Mobility of the IsiA Chlorophyll-binding Protein in Cyanobacterial Thylakoid Membranes*

Mary Sarcina and Conrad W. Mullineaux‡

From the Department of Biology, University College London, Darwin Building, Gower Street, London WC1E 6BT, United Kingdom

We are using fluorescence recovery after photobleaching (FRAP) to probe the dynamics of thylakoid membranes in vivo in cells of the cyanobacterium Synechococcus sp. PCC7942. We have shown previously that the light-harvesting phycobilisomes diffuse quite rapidly on the thylakoid membrane surface. However, the photosystem II core complexes appear completely immobile. This raises the possibility that all of the membrane integral protein complexes in the thylakoid membrane are locked into a rather rigid array. Alternatively, it is possible that photosystem II is specifically anchored in the membrane, with other membrane proteins able to diffuse around it. We have now resolved this question by studying the diffusion of a second integral membrane protein, the IsiA chlorophyll-binding protein. IsiA is induced in starvation to produce some other unidentified component. In iron-stressed cyanobacterial cells, a high proportion of chlorophyll fluorescence comes from IsiA. This makes it straightforward to examine the diffusion of IsiA by FRAP. We find that the complex is mobile with a mean diffusion coefficient of $-3 \times 10^{-11}$ cm$^2$ s$^{-1}$. Thus it is clear that some thylakoid membrane proteins are mobile and that there must be a specific anchor that prevents photosystem II diffusion. We discuss the implications for the structure and function of the cyanobacterial thylakoid membrane.

In plants and cyanobacteria the light reactions of photosynthesis are mediated by protein complexes in the thylakoid membranes. Considerable information is now available on the structure and function of these complexes (1–3). A full understanding of thylakoid membrane function will also require knowledge of the dynamics of the membrane in vivo. The diffusion of proteins in the thylakoid membrane is likely to play a crucial role in processes including photosynthetic electron transport, the regulation of photosynthesis, and the assembly and turnover of the photosynthetic complexes (4–6).

We have been using the cyanobacterium Synechococcus sp. PCC7942 (Synechococcus 7942) as a model system for investigating thylakoid membrane dynamics in vivo (7–9). Our technique of choice is fluorescence recovery after photobleaching (FRAP), which can be used to observe the diffusion of fluoro-

cently tagged membrane components. A laser-scanning confocal microscope is used to bleach fluorescence in a small region of the cell, and the subsequent changes in the bleaching pattern give quantitative information on the diffusion of the fluorophore (10, 11). Synechococcus 7942 has a simple, regular thylakoid membrane organization that makes it ideal for such studies (11), and we have developed a one-dimensional variant of FRAP that has enabled us to measure the diffusion of several thylakoid membrane components in vivo (11–13).

Our previous FRAP studies have shown the mobility of some thylakoid membrane components, including a fluorescently tagged lipid analogue (8) and the light-harvesting phycobilisomes, which diffuse rather rapidly on the membrane surface (7, 9, 12). We have looked previously at the diffusion of only one integral membrane protein complex. This is the photosystem II reaction center, which is amenable to FRAP measurements because its chlorophylls fluoresce significantly at room temperature. Surprisingly, photosystem II appears completely immobile even over long time scales (7), a situation that is very unusual for a membrane protein (14). Two explanations, outlined below, are possible.

The first possibility is that photosystem II is specifically anchored in the membrane. In eukaryotic plasma membranes this situation can arise because of the binding of membrane proteins to components of the cytoskeleton (14). In cyanobacterial thylakoid membranes it is more plausible that photosystem II is rendered immobile by oligomerization into rows of PSII dimers (15) and/or because of the presence of a large domain in the thylakoid lumen (16). The lumen appears to be an extremely crowded environment (17), and, thus, it is possible that protein diffusion in the lumen is greatly restricted.

A second possibility would be that all of the integral membrane protein complexes in the thylakoid membrane are locked into a rather rigid array. Such a situation would be very different from that normally found in eukaryotic plasma membranes (10, 14). However, thylakoid membranes have a different lipid composition and an exceptionally high ratio of protein to lipid (18). Thylakoid membrane lipids are mobile, although their diffusion is slower than that typically found in eukaryotic plasma membranes (8). The lipids could, however, be percolating through a rigid matrix of proteins.

In the present study we resolve this question by observing the diffusion of a second integral membrane thylakoid protein. This is the IsiA protein, which is induced under iron starvation or under oxidative stress conditions (19). IsiA (also called CP43) is a chlorophyll-binding protein whose sequence shows some similarity with that of the CP43 protein of photosystem II (20). During iron deficiency this protein is expressed at a high level and is present in the cells in at least two forms. Some IsiA appears to be free in the membrane, while it may act as a chlorophyll reservoir (19, 20). Some IsiA is bound to photosystem I as a ring of IsiA subunits surrounding the photosystem I.

This paper is available online at http://www.jbc.org

* This work was supported by grants from the Biotechnology and Biological Sciences Research Council and the Wellcome Trust (to C. W. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 44-20-7679-2326; Fax: 44-20-7679-7096; E-mail: c.mullineaux@ucl.ac.uk.

‡ The abbreviations used are: FRAP, fluorescence recovery after photobleaching; PSI, photosystem I; PSII, photosystem II.
trimer (21, 22). The association of IsiA with photosystem II is also a possibility, because there is evidence that IsiA can quench excess PSII excitation (23). IsiA is characterized by strong chlorophyll fluorescence at ∼680–685 nm (20–23), most of which is likely to come from the IsiA that is free in the membrane. The fluorescence of the IsiA that is bound to photosystem I is strongly quenched by efficient energy transfer to the chlorophylls of the photosystem I core complex (22, 24).

The high fluorescence from IsiA makes it amenable to FRAP studies. Here we show that at least the highly fluorescent pool of IsiA is freely mobile in the membrane. We discuss the implications for thylakoid membrane structure and function.

**EXPERIMENTAL PROCEDURES**

**Growth of Cells—**Wild-type cells of *Synechococcus* sp. PCC 7942 were grown in BG11 medium (25) supplemented with 10 mM NaHCO3. Liquid cultures were grown in an orbital shaking incubator at 30 °C with white illumination at ∼10 microeinsteins m−2 s−1. To induce IsiA expression, cells were harvested by centrifugation and washed and inoculated in iron-free BG11 medium. Liquid cultures were grown for at least 8–10 days under iron limitation (26).

**Spectroscopy—**Optical absorption spectra were recorded at room temperature with an Amino DW2000 spectrophotometer. Fluorescence emission spectra were recorded with a PerkinElmer LS50 luminescence spectrometer equipped with a red-sensitive photomultiplier. Chlorophyll excitation was at 435 nm, and the excitation and emission slit widths were 5 nm. Room temperature fluorescence spectra were recorded using cell suspensions at a chlorophyll concentration of 23 μg/ml in 3-ml cuvettes. Spectra were normalized to the optical density of the cell suspension at 750 nm. Low temperature fluorescence spectra were recorded at 77 K using a liquid nitrogen sample holder. Cell suspensions at a chlorophyll concentration of 2–3 μg/ml were injected into quartz capillary tubes (internal diameter 2.5 mm) and frozen by dipping them into liquid nitrogen. 77 K spectra were normalized to the PSI emission peak because their absolute amplitudes are unreliable. Oxygen evolution was measured at 30 °C in a Clark-type oxygen electrode (OxyLab2 from Hansatech, King's Lynn, UK) with saturating illumination from an array of red light-emitting diodes. Cell densities were estimated from optical density at 750 nm measured in a Unicam UV2 spectrophotometer (Unicam, Cambridge, UK). The estimate was calibrated using a hemacytometer. Chlorophyll a concentrations were determined from methanol extracts using the extinction coefficient of Porra (27). FRAP Measurements—FRAP experiments were carried out using a Nikon PCM2000 laser-scanning confocal microscope (Nikon, Tokyo, Japan) equipped with a 50 milliwatt argon laser. The 457-nm laser line was selected using a 475-nm dichroic mirror. A 60× oil immersion objective lens was used with a 50-μm confocal pinhole. Fluorescence emission was selected with a Schott RG665 red glass filter transmitting light of wavelengths longer than 665 nm. Cell suspensions were immobilized by adsorption onto 1.5% agar made up in growth medium. The fluorescence of the IsiA that is bound to photosystem I core complex (22, 24).

**RESULTS**

**Expression of IsiA—**IsiA expression can be induced by iron starvation and typically results in a blue shift of the chlorophyll Qa absorption band accompanied by greatly increased fluorescence at 680–685 nm (20, 26). After 8 days of iron starvation we observed a shift in the chlorophyll Qa absorption band from 680 to 672 nm (Fig. 1). Fig. 2 compares room temperature fluorescence spectra from iron-starved and iron-replete cells. The excitation wavelength is 435 nm, and emission spectra are normalized on the basis of cell density. In iron-replete cells, the main emission peak comes from the chlorophylls of PSII at ∼684 nm. Iron starvation results in greatly increased chlorophyll fluorescence per cell and a slight blue shift in the emission maximum from 684 to 680 nm (Fig. 2). Fig. 3 compares 77 K fluorescence emission spectra for iron-stressed and iron-replete cells. The cellular content of phycobilisomes (indicated by the phycocyanin absorption peak at 625 nm) was slightly reduced after iron stress, but the cells retained significant levels of phycobilisomes (Fig. 1).

**Photosynthetic Performance of Iron-stressed Cells—**To assess whether the iron-stressed cells retained normal photosynthetic function, we measured the light-saturated rate of oxygen evolution. Expressing oxygen evolution on a per cell basis, the rates were 7.5 fmol of O2 per cell per hour for iron-stressed cells and 10.8 fmol of O2 per cell per hour for iron-replete cells. Therefore, the iron-stressed cells retained significant levels of whole-chain photosynthetic electron transport, although their performance was a little lower than that of the iron-replete cells.

**FRAP Measurements on Iron-replete Cells—**In iron-replete cyanobacterial cells, most fluorescence at room temperature comes from photosystem II when the excitation light is at a wavelength absorbed by chlorophyll rather than by the phycobilisins. Hence, FRAP measurements recorded with chlorophyll excitation and emission in the red (>665 nm) report on the mobility of photosystem II (12). We have shown previously that photosystem II appears completely immobile even on a time scale of 20 min in the cyanobacterium *Dactylococcopsis salina* (12) and that no photosystem II diffusion can be detected on short time scales in *Synechococcus* 7942 (7). Fig. 4 shows a typical long term FRAP measurement on a cell of *Synechococ-
We bleached a line across the cell by scanning the confocal spot repeatedly in the \( x \) direction across the center of the cell (11, 12). This generated a bleached line whose profile is approximately Gaussian (12, 13) with a half-width \( (1/e^2) \) of 0.67 \( \mu \)m. Approximately 85% of the fluorescence was bleached at the center of the line, whereas repeated imaging led to no significant bleaching in the remainder of the cell. The images show no obvious recovery of the bleach during the 30-min time course of the measurement (Fig. 4). By contrast, when the light-harvesting phycobilisomes are monitored there is substantial fluorescence recovery on a time scale of a few seconds (7, 9, 12). The bleach depth in Fig. 4 was estimated by extracting fluorescence profiles and fitting Gaussian curves as described under “Experimental Procedures.” The bleach depth (expressed as the fraction of the original fluorescence bleached) was initially 0.85 \( \pm \) 0.02, and after 30 min it was 0.82 \( \pm \) 0.02. Thus, there was no significant recovery of the bleach even after 30 min and, hence, no detectable diffusion of photosystem II (Fig. 4). The fluorescence recovery that would be expected for a given value of \( D \) in the FRAP measurement shown in Fig. 4 can be predicted by using the one-dimensional diffusion equation (12) and the observed half-width of the bleach. A diffusion coefficient of \( 10^{-12} \) cm\(^2\) s\(^{-1}\) would lead to \( \sim \)50% fluorescence recovery in the center of the bleach in 30 min, whereas a diffusion coefficient of \( 2 \times 10^{-13} \) cm\(^2\) s\(^{-1}\) would lead to \( \sim \)10% recovery in 30 min. We can be confident that fluorescence recovery after 30 min was below 10% and, therefore, that the diffusion coefficient for PSII is below \( 2 \times 10^{-13} \) cm\(^2\) s\(^{-1}\).

FRAP Measurements on Iron-stressed Cells—When IsiA is strongly expressed, most of the cell fluorescence at room temperature comes from IsiA when chlorophyll is excited (Fig. 2). Therefore, to a first approximation the confocal fluorescence micrographs recorded with chlorophyll excitation under these conditions show the location of IsiA. This allows us to use FRAP to probe the mobility of IsiA. We generally grow cells of *Synechococcus* 7942 in 0.5% dimethyl sulfoxide for FRAP measurements because this leads to significant cell elongation without detectable perturbation of photosynthetic function (9). Measurements of the diffusion coefficient are more accurate in elongated cells (12). In these measurements, however, we did not need to use dimethyl sulfoxide because a significant proportion of the iron-stressed cells were already elongated. Fig. 5 shows images taken from a typical FRAP sequence recorded for an iron-stressed cell with excitation at 457 nm. A significant spread and recovery of the bleach can be observed on a time scale of \( \sim 1-3 \) min, indicating diffusion of IsiA. IsiA diffusion coefficients were obtained from such image sequences as those described previously (9, 12, 13). IsiA diffusion coefficients were quite uniform in all of the cells measured. The mean diffusion coefficient was \( (3.4 \pm 0.8) \times 10^{-11} \) cm\(^2\) sec\(^{-1}\). There was no indication of an immobile fluorescent fraction, and fluorescence recovery appears nearly complete after \( \sim 3 \) min (Fig. 5). Thus, we conclude that essentially all of the highly fluorescent IsiA population is mobile.
DISCUSSION

The FRAP data presented in this report were obtained by exciting the cells with blue light absorbed mainly by chlorophyll a while monitoring the emission from chlorophyll a in the red region of the spectrum. In iron-replete cells of Synechococcus 7942, fluorescence emission under these conditions is dominated by PSI (Fig. 2). In iron-replete cells fluorescence emission per cell is much higher and is dominated by fluorescence from IsiA (Fig. 2). Therefore, we can use FRAP to monitor the mobility of PSI in iron-replete cells and IsiA in iron-stressed cells. The IsiA present in the thylakoid membranes of iron-stressed cells is likely to be present in several pools, namely free IsiA, IsiA complexed with PSI (21, 22), and possibly also IsiA complexed with PSI, where it may act as a quencher of excess excitation (23). When IsiA is complexed with reaction centers, its fluorescence is strongly quenched because of energy transfer (22, 24). Therefore, our fluorescence measurements in iron-stressed cells report mainly on the pool of IsiA that is free in the membrane rather than specifically complexed to reaction centers. For our purposes, this pool of free IsiA provides a convenient fluorescent probe that allows us to assess the extent to which an integral membrane protein is free to diffuse in the thylakoid membrane.

Previous work on the cyanobacterium D. salina showed that PSI is completely immobile with no diffusion detectable even on long time scales (12). The present study shows that the situation is similar in iron-replete cells of Synechococcus 7942, i.e. no diffusion is detectable even after 30 min, indicating that the diffusion coefficient for PSI must be below 2 \( \times 10^{-13} \text{ cm}^2 \text{ s}^{-1} \) (Fig. 4). This situation is quite unusual for a membrane protein. Most quantitative data for the mobility of membrane proteins in vivo come from eukaryotic plasma membranes. In these membranes, proteins typically have diffusion coefficients in the range of \( 10^{-10} \) to \( 10^{-9} \text{ cm}^2 \text{ s}^{-1} \) (14). Some proteins have diffusion coefficients slower than this, probably because they are anchored or trapped by the cortical cytoskeleton (14). Operationally, proteins with diffusion coefficients below \( 1 - 5 \times 10^{-12} \text{ cm}^2 \text{ s}^{-1} \) are generally defined as immobile (14), and PSI clearly falls into this category. This raises the question as to whether the extreme immobility of photosystem II is a specific property of this complex or whether all of the integral membrane proteins in the thylakoid membrane are locked into a rigid array.

Chlorophyll FRAP measurements on iron-stressed cells show considerable spread and recovery of the bleach on a time scale of a few minutes (Fig. 5). Because chlorophyll fluorescence in these cells is dominated by IsiA (Fig. 2), we conclude that IsiA is mobile with a diffusion coefficient of \( 3 \times 10^{-11} \text{ cm}^2 \text{ s}^{-1} \). Because PSI fluorescence in iron-stressed cells is low compared with IsiA fluorescence, we cannot say whether PSI remains immobile under these conditions, and we have no information on the mobility of other complexes such as PSI.

Compared with a typical eukaryotic plasma membrane protein, IsiA diffusion is slow (14). This may be a consequence of the very crowded environment of the thylakoid membrane with its dense packing of protein complexes (28) and very high ratio of protein to lipid (18). Nevertheless, it is clear that under these conditions the integral thylakoid membrane proteins are not all locked into a rigid matrix preventing diffusion. Photosynthetic function in our iron-stressed cells remains relatively normal, indicating that there is no major loss of photosynthetic complexes; the thylakoid membranes of iron-stressed cells are likely to be as densely packed with protein as the thylakoid membranes of iron-replete cells.

The mobility of IsiA indicates that the immobility of PSI has a specific cause rather than being a general property of all thylakoid membrane proteins. The intact PSIII complex is considerably larger than IsiA, but this cannot account for such an extreme difference in diffusion coefficient. One possibility is that PSI may be immobilized by large scale oligomerization because it is often found arranged as rows of dimers in cyanobacterial thylakoid membranes (15). Alternatively, it is possible that PSI is immobilized by crowding and/or specific interactions in the thylakoid lumen. PSI has a large domain on the luminal side of the membrane mainly composed of the water-oxidizing complex and associated proteins (16). By contrast, IsiA has only minor hydrophilic loops in the lumen (20). The lumen is crowded with protein (17), so it is possible that PSI may be immobilized by its luminal domain. In fact, the most obvious difference between IsiA and the homologous CP43 protein of PSI is that the largest hydrophilic luminal loop of CP43 is greatly truncated in IsiA (20). Burnap et al. postulated that the loss of this loop might facilitate migration of IsiA throughout the thylakoid membrane system and that this might be important for a presumed function as a chlorophyll donor during the assembly of new reaction centers (20).

Our results indicate that thylakoid membrane proteins can be mobile, at least if they do not have major luminal domains. This is particularly important when considering possible mechanisms for the assembly and turnover of the photosystems. A rigid, immobile array of thylakoid proteins would imply that all turnover, repair, and biosynthesis of the integral membrane protein complexes would have to occur in situ and could be mediated only by soluble proteins. There is evidence to suggest that the initial stages of synthesis of the reaction centers may occur in the plasma membrane rather than the thylakoid membrane (29). The mechanism by which nascent photosystems could be transferred to the thylakoid membranes is not known, because the nature of any connections between the plasma and thylakoid membranes is not clear (28). However, our results indicate that it is plausible that repaired or newly synthesized reaction centers could diffuse within the thylakoid membrane system to reach their eventual locations. Alternatively, membrane-bound proteins such as FtsH, which is known to be involved in the PSIII repair cycle (30), could diffuse to mediate turnover and repair in situ.

Acknowledgments—We thank Santiago Garcia for excellent technical support, including the construction of the sample holder used for the FRAP measurements. We also thank Dr. Anne-Lise Etienne for helpful discussions.

REFERENCES

1. Jordan, P., Fromme, P., Witt, H. T., Klukas, O., Saenger, W., and Krauss, N. (2001) Nature 411, 899–917
2. Kurisu, G., Zhang, H., Smith, J. L., and Cramer, W. A. (2003) Science 302, 1009–1014
3. Ferreira, K. N., Iverson, T. M., Maglhaoui, K., Barber, J., and Iwata, S. (2004) Science 303, 1831–1838
4. Kirchhoff, H., Mukherjee, U., and Gall, H.-J. (2002) Biochemistry 41, 4872–4882
5. Allen, J. F., and Forsberg, J. (2001) Trends Plant Sci. 6, 317–326
6. Baena-Gonzalez, E., Barbato, R., and Ara, E.-M. (1999) Planta 208, 196–204
7. Saracina, M., Tobin, M. J., and Mullineaux, C. W. (2001) J. Biol. Chem. 276, 46330–46854
8. Saracina, M., Murata, N., Tobin, M. J., and Mullineaux, C. W. (2003) FEBS Lett. 553, 295–298
9. Aspinwall, C. L., Saracina, M., and Mullineaux, C. W. (2004) Photosynth. Res. 79, 179–187
10. Kubitscheck, U., Wedekind, P., and Peters, R. (1994) Biochim. Biophys. Acta 79, 948–956
11. Mullineaux, C. W., and Saracina, M. (2002) Trends Plant Sci. 7, 237–240
12. Mullineaux, C. W., Tobin, M. J., and Jones, G. B. (1997) Nature 390, 421–424
13. Mullineaux, C. W. (2004) J. Exp. Bot. 55, 1207–1211
14. Zhang, P., Lee, G. M., and Jacobson, K. (1993) BioEssays 15, 579–588
15. Olive, J., Ajlani, G., Astier, C., Recouvreur, M., and Vernet, C. (1997) Biochim. Biophys. Acta 1319, 275–292
16. Zouni, A., Witt, H.-T., Kern, J., Fromme, P., Krauss, N., Saenger, W., and Orth, P. (2001) Nature 409, 739–743
17. Albertsson, P.-A. (2001) Trends Plant Sci. 6, 349–354
18. Szalontai, B., Nishiyama, Y., Gombos, Z., and Murata, N. (2000) Biochim. Biophys. Acta 1509, 409–419
19. Michel, K.-P., and Pistorius, E. K. (2004) *Physiol. Plant.* **120**, 36–50
20. Burnap, R. L., Truan, T., and Sherman, L. A. (1993) *Plant Physiol.* **103**, 893–902
21. Boekema, E. J., Hifney, A., Yakushevska, A. E., Piotrowski, M., Keegstra, W., Berry, S., Michel, K. P., Pistorius, E. K., and Kruip, J. (2001) *Nature* **412**, 745–748
22. Bibby, T. S., Nield, J., and Barber, J. (2001) *Nature* **412**, 743–745
23. Sandstrom, S., Park, Y. I., Oquist, G., and Gustafsson, P. (2001) *Photochem. Photobiol.* **74**, 431–437
24. Andrizhiyevskaya, E. G., Schwabe, T. M. E., Germano, M., D’Haene, S., Kruip, J., van Grondelle, R., and Dekker, J. P. (2002) *Biochim. Biophys. Acta* **1556**, 265–272
25. Castenholz, R. W. (1988) *Methods Enzymol.* **167**, 68–93
26. Sandstrom, S., Ivanov, A. G., Park, Y. I., Oquist, G., and Gustafsson, P. (2002) *Physiol. Plant.* **116**, 255–263
27. Porra, R. J., Thompson, W. A., and Kriedeman, P. E. (1989) *Biochim. Biophys. Acta* **975**, 384–394
28. Mullineaux, C. W. (1999) *Aust. J. Plant Physiol.* **26**, 671–677
29. Zak, E., Norling, B., Maitra, R., Huang, F., Andersen, B., and Pakrasi, H. B. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 13443–13448
30. Silva, P., Thompson, E. P., Bailey, S., Kruse, O., Mullineaux, C. W., Robinson, C., Mann, N. H., and Nixon, P. J. (2003) *Plant Cell* **15**, 2152–2164
