Diffusion of light-harvesting complex II in the thylakoid membranes

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The light-harvesting complex II (LHCII) is the main energy absorber for photosynthesis in green plants, and its translocation between photosystems I and II is the primary means of energy redistribution between them. Using single-particle tracking, we performed the first measurement of the mobility of LHCII in the photosynthetic membranes in both the nonphosphorylated and the phosphorylated (P-LHCII) conformations. These are part of an important, reversible, energy re-equilibration process called the state transition. We found that the population of P-LHCII in unappressed membranes is more mobile than the population of non-P-LHCII from the same regions.

Keywords: light-harvesting complex II (LHCII); membrane diffusion; LHCII phosphorylation; single-particle tracking

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INTRODUCTION

Light harvesting is the primary process in photosynthesis. In green plants, photosynthesis is carried out by two photosystems, PSII and PSI, each associated with antennae that work in series (Buchanan *et al*, 2000; Allen & Forsberg, 2001). PSII and PSI are physically separated, as PSII is mainly found in the appressed regions (grana) of the photosynthetic membranes (thylakoids) and PSI is mainly found in the non-appressed regions (stroma lamellae; Albertsson *et al*, 1990; Hankamer *et al*, 1997; Allen & Forsberg, 2001). The light-harvesting complex II (LHCII)—the main antenna of PSII—accounts for about half of the chlorophyll molecules in nature and is the most abundant integral membrane protein in chloroplasts.

Although the structure of LHCII has been characterized in detail (Liu *et al*, 2004), little is known about the dynamics of this complex. LHCII is a homo- or heterotrimer of three different monomers, LHCB1, LHCB2 and LHCB3 (Peter & Thornber, 1991). LHCB1 and LHCB2 may be reversibly phosphorylated on the amino-terminal threonine by a thylakoid-associated kinase (Depege *et al*, 2003), depending on the redox state of the electron-transport chain, and thus diffuse away from PSII (Allen, 1992; Allen & Forsberg, 2001). This mobile pool of LHCII, subject to lateral diffusion in the thylakoids, associates with PSI, interacting with the small subunits H, L and O (Zhang & Sheller, 2004), and increases the size of the PSI antenna. This condition is named 'state 2', whereas the condition in which all LHCII is associated with PSII is named 'state 1' (Allen, 2003). State transitions are short-term responses of the photosynthetic apparatus that balance the excitation between the two photosystems in varying light conditions (Allen, 2003).

The long-range diffusion coefficient for LHCII has been indirectly estimated from rapid membrane fractionation studies (Drepper *et al*, 1993). Mullineaux *et al* (1997) directly measured the mobility of the accessory light-harvesting complexes of cyanobacteria, the phycobilisomes, by fluorescence recovery after photobleaching (FRAP). Unlike LHCII, which is embedded in the membrane, phycobilisomes are anchored to the cytoplasmic surface of the thylakoids. Furthermore, FRAP is difficult to use in the study of LHCII diffusion because of the broad spectral overlap of the different components of the photosystem antennae, which prevents selective bleaching of LHCII (Jennings *et al*, 1993). Therefore, we used single-particle tracking, which is an alternative method that allows the direct observation of membrane protein diffusion.

RESULTS AND DISCUSSION

We selectively labelled single LHCII complexes with a microsphere (Fig 1), which served as a tag for single-particle tracking. For LHCII labelling, the microsphere was attached to a custom-made 'anti-loop' antibody to a sequence from the stromatic loop between the A and C α -helices of the protein. The specificity of the anti-loop was assayed by western blotting (supplementary Fig S1 online). To make sure that all of the LHCII labelled with the

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Fig 1 | Light-harvesting complex II (LHCII) labelling. (A) The three-dimensional structure of monomeric LHCII is shown in grey. The labelling sites on the threonine at the amino terminus and on the stromatic anti-loop are recognized by the respective biotinylated antibodies (red and blue, respectively), which can then bind to streptavidinated microspheres. The drawing is not to scale. Anti-Pthr, anti-phosphothreonine. (B) An image of LHCII-bound microspheres in intact thylakoid membranes extracted from a whole chloroplast, as described in the Methods. Scale bar, 1 μm .

anti-loop was not phosphorylated, plants were dark adapted before the thylakoid preparation, and western blotting was used to verify the absence of phosphorylated LHCII (P-LHCII; supplementary Fig S2 online). Because of steric hindrance, the microsphere (0.8 μm in diameter) cannot enter between grana stacks, and probably only the LHCII particles in the most superficial grana margins—in the surface-exposed grana end membranes and in the top stroma lamellae, where the membrane is enriched in PSI—were labelled. On the basis of previous studies on the biochemical composition of these regions (Bassi *et al*, 1995; Danielsson *et al*,

2004), it can be concluded that even in the experiments performed in ‘state 1’ conditions, a substantial amount of the labelled LHCII is associated with PSI (Danielsson *et al*, 2004).

Instead, P-LHCII was labelled with a microsphere attached to a commercial anti-phosphothreonine (Finzi *et al*, 2001). Indeed, LHCII becomes phosphorylated on single threonines in the N termini of the LHCBI and LHCBI subunits. Western blot analysis confirmed that, under our experimental conditions, only LHCII became phosphorylated and not other minor antenna complexes (supplementary Fig S2 online).

The motion of LHCII-bound microspheres was analysed in video recordings (supplementary movie online) and the amplitude of motion (σ ; see Methods) was plotted as a function of time. For LHCII (Fig 2A) and P-LHCII (Fig 2B), the amplitudes of motion were constant over time. Each point in the traces represents the accumulation of 4 s of data, which was sufficient for labelled LHCII to explore the whole area available to it. Indeed, plotting the square of the amplitude of motion (σ^2 ; see Methods) as a function of different accumulation times produced a curve that rose linearly, but quickly reached a plateau (R^2 ; see Methods). LHCII motion only seemed Brownian for times too short to explore the boundaries of corrals (Fig 2C,D)—quantified as the height (R^2) of the plateau—which were determined by fitting a function representing confined diffusion. Note that the sizes of the corrals (R) were smaller than that of a granum ($\approx 500 \text{ nm}$).

The frequency distributions of the sizes of the corrals (R^2) confining LHCII and P-LHCII are reported in Fig 3A and B, respectively. In both cases, the distribution can be fitted by a bell-like curve with a long, shallow tail, indicating that most of the complexes move in fairly restricted corrals. The heterogeneity in corral size may reflect physical boundaries associated with stacking of the thylakoids (Boekema *et al*, 2000; Mustardy & Garab, 2003), the fact that LHCII may interact with heterogeneously sized aggregates in the membranes (Finzi *et al*, 1989; Allen & Forsberg, 2001) or that steric hindrance of the microsphere impedes large amplitudes of motion, especially for LHCII in the margins. These phenomena may also determine the shapes of the corrals observed for different LHCII complexes (supplementary Fig S3 online). A few complexes explored considerably larger areas and were probably LHCII trimers or monomers freely diffusing in the membrane. Interestingly, the distribution of R^2 was broader for P-LHCII than for LHCII. The diffusion coefficient, D , of each LHCII or P-LHCII, tracked in the experiments described above, was calculated considering that it equals twice the slope of the initial linear segment in curves such as those in Fig 2C,D. Fig 4 shows the frequency distribution of D values for LHCII and P-LHCII. The average D is larger for P-LHCII ($2.7 \times 10^{-10} \text{ cm}^2/\text{s}$) than for LHCII ($8.4 \times 10^{-11} \text{ cm}^2/\text{s}$). This could be explained by considering that P-LHCII might diffuse more rapidly because of electrostatic repulsion between phosphorylated complexes.

Speculation

Not forgetting that our data probably refer only to LHCII found in end and stromal membranes, which differ in LHCII density from granal membranes, it is interesting that the size of the corrals available to P-LHCII is larger on average than that available to LHCII. This supports data that phosphorylation of LHCII brings about structural changes in the thylakoids (Allen & Forsberg, 2001; Zhang & Sheller, 2004). Furthermore, phosphorylation may

Fig 2 | Analysis of the light-harvesting complex II (LHCII) motion. Amplitudes of motion of LHCII (A) and P-LHCII (B) versus time. Squared amplitude of motion versus data accumulation time (Δt), for LHCII (C) and P-LHCII (D); the error bars represent the standard deviation of the mean.

reduce the amount of membrane stacking. If this were true, there might be a loss in supramolecular membrane organization following phosphorylation of LHCII and grana destacking. We speculate that a higher D might also indicate that P-LHCII was more loosely associated with PSI than LHCII was with either photosystem. The larger D for P-LHCII indicated that it was more

Fig 3 | Histograms of the areas explored by the light-harvesting complex II (LHCII) (A) and phosphorylated LHCII (P-LHCII; B).

mobile, which might guarantee a fast response to unfavourable light conditions that could cause photodamage. Interestingly, using Einstein's equation of diffusion ($\Delta x^2 = 4Dt$), the diffusion coefficient of LHCII/P-LHCII indicates that these two species can traverse a granum in 0.5–2 s. Conversely, state transitions require several minutes, meaning that the slow step of the process is not protein diffusion in the thylakoids (Wollman, 2001).

Our evidence that phosphorylation of LHCII enhances its diffusion in thylakoid membranes shows the utility of single-particle tracking methods in characterizing the state transition of photosynthesis and similar phosphorylation-driven translocations.

METHODS

LHCII labelling. Thylakoid membranes were isolated from leaves of *Spinacia oleracea* following a protocol that preserves these structures (Finzi et al, 2001). Normally, leaves were kept at 4 °C in the dark for 3 h before isolation of thylakoids, and subsequently phosphorylation of LHCII was chemically induced in some samples (Finzi et al, 2001). In brief, isolation was carried out in a medium containing 30 mM tricine, pH 8, 5 mM $MgCl_2$, 10 mM NaCl, 0.2 M sucrose and 10 mM NaF. Kinases were activated by incubating the isolated thylakoids, $[chl] = 500 \mu g/ml$, at 25 °C

Fig 4 | Frequency distribution of the diffusion coefficients determined for the light-harvesting complex II (LHCII) (A) and phosphorylated LHCII (P-LHCII; B). Coefficients were calculated from the experimental data using equation (3).

in the dark for about 10 min in the same medium supplemented with 5 μ M ferredoxin, 1 mM NADP, 2 mM glucose-6-phosphate dehydrogenase in large excess and 1 mM ATP.

P-LHCII was labelled with a 0.8- μ m-diameter microsphere according to previously published methods, using anti-phosphothreonine (Finzi *et al*, 2001). Nonphosphorylated LHCII was labelled similarly using a polyclonal antibody (anti-loop) specific to the stromatic loop of LHCII (Areta Intl. s.r.l., Garenzano (VA), Italy). Western blot analyses showed that anti-phosphothreonine recognized only the phosphorylated threonine in LHCII and proved the specificity of anti-loop (supplementary Figs S1,S2 online). Fig 1 shows, schematically, LHCII labelling and an image of an LHCII-bound microsphere on integral thylakoid membranes.

To reduce the probability that a microsphere might have several crosslinked LHCII complexes, streptavidin-coated microspheres (Bangs Labs. Inc., Fishers, IN, USA) diluted 200-fold in the experimental buffer were passivated with 4 $\times$ 10⁻⁶ g/ml sulphoNHS-*l*c-*l*c-biotin (C₂₆H₄₀O₁₀N₅S₂, 6-((+)-biotinamidocaproilamido)caproic acid *N*-hydroxysuccinimide ester; Uptima Interchim, Montluçon Cedex, France). This solution was

introduced into the thylakoid-containing microchamber. Excess biotin and beads were washed away with several volumes of buffer without biotin. This establishes a non-equilibrium condition in which rare and randomly distributed avidin sites slowly become available for binding to biotinylated LHCII complexes (supplementary information online). Therefore, mobile microspheres were most certainly linked to a single LHCII complex.

Single-particle tracking. We monitored the bead motion by using a differential interference contrast (DIC) microscope. The images were recorded with a CCD camera (Jai CV-A60, Japan) at a rate of 25 frames per second on a videocassette recorder. Sequences of 3,000 frames were acquired using an LG3 frame grabber (Scion Corporation, Frederick, MD, USA). The bead appeared as juxtaposed bright and dark semicircles and its position varied against an almost static thylakoid membrane.

Each frame in the sequence was then analysed to determine the position of the bead. A small, rectangular region $t(x, y)$ including the bead was selected in the first image of the sequence $I_0(x, y)$ and used as a template. Then, the best superposition of the template $t(x, y)$ on each image $I_n(x, y)$ was established by determining the maximum of the correlation function $C_n(\Delta x, \Delta y)$:

$$C_n(\Delta x, \Delta y) = \int [I_n(x + \Delta x, y + \Delta y) - I_{n0}] \times [t(x, y) - t_0] dx dy \quad (1)$$

where $I_{n0} = \langle I_n(x, y) \rangle$ and $t_0 = \langle t(x, y) \rangle$ and $\langle \rangle$ indicates the average over the pixel coordinates x and y .

The maximum of $C_n(\Delta x, \Delta y)$ occurs when the standard deviation from the actual image and the template has the minimum value, regardless of the background intensity fluctuations.

Such analyses produced a time-resolved sequence of X - Y coordinates, which was processed as described in the following section. Tests with simulated data showed that the accuracy of this method is about one-third of a pixel, or ≈ 20 nm.

Data analysis. Sequences of X - Y coordinates were analysed to measure the amplitude and velocity of bead movements. Plotting the position of the bead of each frame produced two-dimensional distributions (supplementary Fig S3 online). Any drift was eliminated by subtracting the best-fitting second-order polynomial curve from the coordinates.

As many position distributions were elongated, although not elliptical, we calculated the length of the two axes independently. To evaluate them, we calculated the standard deviation of the vector $[x(t), y(t)]$, which is a matrix; its eigenvectors are the axis.

We defined the movement amplitude on a time interval $(t, t + \Delta t)$ as the standard deviation of the position:

$$\sigma_{\Delta t}^2(t) = 1/\Delta t \int_t^{t+\Delta t} [x(\tau)^2 + y(\tau)^2] d\tau - \left\{ 1/\Delta t \int_t^{t+\Delta t} x(\tau) d\tau \right\}^2 - \left\{ 1/\Delta t \int_t^{t+\Delta t} y(\tau) d\tau \right\}^2 \quad (2)$$

As the frames were acquired at discrete time steps, the integrations were actually discrete sums. Using a time interval

Δt of 4 s, we observed how the amplitude of the bead movement evolved (Fig 2A,B). Beads with unstable amplitude in plots of $\sigma_{\Delta t}(t)$ against t were discarded from the analysis.

Conversely, the evaluation of $\sigma^2(\Delta t) = \langle \sigma_{\Delta t}^2(t) \rangle$ allows one to measure the dynamics of the bead. For a vanishing time interval Δt , the bead has no time to move, and thus $\sigma^2(\Delta t)$ also vanishes. For small values of Δt (less than 1 s), the movement of the bead is almost Brownian, as confinement has no role; $\sigma^2(\Delta t)$ increases as $2D\Delta t$, where D is the diffusion coefficient of the Brownian motion. For Δt larger than the time required by the bead to traverse the confined region through Brownian motion, $\sigma^2(\Delta t)$ reached a plateau, R^2 , the square of the corral size. For a Brownian motion inside a harmonic confining potential, and neglecting the mechanical drift, the amplitude of movement should have the following form:

$$\sigma^2(\Delta t) = R^2 \Delta t / (R^2 / (2D) + \Delta t) \quad (3)$$

The measured $\sigma^2(\Delta t)$ values were fitted with this expression to evaluate both R and D . From numerical simulations (data not shown), we noted that the value of D derived from the fit is almost independent of the physical model we used, whereas the meaning of R depended on the form of the confining potential.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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