Photochemistry of Phycobiliproteins: First Observation of Reactive Oxygen Species Generated from Phycobiliproteins on Photosensitization

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Phycobiliproteins, including phycoerythrin, phycoerythrocyanin, phycocyanin and allophycocyanin, are brilliantly colored, highly fluorescent components of the photosynthetic light-harvesting antenna complexes of cyanobacteria, red algae, and cryptomonads.[1] In the past decades, studies on phycobiliproteins focused mainly on the photophysical processes of light capture and energy transfer of the different chromophores of these proteins. The refined crystal structures of several phycobiliproteins from several organisms have been determined by X-ray diffraction,[2] and the energy transfer kinetics among the chromophores of phycocyanin have been resolved on the basis of the crystal structural data.[3]

More recently, considerable attention has been paid to the photochemistry of phycobiliproteins.[4] Although phycobiliproteins are considered to be photochemically quite stable, irradiation of Langmuir−Blodgett (LB) films of phycobiliproteins caused the generation of photocurrents and photovoltages in the electrochemical cell; these results showed that the chromophores of phycobiliproteins, especially those chromophores near the surface, exhibit photoinduced charge transfer phenomena in the LB films.[5] Interestingly, preliminary data have shown that phycobiliproteins exert much stronger photodynamic action on tumor cells compared with Photofrin-II and that they might be used as a new type of photodynamic therapeutic agent.[6] Phycocyanin, employed in pioneering photodynamic studies with phycobiliproteins, exhibits several advantages over the presently used hematoporphyrin derivatives (HPD), i.e., ready preparation and easy purification relative to HPD, high molar extinction coefficients, wide UV−visible absorption, no side effects, and significantly reduced normal tissue photosensitivity because of its fast metabolism *in vivo*.[6c] However, the mode of action of these proteins, which was considered to be related to reactive oxygen species,[6a,b] remained unclear. In this communication, we report for the first time the detection of reactive oxygen species, i.e., superoxide radical anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), generated from the photosensitization of the three phycobiliproteins,

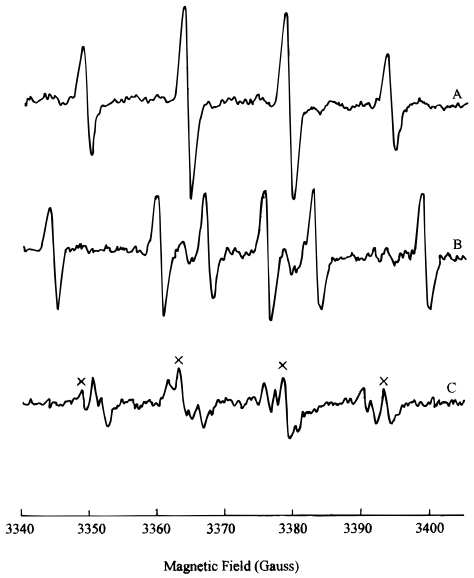

Figure 1. (A) ESR spectrum of trapped hydroxyl radical produced by irradiation of an oxygen-saturated aqueous solution containing *C*-PC (5 mg/mL) and DMPO (45 mM) for 8 min.; (B) Same as A but in the presence of 1% ethanol and incubation of the solution in the dark for 1 min after irradiation; (C) ESR spectrum produced from the irradiation of an oxygen-saturated aqueous solution containing *C*-PC (10 mg/mL), DMPO (0.2 M), and DTPA (1 mM). The signal resulted from the DMPO−$^{\bullet}$OH adduct was marked with crosses. Instrumental settings: microwave power = 10 mW; modulation amplitude = 1 G; receiver gain = 2×10^5.

R-phycoerythrin (*R*-PE), *C*-phycocyanin (*C*-PC), and allophycocyanin (APC).[7] The detection method employed was ESR spin trapping with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as the spin trap.[8]

When an oxygen-saturated aqueous solution containing *C*-PC (5mg/mL) and DMPO (45 mM) was irradiated at wavelengths above 470 nm for 8 min, a four-line ESR spectrum (Figure 1A) was clearly observed with an intensity ratio of 1:2:2:1 and an equal separation of 15.0 G between neighboring lines. This spectrum has been analyzed as due to the DMPO−$^{\bullet}$OH adduct, with a primary nitrogen splitting triplet (a^N = 15.0 G), with each line further splitting into a secondary doublet by the 2−CH proton (a^H = 15.0 G), resulting in a four-line ESR spectrum with the observed intensity distribution.[9] The intensity of the signal increased significantly with prolonged irradiation of the solution (Figure 2). When ethanol (1%, V/V) was introduced into the irradiated system,[10] the ESR spectrum of the DMPO spin adduct of $CH_3{}^{\bullet}CHOH$ generated from the hydrogen abstraction of ethanol by $^{\bullet}OH$ was observed with a^N = 15.8 G and a^H = 23.0 G (Figure 1B).[9] This result confirmed the

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(7) *R*-phycoerythrin (*R*-PE), C-Phycocyanin (*C*-PC), and allophycocyanin (APC) were extracted from dried *Porphyra yezoensis* and purified according to the literature: Zeng, F. J.;Yang, Z. X.; Liu, H. P.; Jiang, L. J. *Sci. China, Ser. B* **1986**, 29, 824. The purities of the proteins were determined by electrophoresis. The powders of lyophilized proteins were stored at −20 °C before use. 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Aldrich Chemical Company (Milwaukee, WI). The bovine liver superoxide dismutase (SOD) and catalase were obtained from Sigma Chemical Company (St. Louis, MO).

(8) Measurements of the ESR spectra were carried out on a Varian E-109 spectrometer operating at room temperature (X band, microwave frequency: 9.47 GHz). Oxygen-saturated solutions of phycobiliproteins (40 μL) were injected quantitatively into specially made quartz capillaries, and were illuminated with a Shoeffel 1kW Xe arc lamp. A cutoff filter was used to eliminate light of wavelengths shorter than 470 nm. The incident fluence rate was approximately 50 W/m^2.

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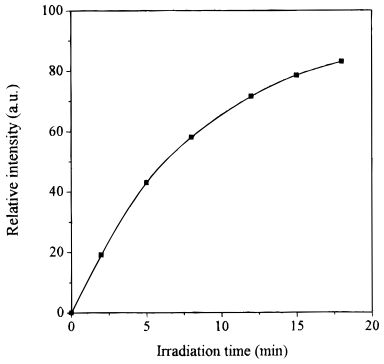

Figure 2. Dependence of the relative intensity of ESR signal of the DMPO−•OH adduct on irradiation time. Other experimental conditions are the same as for Figure 1A.

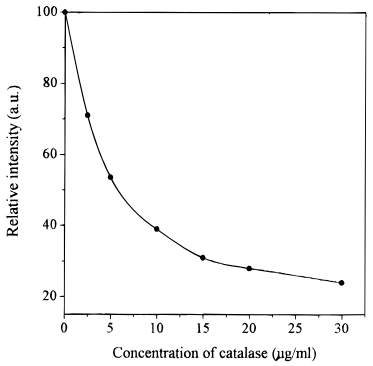

Figure 3. Dependence of the intensities of the ESR signals of the DMPO−•OH adduct on the concentration of catalase. Other experimental conditions are the same as for Figure 1A.

assignment of the spectrum shown in Figure 1A to the DMPO−•OH radical adduct.

The formation of •OH radicals from *C*-PC on photosensitization depends on the *C*-PC and oxygen concentrations and the intensity of irradiation. No ESR signal of the DMPO−•OH adduct was detected in the dark or in the irradiated sample without *C*-PC or oxygen. The presence of catalase in the system inhibited significantly the formation of •OH radicals. As shown in Figure 3, the signal intensity of the DMPO−•OH adduct decreases strongly with increasing amounts of catalase, and 50 μg/mL of catalase eliminate >90% of the ESR signal. Heat-denatured catalase had no effect. These results indicate that H_2O_2 was also generated in the system and acted as a source of •OH radicals.

The formation of superoxide radical anion ($O_2^{\bullet-}$) upon photochemical activation of *C*-PC in oxygen-saturated aqueous solution was also demonstrated. In this experiment, higher concentrations of DMPO (0.2 M) and *C*-PC (10 mg/mL) were used and diethylenetriaminepentaacetic acid (DTPA) (1 mM) was added to the irradiated sample to inhibit the formation of •OH.[11] The ESR spectrum obtained for the DMPO−superoxide adduct is shown in Figure 1C, which also contains a small contribution from the DMPO−•OH adduct (marked with crosses). The hyperfine coupling constants for the DMPO−superoxide adduct determined from Figure 1C are $a^N = 14.1$ G, $a^H = 11.3$ G, and $a^H = 1.3$ G, which are consistent with the reported values for the DMPO−superoxide spin adduct in aqueous solution.[9] When superoxide dismutase (40 μg/mL) was present in the sample, it totally prevented the formation of this spin adduct, confirming its proper identification. Control experiments again ascertained that *C*-PC, oxygen, and light were all necessary to produce the DMPO−superoxide adduct.

When *R*-PE or APC was used instead of *C*-PC as the sensitizer, the photosensitized formation of •OH, H_2O_2, and $O_2^{\bullet-}$ was likewise observed (data not shown). Since the chromophores of phycobiliproteins, especially those chromophores near the surface, can act as electron donors in the presence of a proper acceptor,[5a,b] it was inferred that a photoinduced electron transfer from the phycobiliproteins used (*C*-PC, *R*-PE, and APC) to oxygen might occur in our system and lead to the formation of $O_2^{\bullet-}$. In aqueous solution the $O_2^{\bullet-}$ formed after irradiation underwent rapid dismutation to H_2O_2, which was then transformed into •OH radicals via Fenton type reactions and/or other unidentified pathways.

In summary, we have demonstrated the photosensitized formation of reactive oxygen species (•OH, H_2O_2, and $O_2^{\bullet-}$) upon irradiation of the three phycobiliproteins (*C*-PC, *R*-PE, and APC) with light >470 nm; this may provide an understanding of both their biological functions and their photodynamic action mechanisms. Further studies on the quantitation and on the detailed mechanism of the formation of reactive oxygen species from phycobiliproteins on photosensitization are in progress in our laboratory.

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