Protein Diffusion and Macromolecular Crowding in Thylakoid Membranes[1][W]

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The photosynthetic light reactions of green plants are mediated by chlorophyll-binding protein complexes located in the thylakoid membranes within the chloroplasts. Thylakoid membranes have a complex structure, with lateral segregation of protein complexes into distinct membrane regions known as the grana and the stroma lamellae. It has long been clear that some protein complexes can diffuse between the grana and the stroma lamellae, and that this movement is important for processes including membrane biogenesis, regulation of light harvesting, and turnover and repair of the photosynthetic complexes. In the grana membranes, diffusion may be problematic because the protein complexes are very densely packed (approximately 75% area occupation) and semicrystalline protein arrays are often observed. To date, direct measurements of protein diffusion in green plant thylakoids have been lacking. We have developed a form of fluorescence recovery after photobleaching that allows direct measurement of the diffusion of chlorophyll-protein complexes in isolated grana membranes from *Spinacia oleracea*. We show that about 75% of fluorophores are immobile within our measuring period of a few minutes. We suggest that this immobility is due to a protein network covering a whole grana disc. However, the remaining fraction is surprisingly mobile (diffusion coefficient $4.6 \pm 0.4 \times 10^{-11}$ cm^2 s^{-1}), which suggests that it is associated with mobile proteins that exchange between the grana and stroma lamellae within a few seconds. Manipulation of the protein-lipid ratio and the ionic strength of the buffer reveals the roles of macromolecular crowding and protein-protein interactions in restricting the mobility of grana proteins.

Thylakoid membranes of higher plants have an intricate structure and are laterally segregated into distinct regions known as the grana and the stroma lamellae (for review, see Dekker and Boekema, 2005). The grana are stacked discs of membrane approximately 400 to 500 nm in diameter. The major photosynthetic complexes are heterogeneously distributed in the membrane. PSII and its associated light-harvesting complex II (LHCII) are concentrated in the grana, whereas PSI and the ATPase are excluded from the grana and located instead in the stroma lamellae or at the grana margins (Albertsson, 2001; Dekker and Boekema, 2005). Most likely the cytochrome b_6f complex is evenly distributed. The architecture of the major photosynthetic complexes is now understood in detail, with high-resolution structures for PSI, PSII, LHCII, the cytochrome b_6f complex and the ATPase

(Nelson and Ben Shem, 2004). Electron microscopy and atomic force microscopy provide detail on the organization of complexes in the intact thylakoid membrane (Kirchhoff et al., 2004a, 2004b; Dekker and Boekema, 2005).

Thylakoid membranes in vivo are not static, solid-state structures. There is abundant indirect evidence for protein mobility within the thylakoid membrane system, mostly from biochemical studies in which thylakoids are fractionated into grana and stroma lamellae. This is followed by analysis of the compositions of the fractions under different conditions. For example, phosphorylation of LHCII leads to net migration of LHCII from the grana to the stroma lamellae (Drepper et al., 1993; Allen and Forsberg, 2001). This is essential for state-1/state-2 transitions, a mechanism that regulates photosynthetic light harvesting on a timescale of a few minutes (Allen and Forsberg, 2001). PSII is very susceptible to photodamage and must be continually repaired to maintain safe and efficient photosynthetic electron transport (Barber and Andersson, 1992). The repair of photodamaged PSII complexes takes place in the stroma lamellae, via a complex repair cycle that must involve the movement of photodamaged complexes out of the grana (Baena-Gonzalez and Aro, 2002). Thus protein mobility in the grana must be essential for photosynthetic function.

Grana thylakoids are among the most crowded membranes in nature: 70% to 80% of the membrane area is occupied by proteins (Kirchhoff et al., 2002). It is intuitively difficult to understand how efficient

protein diffusion can take place in this densely packed membrane. Furthermore, Monte Carlo computer simulations predict a very severe restriction of protein (and lipid) mobility in grana thylakoids (Kirchhoff et al., 2002, 2004b; Tremmel et al., 2003). However, direct experimental data are lacking. Measurements of protein mobility are required for proper understanding of photosynthetic function, and of regulation, repair, and biogenesis of the thylakoid membrane. To date, these measurements are only available for cyanobacteria (Mullineaux et al., 1997; Sarcina and Mullineaux, 2004; Sarcina et al., 2006), which have a simpler thylakoid membrane configuration, lacking lateral heterogeneity and grana. Here, we report the use of fluorescence recovery after photobleaching (FRAP) to probe the mobility of chlorophyll-protein complexes in isolated grana membranes from spinach (*Spinacia oleracea*). As with the previous measurements on cyanobacteria (Mullineaux et al., 1997; Sarcina and Mullineaux, 2004; Sarcina et al., 2006), we were able to use the native fluorescence from the photosynthetic pigments to visualize protein movement; there is no requirement for artificial fluorescent tags. Under near-physiological conditions, about 75% of fluorophores are immobile within the 9-min timescale of our measurements. However, the mobile fraction diffuses surprisingly quickly, suggesting that mobile proteins could escape from the grana within a few seconds. This contrasts sharply with computer simulations suggesting an escape time of about 1 h (Kirchhoff et al., 2004b), and suggests that the organization of complexes is optimized for rapid diffusion. By diluting the protein complexes with additional lipid we show the effects of macromolecular crowding on diffusion, and by altering the ionic strength of the surrounding medium we show that protein clusters are stabilized by electrostatic interactions.

RESULTS

Preparation of Spinach Grana Membranes

Grana membranes were prepared from spinach, by a procedure based on that of Berthold, Babcock, and Yocum (BBY), in which grana are isolated by a mild detergent treatment that preferentially solubilizes the more exposed stroma lamellae (Schiller and Dau, 2000). Grana patches prepared in this way consist mainly of pairs of membranes held together by electrostatic interactions at their stromal surfaces, with their lumenal surfaces exposed to the medium (Dunahay et al., 1984; Kirchhoff et al., 2004a). The membranes are flat discs usually about 500 nm in diameter, reflecting the dimensions of grana membranes in vivo (Kirchhoff et al., 2004a). We used membranes either at their native protein density, or diluted by fusion with unilamellar liposomes containing the native lipid mixture (Haferkamp and Kirchhoff, 2008). Ficoll density gradient ultracentrifugation shows that the fused membranes have lower density than the original BBY particles (Haferkamp

and Kirchhoff, 2008). Gradual addition of liposomes in the fusion batch leads to a gradually decreased density of the BBY membranes (Haferkamp and Kirchhoff, 2008). This clearly confirms fusion of the two membranes. The density changes are accompanied by an increase of the F_0 chlorophyll *a* fluorescence level, indicating detachment of LHCII complexes from PSII (Haferkamp and Kirchhoff, 2008).

Optimizing FRAP with Grana Patches

Previous FRAP measurements exploited the relatively simple and uniform configuration of cyanobacterial thylakoid membranes, using elongated cyanobacterial cells whose cell length is much greater than the width of the bleach produced by scanning the laser spot (Mullineaux et al., 1997; Sarcina and Mullineaux, 2004; Sarcina et al., 2006). Adapting this method to isolated grana membranes poses a number of challenges:

1. Measurements will not be possible unless the isolated membrane patches can be immobilized. However, interactions of proteins with a solid support may perturb their mobility.
2. Grana membranes are normally only about 400 to 500 nm in diameter (Dekker and Boekema, 2005), which is not much greater than the minimum diameter of the FRAP bleach. This may make it difficult to bleach a part of the sample: the bleach will tend to extend across the entire membrane area, meaning that no information on diffusion can be obtained.
3. Chlorophyll fluorescence may be less stable in vitro than in vivo, where there are systems for scavenging free radicals and singlet oxygen molecules produced during illumination. For quantitative FRAP measurements it is important that the sample can be repeatedly imaged without further loss of fluorescence due to photobleaching during imaging.

To solve the problem outlined in 1 above we developed a method for immobilizing grana membrane patches by adsorbing them onto an artificial phosphatidylcholine (PC) bilayer. Glass microscope slides were coated with the artificial bilayer, which could be visualized as it was stained with the green lipophilic fluorophore 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoicacid (BODIPY FL C-12; Haugland, 2005; Fig. 1). The suspension of grana membrane patches was layered onto the PC bilayer, where they could be visualized using the red fluorescence from the chlorophylls (Fig. 1). After incubation for about 10 to 30 min, the majority of membrane patches adhere to the PC bilayer surface. Immobile membrane patches could then be selected for FRAP measurements. Note that the grana thylakoids lie on top of the PC bilayer and do not fuse with it (Fig. 1, scheme in inset, top right). This was concluded from vertical scans that showed that the center of gravity of the (green) BODIPY FL C-12 fluorescence is clearly under the (red) chlorophyll fluorescence (not shown). Probably this is due to the mismatch in fatty acid length (C-12 for PC, C-16 and
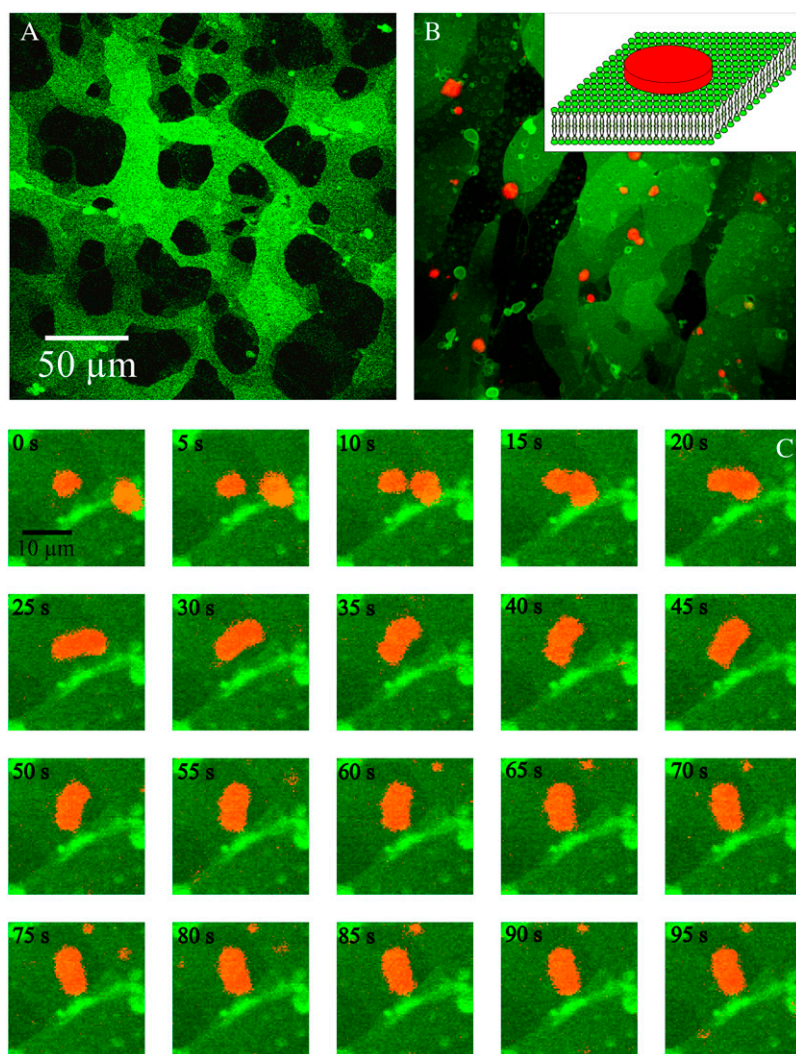
Figure 1. Fluorescence images of labeled PC bilayers (green) and of chlorophyll fluorescence (red) under aqueous conditions. A, Stacks of PC bilayers formed spontaneously after rehydration of dried PC films. Black areas correspond to the glass support. B, Grana patches adhere to the PC bilayer as illustrated in the inset. C, Time series demonstrating the collision and fusion of two grana patches.

C-18 for the thylakoid lipids; Duchêne and Siegenthaler, 2000). FRAP analysis of the diffusion of BODIPY FL C-12 in the PC bilayer shows that the bilayer remains fluid under these conditions, with very rapid diffusion of BODIPY FL C-12 (not shown). Thus the glass-PC system provides a fluid bilayer support for biomembranes under aqueous conditions. It is expected that the diffusion of proteins in the grana membranes is only slightly affected by the fluid PC support.

Fortuitously, we found that the glass-PC system also solves the problem outlined in 2 above. During adhesion to the PC bilayer support, grana membranes tend to move laterally, collide, and fuse into much larger membrane patches (Fig. 1). For FRAP measurements we were able to select membrane patches 1 to 4 μm in diameter. In patches of this size it is straightforward to bleach fluorescence in part of the sample only, allowing subsequent diffusion of fluorophores to be visualized.

As discussed in 3 above, we found that chlorophyll fluorescence of isolated grana thylakoids in untreated buffer decreased rather rapidly during continuous

imaging, with a half-time of a few minutes (not shown). We found that this problem could be solved by reducing the oxygen content of the buffer with Glc, Glc oxidase, and catalase. Under these conditions chlorophyll fluorescence was stable for more than 1 h during exposure to the laser intensity used for imaging. However, chlorophyll fluorescence could still be rapidly bleached by increasing the laser intensity, making FRAP measurements straightforward.

In summary, the conditions used for FRAP analysis ensure a fluid support for the grana membranes, a stable fluorescence intensity, and a size of the samples that is large enough for FRAP measurements.

FRAP Analysis on Grana Membranes

Figure 2 shows chlorophyll fluorescence redistribution after bleaching a line across a grana membrane patch. The appearance of the unbleached patch is shown in the top left picture (prebleach). For each image we extracted a one-dimensional fluorescence profile, defined as the sum of intensities over the x axis. This is

Figure 2. FRAP measurement on a native grana membrane patch. The gray arrow at 0 s indicates the position of the bleach line. The grayscale has been inverted, so that regions of higher fluorescence appear darker. The one-dimensional fluorescence profile (summed in the X-direction) is shown to the right of each image. Bottom part shows fluorescence recovery curve deduced from the fluorescence profiles.
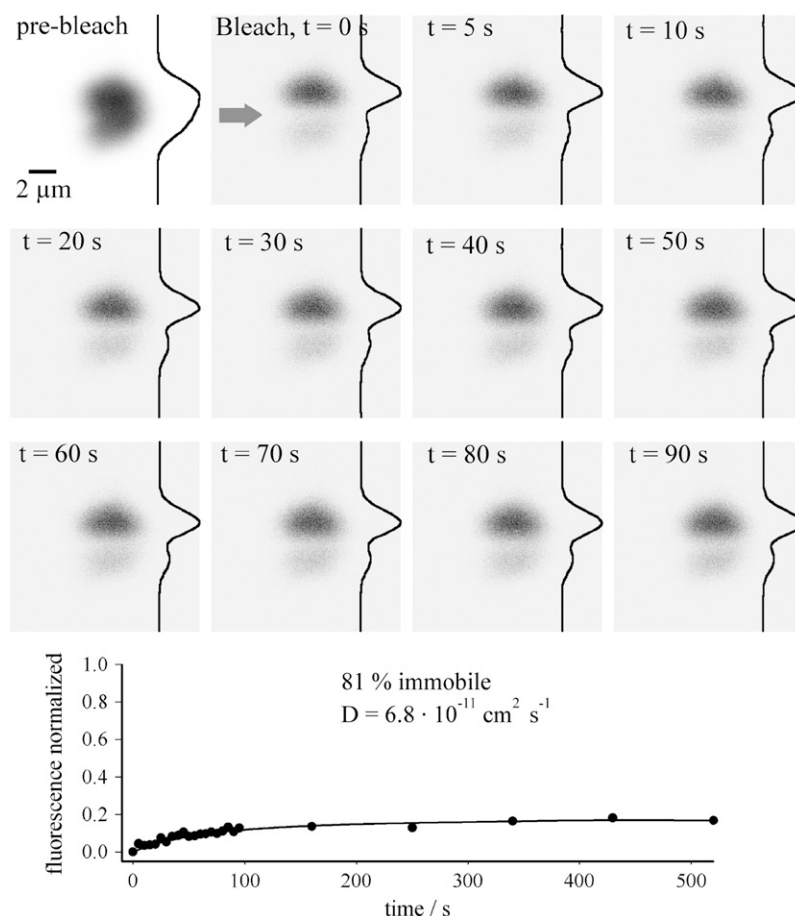
shown to the right of each image. It is obvious that fluorescence recovery is incomplete on the timescale of the measurement because the bleach line remains visible in all pictures (Fig. 2). However, there is some redistribution of fluorescence, with partial recovery of fluorescence at the center of the bleach (kinetics shown at the bottom of Fig. 2). From analysis of the recovery kinetics we estimate that about 80% of the chromophores are immobile, at least within the measurement time of 9 min, and the diffusion coefficient of the remaining mobile fraction is about 7×10^{-11} cm^2 s^{-1}. Measurements on 36 patches gave a mean immobile fraction of 73% $\pm$ 3% (SE) and a mean diffusion coefficient for the mobile fraction of 4.6 $\pm$ 0.4 $\times 10^{-11}$ cm^2 s^{-1}. From the variance of the five last data points in the recovery kinetics (recorded at 60-s intervals as in Fig. 2) we can be confident that the diffusion coefficient of the immobile fraction is below 10^{-12} cm^2 s^{-1}.

Diffusion of Photosynthetic Complexes in Grana Patches with Lower Protein Density

Figure 3 shows an example of a FRAP series for a grana patch fused with liposomes at a lipid:chlorophyll ratio of 10:1. In contrast to BBY membranes at native protein density (Fig. 2) the redistribution of chlorophyll fluorescence is almost complete and much

faster, as indicated by the quick recovery of the bleached line. From analysis of the time series (Fig. 3, bottom) we estimate a diffusion coefficient of about 2.3 $\times$ 10^{-10} cm^2 s^{-1} for the mobile fraction, and an immobile fraction of about 20%. The degree of bleaching in Figure 3 is more efficient than in the undiluted grana membrane (Fig. 2). This may be a consequence of the higher protein mobility in lipid diluted membranes: During the bleaching period, mobile protein complexes move from unbleached regions into the bleached line. However, we checked that the estimation of the immobile fraction and of the diffusion coefficient does not depend on the degree of bleaching (see Supplemental Figs. S1 and S2). Thus the differences in the immobile fraction and in D between Figures 2 and 3 are not caused by differences in bleaching efficiency. Figure 4 shows the dependence of diffusion parameters on the lipid-chlorophyll ratio. With increasing lipid dilution of grana, the immobile fraction decreases and the diffusion coefficient increases. Over the range of dilutions used, the immobile fraction decreases from about 75% (native grana membranes) to about 25%. Over the same range of dilutions, the diffusion coefficient increases roughly 7-fold (4.6 $\pm$ 0.4 $\times 10^{-11}$ to 3.2 $\pm$ 0.9 $\times 10^{-10}$ cm^2 s^{-1}). The changes in both parameters have threshold characteristics. Up to a lipid:chlorophyll ratio of about 4, both parameters
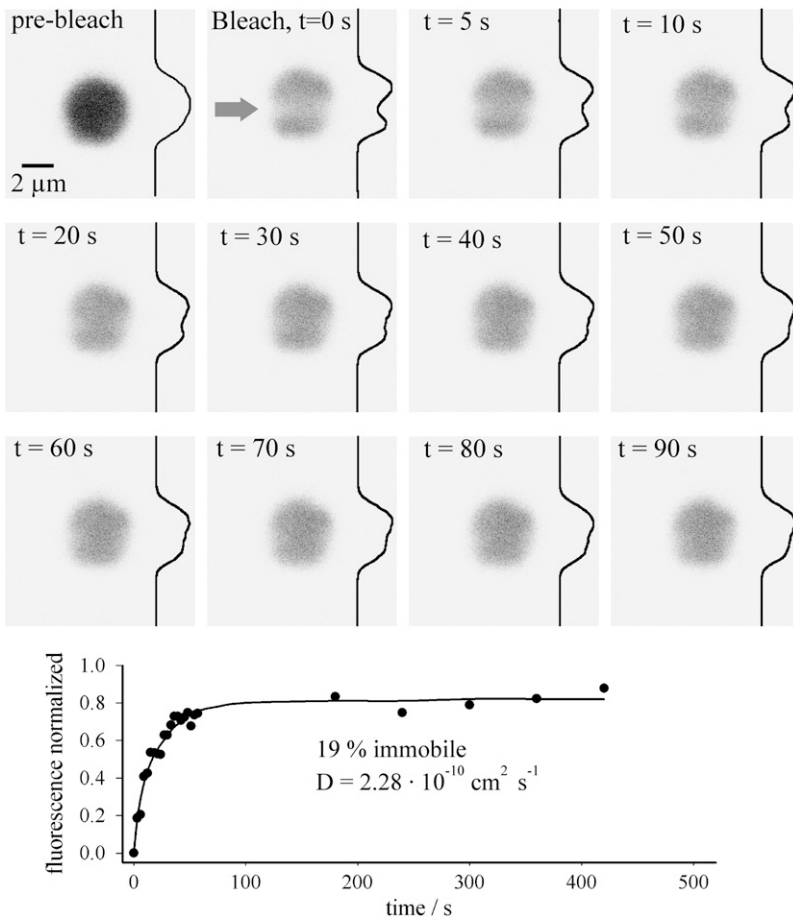
Figure 3. FRAP measurement on a grana membrane-liposome fusion product (added lipid:chlorophyll, 10:1). Presentation as explained in the legend to Figure 2. Note the larger mobile fraction and faster fluorescence recovery as compared to Figure 2.

19 % immobile
$D = 2.28 \cdot 10^{-10}$ cm^2 s^{-1}

did not change significantly. Further addition of lipids induces a decline in the immobile fraction and an increase in the diffusion coefficient of the mobile fraction.

Effects of Salt Concentration

There is strong evidence that protein-protein interactions in thylakoid membranes are stabilized by divalent cations and that removing these cations (giving "low-salt" conditions) leads to a breakdown of the protein network (Barber, 1982; Harrison and Allen, 1992; Kirchhoff et al., 2004a). Thus cation depletion is expected to influence the mobility of chlorophyll-protein complexes. To test this possibility we carried out FRAP measurements under low-salt conditions (no $MgCl_2$ and 10 mM NaCl, as compared to 40 mM NaCl and 7 mM $MgCl_2$ under standard conditions). In grana membranes at native protein density, the low-salt incubation has only minor effects on diffusion characteristics. However, in diluted grana, diffusion characteristics are strongly influenced by salt concentration. At a lipid:chlorophyll ratio of 7:1, low-salt incubation decreases the immobile fraction from about 60% to 33%, and increases the diffusion coefficient by a factor of about 13 (Fig. 4, white circles).

DISCUSSION

Which Chlorophyll-Protein Complexes Are Mobile in Grana Membranes?

Our FRAP measurements show that native spinach grana membranes contain two distinct populations of chlorophyll-protein complexes, each with a distinct mobility. One population (responsible for about 75% of chlorophyll fluorescence) appears completely immo-

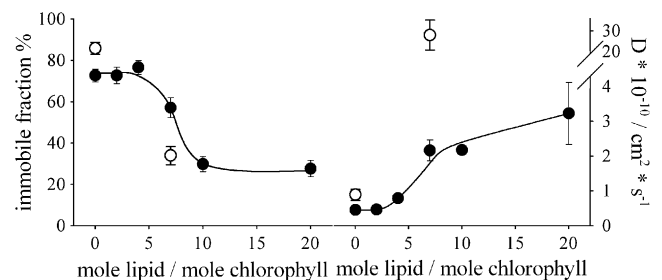**Figure 4.** Effects of dilution of grana membranes with additional lipid on the diffusion coefficient and the immobile fraction. The x axes show the molar ratio of additional lipid-chlorophyll (i.e. zero corresponds to native grana membranes without fusion to liposomes). Black circles indicate standard buffer (7 mM $MgCl_2$, 40 mM NaCl). White circles indicate low-salt buffer (10 mM NaCl, 0.5 mM EDTA).

bile on short (9-min) timescales. A second population (responsible for about 25% of chlorophyll fluorescence) diffuses freely and relatively rapidly (diffusion coefficient about 5×10^{-11} cm^2 s^{-1}). This observation raises the question of which of the various chlorophyll protein complexes in the grana are mobile. The major chlorophyll-protein complexes in grana membranes are the PSII core complex (which is normally dimeric) and the "major" LHCII light-harvesting antenna consisting of trimers of LHCII proteins. In addition there are three "minor" antenna complexes that are normally monomeric. There are usually about eight LHCII trimers for every PSII dimer (Dekker and Boekema, 2005). Electron microscopy and single particle analysis of solubilized protein complexes suggest that at least some of the LHCII trimers are closely associated with PSII, with at least three PSII-LHCII binding sites (designated S, M, and L for strongly, moderately, and loosely bound LHCII; Dekker and Boekema, 2005). In our FRAP measurements we observe the mobility of chlorophyll fluorophores. The chlorophylls must be protein-bound, but we cannot directly distinguish between PSII, the minor antenna complexes, and the various subpopulations of LHCII because the spectral differences between these complexes are small (Jennings et al., 1993).

However, it is likely that the mobile fraction is a subpopulation of LHCII because another approach indicates that part of the LHCII antenna must be mobile, at least under some conditions; some LHCII can redistribute between the grana and the stroma lamellae as a result of the acclimation mechanism known as state transitions (Drepper et al., 1993; Allen and Forsberg, 2001). In addition, FRAP measurements in cyanobacteria have shown that PSII core complexes are normally immobile, indicating that something "anchors" the complex in the membrane (Mullineaux et al., 1997; Sarcina and Mullineaux, 2004). It seems likely that the situation in green plants is similar. However, it is possible that some or all of the PSII of green plants becomes mobile under other physiological conditions, for example, to facilitate the PSII repair cycle during exposure to intense light. Such an effect has been observed in cyanobacteria (Sarcina et al., 2006). Further studies involving specific fluorescent tagging of the grana protein complexes will be needed to show which specific complexes are mobile.

Comparison with Previous Estimates of Protein Diffusion Coefficients in Thylakoid Membranes

Consoli et al. (2005) recently made a direct measurement of the LHCII diffusion coefficient in spinach thylakoid membranes by single-particle tracking of LHCII molecules tagged with antibodies conjugated to relatively large fluorescent microspheres (0.8 μm in diameter). The experimental approach is very different to ours, and it is clear that the fluorescent microspheres will exclude the tagged LHCII from the stacked grana membranes (Consoli et al., 2005). Thus, the LHCII observed in these measurements must be located in the

stroma lamellae, or grana end membranes and margins. Nevertheless, the observed diffusion coefficient (8.4×10^{-11} cm^2 s^{-1}) is rather similar to our estimate for the mobile fraction.

Drepper et al. (1993) used rapid biochemical fractionation to monitor the redistribution of phospho-LHCII between grana and stroma lamellae, and estimated a macroscopic (i.e. long-range) phospho-LHCII diffusion coefficient of 1.4×10^{-13} cm^2 s^{-1} based on these data. This estimate is more than two orders of magnitude lower than our measurement of the diffusion coefficient for the mobile fraction of chlorophyll fluorescence. Although ingenious, the method used by Drepper et al. (1993) is very indirect, and in particular it depends on the implicit assumption that the movement of phospho-LHCII from the grana to the stroma lamellae is essentially irreversible, rather than being a dynamic reequilibration between the two membrane environments. We suggest this as a likely cause of the discrepancy.

Effects of Macromolecular Crowding and Electrostatic Interactions

Granal membranes are densely packed with proteins, which occupy about 80% of the membrane area (Kirchhoff et al., 2002). The dense packing would be predicted to have complex effects on protein diffusion, including a drastic reduction in mobility due to macromolecular crowding (Saxton, 1989; Engelman, 2005; Jin and Verkman, 2007). By diluting the protein density in grana membranes with additional lipid we were able to directly explore the influence of macromolecular crowding on protein mobility. We find that lipid dilution increases the mobile fraction (from about 25% up to 75%) and also increases its diffusion coefficient by up to a factor of about 7. However, neither parameter is a straightforward function of protein density because there is a clear threshold effect. Lipid addition has little effect until a critical value is reached, at a ratio of added lipid:chlorophyll of about 4 (Fig. 4). We can estimate the change in protein density at this critical value. From lipid analysis by two-dimensional thin layer chromatography (Haferkamp and Kirchhoff, 2008) we estimate lipid contents of 1.3 mol/mol chlorophyll for native grana membranes and about 4 to 5 mol/mol chlorophyll for membranes fused at an added lipid:chlorophyll ratio of 4. Thus, the lipid content is roughly quadrupled at the critical point. Based on molecular protein areas and typical PSII and LHCII concentrations measured for our grana membrane preparations (Kirchhoff et al., 2002, 2004b) this increased lipid content corresponds to a decrease in protein area occupation from about 75% to 40% to 50%.

The threshold effect for lipid dilution suggests that diffusion of the majority of the protein complexes is impeded by clustering into larger assemblages. These assemblages remain stable and withstand solubilization by additional lipids up to the threshold value of about four added lipids per chlorophyll. Above this value, where the lipid area fraction in grana membranes

is roughly doubled, the assemblages are destabilized and the majority of protein in the membranes becomes mobile. It is interesting that functional analysis of light harvesting by chlorophyll fluorescence also reveals a threshold effect of lipid dilution, although in this case the threshold appears a little lower (Haferkamp and Kirchhoff, 2008). This suggests that dense packing of PSII and LHCII into the grana membranes plays a major role in maintaining their functional interaction in vivo.

We can roughly estimate the minimum size of the immobile assemblages, on the assumption that the diffusion coefficient is inversely proportional to the radius of the diffusing particle (Gambin et al., 2006). If the mobile fraction ($D = 4.6 \times 10^{-11}$ cm^2 s^{-1}; D, diffusion coefficient) represents trimeric LHCII (radius about 4 nm; Standfuss et al., 2005), and the maximum possible diffusion coefficient for the immobile fraction is 10^{-12} cm^2 s^{-1}, this translates to a minimal radius of about 180 nm for the immobile assemblages. This estimate suggests that the immobile fraction represents a cluster of many protein complexes rather than single LHCII-PSII supercomplexes.

To further explore the role of protein-protein interactions in restricting protein diffusion in grana membranes, we tested the effect of incubation in low-salt buffers. Under these conditions electrostatic screening of negative surface charges by cations is reduced, leading to destabilization of protein-protein interactions (Barber, 1982; Harrison and Allen, 1992; Kirchhoff et al., 2004a). In diluted membranes, protein mobility was markedly increased under these conditions (Fig. 4). In native grana membranes, by contrast, the effects were only small (Fig. 4). The results indicate that both crowding and protein-protein interactions play roles in maintaining the stability of the protein assemblages and restricting protein mobility. In native grana membranes, protein-protein interactions are presumably destabilized in low-salt buffer, but there is little effect on protein mobility unless there is sufficient free membrane area to allow dispersion.

Physiological Implications of Protein Mobility

Our results show that most of the protein in grana membranes has restricted diffusion at native protein density. However, there is a mobile fraction of protein, and the diffusion coefficient of this mobile protein (about 5×10^{-11} cm^2 s^{-1}) is surprisingly high for such a crowded membrane. From the diffusion coefficient we can estimate the escape time, that is, the time it takes for a protein complex to diffuse out of a granum and into the stroma lamellae. The escape time has considerable physiological relevance because it indicates the restrictions that molecular crowding may place on the kinetics of regulation of light harvesting, and the PSII repair cycle. From the Einstein diffusion equation, the mean time required to diffuse a given distance from the starting point is given by $X^2/4D$, where X is the distance and D the diffusion coefficient. Thus, the mean escape time for a protein in the center of the disc

would be about 2 s. This result contrasts very sharply with Monte Carlo simulations suggesting that PSII in stacked regions might require up to an hour to escape from the grana (Kirchhoff et al., 2004b). The simulations were based on a random distribution of particles (Kirchhoff et al., 2004b), so the contrast with the experimental finding indicates that diffusion of a proportion of the protein may be facilitated by large-scale organization of the complexes. Aggregation of a large proportion of the protein may leave open areas or channels allowing relatively rapid diffusion of the mobile fraction of protein complexes. These channels may also facilitate diffusion of the lipid-soluble electron carrier plastoquinone, which is essential for photosynthetic electron transport (Kirchhoff et al., 2002).

The organization of grana into many small discs is probably another adaptation to allow faster exchange of proteins with the stroma lamellae. Larger grana would lead to a very significant increase in the escape time. For example, if the grana were 5 μm in diameter (about the diameter of a typical chloroplast), a diffusion coefficient of 5×10^{-11} cm^2 s^{-1} would lead to an escape time of more than 5 min for a protein in the center of the disc.

CONCLUSION

Diffusion of much of the protein in grana membranes is strongly restricted by a combination of macromolecular crowding and protein-protein interactions. However, there is a pool of mobile protein that diffuses rapidly enough to escape from the grana within a few seconds. Thus, rapid, diffusion-based exchange of proteins between the grana and the stroma lamellae is possible. This is likely to be crucial for rapid regulation of light harvesting, and efficient PSII repair.

MATERIALS AND METHODS

Membrane Preparation

Grana (BBY) membranes were isolated from spinach (*Spinacia oleracea*), according to Schiller and Dau (2000) with some modifications as described in Haferkamp and Kirchhoff (2008). The pellet was finally resuspended in buffer containing 1 M betaine, 25 mM MES (pH 6.2), 15 mM NaCl, 5 mM MgCl$_2$, and 5 mM CaCl$_2$. Large unilamellar liposomes with the native grana lipid composition (Duchêne and Siegenthaler, 2000) were prepared as described in Webb and Green, 1989. BBY membranes (100 μg of total chlorophyll per 1 mL final concentration) were mixed with different amounts of liposomes in 10 mM HEPES (pH 7.6) and 0.3 mM MgCl$_2$. leading to lipid:chlorophyll ratios (w:w) of 2:1, 4:1, 7:1, 10:1, and 20:1. Fusion of the two membranes was induced by 30-s sonication (20-W, model B–12; Branson Sonic Power Company) on ice and a freeze-thaw cycle (Haferkamp and Kirchhoff, 2008). Incorporation of lipids was monitored by Ficoll equilibrium density gradient centrifugation (Haferkamp and Kirchhoff, 2008).

Sample Preparation for FRAP

Twenty microliters of 2,2,2-trifluoroethanol (Sigma-Aldrich) containing 10 mM PC (Sigma-Aldrich) and 10 μM 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid (BODIPY FL C-12; Invitrogen; Haugland, 2005) was spotted onto a glass slide. The lipid film was dried under a stream of nitrogen for 1 h and then rehydrated in anaerobic buffer (5 mM MgCl$_2$, 40 mM NaCl, 30 mM MES [pH 6.5], 11 units mL^{-1} Glc oxidase, 800 units/mL catalase, and 4 mM Glc) at 40°C for 2 min. This generated stacks of PC bilayers labeled

with BODIPY FL C-12 (see Fig. 1, left). Thirty microliters of membrane suspension in anaerobic buffer at a chlorophyll concentration of 10 μg mL^{-1} were placed on the glass-PC support, incubated for at least 10 min in the dark, sealed with a coverslip, and used for FRAP measurements.

FRAP Measurements

FRAP measurements were carried out with a Nikon PCM2000 laser-scanning confocal microscope equipped with a 100-mW Argon laser (Spectra-Physics). The 488-nm line of the Argon laser was selected for excitation. BODIPY FL C-12 and chlorophyll emission were detected simultaneously. BODIPY FL C-12 emission was selected with a 505-nm dichroic mirror and an interference band-pass filter transmitting between about 500 and 527 nm. Chlorophyll emission was selected with a Schott RG665 red-glass filter transmitting wavelengths longer than about 665 nm. For FRAP, a line was bleached across the sample by switching from XY-scanning mode to X-scanning mode, and increasing the laser power by a factor of 32 by withdrawing neutral density filters. After 1 to 2 s, the laser power was reduced again, the microscope was switched back to XY-scanning and a series of 20 postbleach images was recorded, typically at 3-s intervals with five additional images at 60-s intervals.

Mathematical Analysis

Analytical estimation of diffusion parameters is impracticable because the dimensions of the membrane patch, and the placement and depth of the bleach, are different in every measurement. We therefore used a computational method for estimating the diffusion coefficient. The method uses a one-dimensional approximation. One-dimensional fluorescence profiles were extracted from each image in the Y-direction (i.e. perpendicular to the line-bleach), summing fluorescence across the membrane patch in the X-direction (see Figs. 2 and 3). Because the membrane patches are small, bleaching results in a significant decrease in the total fluorescence from the sample. To compare fluorescence distributions before and after the bleach, the profiles were normalized to the same total fluorescence. The postbleach profiles were then subtracted from the prebleach profile to generate a set of difference profiles. The mobile fraction was estimated by comparing the first postbleach profile with the profiles obtained at the longest time points. To estimate the diffusion coefficient, the first postbleach difference profile was taken, and an iterative computer routine was used to predict its evolution due to random diffusion, assuming an arbitrary diffusion coefficient. For the mobile fraction of fluorescence the incremental change in fluorescence at each pixel (δF_n) was estimated according to:

$$\delta F_n = D \cdot P^{-2} \cdot \delta t \cdot (F_{n+1} + F_{n-1} - 2F_n)$$

where F_n is the fluorescence value at the nth pixel, δt is a small time increment, P is the pixel width, and D is the assumed value for the diffusion coefficient. The program generates a series of predicted fluorescence profiles at various times after the bleach. From this, a predicted fluorescence recovery curve at the center of the bleach can be extracted. To obtain an estimate for D, the predicted fluorescence recovery curve was fitted to the experimentally observed fluorescence recovery curve by adjusting the time axis.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Comparison of FRAP analysis of control and diluted membranes with a similar bleaching efficiency.

Supplemental Figure S2. Dependency of the immobile fraction and the diffusion coefficient on the bleaching efficiency.

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