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Direct measurement of singlet oxygen produced by four chlorin-ringed chlorophyll species in acetone solution

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ABSTRACT

We developed an optical system to detect singlet oxygen produced by chlorin-ringed chlorophyll (Chl) species, i.e., Chl *a*, Chl *b*, Chl *d*, and di-vinyl-Chl *a*, with a high sensitivity and examined the relationship between molecular structures and reaction rates. Chl *a* in acetone was the lowest producer of singlet oxygen and the most effective quencher; in contrast, Chl *b* exhibited the opposite properties. These results showed that replacement of side chain(s) from a methyl group to a formyl group on the R7 position on a chlorin ring induced a higher production and lower quenching of singlet oxygen in Chl molecules.

1. Introduction

Light is an energy source that drives photosynthesis; however, it is also a source of light-induced damages to photosynthetic systems. Photosynthetic organisms, especially oxygenic photosynthetic organisms, develop rescue reactions to protect them from these damages [1]. Photoinhibition is one of the damage reactions that occur in the photosynthetic electron-transfer system [2]; in particular, the acceptor-side inhibition of photosystem (PS) II is a main cause of photodamage [3]. This inhibition is referred to as a sum of several reactions; however, the overall reaction process has not been clarified. This process is initiated by over-reduction of PS II, which induces the charge recombination between the primary electron donor (P680 or accessory chlorophyll (Chl) *a*) and the primary electron acceptor (pheophytin *a*, i.e. demetalated Chl *a*) (Scheme 1); this recombination yields the triplet state of Chl *a* and leads to the production of singlet oxygen via a spin-exchange reaction between the triplet state of Chl *a* and molecular oxygen. This singlet oxygen is harmful to cell components and induces the breakdown of the D1 protein, which is a major functional component of PS II, to form degradation products with a molecular mass of 16 and 8 kDa [4,5].

Singlet oxygen is one of active oxygen species as similar to hydroxyl radical, super oxide anion radical, and hydrogen peroxide. Singlet oxygen is produced via a photophysical process, whereas the other three active oxygen species are produced via photochemical reactions. Singlet oxygen is detected using luminescence with an emission maximum at 1275 nm (7843 cm^{-1}). As its yield is not high, i.e., less than 10^{-5} in acetone [6], its detection method has been improved to higher sensitivity in the infrared region.

Cyanobacteria are oxygenic photosynthetic prokaryotes that contain Chl *a* as a common pigment (Scheme 1). Cyanobacteria that contain a novel pigment are produced by introduction of a gene for Chl biosynthesis [7,8]. The mutant species of *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*) accumulates di-vinyl (DV)-Chl *a* instead of mono-vinyl (MV)-Chl *a* [8,9] by suppression of the specific enzyme responsible for chlorophyll synthesis, i.e., protochlorophyllide reductase. This transgenic species is sensitive to high-light conditions. In addition, cellular DV-Chl *a* and isolated photochemical complexes were bleached within 1 day under the high-light condition [8,10]. Furthermore, acceptor-side inhibition was observed in the form of degradation products of the D1 protein [9]. It is reasonable to assume that PS II produces a higher amount of singlet oxygen in the presence of DV-Chl *a* compared with MV-Chl *a*. Therefore, we tried to measure the singlet oxygen directly in Chl solutions using luminescence in the infrared region, as a first step in the analysis of the physiological responses to this oxygen species.

There are several reports on the direct measurement of singlet oxygen produced by Chl *a* or Chl *b* in solution [11–14]. In our study, as it was necessary to detect a signal from singlet oxygen in the DV-Chl *a* solution, we developed a detection system with a sensitivity that was adequate for the estimation of the yield of singlet oxygen. In addition to the three Chl species mentioned above, we adopted Chl *d* as a target species to clarify the effect of side-chain replacement in chlorin-ring Chl species on singlet oxygen production. We also examined the correlation of singlet oxygen yield with the molecular structures of the Chl species. This report represents

Abbreviations: Chl, chlorophyll; DV, di-vinyl; MV, mono-vinyl; PS, photosystem.

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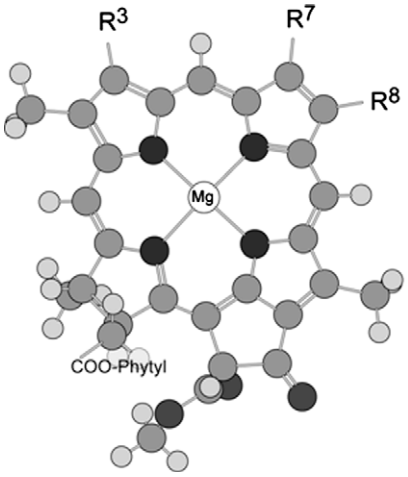

MV-Chl *a*: R^3=CH=CH_2, R^7=CH_3, R^8=CH_2CH_3
DV-Chl *a*: R^3=CH=CH_2, R^7=CH_3, R^8=CH=CH_2
Chl *b*: R^3=CH=CH_2, R^7=CHO, R^8=CH_2CH_3
Chl *d*: R^3=CHO, R^7=CH_3, R^8=CH_2CH_3
Pheophytin *a*: R^3=CH=CH_2, R^7=CH_3, R^8=CH_2CH_3, – Mg (replaced with two H)

Scheme 1. Molecular structure of the four Chl species.

the first description of the properties of production and quenching of singlet oxygen in novel Chl species, i.e., DV-Chl *a* and Chl *d*. We detected species-dependent changes in yield and quenching rates.

2. Materials and methods

2.1. Culture of algal species

Synechocystis sp. PCC 6803 was cultured under the photoautotrophic condition in BG11 medium at 25 °C. The light intensity for growth was adjusted to 25 μmole photon m^{-2} s^{-1} [9]. Air was continuously supplied through a filter (Millex, Millipore, MA, USA). Mutant *Synechocystis* cells containing DV-Chl *a* [8] were cultured in the same medium using the same illumination conditions as those used to culture wild-type cells. *Acaryochloris marina* MBIC 11017 was cultured in IMK medium under continuous illumination from an incandescent light source (15 μmol photons m^{-2} s^{-1}) at 25 °C [15] with continuous supply of air.

2.2. Isolation of Chls

Chl *a*, DV-Chl *a*, and Chl *d* were extracted from the thylakoid membranes of *Synechocystis*, a mutant of *Synechocystis*, and *A. marina*, respectively, and Chl *b* was extracted from the thylakoid membranes of spinach. Pigments were extracted using acetone, which was followed by replacement of the solvent with chloroform and purification using high performance liquid chromatography (HPLC; GULLIVER series, JASCO, Tokyo, Japan). Samples were injected into a Senshupak Silica-5301N column (300 mm × 30 mm; Senshu Science, Tokyo, Japan) after filtration (0.2 μm) and were then fractionated. The mobile phase, which was hexane/2-propanol (100:2), was eluted with a flow rate of 5.0 mL min^{-1}. Pigments were detected using a photodiode-array detector (MD-915, JASCO, Tokyo, Japan). Samples were stored in fused glass vessels and kept in the dark at −80 °C until use.

2.3. Measurements of singlet oxygen

Emission from singlet oxygen was measured using an apparatus developed and improved for high-sensitivity detection, based on a commercially available apparatus (NIR-PII System, Hamamatsu Photonics K.K., Hamamatsu, Japan, Fig. 1A). The oxygen concentration in acetone solutions was not controlled; however, it was equilibrated with air. The excitation pulse was obtained using a dye laser excited by a Nd:YAG laser (Tempest, New Wave Research Inc., CA, USA). Pulse width and intensity were approximately 10 ns and 300 μJ/pulse, respectively, and the repetition rate was 30 Hz. Emission of singlet oxygen was monitored using an infrared-gated image intensifier (NIR-PII, Hamamatsu Photonics K.K., Hamamatsu, Japan) after passage through a polychromator (250is, Chromex, NM, USA). Measurements started 5 μs after application of the excitation pulse, and the exposure time was 500 μs. Signals were accumulated by repeated detection (>300 times) and averaged. Calibration of wavelength was performed using a spectral calibration lamp (Krypton type, Oriel Instruments, CT, USA).

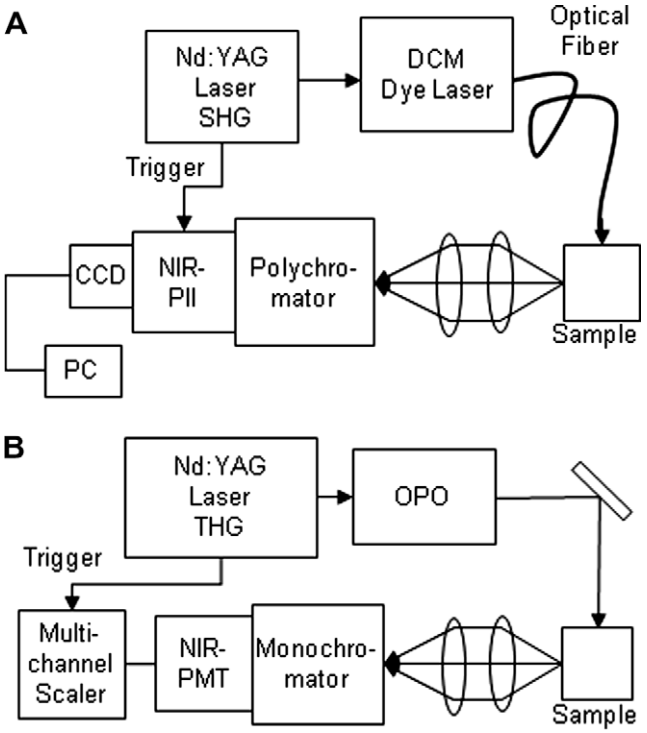

Fig. 1. Experimental setups for the measurement of singlet oxygen. (A) Spectrum measurement setup and (B) decay curve measurement setup.

Decay curves of singlet oxygen were monitored using the apparatus shown in Fig. 1B. The light source was an optical parametric oscillator (OPO) (MOPO-HF, Spectra-Physics, CA, USA) combined with a Nd:YAG laser (PRO-250-10, Spectra-Physics, CA, USA). The