Lipid diffusion in the thylakoid membranes of the cyanobacterium *Synechococcus* sp.: effect of fatty acid desaturation

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Abstract Thylakoid membranes are crucial to photosynthesis in cyanobacteria and plants. In cyanobacteria, genetic modification of membrane lipid composition strongly influences cold tolerance and susceptibility to photoinhibition. We have used fluorescence recovery after photobleaching to measure the diffusion of a lipid-soluble fluorescent marker in cells of the cyanobacterium *Synechococcus* sp. PCC 7942. We have compared the wild-type strain with a transformant with an increased level of fatty acid unsaturation. The transformant showed a six-fold increase in the diffusion coefficient for the fluorescent marker at growth temperature. This is the first direct measurement of lipid diffusion in a photosynthetic membrane.

Key words: Cyanobacterium; Thylakoid membrane; Photosynthesis; Lipid; Diffusion; Membrane fluidity

1. Introduction

The thylakoid membranes of cyanobacteria and chloroplasts are the site of photosynthetic energy conversion. They contain the photosynthetic reaction centres and light-harvesting complexes, electron transport complexes and the proton-translocating ATPase. During photosynthetic electron transport, a proton gradient is generated across the thylakoid membrane, which is used to power ATP synthesis. See [1] for a general introduction to the thylakoid membranes of cyanobacteria. In recent years, considerable progress has been made in determining the structures of cyanobacterial protein complexes in the thylakoid membrane [2]. Cyanobacteria have also proved to be an excellent material for the genetic manipulation of photosynthesis [3]. Genetic approaches have been used to manipulate thylakoid membrane proteins, and also the lipid composition of the thylakoid membranes [4,5]. A transformant of the cyanobacterium *Synechococcus* 7942 has been generated by integrating into the chromosome a copy of the *des*A gene for Δ12 fatty acid desaturase from another cyanobacterium, *Synechocystis* 6803. The *des*A$^+$-transformed cells contain about 20% diunsaturated fatty acids, in contrast to the wild-type, in which diunsaturated fatty acids are absent [6]. In terms of molecular species composition, about 40% of glycerolipids in *des*A$^+$ cells are 16:2/16:0 or 18:2/16:0, whereas both species are absent in wild-type cells [6]. The change in membrane composition results in a faster rate of recovery from photoinhibition [6,7] and the maintenance of photosystem (PS) II activity at low temperatures [7]. Both these processes require the turnover of the D1 protein of PS II [6,7], and it can be postulated that fatty acid unsaturation results in greater membrane fluidity, and that this facilitates the turnover of the D1 protein.

We have little direct information on the dynamics of thylakoid membranes. Fluorescence recovery after photobleaching (FRAP) has been widely used to study the dynamics of eukaryotic plasma membranes. The membrane component of interest is tagged with a fluorophore. A highly focused confocal laser spot is used to bleach the fluorophore in a small area of the membrane. The diffusion of the fluorophore is then measured by observing the recovery of fluorescence in the bleached membrane area [8,9]. Cyanobacterial cells have proved to be a better model system for FRAP than chloroplasts because many species have a regular thylakoid membrane organisation [1], in contrast to the usually convoluted structure of chloroplast thylakoid membranes [10]. Some photosynthetic complexes are naturally highly fluorescent, and specific complexes can be detected by using the appropriate combinations of excitation and emission wavelengths [11]. We have used a one-dimensional form of FRAP in the cyanobacteria *Dactylococcopsis salina* and *Synechococcus* 7942 to show that PS II does not diffuse, but the phycobilisomes (light-harvesting complexes attached to the cytoplasmic surface of the thylakoid membrane) are very mobile [9,11,12]. In this one-dimensional form of FRAP, the confocal spot is used to bleach a line across the cell, and fluorophore diffusion in one dimension along the long axis of the cell is measured [11,12]. *Synechococcus* 7942 has an elongated cylindrical shape, with multiple concentric thylakoid membrane cylinders running parallel to the long axis of the cell [13]. Cells are about 3 μm long on average but can be elongated to up to 15 μm by growth in the presence of cell division inhibitors [14] making them an ideal system for FRAP measurements. Other commonly used cyanobacteria are less suitable for FRAP: for example, *Synechocystis* 6803 has round cells and a rather chaotic thylakoid membrane structure [15].

Here we report the use of FRAP to measure the mobility of lipids in the membranes of *Synechococcus* 7942. We have used BODIPY FL C_{12} (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-dia-

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Abbreviations: BODIPY, BODIPY FL C_{12}, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-5-indacene-3-dodecanoic acid; FRAP, fluorescence recovery after photobleaching; PS II, photosystem II

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za-5-indacene-3-dodecanoic acid; BODIPY) as a fluorescent probe for the measurements. This molecule has a hydrophilic green-fluorescent fluorophore attached to a 12-carbon fatty acid chain [16]. The fluorescence of the chromophore does not overlap with that of the photosynthetic pigments. We have compared the BODIPY diffusion in wild-type and *des*A$^+$ transformant cells for *Synechococcus* 7942. We find that the increased level of fatty acid unsaturation in *des*A$^+$ cells [6] leads to much more rapid diffusion of the BODIPY probe, indicating a more fluid membrane. This may account for the enhanced tolerance of *des*A$^+$ cells to high light and low temperatures [6,7].

2. Materials and methods

2.1. Growth of cells

Wild-type and *des*A$^+$ cells of *Synechococcus* sp. PCC 7942 were grown in BG11 medium [17] supplemented with 10 mM $NaHCO_3$. Liquid cultures were grown in an orbital shaking incubator at 30°C with white illumination at about 10 μE/m^2/s. The growth medium for *des*A$^+$ cells was supplemented with kanamycin at 50 μg/ml.

2.2. TBZ treatment

Prior to FRAP measurements, cells were grown for approximately 16 h in the presence of thiobendazole (TBZ) at 30 μg/ml. This treatment was found to inhibit cell division, thereby increasing cell length, without impairing the photosynthetic function of the cells [14].

2.3. BODIPY labelling

BODIPY FL C_{12} [16] was purchased from Molecular Probes (Eugene, OR, USA). A 10 mM stock solution in dimethyl sulphoxide was added to a cell suspension (about 2 μM chlorophyll) to give a final concentration of 1 μM. Cells were incubated for approximately 30 min in the presence of BODIPY, then harvested by centrifugation at room temperature and washed several times in fresh growth medium to remove unincorporated dye.

2.4. Fluorescence microscopy

Fluorescence microscopy was carried out using an Axiophot confocal microscope (Zeiss) equipped with a mercury lamp (HBO 100). Images were acquired using a digital black and white camera (Hamamatsu) and Openlab 2.5 software (Improvision). Chlorophyll and BODIPY fluorescence emissions were selected using red (590 nm long-pass) and green (515–565 nm) filters, respectively.

2.5. FRAP measurements

FRAP experiments were carried out at CLRC Daresbury Laboratory (Warrington, Cheshire, UK) using the scanning confocal microscope Syclops [11] with a 488-nm argon laser. Cells were spread on 1.5% agar containing BG11 growth medium, covered with a glass coverslip and placed on a temperature-controlled stage under the microscope objective lens. A 40× oil immersion lens (numerical aperture 1.3) was used with 20-μm pinholes to create a confocal spot with full width at half maximum dimensions of about 0.7 μm in the *z*-direction and 0.23 μm in the *xy* plane.

Fluorescence was selected using a combination of Schott OG530 and Ealing 35-5362 filters, transmitting between about 520 nm and 545 nm. Cells aligned in the *y*-direction were selected. The confocal spot was scanned for about 1 s in the *x*-direction across the middle of the cell to create a line bleach. The confocal spot was then scanned in the *xy* plane to record a sequence of images of the cell at 3-s intervals.

2.6. FRAP data analysis

Images were analysed using Optimas software (Optimas Corporation). A one-dimensional bleaching profile was extracted by integrating across the cell in the *x*-direction. The baseline fluorescence from the unbleached cell was subtracted, and the bleaching profile was then fitted to a Gaussian curve using SigmaPlot (Jandel Scientific) to obtain a measurement of the bleach depth [11]. Diffusion coefficients were obtained by plotting maximum bleach depth versus time, using the equation $C_{(t)} = C_0\ R_0\ (R_0^2+8Dt)^{-1/2}$, where $C_{(t)}$ is the bleach depth at time t, C_0 is the initial bleach depth, R_0 is the initial half width ($1/e^2$) of the bleach and D is the diffusion coefficient. The equation describes one-dimensional diffusion in the case where the initial bleaching profile has a Gaussian form [11]. At least three measurements were made at each temperature, and diffusion coefficients are presented as mean values, with standard errors.

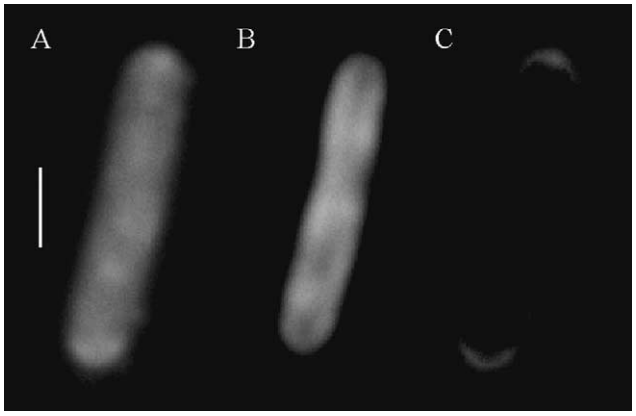

Fig. 1. Fluorescence micrographs showing the localisation of the BODIPY dye in a *Synechococcus* 7942 cell. A: BODIPY fluorescence. B: Chlorophyll fluorescence, showing the thylakoid membranes. C: Difference image (A−B). The scale bar represents 1 μm.

3. Results

3.1. Location of BODIPY in the cell

We have used the green fluorescent membrane probe BODIPY FL C_{12} [16] to investigate lipid mobility in cells of *Synechococcus* 7942. Fig. 1 shows fluorescence micrographs of a wild-type cell, detecting either green fluorescence from BODIPY (panel A) or red fluorescence from the chlorophylls (panel B). The red fluorescence reveals the position of the thylakoid membranes, since the chlorophyll content of the plasma membrane is negligible [18]. The BODIPY fluorescence largely overlaps with the chlorophyll fluorescence, although the dif-

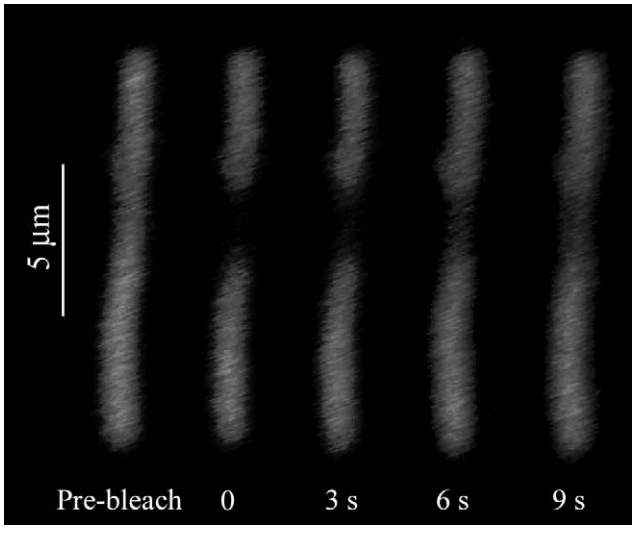

Fig. 2. FRAP image sequence, showing fluorescence from BODIPY. The sequence illustrated is for a wild-type cell of *Synechococcus* 7942 at 39°C. The 'pre-bleach' image shows the cell at the start of the experiment. The image at $t = 0$ was recorded immediately after bleaching a line across the cell. Thereafter, images were recorded at 3-s intervals.

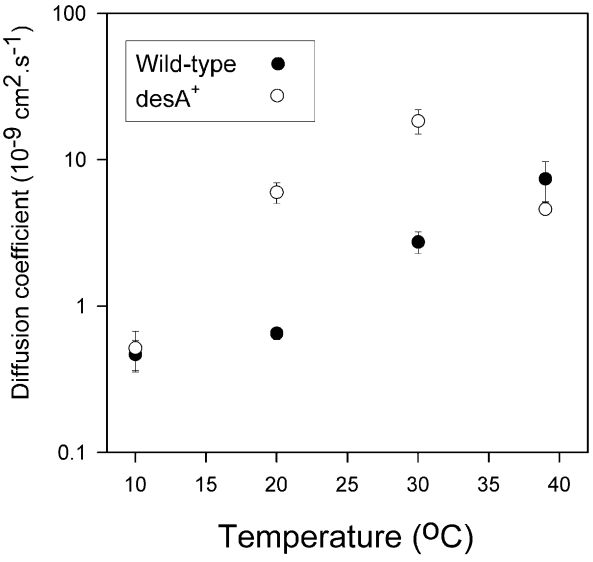

Fig. 3. Temperature dependence of the diffusion coefficient for BODIPY in wild-type and DesA$^+$ cells. Diffusion coefficients were obtained from FRAP image sequences as described in Section 2. Diffusion coefficient is shown on a logarithmic scale. Each point is the mean value obtained from measurements on three cells, with standard error.

ference image (panel C) shows a faint ring of BODIPY fluorescence outside the thylakoid membrane area, particularly at the poles of the cell. This must result from BODIPY incorporation into the plasma membrane and outer membrane. However, most of the dye is located in the thylakoid membranes. When fluorophores are localised in the periplasm or the plasma membrane, fluorescence micrographs show a bright halo around the periphery of the cell and very little fluorescence from the thylakoid membrane region [19] in contrast to the images that we see here. *Synechococcus* cells contain multiple thylakoid membrane layers. Electron micrographs [9] indicate that cells grown under our conditions have three to four concentric double cylinders of thylakoid membranes and therefore six to eight layers of thylakoid membrane as compared to single layers of the plasma and outer membranes. Typically, approximately 90% of the total lipid in the cell is in the thylakoid membranes [20].

3.2. FRAP measurements of BODIPY diffusion

Fig. 2 shows a typical sequence of images from a FRAP measurement of BODIPY diffusion. Values for the diffusion coefficient of BODIPY were extracted from such image sequences as described in Section 2. The results are summarised in Fig. 3. For wild-type cells at the growth temperature of 30°C, the mean diffusion coefficient is $(2.8 \pm 0.5) \times 10^{-9}$ cm^2/s. For *des*A$^+$ cells under the same conditions, the diffusion coefficient is about six times faster: $(1.8 \pm 0.3) \times 10^{-8}$ cm^2/s. The diffusion coefficient is temperature-dependent in both wild-type and *des*A$^+$ cells. In both cell types the diffusion coefficient falls to around 5×10^{-10} cm^2/s at 10°C. However, at 20°C the diffusion coefficient in *des*A$^+$ cells remains considerably faster than in wild-type cells.

4. Discussion

Our results provide the first direct measurement of a lipid diffusion coefficient in a native prokaryotic membrane, or in any photosynthetic membrane. Interpretation of the results is complicated by the fact that cyanobacterial cells contain three membrane systems: the plasma membrane, the outer membrane and the thylakoid membranes. The three membrane systems have very different protein constituents [18]. The spatial resolution of our FRAP measurements is not sufficient to allow us to distinguish between diffusion in the three membrane systems. However, it appears that most of the BODIPY marker dye is located in the thylakoid membranes (Fig. 1), which have much the greatest area and contain approximately 90% of the total lipid in the cell [20]. Our results therefore report mainly on lipid diffusion in the thylakoid membranes. Cyanobacterial thylakoid membranes are the site of photosynthetic energy conversion: they have an exceptionally high protein content [21]. In wild-type cells at their growth temperature of 30°C, we found a diffusion coefficient for the BODIPY marker of about 3×10^{-9} cm^2/s. This is somewhat slower than the diffusion usually observed in eukaryotic plasma membranes. For example, FRAP measurements of lipid dye diffusion in the plasma membranes of human keratinocytes revealed diffusion coefficients of about $10–40 \times 10^{-9}$ cm^2/s [22]. The slower diffusion in cyanobacterial thylakoid membranes may reflect both the high protein content of the membrane [23] and differences in lipid composition. The diffusion coefficient of plastoquinone in chloroplast thylakoid membranes has been estimated using the pyrene fluorescence quenching technique [24]. The estimated diffusion coefficients were of the order of 10^{-9} cm^2/s or less, generally a little slower than the BODIPY diffusion coefficients that we measure. Direct comparison of the diffusion coefficients may be misleading because because of the very different methods used to obtain them. For example, our FRAP measurements yield long-range diffusion coefficients on a scale of micrometres, whereas pyrene fluorescence quenching yields shorter-range diffusion coefficients on a scale of 10–100 nm [24]. However, this work provides convincing evidence that lipid diffusion is slowed due to the high protein content of thylakoid membranes [24,25].

The membranes of wild-type *Synechococcus* 7942 cells contain only saturated and monounsaturated fatty acids [6]. However, in the *des*A$^+$ transformant cells about 20% of fatty acids are diunsaturated and around 40% of glycerolipids contain one diunsaturated fatty acid [6]. This change in membrane lipid composition leads to an enhanced ability to recover from photoinhibition and to tolerate low temperatures, apparently due to a faster exchange of the two isoforms of the D1 protein of PS II [6,7]. Studies on another cyanobacterium, *Synechocystis* 6803, have provided further evidence that the physiological properties of the cells can be altered by genetic manipulation of membrane lipid unsaturation. A mutant lacking two desaturase genes was found to have a higher temperature of lipid phase transition as judged by differential scanning calorimetry [5] and from chlorophyll fluorescence parameters [23], and less lipid disorder as judged by Fourier transform infrared spectroscopy [21].

Our results provide direct evidence that the increased lipid desaturation in *des*A$^+$ cells results in more rapid lateral diffusion of lipids. At the growth temperature of 30°C, the diffusion coefficient for the BODIPY marker is about six times greater in *des*A$^+$ cells than in wild-type cells (Fig. 3). Thus the bulk phase of the membrane is much more fluid in *des*A$^+$

cells, when the cells are at their growth temperature. In wild-type cells, the membranes enter a phase-separated state, with distinct gel and liquid–crystalline phases, about 10°C below the growth temperature [26]. This can account for the slower BODIPY diffusion seen at 20°C and below (Fig. 3). Unexpectedly, we found that raising the temperature from 30°C to 39°C led to a decrease in the BODIPY diffusion coefficient in *des*A$^+$ cells (Fig. 3). No comparable effect was seen in wild-type cells over the temperature range tested. We can only provide a tentative explanation for this effect: it is possible that at high temperatures the membrane segregates into laterally heterogeneous domains, and BODIPY is specifically distributed in one of the more rigid domains.

Transformation with the *des*A gene enhances ability to turn over and exchange isoforms of the D1 protein of PS II, which leads to faster recovery from photoinhibition [6] and greater tolerance of low temperatures [7]. Our results suggest that membrane fluidity may be a critical factor for protein turnover. A more fluid membrane may allow freer access of ribosomes and thylakoid membrane proteases to photodamaged PS II reaction centres, or may assist with the targeting of the new D1 polypeptides and reassembly of the PS II complexes [27].

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