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Photodynamic action of phycobiliproteins: in situ generation of reactive oxygen species

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Abstract

Phycobiliproteins are important photodynamic pigmental proteins. The photosensitized generation of superoxide radical anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^1O_2$) from three phycobiliproteins, R-phycoerythrin (R-PE), C-phycocyanin (C-PC) and allophycocyanin (APC) has been investigated throughout irradiation of these proteins with visible light of wavelengths longer than 470 nm. Upon irradiation of an oxygen-saturated aqueous solution of C-PC, R-PE or APC, the generation of $O_2^{\cdot -}$ and $^{\cdot}OH$ was clearly observed by ESR-spin trapping with 5,5-dimethyl-1-pyrroline-*N*-oxide as the spin trap. The formation of $^{\cdot}OH$ can be significantly inhibited by catalase, indicating that H_2O_2 is also generated in the system and acts as a potential source of $^{\cdot}OH$. Irradiation of the oxygen-saturated aqueous solutions of the phycobiliproteins can also result in the photobleaching of these proteins. Inhibition of the bleaching by superoxide dismutase (SOD) and catalase confirms further the generation of $O_2^{\cdot -}$, H_2O_2 and $^{\cdot}OH$, and their involvements in the photobleaching of phycobiliproteins. Furthermore, the bleaching can be significantly inhibited by histidine and NaN_3, two specific inhibitors of $^1O_2$, suggesting that $^1O_2$ is also generated from C-PC, R-PE or APC on photosensitization and participates in the photobleaching of these proteins. The photobleaching rates of these proteins are sharply promoted in oxygen-saturated ^2H_2O, further verifying the generation of $^1O_2$. Quantitative analyses of the photobleaching rates (K_d) are presented on visible-light-induced ($\lambda > 470$ nm) damage to these phycobiliproteins under some inhibitors of reactive oxygen species, which further confirm that the generation of reactive oxygen species and their participation in the photobleaching of these proteins. Since the fluorescences of the phycobiliproteins are not quenched by oxygen, it seems probably that the triplet states of these proteins are the inductors of $^1O_2$ and $O_2^{\cdot -}$ generated. Therefore, the photodynamic action of phycobiliproteins proceeds by both free radical and singlet oxygen pathways which could contribute to the reported phototoxicity of phycobiliproteins in vivo.

Keywords: Phycobiliprotein; Photodynamic action; Reactive oxygen species; Photobleaching; Triplet state

Abbreviations: PDT, photodynamic therapy; HPD, hematoporphyrin derivative; DHE, dihematoporphyrin ether; PBP, phycobiliprotein; R-PE, R-phycoerythrin; C-PC, C-phycocyanin; APC, allophycocyanin; SOD, superoxide dismutase; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DTPA, diethylenetriaminepentaacetic acid; His, histidine; ESR, electron spin resonance

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1. Introduction

Photodynamic therapy (PDT) is a promising new treatment for light-accessible tumors [1,2]. The initial photochemical processes leading to cell death may follow two principal pathways: Upon light absorption the triplet state of the photosensitizer transfers the energy to triplet state O_2 ($^3O_2$) to yield singlet oxygen ($^1O_2$), a potent oxidizer (Type II mechanism), or alternatively photosensitized formations of $O_2^{\cdot -}$ and $^{\cdot}OH$ engage in charge transfer reactions with biomolecules (Type I mechanism) [3]. Either pathways will require oxygen to propagate the damage via radical chain reactions. The most widely used sensitizers in PDT are hematoporphyrin derivatives (HPD) and the enriched product Photofrin II ® = dihematoporphyrin ether (DHE) [4–6]. Although encouraging results have been obtained with these photosensitizing agents for a wide variety of neoplasms, it appears that several limitations, such as low absorption coefficient in the phototherapeutic region (600–900 nm) and dermal photosensitivity resulted from prolonged retention of these agents in the skin, may adversely affect the therapeutic outcome of PDT [7]. These limitations have led to a search for improved photosensitizers for PDT.

Phycobiliproteins (PBPs), including phycoerythrin, phycoerythrocyanin, phycocyanin and allophycocyanin, are brilliantly colored, highly absorbent and fluorescent components of the photosynthetic light-harvesting antenna complexes of cyanobacteria, red algae and cryptomonads. Previous studies on phycobiliproteins emphasized mainly on the photophysical processes about energy capture and transfer aspects of the different chromophores of these proteins [8–10]. Recently, Morcos et al. and Zheng et al. have demonstrated that phycobiliproteins exert much stronger photodynamic action on tumor cells compared with HPD and they might be used as a new type of photodynamic therapeutic agents [11–13]. Phycocyanin, employed in pioneering photodynamic therapy of 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 reduction of normal tissue photosensitivity because of its fast metabolism in vivo [13]. However, the mechanism of action for these proteins, which was considered to be related to reactive oxygen species [11,12], remains unclear.

In the present research, we report the detection of the reactive oxygen species, i.e., superoxide radical anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^1O_2$), generated from the photosensitization of the three phycobiliproteins, R-phycoerythrin (R-PE), C-phycocyanin (C-PC) and allophycocyanin (APC). In addition, oxygen-dependent photobleaching mechanism of phycobiliproteins is proven by the effects of a series of reactive oxygen inhibitors on the bleaching. In general, since triplet state of photosensitizer is generally considered to be an inductor of these damaging oxygen species in PDT, our results also give evidences for the understanding of both the photodynamic action mechanisms and the triplet state properties of phycobiliproteins.

2. Materials and methods

R-phycoerythrin (R-PE), C-phycocyanin (C-PC) and allophycocyanin (APC) were extracted from dried *Porphyra yezoensis* by extraction with 0.1 M phosphate buffer (pH6.8) and then by fractional precipitation with ammonium sulfate from high to low concentrations (55%, then 50–45%). The crude sample was separated through a Sephadex G-200 column (Pharmacia, Uppsala, Sweden), and finally purified through a Bio Gel P-300 column (Bio-Rad, Richmonnd, VA). The purities of the proteins were examined by absorption spectra ($A_{max}/A_{280} > 4.0$) and electrophoresis. The absorption spectra of three proteins were given in Fig. 1, the characteristic peaks of these proteins are identical with that reported by other researchers [14–16]. The aggregation states of R-PE as $(\alpha\beta)_6\gamma$, and C-PC, APC as $(\alpha\beta)_3$ in neutral medium were ascertained through the measurements of their molecular weights using standard electrophoresis method [17]. The powders of lyophilized proteins were stored at $-20°C$ and dissolved with twice distilled water before use. 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and diethylenetriamine-pentaacetic acid (DTPA) were purchased from Aldrich Chemical Company. Catalase, superoxide dismutase (SOD) and histidine were obtained from Biotech.

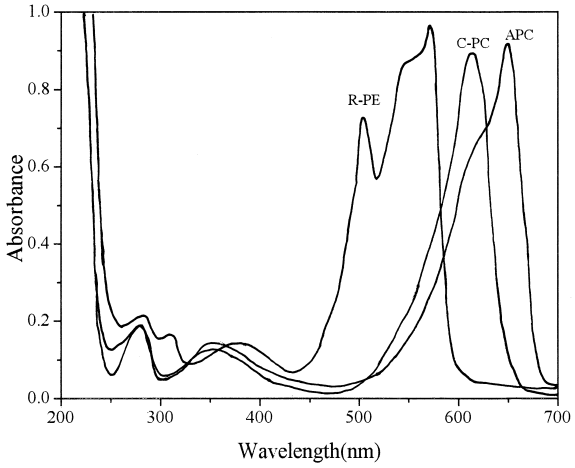

Fig. 1. Absorption spectra of R-PE, C-PC and APC extracted from dried *Porphyra yezoensis* dissolved in twice distilled water. The maximal absorption peaks of R-PE, C-PC and APC are located at 562, 612 and 650 nm, respectively.

Technology Corporation, Chinese Academy of Sciences. Heavy water (^2H_2O) and sodium azide (NaN_3) were purchased from Beijing Chemical Plant, China.

The photobleaching rate was calculated by fitting the experimental data with the equation [18]: $A(t)/A(0) = \exp(-K_d t)$, where $A(0)$ is the absorbance of proteins at time 0, $A(t)$ is the absorbance of proteins after time t of irradiation, and K_d is the photobleaching rate (s^{-1}). Photobleaching experiments were performed under irradiation of a medium-pressure mercury vapour lamp (450 W) on a 'merry-go-round' apparatus. Light of wavelength below 470 nm was cut off by a long-pass filter, and the apparatus was immersed in running water in a thermostat at 20°C. The incident fluence rate was measured to be 18 W/m^2. The solutions examined were put in quartz cuvettes (10 mm × 10 mm) with a long neck. The solutions were purged with argon or oxygen for 10 min before irradiation according to the experimental requirements. The maximum absorbances of the protein solutions were adjusted to be 1.0 before irradiation. UV-visible absorption spectra were recorded on an HP diode array spectrophotometer model 8451A.

Hydroxyl radicals and superoxide radical anions were detected by means of the spin-trapping method with ESR. 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, 5 or 10 mg/ml) were injected quantitatively into specially made quartz capillaries, and were illuminated with a Shoeffel 1 kW Xe arc lamp. A cutoff filter was used to eliminate light of wavelength shorter than 470 nm. The incident fluence rate was measured to be approximately 50 W/m^2.

3. Results

3.1. Free radical reactions

When an oxygen-saturated aqueous solution containing C-PC (5 mg/ml) and DMPO (45 mM) was irradiated at wavelengths longer than 470 nm for 8 min, a four-line ESR spectrum (Fig. 2A) was clearly observed with an intensity ratio of 1:2:2:1 and an equal separation of 15.0 G between neighbouring lines. This spectrum was analysed to be due to the DMPO-·OH adduct, with a primary nitrogen splitting triplet ($a^N = 15.0$ G), with each line further splitting into secondary doublet by the 2-CH proton ($a^H = 15.0$ G), resulting in a four-line ESR spectrum with the observed intensity distribution [19]. The intensity of the signal increased significantly with prolonged irradiation of the solution (Fig. 3). When ethanol (1%, v/v) was introduced into the irradiated system, the ESR spectrum of the DMPO spin adduct of $CH_3\dot{C}HOH$ generated from the hydrogen abstraction of ethanol by ·OH was observed with $a^N = 15.8$ G and $a^H = 23.0$ G (Fig. 2B) [19]. This result confirmed the assignment of the spectrum shown in Fig. 2A to the DMPO-·OH radical adduct.

The formation of hydroxyl radicals from C-PC on photosensitization depends on both the C-PC and oxygen concentrations and the intensity of the irradiation. No ESR signal of the DMPO-·OH adduct was detected in the dark or in the irradiated sample without either C-PC or oxygen. Presence of catalase in the system inhibited significantly the formation of ·OH radicals. As shown in Fig. 4, the signal intensity of the DMPO-·OH adduct decreases sharply with increasing amount of catalase and 50 μg/ml of catalase eliminates > 90% of the ESR signal. Heat-denatured catalase had no effect. These results indicated that H_2O_2 was also generated in the system and acted as a source of ·OH radicals.

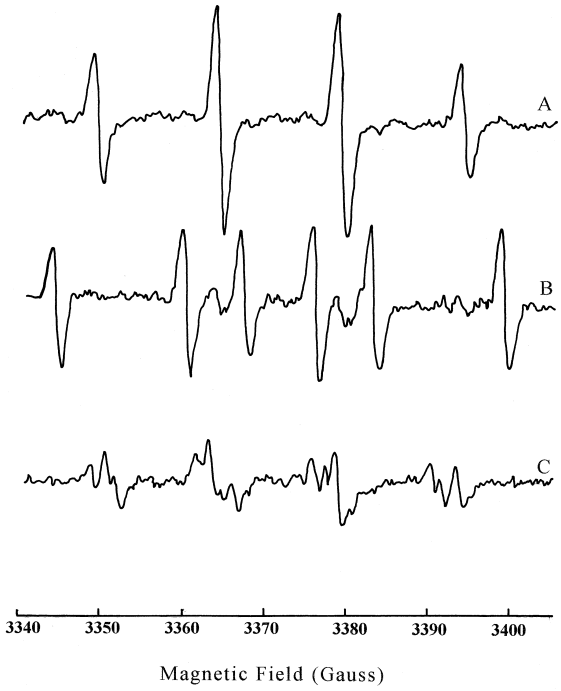

Fig. 2. A: ESR spectrum of Hydroxyl radical produced by irradiation of an oxygen-saturated aqueous solution containing C-PC (5 mg/ml) and DMPO (45 mM) for 8 min; B: same as panel A, but in the presence of 1% (v/v) ethanol and the solution was incubated in the dark for 1 min after irradiation; C: ESR spectrum produced by the irradiation of an oxygen-saturated aqueous solution containing C-PC (10 mg/ml), DMPO (0.2 M) and DTPA (1 mM). Instrumental settings: microwave power, 10 mW; modulation amplitude, 1 G; receiver gain, $2 \cdot 10^5$. Light source of the illumination: > 470 nm visible light from a Shoeffel 1 kW Xe arc lamp at a incident fluence rate of approximately 50 W/m^2.

The possible formation of superoxide radical anion ($O_2^{\cdot -}$) upon photochemical activation of C-PC in oxygen-saturated aqueous solution was also ascertained. In this experiment, higher concentrations of DMPO (0.2 M) and C-PC (10 mg/ml) were used and DTPA (1 mM) was added to the irradiated sample to inhibit the formation of $^{\cdot}$OH. The ESR spectrum obtained from DMPO-superoxide adduct is shown in Fig. 2C, which also contains a small contribution from the DMPO-$^{\cdot}$OH adduct. The hyperfine coupling constants for the DMPO-superoxide adduct determined from Fig. 2C 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 [19]. When superoxide dismutase (40 μg/ml) was present in the sample it completely

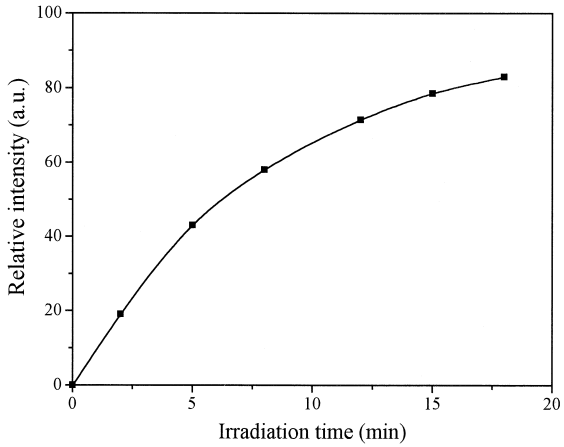

Fig. 3. Dependence of the relative intensity of ESR signal of the DMPO-$^{\cdot}$OH adduct on irradiation time. Other experimental conditions are the same as for Fig. 2A.

prevented the formation of this spin adduct, confirming its proper identification. Control experiments verified that C-PC, oxygen and light were all necessary to produce the DMPO-superoxide adduct.

The formation of $O_2^{\cdot -}$, H_2O_2 and $^{\cdot}$OH from C-PC on photoexcitation were also confirmed by the effects of SOD and catalase on the self-sensitized bleaching of C-PC. C-PC absorbs strongly at 612 nm (see Fig. 1). Irradiation of an argon-saturated aqueous solution of C-PC with light of wavelengths longer than 470 nm resulted in gradually irreversible decrease in the absorbance at 612 nm from C-PC (line 1 of Fig. 5), suggesting the self-sensitized photobleaching of C-PC. The bleaching under argon was probably due to

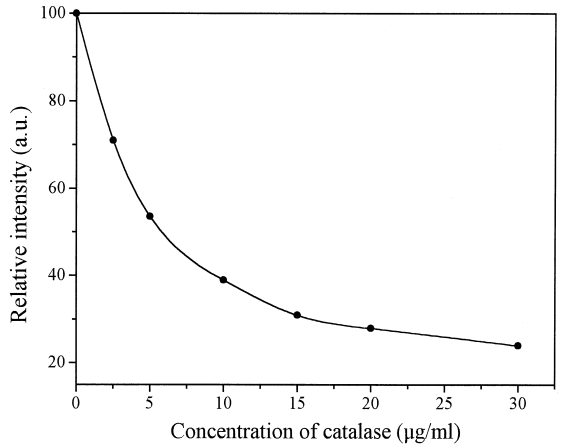

Fig. 4. Plot of the effects of various concentrations of catalase on the relative intensities of ESR signals of DMPO-$^{\cdot}$OH adduct. Other experimental conditions are the same as for Fig. 2A.

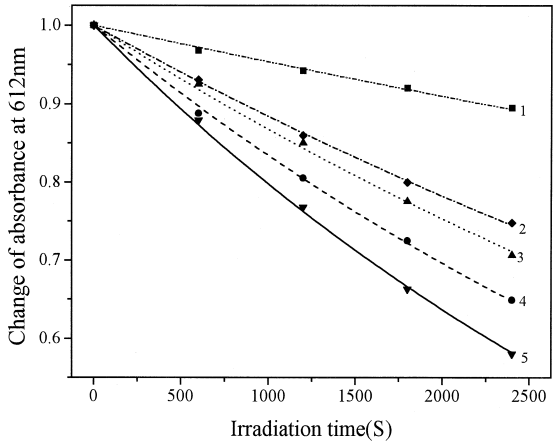

Fig. 5. Changes in absorption of C-PC measured at 612 nm as a function of irradiation time. line 1, C-PC in argon-saturated aqueous solution, $K_d = (4.71 \pm 0.09) \cdot 10^{-5}$ s^{-1}; line 2, same as line 5 but in the presences of SOD (100 μg/ml) and catalase (100 μg/ml), $K_d = (1.23 \pm 0.01) \cdot 10^{-4}$ s^{-1}; line 3, same as line 5 but in the presence of NaN_3 ($1.0 \cdot 10^{-6}$mol/l), $K_d = (1.42 \pm 0.02) \cdot 10^{-4}$ s^{-1}; line 4, same as line 5 but in the presence of histidine ($1.0 \cdot 10^{-5}$mol/l), $K_d = (1.81 \pm 0.02) \cdot 10^{-4}$ s^{-1}; line 5, C-PC in oxygen-saturated aqueous solution, $K_d = (2.25 \pm 0.02) \cdot 10^{-4}$ s^{-1}. The initial absorbances at 612 nm of all samples were adjusted to 1.0 with twice distilled water. The data were fitted with a single exponential decay function $[A(t)/A(0) = \exp(-K_d t)]$ yielding photobleaching rates (K_d) of C-PC under the various additives. The condition of illumination: > 470 nm visible light from a 450 W medium-pressure mercury vapour lamp on a 'merry-go-round' apparatus at a incident fluence rate of 18 W/m^2.

the conformational changes of phycocyanobilins caused by the photoinduced C-PC dissociation from trimer to monomer [20], or due to the direct damages to the aromatic amino acids and the bilins of C-PC similarly to the photodestruction of phycobiliproteins by UV-B radiation [18]. When oxygen was introduced in the system, the photobleaching rate of C-PC was enhanced sharply (line 5 of Fig. 5). Calculation from lines 1 and 5 of Fig. 5, the photobleaching rate in oxygen-saturated solution is about 5.0 times as fast as that in argon-saturated solution (see Table 1), confirming that reactive oxygen species participated in the bleaching of C-PC. From Fig. 5, the fraction of the bleaching rate of C-PC was attributed to the reactive oxygen species which is calculated to be 79%. In the presence of SOD (40 μg/ml) or catalase (50 μg/ml) the bleaching of C-PC was inhibited by 15% or 25% (data not shown), further suggesting in situ formations of $O_2^{\cdot -}$, H_2O_2 and $^{\cdot}OH$, and their involvements in the photobleaching of C-PC.

When R-PE or APC was used instead of C-PC as the sensitizer, similar photosensitized formation of $O_2^{\cdot -}$, H_2O_2 and $^{\cdot}OH$ was also observed by ESR-spin trapping (data not shown) and SOD- and catalase-inhibitions (shown in Fig. 6 for R-PE and Fig. 7 for APC). Recently, it has been demonstrated that phycobiliproteins exhibit photoinduced charge transfer phenomenon in their electrochemical cells, suggesting that these proteins can act as an electron donor or acceptor when a proper acceptor or donor is present [21,22]. Thus, 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 $O_2^{\cdot -}$. In aqueous solution, the $O_2^{\cdot -}$ formed after irradiation underwent rapid dismutation to H_2O_2, which was then transformed to $^{\cdot}OH$ radicals via Fenton reaction and/or other unidentified pathways.

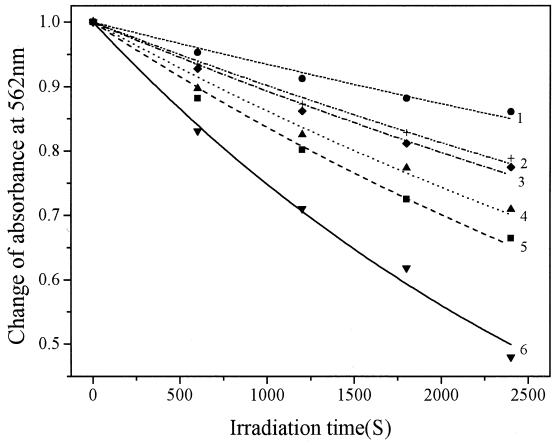

Fig. 6. Changes in absorption of R-PE measured at 562 nm as a function of irradiation time. line 1, R-PE in argon-saturated aqueous solution, $K_d = (6.75 \pm 0.28) \cdot 10^{-5}$ s^{-1}; line 2, same as line 5 but in the presence of NaN_3 ($1.0 \cdot 10^{-6}$ mol/l), $K_d = (1.03 \pm 0.03) \cdot 10^{-4}$ s^{-1}; line 3, same as line 5 but in the presences of SOD (100 μg/ml) and catalase (100 μg/ml), $K_d = (1.13 \pm 0.03) \cdot 10^{-4}$ s^{-1}; line 4, same as line 5 but in the presence of SOD (40 μg/ml), $K_d = (1.48 \pm 0.05) \cdot 10^{-4}$ s^{-1}; line 5, R-PE in oxygen-saturated aqueous solution, $K_d = (1.78 \pm 0.04) \cdot 10^{-4}$ s^{-1}; line 6, R-PE in oxygen-saturated solution of heavy water, $K_d = (2.89 \pm 0.08) \cdot 10^{-4}$ s^{-1}. The initial absorbances at 562 nm of all samples were adjusted to 1.0 with twice distilled water. The fitting method and the condition of illumination are the same as for Fig. 5.

Table 1
Photobleaching rates of three phycobiliproteins at the different conditions used

Samples	K_d (s^{-1}) [a]		
	R-PE	C-PC	APC
Argon bubbling in H_2O	$(6.75 \pm 0.28) \cdot 10^{-5}$	$(4.71 \pm 0.09) \cdot 10^{-5}$	$(4.40 \pm 0.16) \cdot 10^{-5}$
Oxygen bubbling in H_2O	$(1.78 \pm 0.04) \cdot 10^{-4}$	$(2.25 \pm 0.02) \cdot 10^{-4}$	$(1.15 \pm 0.02) \cdot 10^{-4}$
White and Stryer [b]	$8.2 \cdot 10^{-5}$	$8.5 \cdot 10^{-5}$	$6.2 \cdot 10^{-5}$
Oxygen bubbling in ^2H_2O	$(2.89 \pm 0.08) \cdot 10^{-4}$	$(3.56 \pm 0.06) \cdot 10^{-4}$	$(1.59 \pm 0.02) \cdot 10^{-4}$
Oxygen bubbling in H_2O with NaN_3	$(1.03 \pm 0.03) \cdot 10^{-4}$	$(1.42 \pm 0.02) \cdot 10^{-4}$	$(8.36 \pm 0.06) \cdot 10^{-5}$
with His	–	$(1.81 \pm 0.02) \cdot 10^{-4}$	–
with SOD	$(1.48 \pm 0.05) \cdot 10^{-4}$	–	$(7.38 \pm 0.07) \cdot 10^{-5}$
with SOD and Catalase	$(1.13 \pm 0.03) \cdot 10^{-4}$	$(1.23 \pm 0.01) \cdot 10^{-4}$	$(6.79 \pm 0.03) \cdot 10^{-5}$

[a] According to $A(t)/A(0) = \exp(-K_d t)$, the data were fitted with a single exponential decay curve, see text for details. The samples were illuminated with > 470 nm visible light at a incident fluence rate of 18 W/m^2 at 20°C.
[b] The data derived from the work of White and Stryer [27]. Using $K_d = 3.8 \cdot 10^{-21}\ \epsilon I \Phi$, we transform Φ (photodestruction quantum yield) given by White and Stryer into K_d (photobleaching rate) in order to compare with each other.

As mentioned above, superoxide dismutase (SOD, 40 μg/ml) and catalase (50 μg/ml) could totally eliminate $O_2^{\cdot -}$, H_2O_2 and $^{\cdot}OH$ formed from C-PC on photosensitization. When an excess of SOD (100 μg/ml) together with catalase (100 μg/ml) were added to the present system, the photobleaching rate of C-PC was only inhibited by 45% (line 2 of Fig. 5). This result led us to assume that another reactive oxygen mechanism, most probably the singlet oxygen mechanism, could also cause the photobleaching of C-PC, and subsequent experiments were carried out to examine this assumption.

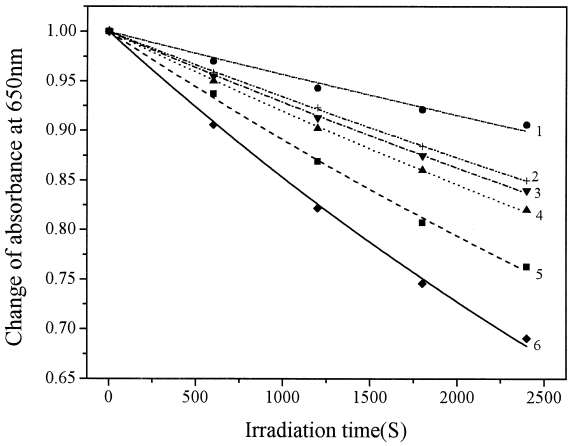

Fig. 7. Changes in absorption of APC measured at 650 nm as a function of irradiation time. line 1, APC in argon-saturated aqueous solution, $K_d = (4.40 \pm 0.16) \cdot 10^{-5}\ s^{-1}$; line 2, same as line 5 but in the presences of SOD (100 μg/ml) and catalase (100 μg/ml), $K_d = (6.79 \pm 0.03) \cdot 10^{-5}\ s^{-1}$; line 3, same as line 5 but in the presence of SOD (40 μg/ml), $K_d = (7.38 \pm 0.07) \cdot 10^{-5}\ s^{-1}$; line 4, same as line 5 but in the presence of NaN_3 ($1.0 \cdot 10^{-6}$ mol/l), $K_d = (8.36 \pm 0.06) \cdot 10^{-5}\ s^{-1}$; line 5, APC in oxygen-saturated aqueous solution, $K_d = (1.15 \pm 0.02) \cdot 10^{-4}\ s^{-1}$; line 6, APC in oxygen-saturated solution of heavy water, $K_d = (1.59 \pm 0.02) \cdot 10^{-4}\ s^{-1}$. The initial absorbances at 650 nm of all samples were adjusted to 1.0 with twice distilled water. The fitting method and the condition of illumination are the same as for Fig. 5.

3.2. Singlet oxygen reactions

Linetsky and Ortwerth have recently reported that histidine (His) reacts hardly with $O_2^{\cdot -}$ and $^{\cdot}OH$ and so can be used as a specific inhibitor for singlet oxygen [23]. When histidine ($1.0 \cdot 10^{-5}$ mol/l) was introduced to the oxygen-saturated sample of C-PC, the photobleaching rate of C-PC was decreased by the extent of nearly 20% (line 4 of Fig. 5). The effects of various amounts of histidine on the bleaching of C-PC was also examined. The results are shown in Fig. 8. It can be seen that the bleaching rate decreases drastically with increasing amount of histidine, strongly confirming the involvement of $^1O_2$ in the photobleaching of C-PC. Since no additional sensitizer except for C-PC was involved in the system, it was evident that $^1O_2$ was generated from C-PC upon photosensitization. Furthermore, when histidine was replaced by NaN_3, another specific inhibitor of $^1O_2$, similar inhibiting effect was also obtained. As shown in line 3 of Fig. 5, $1.0 \cdot 10^{-6}$ mol/l of NaN_3 reduces the photobleaching rate of

C-PC by an extent of 37%. This further confirms the generation of singlet oxygen from the photosensitization of C-PC and its involvement in the photobleaching of C-PC.

Another important evidence for the C-PC photosensitized generation of $^1O_2$ comes from the ^2H_2O effect on the photobleaching of C-PC. As shown in Fig. 9, when measurements were repeated with ^2H_2O in place of H_2O, the bleaching rate of C-PC increased by 58% due to the increased halflife of $^1O_2$ in this medium (lines 2 and 3 of Fig. 9). Histidine ($1.0 \cdot 10^{-5}$ mol/l) inhibited the photobleaching rate of C-PC by 43% in ^2H_2O (line 1 of Fig. 9).

From Fig. 8 it can also be observed that, even at higher concentrations of histidine, inhibition of C-PC photobleaching is not complete. Moreover, the curve reaches a plateau for histidine concentrations between $1.0 \cdot 10^{-3}$ mol/l and $5.0 \cdot 10^{-3}$ mol/l. This incomplete inhibition indicates that, in addition to the $^1O_2$ mechanism, other mechanisms, such as oxygen-independent photodissociation of C-PC and oxygen-dependent photodestruction resulted from $O_2^{\cdot -}$, H_2O_2 and $^{\cdot}OH$ (vide supra), also produce the photobleaching of C-PC. Calculated from Fig. 8, the fraction of C-PC bleaching caused by $^1O_2$ is about 36%.

When R-PE or APC was used instead of C-PC as the sensitizer, the similar photosensitized generation of $^1O_2$ and its involvement in the photobleaching of

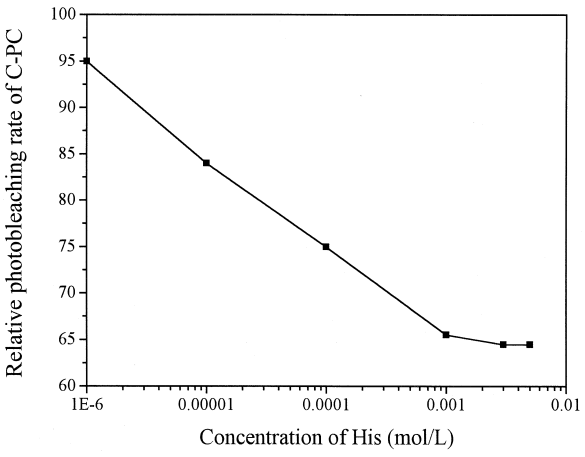

Fig. 8. Dependence of the photobleaching rate of C-PC in the oxygen-saturated aqueous solution (initial $A_{612\ nm} = 1.0$) on the concentrations of histidine. A value of 100 is arbitrarily attributed to the photobleaching rate of C-PC in oxygen-saturated aqueous solution in the absence of histidine.

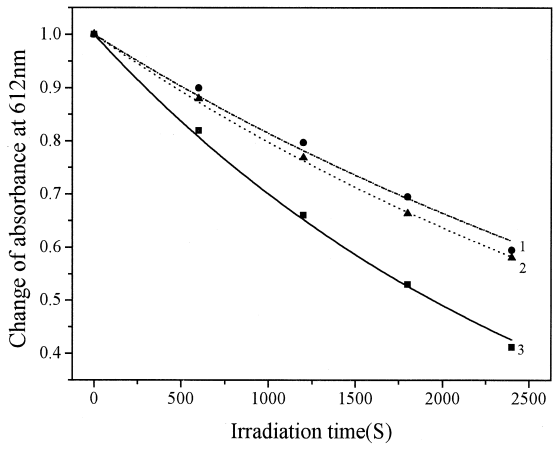

Fig. 9. Changes in absorption of C-PC measured at 612 nm as a function of irradiation time. line 1, same as line 3 but in the presence of histidine ($1.0 \cdot 10^{-5}$ mol/l), $K_d = (2.04 \pm 0.06) \cdot 10^{-4}$ s^{-1}; line 2, C-PC in oxygen-saturated solution of water, $K_d = (2.25 \pm 0.02) \cdot 10^{-4}$ s^{-1}; line 3, C-PC in oxygen-saturated solution of heavy water, $K_d = (3.56 \pm 0.06) \cdot 10^{-4}$ s^{-1}. The initial absorbances at 612 nm of all samples were adjusted to 1.0 with heavy water or twice distilled water. The fitting method and the condition of illumination are the same as for Fig. 5.

R-PE or APC were also observed by NaN_3-inhibiting and ^2H_2O-enhancing experiments (Fig. 6 and Fig. 7). Control experiments showed that oxygen could not quench the fluorescences of phycobiliproteins under our experimental conditions. Thus the photosensitized generation of $^1O_2$ from the phycobiliprotein used (R-PE, C-PC or APC) is considered to proceed via excitation energy transfer from the triplet state of the phycobiliprotein to ground state oxygen and the following reactions may occur:

$$^0PBP(S_0) \xrightarrow{h\nu} {}^1PBP(S_1) \xrightarrow{k_{isc}} {}^3PBP(T_1) \longrightarrow {}^0PBP(S_0)$$
$$\downarrow O_2$$
$$\rightarrow {}^1O_2$$

and then:

$$PB + {}^1O_2 \rightarrow \text{Products}$$

Though no direct experimental evidence for the existing triplet states of phycobiliproteins has been obtained. Several indirect facts, such as the observations of delayed luminescence and phosphorescence of these proteins at 77 K [24,25], show probably the formations of the triplet states when phycobilipro-

teins are excited. Herein, the detection of $^1O_2$ from phycobiliproteins on photosensitization gives another important evidence for the existing triplet states of phycobiliproteins.

4. Discussion

On the basis of our experimental results, the pathways for the generation of reactive oxygen species from phycobiliproteins (PBPs) on photosensitization can be summarized as shown in Fig. 10. It shows that exposure of oxygen-saturated solution of PBP to visible light results in the generation of both singlet oxygen and superoxide. Dismutation of superoxide, either spontaneously or by the enzyme SOD, give rise to hydrogen peroxide, which is then transformed into hydroxyl radicals. Since the fluorescences of PBPs (R-PE, C-PC and APC) can not be quenched by oxygen, it seems probably that the triplet state of PBP [^{3}PBP(T_1)], generated from intersystem crossing (ISC) of the excited singlet state of PBP [^{1}PBP(S_1)], is the inductor of both $^1O_2$ and $O_2^{\cdot -}$. Therefore, the photodynamic action of PBPs proceeds by both free radical and singlet oxygen pathways which could contribute to the reported PBP phototoxicity in vivo [11–13] through their abilities to oxidize a wide variety of biomolecules, including lipids, proteins and nucleic acids [26].

Our studies indicate that reactive oxygen species, including $O_2^{\cdot -}$, H_2O_2, $^{\cdot}OH$ and $^1O_2$ generated from

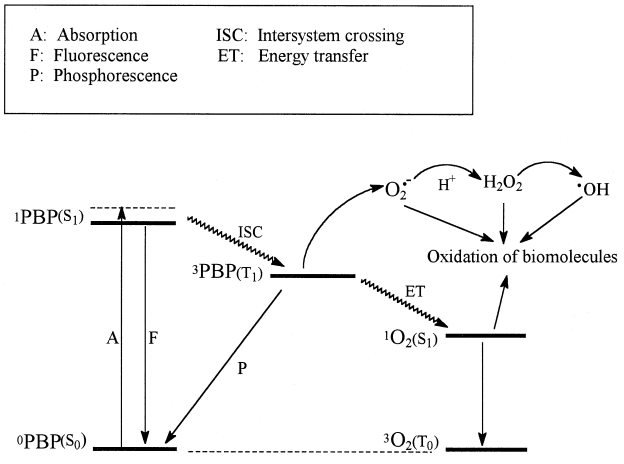

Fig. 10. Proposed pathways for the generation of reactive oxygen species from phycobiliproteins on photosensitization.

three phycobiliproteins on photosensitization, lead to the photobleaching of these proteins by self-sensitized photooxidation. The photobleaching rates of three phyco-biliproteins under the various inhibitors of reactive oxygen species were listed in Table 1. From the table we can see that the photobleaching rates, for R-PE, C-PC and APC extracted from *Porphyra yezoensis*, are on the order of 10^{-5} s^{-1} in argon-saturated solution and 10^{-4} s^{-1} in oxygen-saturated solution. These values of K_d basically coincide with that of White and Stryer [27]. From Table 1 it could be seen that the values of White and Stryer were located at the middle of our results. This is understandable because the samples were bubbled with argon or oxygen before irradiation in our experiments but their samples were simply dissolved in natural aqueous solutions. It must be noted that our results are not completely identical with that of White and Stryer, because many experimental conditions, such as light source and origin of phycobiliproteins, are different.

Also shown in Table 1, the photobleaching of these proteins can be prevented effectively by the treatment with $^1O_2$ quenchers sodium azide and histidine, as well as by inhibitions of $O_2^{\cdot -}$, H_2O_2, $^{\cdot}OH$ with SOD and catalase. On the other hand, heavy water, capable of increasing halflife of $^1O_2$ in this medium, accelerates obviously the photobleaching of three phycobiliproteins. These results strongly confirm that reactive oxygen species are produced from the phycobiliproteins and involve in the photobleaching of these proteins. Nevertheless, White and Stryer [27] reported that neither molecular oxygen, peroxide, nor oxygen radicals seem to be primarily responsible for the photobleaching of phycoerythrin. This conclusion is totally at variance with the results reported here. How to explain this discrepancy? Firstly, our observation agrees with the work of Delange and Glazer [28], where they found that the peroxy radicals could damage phycoerythrin and result in the photodestruction of the protein. In addition, our recent work [29] also verified that reactive oxygen species could be generated from phycobiliproteins on photosensitization and responsible for their photobleaching. Therefore, it can be concluded that reactive oxygen species are at least partially responsible for the photobleaching of phycobiliproteins. Why did White and Stryer not observe the

fluorescent change of phycobiliproteins when addition of reactive oxygen inhibitors? The possible reason is that the sensitivity of the measurement of fluorescent change is not high enough in order to examine so much small changes originated from the inhibition effect of the additives in their experiments. Let us concentration on the details of their experiments. In their illumination experiments of R-PE, the light intensity is $8.6 \cdot 10^{15}$ photons/s (514.5 nm), i.e., 3.3 mW. When such small light energy attached a 100-μl capillary tube, which served as the flow cell for the moving protein solution, it may be inferred that the change of fluorescence is very weak, moreover, the fluorescent changes come from inhibitions of the additives occupied only a little part in comparison with the total fluorescence. Therefore, it is essential that the sensitivity of the detector for probing the fluorescent change must be very high. From their results presented in Fig. 9 in their paper, it could obviously be seen that those data appear differences to some extent. But perhaps some scatter from the sample interfered with the detection, resulting in the sensitivity of the detector is not enough to differentiate the small change.

Phycobiliproteins, as a new type of agent applied in PDT, exhibits several advantages compared with HPD (see Section 1). Nevertheless, the potential immune responses of human to these proteins are likely to limit their application in PDT. Therefore, it is necessary and beneficial to investigate the immune reactions of phycobiliproteins. In the previous studies, the immuno-chemical properties of various phycobiliproteins that contribute to the understanding of evolution had been presented [30]. As a photodynamic therapeutic agent, the studies on the immune reactions of phycobiliproteins and the effects of these reactions on human are not carried out until now. According to the works of Morcos et al. and Zheng et al. [11–13], phycocyanin exhibits much strong photodynamic effect on atherosclerosis and tumor, and no side effects were observed. The previous results of our laboratory also indicated that R-phycocyanin from *Porphyra haitanensis* stimulated human B lymphatic cell reproduction, and this effect of R-PC can enhance the immunological competence of human [31]. Moreover, phycobiliproteins were extracted from various algae, such as *Porphyra yezoensis*, *Polysiphonia urceolata* etc, most of which are eatable. Therefore, phycobiliproteins, as a kind of natural photosensitizer, seem not to bring harmful immune reactions to human body when they are applied in PDT. Of course, the studies on the application of phycobiliproteins in PDT only just begin, we think the researches about the immune reactions of these proteins to human are of importance in the future clinical practice.

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References

[1] T.J. Dougherty, Photochem. Photobiol. 45 (1987) 837–841.
[2] B.W. Henderson, T.J. Dougherty, Photochem. Photobiol. 55 (1992) 145–157.
[3] C.S. Foote, Photochem. Photobiol. 54 (1991) 659.
[4] C.J. Gomer, T.J. Dougherty, Cancer Res. 39 (1979) 146–151.
[5] D. Kessel, P. Thompson, B. Musselman, C.K. Chang, Photochem. Photobiol. 46 (1987) 563–568.
[6] D.A. Bellnier, Y.-K. Ho, R.K. Pandey, J.R. Missert, T.J. Dougherty, Photochem. Photobiol. 50 (1989) 221–228.
[7] M. Kreimer-Birnbaum, Semin. Hematol 26 (1989) 157–173.
[8] H. Scheer, Angew. Chem. Int. Ed. Engl. 20 (1981) 241–261.
[9] A.R. Holzwarth, Physiol. Plant. 83 (1991) 518–528.
[10] Sidler, W.A. (1994) in The Molecular Biology of Cyanobacteria (Bryant, D.A., ed.), pp. 139–216, Kluwer Academic Publishers, Dordrecht, Netherlands.
[11] N.C. Morcos, M. Berns, W.L. Henry, Lasers Surg. Med. 8 (1988) 10–17.
[12] Morcos, N.C. and Henry, W.L. (1987) US Patent, 4, 886, 831; Chem. Abstrac. 1990, 113, 553 j.
[13] S. Zheng, X.H. Chai, L.M. He, Faming Zhuanli Shenqing Gongkai Shuomingshu CN 1, 091, 976; Chem. Abstr. 1995 122 (1994) 182179h.
[14] Q.S. Lin, J.P. Zhang, F.J. Zeng, L.J. Jiang, Acta Phytophysiol. Sinica 18 (3) (1992) 253–258.
[15] H.J. Wu, J.P. Zhang, A.D. Xia, L.J. Jiang, Acta Biochim. Biophys. Sinica 26 (5) (1994) 491–49716.
[16] Q.S. Lin, J.P. Zhang, F.J. Zeng, L.J. Jiang, Kexue Tongbao 11 (1992) 869–872.
[17] (1980) Pharmacia Fine Chemicals AB, Polyacrylamide Gel Electrophoresis Laboratory Techniques, Pharmacia, Sweden.
[18] K.Q. Lao, A.N. Glazer, Proc. Natl. Acad. Sci. USA 93 (1996) 5258–5263.
[19] E. Finkelstein, G.M. Rosen, E.J. Rauckman, Arch. Biochem. Biophys. 200 (1980) 1–16.

[20] K. Ohki, Y. Fujita, Plant Cell Physiol. 20 (1979) 1341–1348.
[21] D. Frackowiak, L.G. Erokhina, C. Jadzyn, L.M. Shubin, A.Y. Shkuropatov, Photosynthetica 15 (1981) 36–48.
[22] J.A. He, L.J. Jiang, Z.C. Bi, L. Jiang, Science in China (Series B) 39 (4) (1996) 386–397.
[23] M. Linetsky, B.J. Ortwerth, Photochem. Photobiol. 63 (1996) 649–655.
[24] I.N. Stadnichuk, Y.V. Kovalev, A.A. Krasnovski, J. Photochem. Photobiol., B: Biol. 19 (1993) 15–18.
[25] D. Frackowiak, A. Planner, A. Dudkowiak, Photochem. Photobiol. 57 (1993) 81–85.
[26] Straight, R.C. and Spikes, J.D. (1985) in Singlet Oxygen (Frimer, A.A., ed.), Vol. IV, pp. 91–143, CRC Press, Boca Raton, FL.
[27] J.C. White, L. Stryer, Anal. Biochem. 161 (1987) 442–452.
[28] R.J. DeLange, A.N. Glazer, Anal. Biochem. 177 (1989) 300–306.
[29] J.A. He, Y.Z. Hu, L.J. Jiang, J. Am. Chem. Soc. 118 (1996) 8957–8958.
[30] MacColl, R. and Guard-Friar, D. (1987) in Phycobiliproteins, pp. 199–206, CRC Press, Boca Raton, FL.
[31] F.J. Zeng, Q.S. Lin, L.J. Jiang, L.P. Zhu, Acta Biochim. Biophys. Sinica 24 (6) (1992) 545–552.