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Review Article (Invited)

Recent advances in single-molecule spectroscopy studies on light-harvesting processes in oxygenic photosynthesis

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Photosynthetic light-harvesting complexes (LHCs) play a crucial role in concentrating the photon energy from the sun that otherwise excites a typical pigment molecule, such as chlorophyll-*a*, only several times a second. Densely packed pigments in the complexes ensure efficient energy transfer to the reaction center. At the same time, LHCs have the ability to switch to an energy-quenching state and thus play a photoprotective role under excessive light conditions. Photoprotection is especially important for oxygenic photosynthetic organisms because toxic reactive oxygen species can be generated through photochemistry under aerobic conditions. Because of the extreme complexity of the systems in which various types of pigment molecules strongly interact with each other and with the surrounding protein matrixes, there has been long-standing difficulty in understanding the molecular mechanisms underlying the flexible switching between the light-harvesting and quenching states. Single-molecule spectroscopy studies are suitable to reveal the conformational dynamics of LHCs reflected in the fluorescence properties that are obscured in ordinary ensemble measurements. Recent advanced single-molecule spectroscopy studies have revealed the dynamical fluctuations of LHCs in their fluorescence peak position, intensity, and lifetime. The observed dynamics seem relevant to the conformational plasticity required for the flexible activations of photoprotective energy quenching. In this review, we survey recent advances in the single-molecule spectroscopy study of the light-harvesting systems of oxygenic photosynthesis.

Key words: conformational dynamics, spectral diffusion, fluorescence blinking, nonphotochemical quenching, cryogenic microscope

Significance

The light-harvesting processes in natural photosynthesis relies on the dense packing of pigment molecules which ensures the efficient energy transfer. At the same time, conformational changes in the protein can switch the pigment system to the energy quenching state under excessive light condition. Revealing the molecular mechanism of this photoprotective function requires accurate understanding of the excited states of densely packed pigments and how they are affected by the surrounding environment. Single-molecule spectroscopy on photosynthetic complexes have provided unique information on how the photophysical properties of the bound pigment molecules are affected by the conformational dynamics of proteins.

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Introduction

Photosynthetic light-harvesting systems are so efficient that they feed the absorbed photon energy to the reaction center with almost 100% quantum efficiency. This highly efficient light harvesting is achieved through the dense packing of pigments to maintain the distances between pigments suitable for the rapid energy transfer. However, the dense packing of pigments creates a risk of unwanted chemical reactions, such as charge separations between inappropriate adjacent pigments. Such reactions can not only result in the loss of the excited state but also generate harmful reactive-oxygen species (ROS) [1]. This kind of difficulty can be seen in early challenges to the artificial photosynthesis. For example, Itoh et al. reported that densely packed chlorophyll-*a* molecules in the pores of porous silica material performed efficient energy transfers among pigments, unfortunately together with rapid quenching of the excitation due to the charge separations between randomly packed pigments [2]. The natural light-harvesting systems elegantly avoid such unwanted reactions by locating pigments in appropriate positions.

Figure 1 shows the pigment arrangements in photosystem I (PSI) and photosystem II (PSII) of cyanobacteria (left column) and eukaryote (right column). Cyanobacterial PSI usually forms a trimer, whereas that of eukaryote is a monomer and attach several peripheral light-harvesting chlorophyll complexes (LHCs) called LHCI. PSII forms a dimer and bind various types of peripheral antenna depending on species. Cyanobacterial and red algal PSII have phycobilisomes, and other eukaryote PSIIs bind LHCII. In both systems, there are spaces between the central and outer clusters of pigments. The former including the special pair shown in magenta in Figure 1 is involved in the photoinduced electron transfer, and the latter functions exclusively as an antenna. The space between the two clusters is necessary to avoid unwanted electron transfer in the antenna cluster.

On the other hand, it is known that natural photosynthetic systems utilize the quenching of the excited state as photoprotective mechanisms, which are critically important for the survival of organisms under the fluctuating intensity of natural light. The enhanced photoprotective quenching ability produced by bioengineering has been demonstrated to increase biomass production [3]. The efficient light-harvesting state of natural light-harvesting systems seems to have marginal stability and can be readily converted to highly quenched states in response to external perturbations.

For decades, photoprotective mechanisms, especially in the oxygenic photosynthesis of plants and algae, have attracted many researchers in the fields of biophysics, biochemistry, and photochemistry. Despite intensive studies, the molecular basis underlying the switching from the light-harvesting to the quenching state has not been fully understood. This is partly due to the difficulty in understanding the nature of the excited states of pigment aggregates. Studies based on single-molecule spectroscopy (SMS) of photosynthetic systems have made unique contributions to this field by clarifying the importance of the conformational plasticities of the systems, which should be closely related to their responsiveness to the environmental changes [4-9].

Figure 1 clearly shows that the pigment arrangements in oxygenic photosynthetic complexes contrast sharply with those in photosynthetic bacteria. While the latter seem to prefer rather periodic and ordered pigment arrays (see, for example, [10-12]), the former rarely show periodic arrangements of pigments. In these systems, chlorophylls (Chls) seem to be placed at random positions with random orientations. The lack of periodicity in the structure raises specific difficulties in understanding the photophysics of oxygenic light-harvesting systems. One difficulty is in inferring the excited-state energy of each Chl molecule bound to a specific site of a protein (called “on-site energy” or simply “site energy”). The site energy of a pigment molecule is modified by several factors, such as electrostatic interactions, hydrogen bonding with the surrounding charged atoms, side-chain conformation, deformation of the molecular plane of the pigment molecule induced by the interactions with the surrounding protein residues, and so on. It has not been feasible to determine the site energy of each Chl using a quantum-chemical approach, taking the effect of surrounding protein residues into account, although several studies based on powerful computational methods have recently been conducted to try to break the barrier [13].

Delocalization of the excited state over densely packed pigments poses another difficulty in understanding the photophysics of the system. Since the degree of delocalization depends on both the electric interactions and the difference in site energies between pigments, rational determination of the site energy is again of crucial importance for understanding the photophysics of the system. SMS studies have also made contributions to this issue.

In this article, we briefly review recent advances in the SMS study of photosynthetic systems. We focus on recent SMS studies that are relevant to the photoprotective mechanisms in the oxygenic photosynthesis.

The Light Reaction in Oxygenic Photosynthesis

Electron Transfer in Oxygenic Photosynthesis

Oxygenic photosynthesis is driven by two photosystems, photosystem I (PSI) and photosystem II (PSII), which function as tandem solar cells. The two PSs are embedded in the thylakoid membrane that is contained in the chloroplasts of

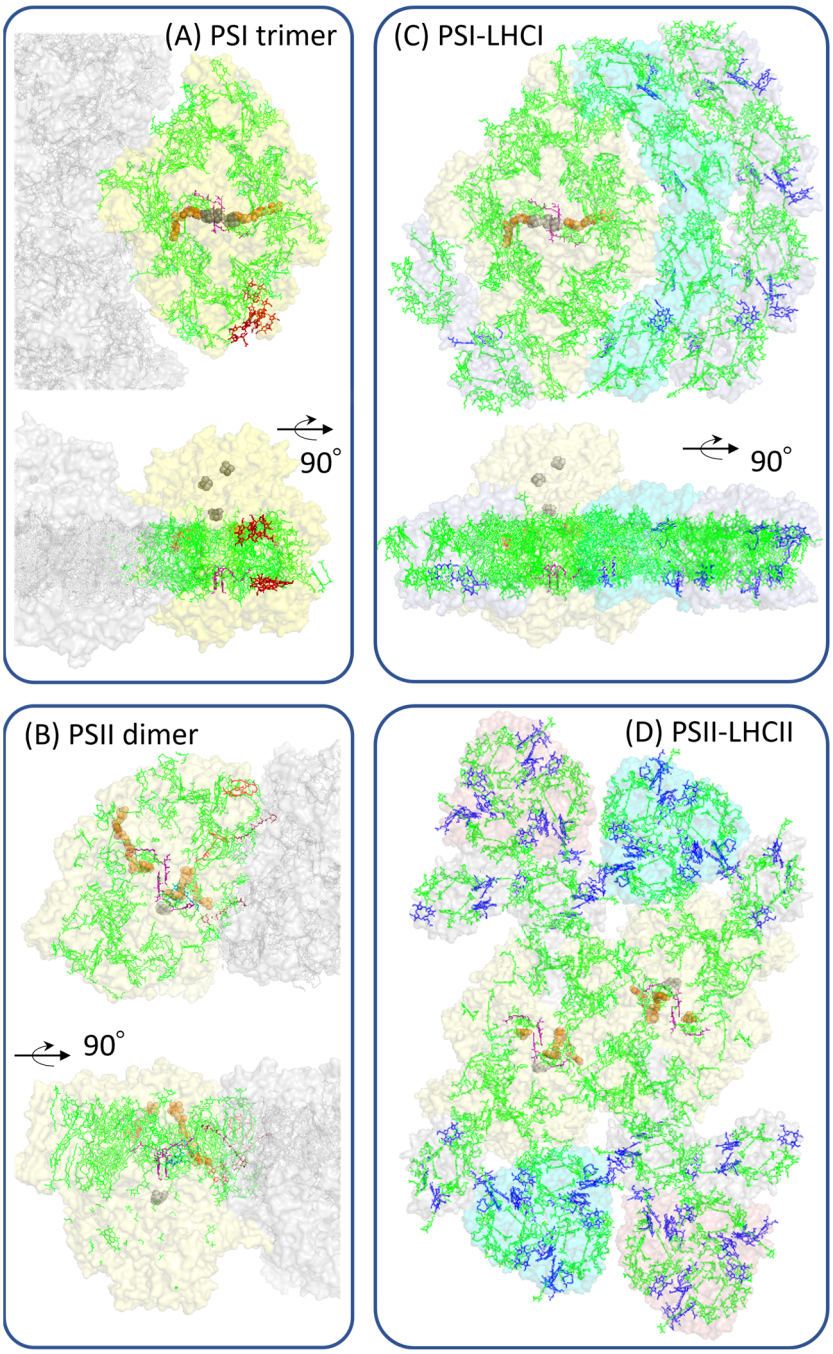

Figure 1 Cofactor arrangements in the PSI trimer [PDB ID: 6pgk] (A) and PSII dimer [PDB ID: 3wu2] (B) of *T. elongatus*, the PSI-LHCI supercomplex of *C. reinhardtii* [PDB ID: 6jo5] (C), and the PSII-LHC supercomplex of the pea [PDB ID: 5xnl] (D). The structures in (A, B) and (C, D) were those determined by X-ray diffraction and by single-particle analysis of the cryo-electron microscopy, respectively. In (A, B), one of the two monomeric units is highlighted with pale yellow, and in (C, D), the core part is highlighted with pale yellow. Cyan-colored parts in (C) are the LHCIs that are conserved in plant PSI, whereas the light-blue-colored parts are the additional outer LHCIs found in green algae. The cyan-colored and pink-colored parts in (D) are the S-LHCII and M-LHCII trimers, respectively. Chls-*a* are shown in green except for those comprising the special pairs shown in magenta and those indicated as the red Chl candidates in (A, B) in red. Pigments assigned to Chl-*b* are shown in blue. The β-carotene that was indicated to be lost in the monomeric PSII is shown in pink in (B). Quinone molecules are depicted in the orange spherical model with the van der Waals radius. The iron-sulfur complexes in (A, C) and the OEC in (B, D) are depicted as gray spheres with the van der Waals radius.

eukaryotic photosynthetic organisms or in the cells of prokaryotic photosynthetic organisms. The thylakoid forms a pouch and separates the spaces inside the pouch (the lumen) from the space outside the pouch (the stroma). The light reaction in PSII starts with a light-induced electron release from a special Chl molecule called the primary donor. The dimeric Chl P680 at the center is structurally similar to its counterpart in the purple bacterial "special pair" that is assigned to the primary donor. However, P680 does not actually serve as the primary donor. Studies using time-resolved infrared spectroscopy [14] and following transient absorption spectroscopy [15] suggested that the primary donor in PSII is a monomeric Chl, called an accessory Chl, neighboring the P680 and bound to the D1 subunit. The assignment of the primary donor was not straightforward because of the spectral crowding of the absorption bands of pigments bound to PSII. Although two electron-transfer paths exist in PSII due to its pseudo twofold symmetry, the electron flows through the one path containing the primary donor.

The cationic form of the primary donor is reneutralized by the electron provided by the water-oxidation reactions catalyzed by the oxygen-evolving complex (OEC). The electron from the primary donor is transferred sequentially to the pheophytin and two plastoquinone molecules, Q_A and Q_B. Q_B accepts two electrons, together with two protons from the stromal side. Since the resulting plastoquinol has a lower affinity to the binding site, it is released from PSII and diffuses into the thylakoid membrane. The plastoquinols diffuse to the site of the cytochrome b_6f complex, where they pass the electron to cytochrome b_6f, release the protons to the lumen, and then are converted again to plastoquinone. Thus the plastoquinone and plastoquinol in the thylakoid constitute the so-called quinone pool, through which electrons are carried from PSII to cytochrome b_6f and protons are pumped from the stroma to the lumen to generate the proton motive force necessary to drive the ATP synthesis.

The electrons are carried from cytochrome b_6f to PSI by water-soluble cytochrome c_6 or plastocyanin. PSI uses the photon energy to pump the electrons up to $NADP^+$ to generate NADPH. The primary electron transfer in PSI is considered to take place from the dimeric Chls called P700 at the center. Some researchers have argued that the primary donor in PSI is also a monomeric Chl neighboring P700 as in PSII [16]. The electron from the primary donor goes through the path consisting of Chl, phylloquinone, and three iron–sulfur clusters called F_X, F_A, and F_B. Interestingly, the electron transfer in PSI takes place through both of the existing paths [17,18], whereas only one path is active in PSII. Finally, the electron is transferred to the ferredoxin-$NADP^+$ reductase to generate NADPH through a water-soluble protein, ferredoxin. In addition to the above linear electron flow, some electrons from PSI are carried back to the plastoquinone pool and again to cytochrome b_6f. This cyclic electron transport enhances the proton pumping from the stroma to the lumen.

The Importance of Photoprotection

There are several risky processes that generate poisoning compounds under strong sunlight exceeding the photosynthetic capacity. A triplet excited state of a Chl is formed with a certain probability. The triplet formation is known to be enhanced through the charge-recombination at the primary donor [1]. In PSII, high-light condition results in a "closed" situation where the reduced anionic form of the secondary acceptor Q_A is accumulated. In a closed PSII, the blocking of the charge transfer to Q_A enhances the charge-recombination processes at the primary electron donor, and the probability of the triplet excited-state formation is enhanced. The energy transfer from a Chl in the triplet excited state to an oxygen molecule causes generation of a singlet oxygen, one form of ROS. At the downstream of the electron transfer in PSI, too much excitation will cause the electron donation to oxygen molecules to generate another ROS, superoxide (O_2^-).

In order to avoid the generation of ROS, natural photosynthetic systems have developed several photoprotective mechanisms. Rapid triplet transfer to carotenoids (Cars) suppresses the generation of singlet oxygen [19]. Most of the other mechanisms function by quenching the excited state under excessive excitation through processes other than the photosynthetic photochemistry. Thus, these processes are called non-photochemical quenching (NPQ) [20-22]. NPQ is triggered by several environmental inputs: lumen acidification, enzymatic modifications of the Cars bound to the light-harvesting systems, and so on. Although several mechanisms underlying NPQ have been proposed so far [22-28], it remains elusive which molecular process plays the essential role in the rapid switching of NPQ. The dynamical natures of light-harvesting systems have been clarified via SMS studies and often argued in the context of the NPQ mechanisms proposed so far.

Photosystems

Light-Harvesting Systems in Photosystem I

The PSI core binds about 100 Chl-*a* molecules [29-32], the majority of which function as antennae. The antenna systems of PSI often contain specific Chls with conspicuously red-shifted excited states. Here, we designate the red-shifted Chls whose site energies are lower than that of the primary donor as red Chls. At low temperature, an isolated PSI sample typically shows its fluorescence peak at around 710–760 nm, depending on the species [33-35]. This low-temperature fluorescence is emitted from the red Chls, which are much redder than those (around 670–690 nm) of a typical Chl solution in an organic solvent. Since an energy transfer from an excited red Chl to a typical Chl requires thermal energy

to overcome the energy gap, an exciton trapped by the red Chl cannot escape. At room temperature, such an uphill energy transfer can take place, and then red Chls function as efficient antenna pigments.

Plant and algal PSI core complexes are monomeric, whereas those of cyanobacteria usually form trimers. In some cyanobacterial species, PSI is known to form a tetramer [36]. In plants and algae, PSI forms a larger complex containing antenna complexes, called LHCIs, bound to the periphery of the core complexes. Algal PSIs have a larger composition of peripheral antennae (see Fig. 1). Both the PSI core complexes and the LHCIs of many organisms contain red Chl [37,38], whereas the PSI core complex of *Chlamydomonas* does not [39]. The trimeric or dimeric Chls-*a* in the PSI structure have often been assigned to the red Chl based on the assumption that the strong excitonic coupling and/or CT interaction between pigments is the main cause of the red shift [40-44]. However, there has been no authentic agreement on the assignment.

Interestingly, recent investigations have revealed that PSI is specifically damaged under intermittently fluctuating light [45,46]. Later studies clarified a crucial role of $P700^+$ in protecting PSI against the fluctuating light [47,48]. $P700^+$ has an absorption maximum at around 800 nm [49,50] and can be an effective energy acceptor from red Chls through a Förster-type energy transfer. NPQ by $P700^+$ plays a role in photoprotection. The red Chls in PSI may function as mediators of the excitation energy from Chls to the quenching site ($P700^+$) [51]. Another study suggested the importance of the formation of PSI–PSII megacomplexes [52-55]. In such a megacomplex, the excitation energy flows from PSII to PSI (called "spillover") according to the gradient of the site energy. Quenching by $P700^+$ may play a photoprotective role in the megacomplex [54,55].

SMS Studies on Photosystem I

Since the efficient photochemical reaction significantly quenches the fluorescence emission at room temperature, many SMS studies on PSI have been performed at low temperatures. The fluorescence quantum yield of PSI was reported to increase from ca. 0.5% at room temperature [56] to 5-10% below 100 K [57]. Jelezko et al. reported for the first time an SMS study on PSI. The study was done for PSI isolated from a thermophilic cyanobacterium, *Thermosynechococcus* (*T.*) *elongatus* [58]. Complexes purified from thermophilic organisms have often been chosen for use because of their high stability. In that study, Jelezko et al. used a self-built laser-scanning confocal microscope system operating at cryogenic temperatures. In their system, the objective lens with a numerical aperture of 0.85 was immersed in the liquid He together with the sample. This brief report showed that the fluorescence signal of single PSI can be obtained even at 240 K in spite of the drastic reduction in the fluorescence signal at temperatures higher than 100 K. Although fluorescence emission spectroscopy has been the main mode used in later SMS studies on PSI, this first study reported the excitation spectrum of PSI at 2 K together with the emission spectra.

Since this initial study, the SMS studies on PSI have been done mainly at cryogenic temperatures below 4 K [4,59-62]. These studies have revealed the nature of the red Chl excited states. At such a cryogenic temperature, the fluorescence spectrum of a pigment is typically composed of a sharp zero-phonon line (ZPL) and a broad phonon-side band (also called a phonon wing; PW) on the lower energy side. The ZPL reflects the purely electronic transition, whereas the PW reflects transitions that excite the phonon modes (or low-frequency vibrational modes of the surrounding protein) on the electronic ground state (see Fig. 2). Low-frequency vibrational modes of protein in the range of 5–200 cm^{-1} were often reflected in the shape of the PW. A sharp ZPL is observable only at cryogenic temperatures because of the spectral broadening at higher temperatures due to rapid energetical modulation by the environment. In the initial SMS report on PSI by Jelezko et al., a sharp ZPL was observed for both the fluorescence emission and the excitation spectra [58].

However, it was reported that the fluorescence bands of a single PSI in the 710-730 nm region often lacked a visible sharp ZPL [62]. This was because of a phenomenon called spectral diffusion, which is rapid modulations of the site energy caused by the conformational fluctuation around the emitting pigment. Spectral diffusion reflects the jumps between the conformational substates, which are the states of a protein with slightly different conformations and divided by potential energy barriers with various heights. It is surprising that conformational changes are still possible at 2 K. Brecht et al. revealed that the spectral diffusion of a single PSI emission can be slowed by using heavy water as a solvent [62]. This clearly demonstrated that the fine motions of protons are mainly responsible for the observed spectral diffusion. The extreme sharpness of the ZPL makes it highly sensitive to the slight proton motions. The observations of spectral diffusion even at cryogenic temperatures suggested that the site energies of antenna pigments are inherently subject to the conformational motions of the surrounding protein residues. Thus, the protein dynamics at physiological temperatures will have an even larger impact on the light-harvesting dynamics.

The slowed spectral diffusion resulting from the replacement of water with a deuterated water also enabled accurate determinations of the parameter called Huang–Rhys factor S using the formula where I_{ZPL} and I_{pw} are the integrated

$$e^{-S} = \frac{I_{ZPL}}{I_{ZPL}+I_{PW}} \quad (1)$$

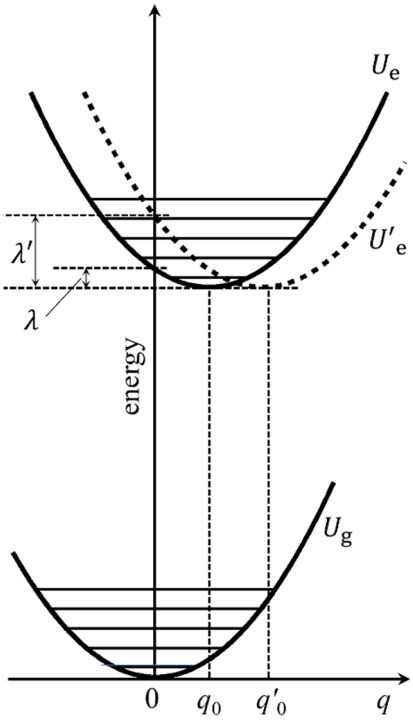

Figure 2 Schematic diagram of the adiabatic potential-energy surfaces of the electronic ground state (U_g) and excited states (U_e and U'_e) of a pigment molecule embedded in a protein. The horizontal axis indicates a normal-mode coordinate q of the protein, and the horizontal lines indicate the vibrational quantum states of q. U_e and U'_e are the excited-state adiabatic potential with the Huang–Rhys factors S=0.9 and 3.6, respectively. In the latter case, the stable configuration of the protein is more distorted (q'_0) than the former (q_0) upon photo-excitation of the pigment. λ and λ' are the reorganization energies for S=0.9 and 3.6, respectively. S follows the relation $S = \lambda/\hbar\omega$, where $\hbar$ and ω are the reduced Planck constant and the angular frequency of the vibrational mode, respectively. Thus, S is a rough measure of the average vibrational quanta emitted during the electronic transition. At cryogenic temperatures, the fluorescence-emitting transition starts from the bottom of the excited-state adiabatic potential surface. Therefore, when the curvature of the potential surface can be assumed to be unchanged during the transition, the reorganization energy is the same between the absorption and emission processes.

intensities of ZPL and PW, respectively. It should be noted that the above formula is valid only under the low-temperature limit. S indicates the average number of phonons emitted during the electronic transition and reflects how the electronic transition deforms the surrounding medium (Fig. 2). S increases when the deformation is greater; in other words, the electronic transition has a strong coupling with the surrounding medium. Brecht et al. estimated an S value of 2.9 for a red Chl emission band at around 710 nm [62]. This value is very large compared to other bands at shorter wavelengths, indicating strong coupling of the red Chl to the surrounding protein matrix. One explanation for the large S value is the mixing of the charge-transfer (CT) nature with the excited state. The CT mixing results in a large permanent electric dipole moment, which causes significant deformation in the surroundings after the transition. This interpretation is in line with the earlier observations of the large Stark effect of the red-Chl emission bands [63,64]. In measurements of the Stark effect, spectral variations caused by an induced static electric field are observed. A large spectral variation indicates a large difference in the permanent dipole moment between the ground and excited states of the pigment molecule. The measurements of the stark effect also implied the mixing of the CT state.

Although the initial SMS study on PSI was done over a wide temperature range [58], the following studies have been performed mainly at cryogenic temperatures below 4 K. Recently, Jana et al. reported the spectral diffusion and blinking of PSI in the higher temperature range from 80 K to 120 K [8,9], where one expects activations of larger conformational fluctuations frozen out at cryogenic temperatures. The study revealed that the dynamical changes in the spectral properties of a single PSI are mainly observed as the blinking, and rarely as the spectral diffusion. The scarce observation of spectral diffusion at higher temperatures is due to the drastic broadening of the ZPL. The rarely observed spectral diffusions seemed to be interpretable as blinking: the fluorescence spectrum of the molecule was contributed by multiple pigments with different emission wavelengths, and the blinking of one pigment resulted in the apparent spectral diffusion.

The blinking of a single PSI has been explained based on the fluctuating energy-transfer pathway [4,8,9]. Brecht et al. observed time courses of fluorescence spectra of single PSIs at 1.4 K and found anticorrelation of fluorescence intensities from multiple emitters within one PSI complex. This anticorrelation is direct evidence of the fluctuating energy-transfer pathway resulting in the temporally changing energy flow to different emitters. The blinking observed by Jana et al. at 80–120 K can be also explained by the changing energy-transfer pathway: during the light period, the excitation energy flows more to the emitting pigment, whereas during the dark period, it flows more to $P700^+$ and is quenched. It should be noted here that P700 in the observed complex experiences repeated photo-oxidizations by the excitation light and charge recombinations from one of the electron carriers. Due to the high excitation light in the SMS measurement, P700 remains in its oxidized form most of the time, even if the addition of a reducing agent makes P700 neutral in the dark. The larger conformational changes allowed at $T \geq 80$ K cause the change in the energy-transfer pathway, which switches the energy flow into the emitting Chl to an energy flow into $P700^+$.

Interestingly, the blinking of the single PSI was found to be enhanced when P700 was pre-oxidized by the addition of oxidizing agents. As described above, P700 is in its oxidized form during the SMS measurement even when a reducing agent is added. However, the redox state of the secondary acceptor, phylloquinone, during the measurement changes if a P700-pre-oxidizing agent is added. When P700 is pre-reduced, $P700^{+}$-A_1^{-} is effectively formed by the repeated charge-separations caused by the laser excitations. On the other hand, when P700 is pre-oxidized, the state of $P700^{+}$-A_1 remains during the laser excitations. The enhanced blinking due to P700 pre-oxidation suggested that the neutral form of phylloquinone is more effective to cause the fluctuation of the excitation-energy pathway. Thus, the observation suggested that the energy-transfer pathway is affected by the change in the redox state of phylloquinone. Indeed, phylloquinone is placed near the Chl-*a* molecule, which has been considered to mediate the excitation energy from other antenna pigments to the central electron-transfer active pigments [43,65] (see Fig. 1).

SMS studies on PSI have also contributed to the deduction of which Chl-*a* on the known structure is responsible for the red Chl. The spectral positions of the red Chls of PSI depend on the species from which the complex is isolated. The red Chls of PSIs have been studied most intensively for cyanobacterial complexes. Those of *T. elongatus* PSI are denoted C708, C715, and C719 according to their absorption peaks at cryogenic temperatures [66,67]. *Synechocystis* sp. PCC6803 is another non-thermophilic cyanobacterium often used for photosynthesis study because of its ease of gene manipulation. PSI from *Synechocystis* sp. PCC6803 has the red Chls denoted as C706 and C714 [63]. Their emission peaks are red-shifted from the wavelengths in their names due to the Stokes shift. The Stokes-shift values of these red Chl bands were estimated to be 200–300 cm^{-1} [64]; these large Stokes shifts again indicate strong coupling of the transition to the surrounding medium. Skandary et al. estimated the relative directions of the transition dipole moments of the emitting red Chls of the monomerized PSI from *T. elongatus* by observing the dependence of the fluorescence signal on the direction of the polarizer [68]. They used a combination of a rotating $\lambda/2$ plate and a polarizing beam separator. By detecting both of the separated fluorescence beams, they could detect the polarizer-angle dependence of the fluorescence without losing the signal of the crossed polarization. They concluded that the transition dipole moments of C708 and C719 lie almost at right angles to each other. From this observation, together with the assumption that the red Chls are dimeric or trimeric Chls, they deduced that Chl pairs B18–B19, B31–B32, and B32–B33 (see Fig. 1) are the most likely candidates for C708 and C719. These Chl pairs are located at a distance of ca. 4–6 nm from P700 and can be efficient energy donors to $P700^{+}$ through the Förster mechanism.

Photosystem II

The isolated PSII reaction center (RC) is composed of the D1–D2 heterodimer and cytochrome b_{559}. It contains six Chl-*a*, two pheophytin-*a*, and two β-carotene molecules, which are arranged in pseudo-C_2-symmetric configuration [69-71]. Accordingly, there are apparently two equal electron-transfer paths starting from the central special pair (P680). However, only one path is active in PSII, probably because the primary charge separation takes place at the accessory Chl bound to the D1 (Chl_{accD1}) protein instead of P680. The site energy of Chl_{accD1} was indeed estimated to be lower than that of the other pigments, so it accumulates the excitation energy [72]. The complex composed of the PSII RC, antenna complexes CP43 and CP47, several other subunits stabilizing the OEC, and so on, is called the PSII core complex (PSII CC). It contains 35 Chl-*a*, two pheophytin-*a*, and ca. 10 β-carotene molecules. The PSII CC is biochemically separable into the PSII RC, CP43, and CP47. This is in sharp contrast to the situation of PSI, where the ca. 100 Chl-*a* molecules are mainly included in the psaA–psaB dimer, whose biochemical separation with intact forms has never succeeded. CP43 and CP47 contain 13 and 16 Chl-*a* molecules as antenna pigments, respectively. In the thylakoid membrane, the PSII CC forms a dimer.

The PSII CC structure is almost conserved for all oxygenic photosynthesis organisms, including cyanobacteria, algae, and higher plants. On the other hand, the peripheral antenna complexes optionally attached to the PSII CC show wide variability. Cyanobacteria have phycobilisomes that are not embedded in the thylakoid but are attached to the stromal side of PSII and sometimes PSI [73]. Green algae and higher plants have a light-harvesting complex II (LHCII) that forms a trimer and surrounds the PSII CC. The PSII peripheral antenna systems of these organisms also contain other minor light-harvesting components, CP24, CP26, and CP29, the composition of which depends on the species. The trimeric LHCII has been considered to play sophisticated roles in the regulation mechanism of light harvesting. State transition is a mechanism to maintain the excitation balance between PSI and PSII under illumination of light with a fluctuating spectrum [74]. The shuttling of LHCII between the two PSs is considered to be responsible for the state transitions. NPQ has also been considered to take place in LHCII. However, a recent intensive study using a set of mutant plants revealed substantial contributions of the PSII CC to NPQ [75]. NPQ operating in the PSII CC was also proposed by Heber et al. [76,77].

Thanks to the availability of isolated pigment-protein complexes, estimations of the site energies of Chls in the PSII CC have been conducted in more detail than those of PSI. However, only partial consensus about the site energies has been obtained. PSII, like PSI, has red Chls with site energies lower than that of the primary donor. The red Chls in PSII are responsible for the fluorescence at low temperature [78,79]. Although researchers have agreed that the reddest Chl belongs

to CP47, they have not reached consensus on which Chl-*a* in CP47 corresponds to the reddest pigment [13,80-86]. Raszewski and Renger proposed that B16 has the reddest site energy, based mainly on the results of linear dichroism (LD) spectroscopy [80]. In the present article, the numbering of Chl follows that proposed recently by Müh and Zouni [87]. Recently, several other assignments of the reddest Chl have been proposed: Hall et al. proposed B1 based on the circularly polarized luminescence, and Reinot et al. proposed B11 and B13 as additional potential candidates [84]. While the above proposals were based mainly on the fitting of spectroscopic data, Sirohiwal et al. recently proposed, based on the powerful QM/MM technique, that B3 is the most probable candidate [13]. Their calculation also suggested that B16 is not likely to be the reddest Chl. This kind of extensive computational work will be needed to reach final agreement.

SMS Studies of Photosystem II

The Brecht group also pioneered SMS studies for PSII [88,89]. As for PSI, they determined the intensity ratio of $I_{ZPL}/(I_{ZPL}+I_{PW})$ to estimate the Huang–Rhys factor S of the emitting pigment. They used a similar algorism to correct the spectral diffusion to that used for the analysis of the single PSI spectra. The estimated S values were distributed over a wide range from 0.03 to 0.8, suggesting wide inhomogeneity in the environment of the emitting pigment [89]. On the other hand, the S values estimated for the PSII CC were much smaller than those for PSI and never exceeded 1.0 for the ZPL located at up to 700 nm. This suggested that the red Chl responsible for the reddest emission band of the PSII CC (called F695) has neither very strong coupling to the surroundings nor mixing of the CT state.

The summed fluorescence spectrum over all of the single PSII CC was significantly different from the ensemble spectrum observed by a conventional instrument [86,88]. The summed single PSII CC spectrum showed a profile with a drastically reduced long-wavelength side as compared with the ensemble spectrum. The authors explained the discrepancy by assuming the accumulation of the triplet state of the carotenoid (^{3}Car) in the vicinity of the Chl with the lowest site energy (red-most Chl). The PSII CC binds many β-carotene molecules, which mainly function as quenchers of the Chl triplet state (^{3}Chl). The red-most Chl is most frequently converted to the triplet state due to its highest probability of excitation. Thus, it is likely that the high excitation power used in the SMS experiment results in accumulation of the ^{3}Car in the vicinity of the red-most Chl. In this situation, the triplet state of the red-most Chl remains for a long time due to the lack of quenching activity by the neighboring β-carotene. Then the emission from the red-most Chl is reduced.

On the other hand, one report [90] argued that damage to the PSII CC sample may be the cause of the deformed spectrum of the SMS study. Indeed, the PSII-CC low-temperature emission band at 695 nm has been reported to be highly sensitive to harsh treatments of the sample, such as the freeze–thaw process [81]. Chen et al. recommended using the PSII CC samples obtained by re-solubilization of the PCII CC crystals. However, Skandary et al. later reported an SMS study using the re-solubilized PSII CC crystal sample and confirmed no difference from the previous study [89]. Thus, the reduced red-most Chl emissions in the single PSII CC spectra are most probably due to the accumulation of the triplet state of the neighboring β-carotene molecule.

Although the PSII CC usually forms a dimer, as shown in Figure 1, a monomeric PSII CC sample is available. Comparison of the SMS data for the monomeric PSII CC with those of the dimeric one provided a hint to the quest for which Chl is the red-most Chl in the PSII CC [86]. Skandary et al. showed, by using re-solubilized crystal samples, that the summed spectrum over single monomeric PSII CCs has a further reduced 695 nm intensity than that of the single dimeric PSII CC. They found that one specific β-carotene, designated as BCR528 (numbering according to Guskov et al.; see Fig. 1), is missing from the crystal structure of the monomeric PSII CC [91]. Then they assumed that the reduced 695 nm fluorescence in the monomeric PSII CC came from the loss of the β-carotene. Accordingly, they proposed Chl B7, which is located near the missing Car, to be the most likely candidate for the red-most Chl. However, this new proposal was in addition to the previous ones, thus adding one more to the list.

A relatively high fluorescence quantum yield of PSII at room temperature as compared with PSI makes the detection of single PSII fluorescence at room temperature feasible. Gruber et al. reported the SMS study of the PSII supercomplex purified from *Arabidopsis thaliana* containing peripheral antennae at room temperature [5]. They obtained the fluorescence-decay kinetics of single particles by using a femtosecond pulse laser for the excitation and a time-correlated single-photon counting setup for the fluorescence detection. Two types of supercomplexes, called $C_2S_2M_2$ and C_2S_2, adsorbed on a poly-L-lysine-coated glass surface were observed. The former is composed of the dimeric PSII CC (C_2), two strongly bound LHCII trimers (S trimer), and two mildly bound LHCII trimers (M trimer), and the latter unbinds the M trimers. The C_2S_2 supercomplexes excited by the fs pulse at 633 nm showed a rapid fluorescence decay with a time constant fluctuating in the range of 100–200 ps. The correlated fluctuations of the fluorescence lifetime and intensity are typical observations for SMS study. The signal from the contaminating free M-trimer LHCIIs could be eliminated based on their significantly slower fluorescence decay.

Interestingly, Gruber et al. found that the fluorescence-decay dynamics of the single C_2S_2 supercomplexes contain a much slower component with a lifetime of ca. 3.5 ns just after the start of the excitation. This finding was obtained by using an acousto-optic modulator as a rapid shutter operating on a millisecond time scale. The fluorescence-decay profile acquired within a few hundred μs after the shutter opened was expressed by the sum of 150-ps decaying and 3.5-ns

decaying components. The former component was attributed to the quenching by the Car triplet state that was inferred to be rapidly accumulated within the μs timescale. The 3.5-ns decaying component disappeared within 0.5 ms after the shutter opened and was assigned to a quenching of the singlet excited state of Chls. The authors proposed a model assuming the quenching caused by the random blinking of LHCs. In the model, the rate of switching from the emitting to the dark states was assumed to be enhanced by the laser excitation, and then the unquenched 3.5-ns lifetime component disappeared quickly within 0.5 ms after the start of the laser excitation. The correlated fluctuation in the fluorescence lifetime and intensity can be also explained by the random switches between the emitting and dark states of the bound LHC. Although the model was consistent with the previous observation that the blinking of isolated LHCII depends on the excitation power [92], it was not strictly proven experimentally. Nevertheless, the model is attractive, since it can properly explain an aspect of the photoprotective NPQ mechanism in PSII.

Antenna Complexes

Light-Harvesting Chlorophyll Complex

A photosystem requires light energy for photoelectric conversion, so it creates its own light-harvesting system as described above. Nevertheless, the photon density of sunlight is so low that additional light-harvesting antenna proteins are needed to expand the absorption cross section and concentrate solar energy to the RC. The structures and bound pigments of antenna systems are highly diverse in order to carry out efficient and stable photosynthesis under various light environments. In oxygenic phototrophs, light-harvesting chlorophyll complexes (LHCs, see Fig. 1C, D) assist the PSII and PSI RCs to gather light energy by surrounding them with a thylakoid membrane. LHCII and LHCI are the representative LHCs directly bound to the core parts of PSII and PSI, respectively. Meanwhile, under high light, LHCs dissipate excess light energy that causes photodamage to the RC. The NPQ of LHCs is a significant function for the photoprotection of photosynthetic organisms. While the molecular mechanism of the NPQ remains ambiguous, recent SMS studies of LHCs have revealed that the quenching property is strongly associated with protein conformational dynamics.

The major LHC of green plants, LHCII, is a trimeric transmembrane protein, each monomer of which contains eight Chls-*a*, six Chls-*b*, and four Cars. The Chls-*a* that strongly interact with each other are clustered into three groups with lower energy levels than a monomeric Chl-*a*. The clusters of Chls-*a* are emissive sites observed by the fluorescence measurement [93-95]. The Car located near the Chl-*a* is known to serve as the energy quencher, i.e., to draw excitation energy from Chl-*a* and then dissipate it as heat. Although the photophysical mechanism of the quenching is still under debate, the efficiency should depend on the mutual configuration between Car and Chl-*a* and hence can be perturbed by local conformational changes [96]. The light energy absorbed by pigments in the LHC is eventually radiated from Chls-*a* as fluorescence. When the relative arrangement of Chl-*a* and Car varies to enhance the quenching, the fluorescence intensity and lifetime are both reduced. Furthermore, the fluorescence spectral peak is shifted in response to local conformational and environmental changes in the emissive site. Therefore, by analyzing variations in the fluorescence properties and constructing their histograms, we can visualize a distribution of the local conformational substate. Moreover, the temporal changes in the fluorescence properties, i.e., fluorescence fluctuations, also give information on the dynamics between conformational substates.

The local conformational dynamics reflected in the fluorescence fluctuations is averaged out in ensemble measurements. To perform the ensemble-averaging free measurements, SMS has been applied to LHCs [97]. Individual LHCs exhibited fluorescence fluctuations between higher and lower intensities. A histogram of the intensity displayed two distribution peaks corresponding to the fluorescent and quenching states of the LHC, respectively [98]. From a 2D distribution constructed from fluctuations in two different fluorescence parameters, such as the intensity, lifetime, spectral peak, and so on, one can resolve multiple conformational substates that are obscured by overlapping in the 1D distribution along one parameter. The 2D distribution analysis of the fluorescence intensity and spectral peak of LHCII revealed two discrete quenching states, one of which showed a red-shifted peak suggesting a conformational change in the emissive site [99,100]. In addition to these approaches, various analytical methods have been applied to derive fruitful knowledge from the fluorescence fluctuation [97]. In particular, this review introduces the 2D-distribution analysis, recently applied to LHCs, with respect to the fluorescence intensity and lifetime.

Quenching Dynamics Revealed by Fluorescence Intensity-Lifetime 2D-Distribution Analysis

The first step of the distribution analysis is to identify conformational substates according to the differences in fluorescence intensity and lifetime. In this approach, both the arrival times and the delays from the excitation pulse of all detected fluorescence photons are recorded and analyzed. The intensity-based identification is performed via change-point analysis based on a generalized likelihood-ratio test, where a substate is detected as a state exhibiting a constant fluorescence intensity [101]. Then the fluorescence lifetime is estimated for each substate to construct a 2D histogram along the intensity and lifetime [102]. In the 2D distribution graph, the fluorescent and quenching states of the LHC are

clearly separated as a population with a higher intensity and longer lifetime and that with a lower intensity and shorter lifetime, respectively [6,103]. Furthermore, the detected transitions between substates were statistically analyzed. By creating the 2D histogram of changes in intensity and lifetime due to the transitions, the transition probability between substates was visualized. It corresponds to a density map of the conformational dynamics. The analysis was conducted on an LHC of green algae and mosses called LHCSR1, which is known to play a role in photoprotective quenching in these organisms. The analysis revealed how frequently the switching between fluorescent and quenching states occurs in this antenna complex [6].

The intensity-based 2D distribution analysis described above is useful to visually understand the conformational heterogeneity and dynamics. On the other hand, it presents difficulties in (i) the quantitative estimation of the rate of substate transitions; (ii) the identification of fast dynamics occurring within the bin time, which is the time interval for counting the fluorescence photons to estimate intensity; and (iii) resolving multiple distinct dynamics likely existing in a chromoprotein with multiple pigments, such as LHCs. In order to overcome these difficulties, the SMS data of LHCs was recently subjected to 2D fluorescence-lifetime correlation (2D-FLC) analysis, where the conformational substate was identified based on the lifetime, and then the inter-substate transition was quantitatively evaluated through correlation analysis [104,105]. Because this technique is based on photon-by-photon analysis, the time resolution is given by the detection frequency of fluorescence photons, not by bin time, allowing for the identification of fast dynamics taking place on a microsecond time scale [106].

Using a suitable model function in the correlation analysis, it is also possible to distinguish multiple dynamic components in an individual LHC and to evaluate the number of components as well as the relative fluorescence efficiency and the transition rate between the fluorescent and quenching states [7]. The results from the 2D-FLC analysis are summarized in a 2D diagram of the fluorescence intensity and lifetime. Furthermore, it is possible to calculate the free-energy difference between the detected substates by assuming the thermal equilibrium. Thus, the lifetime-based 2D-FLC analysis has significant advantages in providing quantitative estimations of dynamical parameters with improved time resolution, enabling the identification of multiple conformational dynamics in the LHC. Recently, this technique was applied to clarify the conformational dynamics of LHCSR1 [7] and LHCSR3 [107], both of which are known to be relevant to the NPQ in green algae [54].

The 2D-FLC analysis of the LHC revealed two dynamic components likely associated with two luteins (xanthophyll carotenoid) located in the central part of the LHC [7]. One of the two dynamics is biased toward the fluorescent state, and the other is inversely biased toward the quenching state with a free-energy difference of ~200 cm^{-1}. These quantitative experimental results will facilitate comparative verification with theoretical and computational results, e.g., using molecular dynamics simulation [108,109], and encourage further studies using site-directed mutation analysis [107,110]. In this manner, the recent progress with fluorescence intensity–lifetime 2D distribution analysis provides an opportunity to discuss not only whether the protein conformation fluctuates but also where the local conformational dynamics occur and how they are regulated.

NPQ in LHCs Observed Through Local Conformational Dynamics

The NPQ in LHCs is activated under high light by two factors, the pH drop in the lumen of the thylakoid membrane and the Car conversion from violaxanthin into zeaxanthin [22,111]. The pH drop is induced by excess photoreaction coupled with the oxygen evolution reaction in PSII and the proton pumping into the lumen. The acidification on the luminal side is suggested to cause the protonation of pH-sensing residues at the C-terminus of the LHC, leading to a conformational change in the protein scaffold [110,112]. The fluorescence intensity–lifetime 2D distribution analysis showed that the LHC is stabilized in the quenching state upon the drop in pH [6,103]. The stabilization was quantitatively evaluated as changes in the free energy [7]. The mutant lacking the pH-sensing residues exhibited no change in free energy with the pH drop [107]. These results clearly indicate that the pH-dependent deformation of the protein scaffold regulates the local conformational flexibility to stabilize the quenching state and enhance the NPQ efficiency. The other NPQ trigger, Car conversion, also caused stabilization of the quenching state and enhancement of the energy dissipation [6,7,103,107], consistent with the results from the intensity-based distribution analysis [113,114]. Thus, the fluorescence-fluctuation analysis using SMS makes it possible to disentangle multiple quenching sites in an individual LHC and quantitatively analyze them, enabling detailed discussion of the NPQ mechanism associated with the pH drop and Car conversion.

Conclusion

Here, we provided an overview of how the hidden dynamics of LHCs can be observed using the single-molecule detection technique. Cryogenic optical microscope techniques offer the ability to detect the fluorescence signal from single photosystem complexes that emit very weak fluorescence at room temperature due to the efficient photochemistry. The studies reviewed here clarified that conformational dynamics are active even at cryogenic temperatures. The time-resolved

detection mode has provided new insight on the conformational dynamics of LHCs. Since the information on fluorescence-decay kinetics is directly related to the energy-transfer and quenching dynamics, the time-resolved detection technique will become more and more important. Development of statistical analysis methods will be more important to extract the information of interest. On the other hand, the observation mode of the excitation spectra has not been frequently chosen so far. In the near future, studies will be targeted at larger and more intact complexes such as photosystem–LHC supercomplexes. Since excitation spectral detection allows us to distinguish Chl-*b* from Chl-*a*, the SMS studies with excitation spectral detection will be one way to examine these supercomplexes with increased complexity. The combination of several detection modes will be also more important. The development of a powerful quantum mechanical method is desired to obtain more accurate information on the excited-state properties of pigments clustering in LHCs.

Conflict of Interest

The authors have no conflict of interest to declare.

Author Contributions

T. K. and Y. S. reviewed the recent SMS studies on oxygenic photosynthesis complexes. T. K. and Y. S. mainly wrote the antenna part and the photosystem part of the manuscript, respectively.

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