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REVIEW

Mobility of photosynthetic proteins

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Abstract The mobility of photosynthetic proteins represents an important factor that affects light-energy conversion in photosynthesis. The specific feature of photosynthetic proteins mobility can be currently measured in vivo using advanced microscopic methods, such as fluorescence recovery after photobleaching which allows the direct observation of photosynthetic proteins mobility on a single cell level. The heterogeneous organization of thylakoid membrane proteins results in heterogeneity in protein mobility. The thylakoid membrane contains both, protein-crowded compartments with immobile proteins and fluid areas (less crowded by proteins), allowing restricted diffusion of proteins. This heterogeneity represents an optimal balance as protein crowding is necessary for efficient light-energy conversion, and protein mobility plays an important role in the regulation of photosynthesis. The mobility is required for an optimal light-harvesting process (e.g., during state transitions), and also for transport of proteins during their synthesis or repair. Protein crowding is then a key limiting factor of thylakoid membrane protein mobility; the less thylakoid membranes are crowded by proteins, the higher protein mobility is observed. Mobility of photosynthetic proteins outside the thylakoid membrane (lumen and stroma/cytosol) is less understood. Cyanobacterial phycobilisomes attached to the stromal side of the thylakoid can move relatively fast. Therefore, it seems that stroma with their active enzymes of the Calvin–Benson cycle, are a more fluid compartment in comparison to the rather rigid thylakoid lumen. In conclusion, photosynthetic protein diffusion is generally slower in comparison to similarly sized proteins from other eukaryotic membranes or organelles. Mobility of photosynthetic proteins resembles restricted protein diffusion in bacteria, and has been rationalized by high protein crowding similar to that of thylakoids.

Keywords Photosynthesis · Protein mobility · FRAP · Photoprotection · Thylakoid membrane · Confocal microscopy

Abbreviations

BODIPY	4,4-Difluoro-4-bora-3a,4a-diaza-*s*-indacene
D	Diffusion coefficient
FLIP	Fluorescence loss in photobleaching
FRAP	Fluorescence recovery after photobleaching
GFP	Green fluorescence protein
LHCII	Light-harvesting antennae of photosystem II
NPQ	Non-photochemical quenching
PBS	Phycobilisome
PC	Plastocyanin
PSI	Photosystem I
PSII	Photosystem II
IsiA	Iron-stress induced protein A
ROI	Region of interest

Mobility of photosynthetic proteins

The light reactions of photosynthesis are mediated by pigment-binding proteins and their complexes located inside the thylakoid membrane (e.g., chlorophyll-binding proteins of both the photosystems) or on its surface [e.g.,

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phycobilisomes (PBS) in cyanobacteria and red algae]. Recent and fast progress in structural biology has provided high-resolution structural models of most photosynthetic protein complexes [see (Fromme 2008) for book of reviews] as well as their large-scale organization into supercomplexes (Dekker and Boekema 2005; Folea et al. 2008). Based on abundant but mostly indirect evidence for protein mobility, organization of proteins in the thylakoid membrane is not considered to be a static, "solid-state structure", but a very flexible and dynamic compartment. Mobility of proteins seems to be essential for photosynthetic function, it plays a role in de-novo protein biosynthesis and their turnover after stress [e.g., during the PSII repair process after stress, reviewed in (Nixon et al. 2010)]. It also regulates light-harvesting efficiency in a process of state transitions (Kirilovsky et al. 2013) and electron transport reactions through plastocyanin (PC) diffusion between cytochrome b6f complex and photosystem I [reviewed in (Kramer et al. 2004)]. However, our knowledge about photosynthetic protein mobility in these processes is still rather fragmented, and only a few direct measurements of photosynthetic protein diffusion have been performed so far (see Table 1). Therefore, further research including new experimental approaches is necessary to understand this process together with its controlling factors [see recent review for (Mullineaux 2008a) for details]. This review will focus mostly on the mobility of photosynthetic proteins important for primary photosynthetic reactions (photosystems, light-harvesting antennae, and PBS), it will describe experimental approaches and how to measure protein mobility with the main focus on fluorescence recovery after photobleaching (FRAP) including the possible pitfalls during measurements.

Experimental methods to study protein mobility

Photosynthesis research is based on multidisciplinary experimental approaches that require the application of many noninvasive techniques for monitoring primary and secondary photosynthetic reactions in living cells (Thijs and Matysik 2008). There are various fluorescence methods suitable for kinetics measurements of light-driven electron transport processes and for measurements of light-harvesting efficiency in the thylakoid (Papageorgiou and Govindjee 2004), the processes of which are dependent on the mobility of the thylakoid membrane proteins (see "Protein mobility on the thylakoid membrane surface—phycobilisome mobility"). These experiments are mostly carried out in a cuvette (on a cell suspension), thus they reflect only an average behavior of whole bulk of photosynthetic proteins in thylakoid membranes. This experimental approach does not allow for direct measurements of particular protein mobility, which is an entirely microscopic phenomenon. However, currently, there is

Table 1 Diffusion coefficients of particular proteins from different membranes and cell compartments including thylakoid membrane

Diffusion rates of protein (lipid) in various compartments	D (μm^2 s^{-1})	Ref.
Cell compartments		
Water (GFP)	87	[1]
Plant cytoplasm (GFP)	40	[2]
Animal cytoplasm (GFP)	25	[1]
Mitochondrial matrix (GFP)	20–30	[3]
Prokaryotic cytoplasm (GFP)	6–9	[4]
Prokaryotic periplasm (GFP)	2.6	[4]
Chloroplast stroma (GFP)	0.9	[2]
Cyanobacteria cytoplasm (based on PBS)	0.03	[5]
Thylakoid lumen (Phycoerythrin)	~0	[6]
Eukaryotic membranes		
Plasma membrane (protein)	0.25–0.75	[7]
Endoplasmatic reticulum (protein)	0.26–0.49	[8]
Lipids	1–4	[9]
Mitochondria membranes		
Cytochrome bc_1	0.1–1	[10]
F_1F_0-ATPase	0.1	[10]
Cytochrome *c*	0.016	[11]
Photosynthetic proteins of thylakoid membranes		
Cytochrome b_6f	Unknown values	
F_1F_0-ATPase		
Plastocyanin		
Photosystem 1		
Phycobilisomes core	0.07	[5]
Phycobilisomes	0.004–0.03	[5, 12, 13]
Phycoerythrins	~0	[6]
LHCIIp—in stroma	0.03	[14]
LHCII in stroma	0.003	[14]
LHCII in grana	0.005	[15]
IsiA—cyanobacteria	0.003	[16]
Photosystem II—red light	0.023	[17]
Photosystem II—dark	~0.00002	[16, 12, 18]
Lipids	0.06–1	[19]

Values were taken from literature according the following list: [1] (Swaminathan et al. 1997);[2] (Kohler et al. 2000);[3] (Partikian et al. 1998);[4] (Mullineaux et al. 2006);[5] (Sarcina et al. 2001);[6] (Kaňa et al. 2009b);[7] (Frick et al. 2007);[8] (Nehls et al. 2000);[9] (Fulbright et al. 1997);[10] (Gupte et al. 1991);[11] (Hochman et al. 1982); [12] (Mullineaux et al. 1997);[13] (Kaňa et al. unpublished);[14] (Consoli et al. 2005);[15] (Kirchhoff et al. 2008);[16] (Sarcina and Mullineaux 2004);[17] (Sarcina et al. 2006);[18] (Kirchhoff et al. 2004b);[19] (Sarcina et al. 2003)

whole range of various fluorescence microscopic methods, such as FRAP, fluorescence correlation spectroscopy (FCS), or fluorescence resonance energy transfer (FRET), which are all suitable for monitoring protein–protein interaction and protein mobility on a single cell level (Joo et al. 2008; Lippincott-Schwartz et al. 2001). Since the advent of the green fluorescence protein (GFP) and progress in molecular biology methods, mobility of GFP-tagged proteins can be measured during various cellular processes in vivo. The first application of microscopic methods for the detection of photosynthetic protein mobility came with the pioneering work of Conrad Mullineaux that made use of the autofluorescence of photosynthetic proteins for FRAP measurements (Mullineaux et al. 1997). It was in the work of Conrad Mullineaux and coworkers that the strict difference between immobile chlorophyll-binding proteins in the membrane and mobile PBS on the membrane surface was pointed out (for details see "Lateral protein diffusion inside the thylakoid membrane and Protein mobility on the thylakoid membrane surface—phycobilisome mobility" sections).

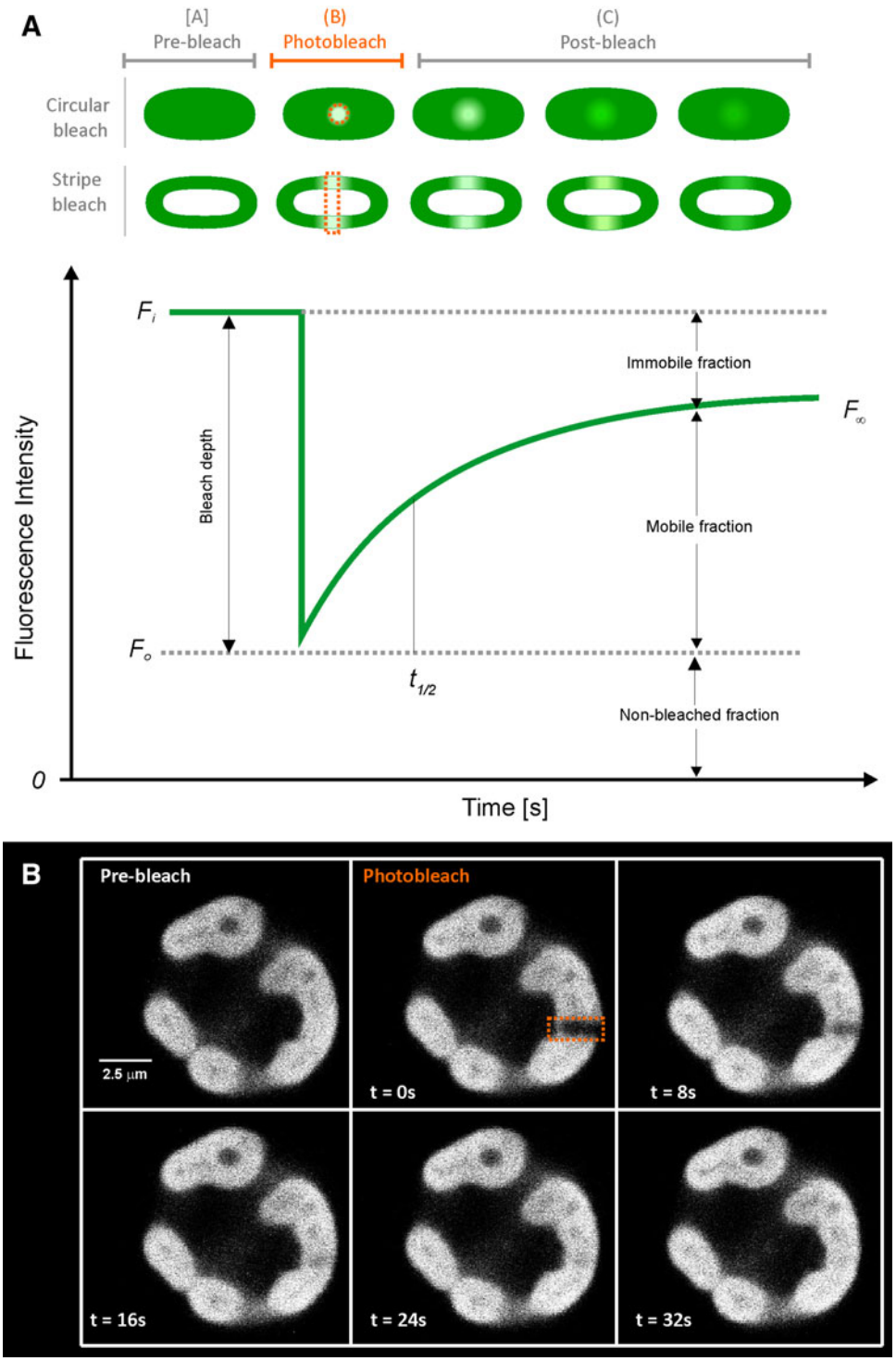
FRAP—fluorescence recovery after photobleaching

FRAP method represents a classical technique for the detection protein mobility in biological systems (Axelrod et al. 1976; Edidin et al. 1976). FRAP together with FCS and single particle tracking allows for the experimental estimation of diffusion coefficients (Lippincott-Schwartz et al. 2001; Reits and Neefjes 2001). The FRAP protocol is composed of three basic phases (see Fig. 1): (1) Pre-bleach phase—fluorescence of cell is simply monitored by low-intensity laser power, a small region of interest (ROI) has an initial value of fluorescence (F_i); (2) Photobleaching phase—a high-powered laser beam is applied to the ROI for a short period and fluorescence in the ROI is reduced to its minimal value (F_0); (3) Post-bleach phase is represented by recovery in fluorescence intensity in the ROI, the final value (F_∞) is a raw estimate of mobile protein fraction. For an optimal experimental set-up, fluorescence recovery during the post-bleach period reflects the movement of the surrounding non-bleached proteins into the bleached area and requires irreversible photobleaching of protein chromophores.

There are several potential complications and pitfalls that have to be taken into account during FRAP measurements. One of them is reversible photobleaching or fluorochrome intermittency (blinking) that may cause flawed FRAP results. This effect is especially known for commercially produced fluorescence proteins (Mueller et al. 2012). In these proteins, the bleaching period causes not only irreversible bleaching of chromophore required for FRAP, but a certain fraction of protein chromophores is reversibly photoswitched into a non-fluorescent dark state. Then fluorescence recovery from this dark state after the bleach period is diffusion independent (Mueller et al. 2012; Sinnecker et al. 2005). This process has already been described for several fluorescence proteins including GFP (Dickson et al. 1997; Nifosi et al. 2003; Henderson et al. 2007), and it seems to be present in photosynthetic proteins as well (Zehetmayer et al. 2002). In fact, photosynthetic light-harvesting antennae have very complex fluorescence spectral dynamics (Kruger et al. 2010, 2011). In the case of GFP, the reversible bleaching caused by blinking depends on excitation intensity (Garcia-Parajo et al. 2000). The fluorescence recovery after bleaching used in FRAP is for every laser setup partially affected by dark state of chromophores, its importance is higher for lower laser power used for bleaching (Mueller et al. 2012).

The reversible photobleaching can be highly stimulated at certain experimental setup for photosynthetic proteins (Liu et al. 2009). Therefore, the extent of photoswitching has to be estimated (or excluded) every time for particular experiments including photosynthetic proteins. One of the best methods to do so is the detection of fluorescence loss in photobleaching (FLIP) together with FRAP. During FLIP measurements, fluorescence loss outside the bleach area is measured. The fluorescence decrease reflects protein movement from area outside of bleach into the bleach area (Kaňa et al. 2013a; Yang et al. 2007). Therefore, fluorescence decrease during FLIP confirms the diffusional origin of fluorescence changes during FRAP and excludes the effect of photoswitching. Alternatively, the effect of photoswitching can be estimated using a special experimental approach (Mueller et al. 2012) or with the application of inhibitors that stop protein movement. It includes glycin betaine (it stops phycobilisome movement) or glutaraldehyde, an effective protein crosslinker (Habeeb and Hiramoto 1968). Another test can be done using bleaching of the entire fluorescent part of the cell; in this case, presence or absence of fluorescence recovery after bleaching is a proof of the importance or unimportance of reversible fluorescence chromophore photoswitching during FRAP routines [see example of application in (Goral et al. 2010)]. However, applicability of this control is limited only to smaller cells where conical-like 3D bleach volume is able to irreversibly bleach all chromophores in the cell including those above and below the focal plane. Photoswitching dominates for low-power laser bleaching (Mueller et al. 2012); therefore, its role in FRAP measurements done with photosynthetic proteins using 1-s long bleach of high power is rather minimal.

A higher laser power during bleaching period eliminates the effect of reversible chromophore photoswitching, but on the other hand, it increases the possibility of some other FRAP's artifacts such as photo-induced proteins cross-



linking and membrane photodamage. Therefore, it is important to check the dependency of the recovery rate on different bleaching intensities and repeat FRAP on the same spot twice. It provides an important control that can exclude presence of protein photo-damage that could cause immobile fiction in FRAP recovery (see Fig. 1). A higher recovery observed for repeating FRAP at the same spot shows the presence of a "real" immobile fraction as immobile fraction has been partly bleached out during the first bleaching period, and thus it is less fluorescent and less contributes to FRAP recovery. On the other hand, in the case of similar recovery for repeating FRAP, the observed protein immobility is the artifact caused by photo-damage. The intactness of lipid membranes during and after

◀**Fig. 1** Schematic figure of typical fluorescence recovery after photobleaching (FRAP) measurements. **a** Shows three phases of experimental routine and kinetic behavior of cell fluorescence in the area of interest (ROI) after application of bleach, circular bleach (see *upper cells*), or stripe bleach (*bottom cells*). *A—Pre-bleach phase* is characterized by initial value of fluorescence (F_i) induced by low laser power. The fluorescence change in this phase reflects acquisition bleaching caused by low laser power used for cell scanning. *B—Bleach phase*—high laser power is applied to ROI (see *red cycle* or *rectangle*) to bleach protein chromophores. Intensity of fluorescence in ROI is characterized by minimal fluorescence (F_0) that reflects depth of bleach. *C—Post-bleach phase*—low laser power is used for cell scanning, recovery in fluorescence in ROI reflects protein diffusion into bleached area. Steady state value of fluorescence (F_∞) reflects mobile fraction of proteins, $t_{1/2}$ is time when fluorescence recovers to its half value. **b** A typical example of FRAP images during phycobilisomes mobility measurements with red algae *Rhodella violacea*. FRAP routine started with measurements of pre-bleach picture (see "Pre-bleach" image) before application of short bleach (2 s long) to *rectangular area* of cell (see "Photobleach" image, $t = 0$). "Post-bleach" images are then characterized by time after the bleaching. Scale-bar is same for all recorded pictures (2.5 μm). Fluorescence was excited to phycobilisomes (630 nm) and detected between 640 and 680 nm

bleaching can be checked by FRAP measurements using the fluorescence lipid dye 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY) [see e.g., (Goral et al. 2010)]. The complete recovery of BODIPY fluorescence during FRAP measurements indicates relatively rapid lipid diffusion within the thylakoid membrane system, and is a control showing intactness of the basic structure of the membrane after photobleaching.

FRAP method is limited by its spatial resolution due to the constraints of confocal microscopy that is about 250 nm for blue light excitation. Therefore, there is a minimal bleach spot size that can be applied making the protein mobility measurements by FRAP more reliable for long-range diffusion processes on scales of about a 1 μm or more. The FRAP protocols for small photosynthetic organisms (with a size between 0.5 and 5 μm) need to be tailored to obtain satisfactory data. Optimal setup includes reduction of the laser bleach size and its intensity to the useful minimum, leaving a significant portion of photosynthetic proteins unbleached to see recovery in fluorescence. However, number of photosynthetic protein dynamics proceeds on a smaller scale [e.g., diffusion of photosynthetic electron carriers (Kirchhoff et al. 2004a)], and therefore, it is undetectable by FRAP.

There are two processes that generally affect fluorescence recovery during FRAP measurements, one is a diffusional exchange of proteins between the bleached and non-bleached area, the other is protein–protein interaction. Therefore, FRAP recovery kinetics can be used for quantitative estimation of both, protein diffusion—so-called "diffusion coupled FRAP recovery" and for protein–protein interaction—so-called "diffusion uncoupled FRAP recovery" (Sprague and McNally 2005). Many authors propose FRAP measurements with photosynthetic proteins as "diffusion coupled FRAP" and use it to calculate diffusion coefficients. Indeed, our measurements with light-harvesting antennae in diatoms have shown independence of FRAP recovery to protein–protein interaction (Kaňa, Krafl, Papáček, Matonoha, *unpublished results*). Even though the prerequisite seems to be valid, it needs to be tested for all possible photosynthetic proteins. Moreover, fully quantitative FRAP requires a known membrane structure throughout an area of at least 1 μm, a parameter that is rarely met in thylakoid membranes. Therefore, diffusion coefficients calculated from FRAP measurements often represent arbitrary value rather than a real rate of diffusion.

Diffusion-coupled FRAP can be used to calculate the arbitrary diffusion rate of autofluorescencing photosynthetic proteins either in the thylakoid membrane (e.g., light-harvesting antennae, see "Lateral protein diffusion inside the thylakoid membrane" section) or on its surface (e.g., PBS, see "Protein mobility on the thylakoid membrane surface—phycobilisome mobility" section). After acquisition bleaching compensation [see e.g., (Phair et al. 2003)], several parameters can be estimated directly from FRAP recovery curves (see Fig. 1). The extent of protein mobile fraction (M) can be determined as:

$$M = (F_\infty - F_0)/(F_i - F_0)$$

where F_i is intensity of fluorescence in the bleached region before bleach, F_0 is intensity of fluorescence in the bleached region just after bleach, and F_∞ represents intensity of fluorescence in the bleached region after final recovery. We can also estimate photobleaching depth.

$$B = F_i - F_0/F_0$$

This is an important experimental parameter for maintaining the measurement reproducibility (Klein and Waharte 2010). The diffusion coefficient can be estimated by fitting the FRAP recovery curves with analytic formulas (Klein and Waharte 2010). The analytic formula has to be selected based on cell geometry and the type of bleach. In fact, geometry of the cell together with bleach size and shape (circular or stripe-like) affect FRAP curves greatly. This dependency is clear from the following equation (Axelrod et al. 1976)

$$D \sim \omega^2/\tau_D$$

showing dependency of D on bleach size ω (represented by radius for circular bleach), and on characteristic diffusion time (τ_D), a time required for diffusion across bleach spot. Therefore, size/shape of the bleached spot, intensity of the bleach, and its duration are parameters that have to be kept constant among different samples to maintain a measurement reproducibility especially if we are not sure about the involvement of protein–protein interactions on FRAP

recovery. In some models, there is a simple relation between τ_D and half-time of recovery ($t_{1/2}$) that allows direct estimation of the diffusion coefficient from $t_{1/2}$ (Klein and Waharte 2010). However, in most other cases, the empirical formula of Ellenberg et al. 1997 has to be used for fitting the FRAP curve made by a stripe-like bleach and the model of Soumpasis 1983 for a circular bleach. For the calculation of phycobilisome diffusion in cyanobacteria by FRAP with a stripe-like bleach (Mullineaux et al. 1997), a simple model has been developed allowing for the calculation of D based on the construction of a one-dimensional bleaching profile [see e.g. (Mullineaux 2004) for details]. These analytic approaches have several limitations as they can only be used for full recovery of certain geometries of bleach with a Gaussian-like profile [see e.g., model of (Mullineaux 2004; Mullineaux et al. 1997)]. Some from these restrictions can overcome using numerical methods, for a direct solution of Fick's equation for protein diffusion (Papáček et al. 2013; Irrechukwu and Levenston 2009; Sukhorukov et al. 2010). However, this approach requires a more robust mathematical apparatus as a numerical solution of Fick's equations represents an ill-posed problem (Papáček et al. 2013; Kaňa et al. 2013b).

Protein displacement through diffusional processes does not only depend on values of diffusion coefficients, but on the type of diffusion as well (Wang et al. 2009, 2012). In a real system, such as cell membrane or viscous cytosol, the diffusional process represents a combination of Brownian diffusion with anomalous diffusion (Dix and Verkman 2008). Anomalous diffusion is caused not only by protein crowding typical for thylakoids (Kirchhoff 2008a, b), but it is also affected by lipid–protein and protein–protein interactions. Anomalous diffusion is characterized by the fact that the mean square displacement (MSD), a real measure of change in protein position caused by diffusional process, does not follow the equation typical for normal, Brownian diffusion:

$$\mathrm{MSD} = \langle x \rangle = (4Dt)^{1/2}$$

where D is a two dimensional diffusion coefficient (in $\mu m^2\ s^{-1}$), and $\langle x \rangle$ is average displacement of particular protein (in μm) in time t (in seconds). Therefore, average protein displacement during diffusion represented by MSD depends not only on the value of the diffusion coefficient, but also on the surrounding obstacles causing anomalous diffusion (Dix and Verkman 2008).

Lateral protein diffusion inside the thylakoid membrane

It is generally accepted that mobility of transmembrane proteins is slower in comparison to diffusion of free proteins in cytoplasm [see Table 1 and review (Lippincott-Schwartz et al. 2001)]. The photosynthetic thylakoid represents a special type of membrane that restricts diffusion of proteins even more than other typical membranes as it is a densely packed environment with a high protein abundance [w/w ratio of protein/lipid is 86:14 and 61:39 for granal and stromal thylakoids, respectively, (Murphy 1986)]. It makes most of the photosynthetic protein complexes almost immobile, the mobile fraction represents only about 20–40 % of chlorophyll-binding proteins with a diffusion rate between ~ 0.01 and $0.001\ \mu m^2\ s^{-1}$ (Table 1). This means that free diffusion of photosynthetic proteins is more impeded in comparison to proteins from the plasma membrane or membranes in the endoplasmatic reticulum or other eukaryotic membranes in general (see Table 1). It is also slower in comparison to other energy transducing membranes (compare diffusion of cytochrome bc_1 or F_0-ATPase ~ 0.1–$1\ \mu m^2\ s^{-1}$ in the inner mitochondria membrane, see Table 1). The low mobility of photosynthetic membrane proteins has been rationalized by macromolecular crowding of proteins (Kirchhoff 2008a, b). A similar restriction in diffusion of proteins due to protein crowding can be observed in all prokaryotic bacterial membranes (Dix and Verkman 2008; Mika and Poolman 2011) as a result of the limited size of these organisms. In the case of thylakoid membranes, the protein crowding is required for efficient photosynthesis. It has been clearly shown that "dilution" of the membrane with additional lipids results in a reduction of the efficiency of energy transfer from external light-harvesting antennae to PSII (Haferkamp et al. 2010). In higher plants, protein crowding is dominant in granal thylakoids and less dominant in the agranal (stromal) part of the membrane, which is also reflected in the different mobility of proteins in these two compartments (Kirchhoff et al. 2013). The extent of protein crowding is controlled by the protein/lipid ratio that highly correlates with proteins mobility—higher protein content is connected with a lower mobility due to macromolecular crowding of proteins (Kirchhoff et al. 2013). Protein crowding in other photosynthetic organisms without typical granal organization of thylakoid (e.g., cyanobacteria, green algae, red algae, and in chromalveolate algae-like diatoms) has not been explored in detail yet. However, our unpublished data indicates that protein crowding is dominant in cyanobacteria, but less pronounced in chromalveolate algae (R. Kaňa, M. Lukeš, J. Krafl unpublished).

Not only protein diffusion, but also lipid diffusion in the thylakoid is slower when compared to other eukaryotic membranes (see Table 1). It is obvious that there is a tight interaction between lipids and proteins in membrane bilayers. In fact, a protein diffusion changes with lipid composition and is affected by membrane fluidity (Mullineaux and Kirchhoff 2009). It has already been shown that protein

phosphorylation can switch membranes into a more fluid state, resulting in higher protein mobility (Goral et al. 2010). Protein mobility also tends to increase with decreasing prevalence of membranes in a form of 2D semi-crystalline arrays of PSII–LHC-II protein complexes in granal thylakoid (Goral et al. 2012). These semi-crystalline arrays represent less fluid membrane areas with low protein mobility. This seemingly contradicts the earlier proposal, suggesting a stimulatory effect of semi-crystalline arrays on protein mobility (Kirchhoff et al. 2007). However, this effect has been detected for low light adapted plants, where almost all semi-crystal arrays are represented only by a "single array" not wider 2D arrays [see discussion in (Kouřil et al. 2013)], that in fact do not reduce whole membrane fluidity. Interestingly, it has been proposed that the PsbS protein could act as a membrane fluidization factor that reduces profusion of 2D semi-crystalline arrays (Goral et al. 2012).

There is a limited number of studies addressing the mobility of a particular thylakoid protein, authors focused only on pigment–proteins and studies on mobility of non-fluorescence proteins are still missing (see Table 1). The current model proposes mobility of small thylakoid membrane proteins (up to ~50 kDa, e.g., LHCII or IsiA see Table 1), and other photosynthetic supercomplexes with a higher molecular mass (e.g., Photosystem I or Photosystem II) seem to be immobile based on FRAP measurements. Such an immobility of Photosystem II (PSII) has been proved in many photosynthetic organisms including cyanobacteria, higher plants, and some chromalveolate algae by using FRAP (Mullineaux et al. 1997; Kaňa et al. 2009b; Sarcina and Mullineaux 2004). Recently, rather slow mobility of cyanobacterial NDH complexes has been shown (Liu et al. 2012). However, the NDH complex must be mobile under some conditions because it can redistribute in the thylakoid membrane (Liu et al. 2012). In contrast, there is no data on other photosynthetic complexes such as PSI and cytochrome b_6f. In higher plants, PSI abundance in agranal thylakoids could indicate a higher mobility of PSI, as it is an area with faster protein diffusion (Kirchhoff et al. 2013). However, this hypothesis requires verification through direct measurements. For other photosynthetic supercomplexes without autofluorescence like cytochrome b_6f and F_0-ATPase, we lack even any indirect data. Their mobility can only be indirectly deduced from their mitochondrial analogs, cytochrome bf_1, and F_0-ATPase which diffuse relatively fast (D ~0.1–1 $\mu m^2\ s^{-1}$, see Table 1). As thylakoid membrane crowding by proteins is similar to that in inner mitochondrial membrane, one could expect the similar mobility of cytochrome b_6f and F_0-ATPase also in thylakoid. However, this hypothesis requires to be tested by future experiments.

Mobile fractions of chlorophyll-binding proteins in thylakoid membranes usually represent 15–40 % of all autofluorescent proteins [see e.g., (Kirchhoff et al. 2013, 2008)]. This fraction has been attributed to the light-harvesting antennae of PSII [LHCII in higher plants (Consoli et al. 2005; Kirchhoff et al. 2008) and PSI (IsiA proteins from cyanobacteria (Sarcina and Mullineaux 2004)]. These proteins are smaller (up to ~25–50 kDa) which allows them to move relatively fast inside the thylakoid membrane (see D ~0.01–0.001 $\mu m^2\ s^{-1}$ in the Table 1). Surprisingly, their mobility can also be observed in overcrowded protein areas such as granal thylakoids, (Kirchhoff et al. 2008). Therefore, it has been suggested that thylakoid membrane proteins in that area have a special organization (Kirchhoff 2008a) that facilitates the movement of small proteins. Indeed, theoretically calculated diffusion of high-molecular mass supercomplexes, such as PSII out of overcrowded granal disk (with a diameter of 500 nm), might take as long as 1 h (Kirchhoff et al. 2004b) which highly contrasts with only about 1 or 2 s for small proteins (Kirchhoff et al. 2008). The diffusion rate of small membrane proteins is only slightly affected by their size (Gambin et al. 2006) and more by the organization of other proteins in the membrane. For instance, protein diffusion of small proteins may be limited by the presence of lumenal or stromal domains which stick out of the membrane which could be the case for PSII and PSI subunits. As protein diffusion in the lumen seems to be highly restricted (Kaňa et al. 2009b), these hydrophilic domains could act as a protein anchor which reduces mobility (Mullineaux 2008a; Oguchi et al. 2008).

Another important factor controlling protein mobility is their mutual interaction (see "Experimental methods to study protein mobility" section) which has not been tested for thylakoid proteins in detail. There are some model systems where protein–protein interaction not diffusion is a limiting factor controlling lateral protein transport in membranes (Mueller et al. 2010). In the thylakoid, there is still only indirect data; for instance detachment of the highly mobile LHCII from the PSII supercomplex during light-induced NPQ induction is not accompanied by higher protein mobility, but by a decrease in mobility due to LHCII aggregation (Goral et al. 2012; Johnson et al. 2011). The opposite effect, an increase in proteins mobility, can be observed when protein–protein interaction is reduced when a low salt buffer is used (Kirchhoff et al. 2008). This indicates that not only protein crowding, but also protein–protein interactions play a role in maintaining low proteins mobility in the thylakoid.

Protein mobility on the thylakoid membrane surface—phycobilisome mobility

The restricted mobility of internal membrane supercomplexes (photosystems) contrasts to relatively mobile PBS

(Mullineaux et al. 1997; Sarcina et al. 2001) attached to the thylakoid membrane surface. PBS represents large macromolecular complexes associated with photosynthetic membranes of prokaryotic cyanobacteria and specific eukaryotic algae (red algae and cryptophytes). PBS diffusion was first described more than 15 years ago using FRAP (Mullineaux et al. 1997) revealing the diffusion coefficient of PBS to be between 0.03 and 0.007 $\mu m^2\ s^{-1}$ (Mullineaux et al. 1997; Sarcina et al. 2001). Recently, synchronous monitoring of FRAP and FLIP proved that PBS mobility is not an artifact of high-intensity laser power usually used during FRAP measurements (Yang et al. 2007). PBS diffusion depends on their size (it is faster in smaller PBS without external rods) and is not influenced by temperature (Sarcina et al. 2001). High efficiency of energy transfer from PBS to reaction centers suggests that during short periods, PBS can move even faster than was estimated by FRAP (Mullineaux et al. 1997).

PBS represents extramembrane hydrophilic proteins without integral membrane domains; therefore, their diffusion mostly reflects the fluidity of the cytoplasm and interaction with the thylakoid membrane surface (Sarcina et al. 2001). The nature of PBS interaction with photosystems and with thylakoid membrane is only poorly understood [for recent review, see e.g., (Mullineaux 2008b)]. Due to steric hindrance of thylakoid membrane complexes (e.g., Photosystem I that protrudes more than 3 nm into the cytoplasm), a long-distance PBS diffusion requires PBS decoupling from the membrane surface [reviewed in (Kirilovsky et al. 2013)]. As the PBS decoupling can be induced either by excessive irradiance or by short-term heat stress, it has been suggested that it is based on the so-called "thermo-optic effect" (Tamary et al. 2012) (Stoitchkova et al. 2007). However, PBS decoupling is stimulated also at low temperature (Li et al. 2001; Manodori and Melis 1985); therefore, understanding of PBS decoupling mechanism requires additional research (for details see discussion in Kirilovsky et al. 2013).

There are several factors that influence PBS interaction with photosystems or thylakoid membranes: (1) Energetic coupling of the PBS to the PSII reaction center requires formation of an active oxygen evolving complex (Hwang et al. 2008), and is generally affected by the presence of the PsbU protein on the donor side of PSII (Veerman et al. 2005); (2) PBS–PSII interaction is controlled by RpaC protein—without RpaC, PBS is locked to Photosystem II (Joshua and Mullineaux 2005; Emlyn-Jones et al. 1999); (3) PBS–Photosystems complex is stabilized when the water activity is lowered (Mullineaux 2008b); and (4) Some experiments have also suggested that lipid saturation plays a role in PBS-Photosystems interaction (Sarcina et al. 2001). Even though PBS interaction with membrane proteins (Photosystems) seems to be essential for their mobility (measured through FRAP), the importance of this interaction for phycobilisome mobility has not yet been estimated.

PBS is present not only in prokaryotic cyanobacteria, but can also be found on the stromal surface of thylakoid membranes in red algae and also in the lumen of cryptophytes. Hemispherical PBS in red algae were initially proposed to be immobile; the observed recovery in FRAP experiments have been ascribed rather to an intrinsic photoprocesses of the bleached PBS in situ (Liu et al. 2009). However, our recent experimental results (Kaňa et al. 2013a) have shown the opposite, we have conclusively proven PBS diffusion in red algae with a rate of approximately $\sim 0.004\ \mu m^2\ s^{-1}$. On the contrary, the phycobiliproteins in the Cryptophyte algae *Rhodomonas salina* (that are situated in the lumen side of the thylakoid) are fully immobile (Kaňa et al. 2009b). Thus, there is species variability in mobility of phycobiliproteins which is reflected by the different regulation of their light harvesting and photoprotection mechanism. For instance in cyanobacteria, excessive light is dissipated in blue light-induced non-photochemical quenching (NPQ) that take place in PBS and is dependent on a special Orange carotenoid protein (OCP) (Kirilovsky et al. 2013; Kirilovsky 2007). This protein is missing in red algae where NPQ is still only a poorly understood process (Delphin et al. 1996, 1998). Recently, NPQ in reaction centrum (Krupnik et al. 2013) and by spill-over mechanism (Kowalczyk et al. 2013) have been suggested for extremophilic and mesophilic red algae, respectively. NPQ in cryptophytes have been described in details, photoprotective NPQ proceeds on a level of light-harvesting antennae (LHCII), and there is no excessive light dissipation in immobile phycobiliproteins (Kaňa et al. 2012b, c).

Protein mobility outside the thylakoid membrane

Chloroplast stroma in eukaryotic phototrophs or cytoplasm in cyanobacteria represents a key compartment for activity of many photosynthetic enzymes including enzymes of the Calvin–Benson cycle. Diffusion of photosynthetic proteins in these compartments, outside the thylakoid membrane is only poorly understood. The chloroplast stroma seems to be quite a viscous compartment in comparison to the cytosol (Kohler et al. 2000) as has been indicated by experiments with stromules, stroma-filled extensions, and protrusions of the chloroplast envelope (Kohler et al. 1997; Hanson and Sattarzadeh 2011). In fact, results obtained by FCS have proven that GFP diffuses 50 times slower in stroma ($D \sim 0.9\ \mu m^2\ s^{-1}$ in stroma) than in the cytosol ($D \sim 4\ \mu m^2\ s^{-1}$). It is faster in comparison to thylakoid membrane proteins (see Table 1), but slower in comparison to other non-membrane proteins. However, these experiments were only carried out in stromules and direct

measurements of photosynthetic proteins mobility in stroma, outside these structures, is still lacking. The mechanism of stromal protein diffusion in stromules seems to be either an anomalous or active diffusion (Kohler et al. 2000).

It is also questionable how stromules affect internal chloroplast morphology during active photosynthesis. The mechanism of stromule formation seems to be connected to a "plastoskeleton", a proposed cytoskeleton-like network in chloroplasts (Kiessling et al. 2000). This structure is supported by chloroplast and cyanobacterial orthologs of eukaryotic tubulins—the FtsZ protein found inside chloroplast. The plastoskeleton probably acts, not only in chloroplast division or in maintaining chloroplast shape but also it may provide a filamentous network inside chloroplasts (Reski 2009). Even though it is still not fully certain whether the endogenous FtsZ really forms a complex plastoskeleton structure in chloroplasts (Martin et al. 2009a, b), the role of this plastoskeleton in diffusion of stromal or thylakoid membrane proteins needs to be tested as, like in the other eukaryotic cells, protein diffusion in the cytosol as well as in the membrane is controlled by the cytoskeleton matrix (Koppel et al. 1981).

The thylakoid membrane lumen is an even more viscous compartment than the chloroplast stroma. This photosynthetic compartment forms a continual aqueous space separated from the stroma of the chloroplast (or from the cytosol in cyanobacteria) by the thylakoid membrane. The thylakoid lumen has several functions; it acts as a reservoir of protons for photosynthetic ATP synthesis; contains various proteins including chaperones (Schlicher and Soll 1996) and proteases (Kapri-Pardes et al. 2007), and is the site of photosynthetic electron transport between the cytochrome b_6f complex and Photosystem I mediated by PC. Currently, there is no direct data on protein mobility in a typical higher plant or in the cyanobacterial thylakoid membrane lumen. In cyanobacteria, application of the TAT system to import GFP into the lumen resulted only in GFP accumulation in the periplasm (Spence et al. 2003). In *Arabidopsis*, GFP was successfully imported into the lumen using the TAT system; however, it could not be visualized in a continuously illuminated chloroplast, perhaps due to its destabilisation at low pH (Marques et al. 2004). Therefore, the only direct measurements by FRAP came from *cryptophytes* that contain autofluorescent phycoerythrins inside the lumen. These experiments have shown almost no protein diffusion in the thylakoid membrane lumen of cryptophytes (Kaňa et al. 2009b). This result indicates restricted mobility of PC between cyt b6f and PSI that is in line with previous experimental results for higher plants where PC diffusion has been proposed to occur only in the grana margins, where PSI and cyt b6f could be in close proximity (Kirchhoff et al. 2004a). PC diffusion in the thylakoid lumen is restricted by the lumenal part of membrane supercomplexes, especially by the oxygen evolving complex of PSII that protrudes more than 3 nm through a very thin lumen. Hence, it has been suggested that lumenal proteins are packed so tightly that they form almost a crystalline gel (Mullineaux 2008a). Should this hypothesis be valid, diffusion of mobile electron carriers in the lumen would be less important for electron transport than has been generally assumed. It has been hypothesized that PC-mediated electron transport could act through ''molecular wires" composed of supramolecular PC assemblies (Kaňa et al. 2009b) that have recently been observed (Crowley et al. 2008). An alternative theory suggests that PC diffusion on illumination is facilitated by light-induced lumen expansion (from 5 to about 9 nm) that opens free diffusion space for PC allowing faster electron transport between cyt b_6f and PSI (Kirchhoff et al. 2011). However, both models propose restriction in PC diffusion close to thylakoid membranes; in fact this is a limiting step in the whole photosynthetic electron transport chain. Interestingly, the same effect (slow diffusion of a small soluble protein-like PC) can be observed in the mitochondria analog of PC, cytochrome-*c* (Hochman et al. 1982) that is a soluble protein transporting electrons from bc_1 complex to the cytochrome c oxidase complex. This small protein diffuses much more slowly in comparison to other mitochondrial proteins (see Table 1).

The model of light-induced lumen expansion presented by Kirchhoff et al. (2011) contradicts with the formerly proposed model of light-induced lumen shrinkage (Murakami and Packer 1970a, b). Kirchhoff et al. 2011 suggested that the discrepancy likely reflects the differences between sample preparations used in older works (Murakami and Packer 1970a, b) where former experiments were done with less native thylakoid membranes [see discussion in (Kirchhoff et al. 2011)]. However, the light-induced changes in the lumen requires more research, as recent results with native cells obtained by small-angle neutron scattering have supported both models, the light-induced thylakoid membrane expansion in living cyanobacteria and diatoms (Nagy et al. 2012; Liberton et al. 2013; Krupnik et al. 2013) and lumen shrinkage in higher plant thylakoids (Krupnik et al. 2013; Posselt et al. 2012).

Role of protein mobility in physiological processes

There are several physiological processes that are connected with thylakoid membrane re-organization and with protein mobility. One of the most important is represented by a fast response of photosynthetic apparatus to excessive irradiation, NPQ [see e.g., (Ruban et al. 2012) for recent

review] that dissipates excessive light to heat (Kaňa and Vass 2008). Longer exposure to high light can induce photoinhibition of PSII, complex process that requires relatively fast D1 protein repair and turnover [see e.g., (Komenda et al. 2012)]. Finally, high efficiency of primary photosynthetic reactions is maintained by physical redistribution of external light-harvesting antennae during so-called state transitions (Mullineaux and Emlyn-Jones 2005; Minagawa 2011; Kirilovsky et al. 2013). The importance of photosynthetic protein mobility in all these processes is described in detail in the following paragraphs.

Photoprotection and protein mobility

The role of protein mobility in photoprotection has recently been intensively studied for higher plants (Goral et al. 2010, 2012; Herbstova et al. 2012; Johnson et al. 2011). Interestingly, the stimulation of photoprotection by NPQ, a typical fast response to a fast excess in absorbed light ($\sim$5 min), is accompanied by decreasing protein mobility (Goral et al. 2012; Johnson et al. 2011) even though all other observed effects [increase in PSII distances, membrane fluidization see (Johnson et al. 2011) for details] would indicate the opposite—a higher protein mobility. Decrease in protein mobility was explained as a result of LHCII aggregation that stops movement of other occasion mobile LHCII (Kirchhoff et al. 2008). This result shows that LHCII aggregation is a dominant effect for the fast phase of NPQ stimulation. The decrease in proteins mobility is further increased by the formation of zeaxanthin (Johnson et al. 2011), a chemical compound that increases NPQ. Zeaxanthin can act on two levels; it facilitates LHCII aggregation when it binds to light-harvesting proteins, but it can also acts as a membrane rigidifier (Havaux 1998) directly in the lipid bilayer. Both these effects are capable of reducing protein mobility. Protein mobility in the thylakoid is also affected by the presence of the PsbS protein (Goral et al. 2012), a key factor of the fast and flexible NPQ stimulation and recovery (Ruban et al. 2012). It has been suggested that PsbS acts as an "membrane lubricant" that accelerates all changes in the PSII macrostructure affecting protein mobility—higher PsbS content results in higher proteins mobility (Goral et al. 2012).

After prolonged periods of excessive irradiation (>1 h), light-induced membrane fluidization (Johnson et al. 2011) increases together with membrane fluidity and protein mobility (Goral et al. 2010; Herbstova et al. 2012). This process in higher plants is conditional to protein phosphorylation; it is inhibited in mutants with protein kinases (Goral et al. 2010; Herbstova et al. 2012). It is in line with the observation that phosphorylation enhances the structural flexibility of the thylakoid membrane architecture (Varkonyi et al. 2009). Interestingly, there seems to be a "stromal factor" required for protein mobilization on illumination as it is not visible in isolated thylakoid membranes without chloroplast stroma (Goral et al. 2012). Protein mobilization after prolonged period of excess light is also accompanied by more severe changes in thylakoid membrane organization: (1) Reduction in the diffusion path caused by smaller grana size (Goral et al. 2010; Herbstova et al. 2012); (2) Decrease in protein overcrowding due to redistribution of PSII and LHCII from grana to stroma lamellae (Herbstova et al. 2012); and (3) Protein phosphorylation (Goral et al. 2010; Herbstova et al. 2012) that facilitates protein diffusion (Consoli et al. 2005). All these processes together increases protein diffusion from overcrowded granal disks, and thus facilitates the PSII repair cycle in grana margins.

In contrast to higher plants, the connection between protein mobility and photoprotection in other organisms (e.g., cyanobacteria and algae) is still poorly understood. Light-induced increase in protein mobility has already been shown for cyanobacteria (Sarcina et al. 2006). It has been suggested that this process could be accompanied by the formation of "PSII repair zones" responsible for D1 protein turnover (Sarcina et al. 2006). Cyanobacterial thylakoid membranes seem to be a heterogeneous compartment with specific regions (Vermaas et al. 2008; Nevo et al. 2007). The similar zones of *de-novo* protein synthesis and repair were also detected in the green alga *Chlamydomonas reinhardtii* (Uniacke and Zerges 2007); however, the locus of PSII repair still remains controversial. It is also because we still lack any direct data on protein mobility in green algae (Mullineaux and Sarcina 2002). Therefore, a more detailed study is required to understand the connection of protein mobility and photoprotection in algae and cyanobacteria.

Role of protein mobility in state transitions

The importance of protein diffusion in photosynthesis was first revealed in the process of "state transitions". It is a regulatory mechanism that controls the distribution of excitation energy between both photosystems connected with physical movement (diffusion) of external antennae of photosystems. In State II, antennae move toward PSI, in State I they are transferred toward PSII. This process has already been described for various organisms including higher plants (Minagawa 2011) and cyanobacteria (Kirilovsky et al. 2013). In higher plants and green algae, the physical redistribution of LHCII between photosystems (Kouřil et al. 2005; Iwai et al. 2010) together with membrane reorganization (Chuartzman et al. 2008) have been indirectly confirmed by biochemical and biophysical methods. The mobile antennae, light-harvesting complex of PSII (LHCII), are phosphorylated during their transfer from PSII to PSI (Iwai et al. 2010). It has already been

shown that phosphorylation switches the thylakoid membrane into a more fluid state (Goral et al. 2010; Varkonyi et al. 2009). In fact, a fourfold increase in LHCII diffusion rate after phosphorylation has already been directly proven [see (Consoli et al. 2005) and Table 1]. Therefore, as LHCII can diffuse from thylakoid grana (occupied mostly by PSII) to stroma lamellae in a few seconds (see diffusion coefficient in Table 1) LHCII diffusion is not limiting for state transition in higher plants.

The mechanism of state transitions in cyanobacteria is different, as external antennae of photosystems are represented by PBS, situated on thylakoid membrane surfaces and moreover, there is no phosphorylation involved in state changes (Mullineaux and Emlyn-Jones 2005; Kirilovsky et al. 2013). PBS can act as a light-harvesting antenna of both Photosystems (Mullineaux 1992, 1994; Mullineaux and Holzwarth 1991), and thus can redistribute absorbed energy between photosystems during state transitions [see e.g., (Kirilovsky et al. 2013) for recent review]. The fact that PBS mobility is essential for one from the proposed mechanism of state transitions (Kirilovsky et al. 2013) was confirmed by using the experiments with treatments that inhibits PBS mobility (e.g., high glycin betaine concentration) which have provably abolished state transitions in cyanobacteria (Mullineaux et al. 1997; Joshua and Mullineaux 2004). The PBS mobility does not necessarily represent a long-distance PBS diffusion, it is more probable only a very short-distance (in tens of nanometers) PBS movement (a shuttle) between photosystems. Even though PBS sits on the membrane surface with supposedly less protein crowding (in comparison to LHCII in membrane), the final diffusion coefficient of PBS (estimated to be 0.004–0.03 $\mu m^{-2}\ s^{-1}$) is very close to LHCII (compare with D for LHCII, 0.005–0.03 $\mu m^{-2}\ s^{-1}$, see Table 1). Therefore, phycobilisome movement, like LHCII, is not limiting for state changes. It has been suggested (Mullineaux et al. 1997) that PBS interacts with reaction centers of PSII and PSI only transiently and state transition represents a change in the dynamic equilibrium of their interaction with PSII and PSII; however this model should be tested as we do not know strength of phycobilisome coupling to photosystems. However, only little is known about the mechanism involved in PBS disconnection from PSII [see e.g., (Kirilovsky et al. 2013) for recent review] that is visible in variability in PBS fluorescence (Kaňa et al. 2009a, 2012a). In fact, PBS decoupling from the membrane surface seems to be limiting for free diffusion as sterical hindrance of PSI extrinsic subunits represents obstacles that prevent free diffusion on the membrane surface (Kirilovsky et al. 2013). Therefore, a complex model explaining role of PBS mobility in state transitions requires further research.

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