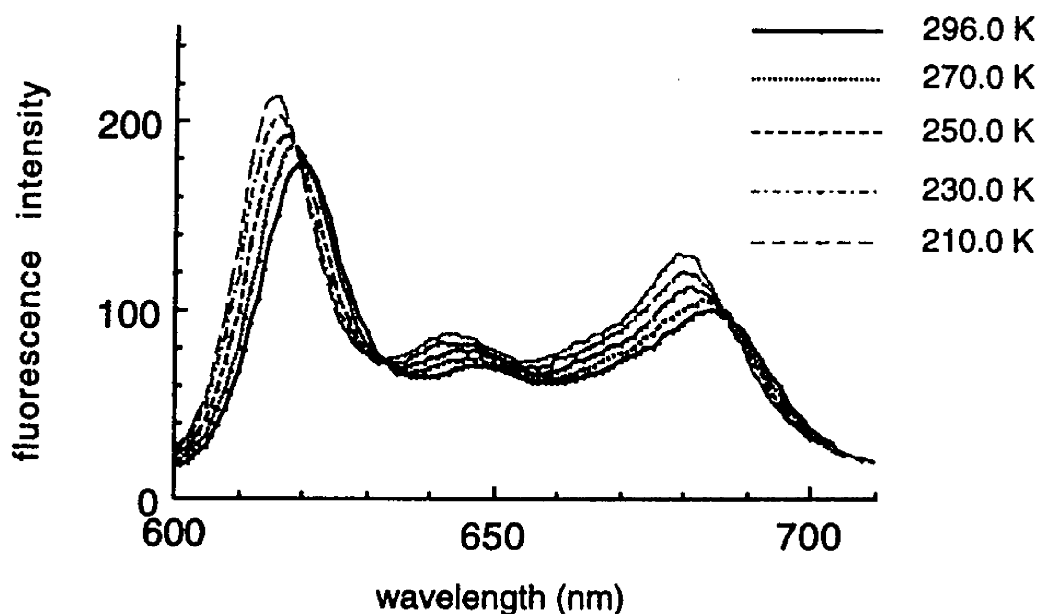

Figure 3 : Fluorescence spectra of porphyrin cytochrome c-553 in glycerol-water (5:1 v/v) at different temperatures. Excitation wavelength is 502 nm.

constants of 2.8, 2.6 ns and 13.0, 12.8 ns, respectively. The time constants of a major component in fluorescence decay agreed well with those of transient absorption. Therefore, it can be concluded that the main relaxation process from the excited state of porphyrin cytochrome c-553 is not by way of the triplet state, but rather directly from the first excited singlet state. The existence of two time constants suggests that there are different microenvironments around porphyrin in the case of iron-free cytochrome c-553. When iron atoms were removed from cytochrome c-553, heme-liganding histidine and methionine residues are released from coordinate bonds with iron, and are stabilized by having new interaction with other parts in this protein. This would be a reason that two different circumstances around a porphyrin moiety are produced and the molecular size of porphyrin cytochrome c-553 slightly changes.

Now the nature and dynamics of the excited states of porphyrin cytochrome c-553 are clarified, it will be possible to apply it to optical data processing devices including PHB memory.

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