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Replacement of chlorophyll with di-vinyl chlorophyll in the antenna and reaction center complexes of the cyanobacterium *Synechocystis* sp. PCC 6803: Characterization of spectral and photochemical properties

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ABSTRACT

Chlorophyll (Chl) *a* in a cyanobacterium *Synechocystis* sp. PCC 6803 was replaced with di-vinyl (DV)-Chl *a* by knock-out of the specific gene (*slr1923*), responsible for the reduction of a 8-vinyl group, and optical and photochemical properties of purified photosystem (PS) II complexes (DV-PS II) were investigated. We observed differences in the peak wavelengths of absorption and fluorescence spectra; however, replacement of Chl *a* with DV-Chl *a* had limited effects. On the contrary, photochemical reactions were highly sensitive to high-light treatments in the mutant. Specifically, DV-Chl *a* was rapidly bleached under high-light conditions, and we detected significant dissociation of complexes and degradation of D1 proteins (PsbA). By comparing the SDS-PAGE patterns observed in this study to those observed in spinach chloroplasts, this degradation is assigned to the acceptor-side photoinhibition. The delayed fluorescence in the nanosecond time region at 77 K was suppressed in DV-PS II, possibly increasing triplet formation of Chl molecules. Our findings provide insight into the evolutionary processes of cyanobacteria. The effects of pigment replacement on the optimization of reactions are discussed.

1. Introduction

Photosynthetic pigments, particularly chlorophylls (Chls), play crucial roles in the primary processes of photosynthesis of oxygenic photosynthetic organisms. Light absorption, resonance energy transfer, photochemical reaction, and quenching of excess light energy are typical examples of these processes. Nine Chl species [i.e., Chl *a*, Chl *b*, Chl *c*, Chl *d*, Chl *a*′ (13^2 epimer of Chl *a*), Chl *d*′ (13^2 epimer of Chl *d*), pheophytin *a* (Mg-free Chl *a*), di-vinyl (DV)-Chl *a* (Chl a_2), and DV-Chl *b* (Chl b_2)] are known to be confined in pigment–protein complexes [1,2]. With the exception of one taxonomic group (i.e., *Prochlorococcus* sp.), all other oxygenic photosynthetic organisms contain mono-vinyl (MV)-Chl *a*. Reduction of the 8-vinyl group on the pyrrole ring II of 3,8-DV-chlorophyllide (i.e., or 3,8-DV-protochlorophyllide) is a prerequisite for the synthesis of MV-Chl (i.e., Chl *a*, Chl *b*, Chl *c*, and Chl *d*), and a gene encoding the reducing enzyme is present in most oxygenic photosynthetic organisms [3]. Among these nine Chl species, Chl *a*, Chl *a*′, Chl *d* and DV-Chl *a* can serve as electron donors in photochemical reaction center (RC) complexes. In addition, several minor pigments species [i.e., Chl *a*′, Chl *d*′, and pheophytin (Pheo) *a*] constitute a frame for photochemical reactions in the RC. Furthermore, intermediates of Chl metabolism accumulate to detectable amounts under certain growth conditions.

The properties of pigments are modified via interactions with proteins. Energy levels and redox potentials in the ground and the excited states are directly affected by interactions with specific groups of amino acids and also with other Chls, and electric fields formed by protein moieties may confer additional effects. It is well known that hydrogen bonds affect the redox potential of primary electron donors in the RC of anoxygenic purple bacteria [4,5]. The stability of pigments in the complexes has not been well studied; however, stability is a primary requisite for function. Accommodation of pigments by protein moieties is accomplished via strict molecular interactions between pigments and amino acid residues.

In the course of acquisition of oxygenic photosynthesis in cyanobacteria, bacteriochlorophyll pigments are replaced with Chl pigments. This replacement occurs prior to the establishment of oxygenic photosynthesis, because the high potential (i.e., approximately

Abbreviations: Chl, chlorophyll; DF, delayed fluorescence; DV, di-vinyl; MV, mono-vinyl; Pheo, pheophytin; PS, photosystem; RC, reaction center; TDDFT, time-dependent density functional theory; TRFS, time-resolved fluorescence spectrum

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+1.2 V) [6–8] is required for oxygenic photosynthesis. The oxidation potential of Chl *a* in CH_2Cl_2 is estimated to be 0.80 V [9–11], and additional potential is conferred via interactions with protein moieties. However, replacement of pigments always induces a partial loss in integrity of complexes. For switching of the biosynthesis pathway from bacteriochlorophyll *a* to Chl *a*, loss of several enzymes and acquisition of one enzyme are required. The second event in acquisition of oxygenic photosynthesis involves the modification of protein moieties, which stabilizes the replaced pigments in the complexes. Therefore, the replacement of pigments precedes stabilization with the protein moieties.

We have developed several experimental systems to reproduce the process of pigment replacement during oxygenic photosynthesis. For example, we introduced Chl *b* into a cyanobacterium (i.e., *Synechocystis* sp. PCC 6803, hereafter referred to as *Synechocystis*) [12], and introduced DV-Chl *a* into *Arabidopsis* [13]. The former example constitutes an experimental reproduction of the evolution from *Synechococcus* sp. to *Prochlorococcus* sp. [14,15]. These experiments successfully incorporated newly acquired pigments into pre-existing proteins, and the pigments retained their functions (e.g., light absorption and energy transfer in the antenna [12,15] or electron transfer in RC complexes [16]). However, introduction of DV-Chl *a* reduced tolerance to strong illumination [13,17,18], perhaps reflecting a partial loss in the integrity of the complexes. To identify the reasons for this loss in integrity, we isolated PS II complexes from DV-Chl *a* introduced into *Synechocystis*, and compared the properties of these complexes with those isolated from control cells. We observed acceptor-side photoinhibition in the mutant cells. Based on our observations, the integrity of pigments in pre-existing proteins and the evolution of cyanobacteria are discussed.

2. Materials and methods

2.1. Cyanobacterial culture

Synechocystis sp. PCC 6803 was engineered to express a 6×His-tag at the C-terminus of a 47 kDa chlorophyll-protein (i.e., CP47, PsbB) for use as a control. The control cells were constructed by site-directed mutagenesis [19]. A *slr1923* knock-out mutant of *Synechocystis*, which lacked a component of DV-protochlorophyllide reduction, was obtained on the basis of the above control cells using a method described previously [17]. Control and mutant cells were cultured under autotrophic conditions in BG 11 medium at 298 K, with illumination from an incandescent light (i.e., 20 μmol photons m^{-2} s^{-1}). Air was continuously supplied through a Myrex filter (Millipore, USA).

2.2. Pigment preparation

MV-Chl *a* and DV-Chl *a* were isolated from the thylakoid membranes of control and mutant cells of *Synechocystis*, respectively. All pigments were purified by HPLC with a JASCO GULLIVER series instrument (JASCO, Tokyo, Japan). Pigments were extracted with acetone, and the solvent was replaced with chloroform. Samples were injected into a Senshupak Silica-5301N column (300 mm×30 mm, Senshu Science., Tokyo, Japan) after filtration (0.2 μm). The mobile phase was hexane/2-propanol (100:2) with a flow rate of 5.0 ml/min. Pigments were detected by a photodiode-array detector (JASCO, MD-915, Tokyo, Japan). The absorption spectra of Chls were measured using a Cary 500 spectrophotometer.

2.3. Isolation of PS II complexes

We isolated PS II complexes from control and mutant cells using a His-tag introduced at the C-terminus of CP47. Thylakoid membranes were isolated via mechanical disruption and differential centrifugation. The resulting PS II was solubilized using a detergent (i.e., dodecyl-β-D-maltoside, 0.8%) for 20 min at 277 K in the dark, and purified by Ni^{2+}-affinity column chromatography [20].

2.4. Assay of oxygen-evolving activity

Oxygen-evolving activity was measured using a Clark-type oxygen electrode (Rank Brothers, England) at 298 K with 6 mM potassium ferricyanide as an electron acceptor. The measurements were carried out in a buffer solution (50 mM MES, pH 6.0) containing 1.0 M sucrose, 20 mM $CaCl_2$, and 10 mM NaCl.

2.5. Photochemical reactions

Pigment photobleaching was monitored using a Hitachi photodiode-array spectrophotometer (Hitachi 0080D, Japan). For actinic light, blue light was provided from a slide projector with heat- and UV-absorbing filters (HA-50, Toshiba, Japan) and spectral differences in the red region were monitored. The intensity of actinic light was 1000 μmol photons m^{-2} s^{-1}. The Chl contents were spectroscopically determined using a combination of 80% acetone extraction with the reported extinction coefficient by Porra et al. [21].

2.6. Degradation of D1 proteins

Degradation of D1 proteins was monitored via gel-electrophoresis of component proteins. After illumination of red light over a certain time period (i.e., from 0 min to 60 min, 2500 μmol photons m^{-2} s^{-1}), aliquots were sampled and SDS-PAGE patterns were compared. The D1 protein and its degradation products were identified using an anti-D1 antibody (AgriSera, Sweden) raised against the C- and N-termini of the D1 protein [22]. An antibody raised against the 43 kDa chlorophyll-protein (i.e., CP43, PsbC) (AgriSera, Sweden) was also used for protein identification.

2.7. Spectroscopy

Absorption spectra were measured using a Cary 500 spectrophotometer; for low-temperature spectra, a cryostat for liquid nitrogen temperatures (OptistatDN, Oxford Inst. Oxford, UK) was used in conjunction with a controller (Oxford ITC-601PT). Fluorescence spectra at 77 K were measured with a Hitachi 850 spectrofluorometer (Hitachi, Japan) with a custom-made Dewar bottle [23,24]. To assess low-temperature fluorescence spectrum, polyethylene glycol (i.e., PEG, average molecular weight 3350, final concentration 15% (w/v), Sigma-Aldrich) was added to obtain a homogeneous glass. The spectral sensitivity of the fluorometer was corrected using a sub-standard lamp with a known radiation profile. Time-resolved fluorescence spectrum and fluorescence decay curves were measured using time-correlated single-photon counting methods [22], with an excitation wavelength of 425 nm (i.e., the second harmonic of the 850-nm oscillation). Fluorescence lifetime was estimated via the convolution calculation [25].

2.8. Calculation of geometry optimization and energy levels for MV- and DV-Chl a

All calculations were performed using GAUSSIAN 03 [26]. The initial coordinates of atoms in the model Chl compound were obtained from the crystal structure of MV-chlorophyllide *a* [27]. Geometry optimization of MV- and DV-Chl *a* was performed using the DFT method, with Becke's three-parameter hybrid functional set [28] combined with the Lee–Yang–Parr correlation functional set (B3LYP) [29] and the 6–31G(d) basis set. For these molecules, the energy levels of singlet and triplet states were estimated via the