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Reevaluating the mechanism of excitation energy regulation in iron-starved cyanobacteria

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ABSTRACT

This paper presents spectroscopic investigations of IsiA, a chlorophyll *a*-binding membrane protein produced by cyanobacteria grown in iron-deficient environments. IsiA, if associated with photosystem I, supports photosystem I in light harvesting by efficiently transferring excitation energy. However, if separated from photosystem I, IsiA exhibits considerable excitation quenching observed as a substantial reduction of protein-bound chlorophyll *a* fluorescence lifetime. Previous spectroscopic studies suggested that carotenoids are involved in excitation energy dissipation and in addition play a second role in this antenna complex by supporting chlorophyll *a* in light harvesting by absorbing in the spectral range inaccessible for chlorophyll *a* and transferring excitation to chlorophylls. However, this investigation does not support these proposed roles of carotenoids in this light harvesting protein. This study shows that carotenoids do not transfer excitation energy to chlorophyll *a*. In addition, our investigations do not support the hypothesis that carotenoids are quenchers of the excited state of chlorophyll *a* in this protein complex. We propose that quenching of chlorophyll *a* fluorescence in IsiA is maintained by pigment-protein interaction via electron transfer from an excited chlorophyll *a* to a cysteine residue, an excitation quenching mechanism that was recently proposed to regulate the light harvesting capabilities of the bacteriochlorophyll *a*-containing Fenna-Mathews-Olson protein from green sulfur bacteria.

1. Introduction

Cyanobacteria are oxygenic photosynthetic organisms that are responsible for a significant portion of global biomass production. They are genetically and morphologically diverse and are found in various environments across a wide range of altitudes and latitudes [1]. Cyanobacteria have survived many geological and climatic changes on Earth during the past ~3.5 billion years, and have evolved to overcome severe environmental conditions, such as nutrient deficiencies [2]. Iron deficiency is a common nutrient-deficient condition in cyanobacterial habitats. Although iron is one of the most abundant elements on Earth, it is usually found in the form of insoluble ferric oxides [3,4]. Cyanobacteria need significant amounts of ferric iron (Fe^{3+}) for assembly of iron-sulfur complexes, which are necessary for maintaining light-dependent photochemical reactions in protein complexes like photosystem I (PSI) [5]. If the surrounding environment lacks iron, cyanobacteria cannot produce sufficient levels of PSI or other essential iron-sulfur proteins, which may have lethal consequences.

IsiA is a chlorophyll *a* (Chl *a*)-binding protein produced by cyanobacteria living in iron-deficient conditions [6]. Given that iron limitation is common in natural environments, under such conditions the IsiA protein is produced and associated with PSI [7–9], where it participates in the process of light harvesting. IsiA is a 36 kDa membrane protein with high protein sequence homology to CP43, a core light-harvesting antenna protein of photosystem II (PSII) [7]. A major difference between these proteins is their pigment content. While CP43 binds 13 Chl *a* and three molecules of carotenoid β-carotene, IsiA contains between 13 and 16 Chl *a* and four carotenoids: three β-carotenes and one echinenone [10,11]. A high-resolution crystal structure of the IsiA protein is not available, but top view images obtained by electron microscopy analysis of single particles of PSI-IsiA supercomplexes showed that the PSI trimer is surrounded by 18 IsiA subunits forming a $(PSI)_3(IsiA)_{18}$ supercomplex [8]. This ring-shaped supercomplex is a preferred formation adapted by $(PSI)_x(IsiA)_y$ supercomplexes [12].

Since the identification of the IsiA protein in the 1980s [6], several hypotheses have been proposed to explain its biological function. IsiA is highly homologous with CP43, but is produced when the cyanobacterial cells are grown in iron-deficient environments where adequate quantities of PSI cannot be assembled [13]. It was suggested that the protein is synthesized to compensate for the loss of PSI and maintain light harvesting capacity. From this perspective, the IsiA

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rings formed around PSI may act as huge light harvesting antennas, similar to phycobilisomes associated with PSII [14]. Due to a high mobility of IsiA in thylakoid membranes and a large pigment capacity, a function in Chl *a* storage has been also proposed [15].

Studies using time-resolved optical spectroscopies [11,16] suggested that in PSI-IsiA supercomplexes, IsiA very efficiently transfers the excitation energy of absorbed light to PSI. However, antenna proteins that are separated from PSI and freely float in the thylakoid membrane show a protective, dissipative mechanism that mitigates potential photo-oxidative damage. Excitation quenching has been clearly observed as a substantial shortening of the excited state lifetime of Chl *a* [10]. Further investigations proposed that a quenching mechanism based on non-photochemical quenching was present at the level of the protein monomer and maintained by carotenoids. It was argued that one of the carotenoids, preferentially echinenone, quenches the singlet excited state of Chl *a* via direct energy transfer from the Chl *a* Q_y state to the carotenoid S_1 state [17,18].

Efficient quenching of the Chl *a* excited state via the carotenoid S_1 state can compete with the intrinsic decay of the carotenoid S_1. If the pool of carotenoids excited to their S_1 state via chlorophyll-to-carotenoid energy transfer is populated faster than the intrinsic decay of the S_1 state, the state can be detected by time-resolved absorption spectroscopy by recording the $S_1 \rightarrow S_n$ excited state absorption band. This provides direct evidence of carotenoid involvement in the quenching process. Recently, this spectroscopic method directly demonstrated the involvement of a carotenoid in Chl *a* quenching in another class of cyanobacterial proteins called High-Light Inducible Proteins [19,20]. No such spectral signature of the carotenoid excited S_1 state has ever been experimentally observed for IsiA. An explanation that has been provided was based on the hypothesis that the populating rate of the quencher (echinenone in the S_1 state) is not fast enough to compensate for a subsequent, immediate decay of its excited state. The excited carotenoid will be only a "virtual" element in the excitation decay pathway. Thus carotenoid involvement was simply anticipated and built into kinetic models of the Chl *a* excitation decay path [17,18].

Furthermore, the absorption spectrum of the IsiA sample used in the previous studies [17,18] showed a maximum of absorption of the Chl *a* Q_y band shifted to 675 nm, which according to other spectroscopic studies is more characteristic of the IsiA-PSI supercomplex [11,16,21,22]. This is strongly suggestive of a sample that could be substantially contaminated by PSI or to some extent contains a mixture of IsiA and IsiA-PSI supercomplexes. Because the effect of the quenching of Chl *a* fluorescence in the IsiA protein could be undermined by hypothetically possible IsiA-to-PSI energy transfer, this questions the conclusions of the previous studies.

The work presented here strongly suggest that carotenoids do not play a role in the energetics of this pigment protein complex, either as quenchers or supporters of Chl *a*. The results of this study strongly suggest that the quenching mechanism is merely governed by Chl *a*-protein interactions via electron transfer from an excited Chl *a* to a cysteine residue. Such a novel energy-quenching mechanism was very recently proposed to regulate the light harvesting capabilities of the bacteriochlorophyll *a*-containing Fenna-Mathews-Olson (FMO) protein from green sulfur bacteria [23]. However, the current study suggests that this mechanism could be more broadly utilized by photosynthetic organisms than initially anticipated.

2. Materials and methods

2.1. Strain growth and thylakoid membrane preparation

The IsiA-His strain of *Synechocystis* sp. PCC 6803 was constructed by oligonucleotide-directed mutagenesis to introduce six histidyl codons at the carboxy terminus of *isiA*. IsiA-His cells were grown phototrophically in BG11 medium containing kanamycin at 30 °C. The liquid cultures were shaken in Erlenmeyer flasks at 60 rpm with illumination of 30 μmol photons m^{-2} s^{-1}. After 5 days of growth, cells were washed with YBG11-Fe [24] medium three times, and inoculated into 1 L YBG11-Fe_3 medium. After about 2 weeks, the cells were then harvested and broken by bead-beating as described previously [25]. Thylakoid membranes were resuspended in Buffer A (50 mM HEPES-NaOH [pH 7.8], 10 mM $MgCl_2$, 5 mM $CaCl_2$, 25% glycerol). Membranes were solubilized by addition of β-D-dodecyl maltoside (DDM) to a final concentration of 1%. After incubation on ice in dark for 30 min, the solubilized membranes were separated from the insoluble material by centrifugation at gradually increasing speed from 120 ×*g* to 27,000 ×*g* at 4 °C for 20 min. The solubilized membranes were then stored at −80 °C for future use.

2.2. IsiA protein purification

The IsiA and PSI-IsiA complexes were purified using nickel affinity chromatography [26] with some modifications. Ni-NTA slurry was precharged with 50 mM nickel sulfate overnight and loaded into an open column. The resin was washed with 25 column volumes of water, and then twice with 5 column volumes of Buffer A plus 0.04% DDM and 5 mM histidine to remove ethanol and nickel sulfate. After continuous mixing of the washed resin with the previously prepared solubilized membranes at 4 °C for 2 h, the flow through material was collected. The resin was then washed with 1 column volume Buffer A plus 0.04% DDM and 5 mM histidine. To remove all other unbound proteins, 12 column volumes of Buffer A plus 0.04% DDM was used to wash the resin. The eluents were collected and absorption was measured using a DW2000 spectrophotometer (OLIS, USA) to verify that any residual unbound chlorophyll-containing proteins had been washed from the column. The target proteins, PSI-IsiA supercomplexes and IsiA proteins, were eluted with 6 column volumes of buffer A plus 0.04% DDM and 100 mM histidine. To concentrate the proteins, 80% (v/v) PEG8000 in 30 mM HEPES-NaOH (pH 7.8) was added into the elution, and the proteins were precipitated by centrifugation at 31,000 ×*g* for 15 min. The precipitated proteins were resuspended in Buffer A plus 0.04% DDM.

Sucrose gradient ultracentrifugation was used to obtain highly purified IsiA aggregates that do not contain PSI. The PEG-concentrated protein sample was diluted in glycerol-free Buffer A plus 0.04% DDM and then loaded on the top of a 10–35% sucrose gradient in glycerol-free Buffer A plus 0.04% DDM. Centrifugation was performed using a swinging bucket type Beckman-Coulter SW41 rotor at 4 °C and relative centrifugal force of 186,000 ×*g*. After 18 h of ultracentrifugation, green bands were collected. The first green band from the top of the gradient was determined spectroscopically to contain IsiA only, and was stored at −80 °C until future use.

2.3. SDS-PAGE and immunoblot analysis

SDS-PAGE was performed by loading the isolated PSI-IsiA supercomplexes and IsiA protein samples (adjusted to Chl *a* mass weight of 0.75 μg) on a 12.5% acrylamide resolving gel. After transfer of the proteins onto a PVDF membrane, IsiA and PsaA were detected by using specific antisera. Bands were visualized using chemiluminescence reagents (EMD Millipore, Billerica, MA, USA) with an ImageQuant LAS-4000 imager (GE Healthcare).

2.4. Spectroscopic techniques

For all low-temperature spectroscopic measurements, the IsiA or IsiA-PSI samples were mixed with glycerol in a 1:1 (v/v) ratio, placed in 1 cm square plastic cuvettes and frozen in a VNF-100 liquid nitrogen cryostat (Janis, USA). Steady-state absorption measurements were performed using a Shimadzu UV-1800 spectrophotometer. Fluorescence and fluorescence-excitation spectra were recorded at room temperature using a Horiba-Spex Nanolog fluorometer. The spectra were