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Supermolecular structure of photosystem II and location of the PsbS protein

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This paper addresses the question of whether the PsbS protein of photosystem two (PS II) is located within the LHC II–PS II supercomplex for which a three-dimensional structure has been obtained by cryoelectron microscopy and single particle analysis. The PsbS protein has recently been implicated as the site for non-photochemical quenching. Based both on immunoblotting analyses and structural considerations of an improved model of the spinach LHC II–PS II supercomplex, we conclude that the PsbS protein is not located within the supercomplex. Analyses of other fractions resulting from the solubilization of PS II-enriched membranes derived from spinach suggest that the PsbS protein is located in the LHC II-rich regions that interconnect the supercomplex within the membrane.

Keywords: photosystem II; supermolecular structure; PsbS

1. INTRODUCTION

It is very evident that one of the great challenges of photosynthesis research is to determine the structure of photosystem II (PS II) at the highest resolution possible. In so doing a structural framework will be available to interpret the very large amount of existing data obtained through a wide spectrum of approaches ranging from optical and resonance spectroscopy to molecular genetics and physiology. The structure of PS II will also allow new hypotheses to be formulated and tested by experimentation. The overall goal, of course, is to explain the molecular basis of PS II function and in particular elucidate the details of its unique features; namely water oxidation, turnover of the D_1 protein, and associated protection mechanisms against photoinhibition such as non-photochemical quenching.

The water oxidation process takes place within a cluster of four manganese atoms bound to the lumenal surface of the reaction centre (Nixon & Diner 1992). The latter is composed of the D_1 and D_2 proteins that contain the redox active cofactors responsible for bringing about primary and secondary charge separation. The initial event is the formation of the radical pair P^+_{680}·Pheo·$^-$, where P_{680}· and Pheo are molecules of chlorophyll *a* and pheophytin *a*, respectively. Electron transfer from Pheo·$^-$ to plastoquinone Q_A and then Q_B results in charge stabilization on the acceptor side (Diner & Babcock 1996). Meanwhile, electron donation to P^+_{680}· occurs from a redox active tyrosine (Tyr161 of the D_1 protein) and then from the $(Mn)_4$ cluster (Debus 1992). Two electron transfer on the acceptor side fully reduces Q_B to plastoquinol, which then diffuses into the lipid matrix of the membrane. The transfer of four electrons on the donor side allows four oxidizing equivalents to be accumulated within the $(Mn)_4$ cluster that are then used to oxidize two water molecules to molecular oxygen.

The water oxidation process requires a very high oxidizing potential that is generated by P^+_{680}·. The redox potential of P^+_{680}· is one volt or more and thus is sufficient to oxidize other molecules in its vicinity including the pigments and amino acids that make up the reaction centre. Moreover, the splitting of water itself and associated reactions may also produce toxic forms of oxygen and, indeed, PS II is known to generate singlet oxygen via the P_{680} triplet state (Telfer *et al.* 1994). It is for these reasons that the D_1 protein has a tendency to become damaged by oxidations (Sharma *et al.* 1997; Barber 1998) and must be replaced from time to time (Barber & Andersson 1992; Barber 1995). This energy demanding and inconvenient 'turnover' of the D_1 protein represents an inherent but unavoidable imperfection in PS II function. It is therefore not surprising that oxygenic photosynthetic organisms have developed a range of mechanisms to minimize the rate of damage of PS II especially under severe conditions such as high light intensities (Andersson & Barber 1996). One such mechanism is known as non-photochemical quenching (NPQ) whereby excess light is channelled into a heat-dissipating pathway (Horton *et al.* 1996). Indeed, when NPQ is diminished by a partial dissipation of the transthylakoid pH gradient using nigericin, or by inhibition of the xanthophyll cycle using dithiothreitol, the concentration of functional PS II complexes declined to a greater extent for a given dosage of light (Park *et al.* 1996).

As with other photosystems, PS II contains a light-harvesting system that transfers excitons, resulting from photon absorption, to the reaction centre. In higher plants and green algae, this antenna is mainly composed of the chlorophyll *a*–chlorophyll *b*–xanthophyll CAB binding proteins Lhcb1, 2 and 3 that constitute LHC II (Jansson 1994). The LHC II complexes exist as trimers

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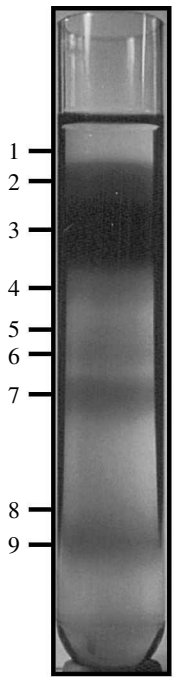

Figure 1. A sucrose density gradient showing the separation of different chlorophyll- containing bands resulting from the solubilization of PS II enriched BBY membranes by 25 mM β-D-dodecyl maltoside. The bands have been given numerical nomenclature.

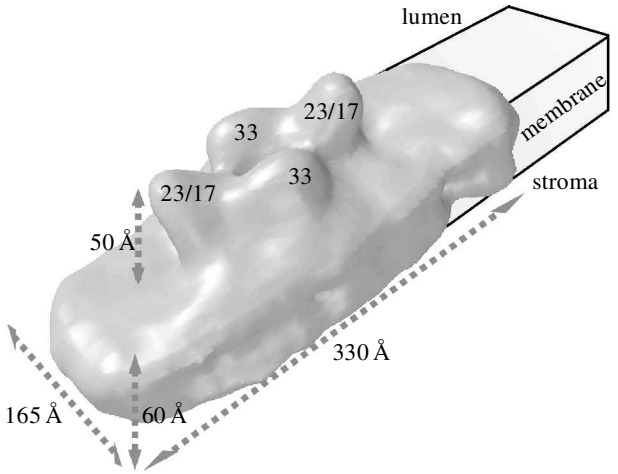

Figure 2. Three-dimensional structure of the spinach LHC II–PS II supercomplex (taken from fraction nine, see figure 1) at 2.4 Å determined by single particle analysis of cryoimages recorded using a Philips CM200 FEG electron microscope with liquid nitrogen stage (from Nield *et al.* 2000).

(Kühlbrandt *et al.* 1994) and transfer energy to the D_1–D_2 reaction centre complex via the minor chlorophyll *a*/*b* binding proteins Lhcb4 (CP29), Lhcb5 (CP26) and Lhcb6 (CP24) and the inner chlorophyll *a* binding proteins CP47 and CP43. Within the light-harvesting system lies the site and mechanism for NPQ and various studies of this phenomenon have implicated the 'xanthophyll cycle' (Demmig-Adams 1990), reversible LHC II aggregation (Horton *et al.* 1991) and the light-induced pH gradient across the thylakoid membrane (Horton *et al.* 1996). Very recently a genetic approach has shown that the 22 kDa PsbS protein plays an important role in non-photochemical quenching (Li *et al.* 2000). This protein binds chlorophyll *a*, chlorophyll *b* and xanthophylls (Funk *et al.* 1994, 1995*a*) and is related to the Lhcb proteins (Wedel *et al.* 1992). However, it is predicted to have four transmembrane helices rather than three (Kim *et al.* 1994). Indeed in terms of evolution PsbS may be a precursor to the Lhcb proteins being derived by gene duplication from related proteins found in cyanobacteria (Green & Pichersky 1994; Dolganov *et al.* 1995; Green & Kühlbrandt 1995; Funk & Vermaas 1999).

Due to the fact that PsbS is present in mutants that are deficient in other Lhcb proteins, the possible importance of PsbS in NPQ was already suggested by Funk *et al.* (1995*b*). This work and the more recent findings of Li *et al.* (2000) open up many questions about the dynamics, pigment content and position of this protein within PS II. Fortunately, structural details of PS II are now emerging (Barber & Kühlbrandt 1999). Electron crystallography has revealed the positioning of transmembrane helices of the major protein subunits of the core complex (Rhee *et al.* 1997, 1998; Hankamer *et al.* 1999). Ten transmembrane helices have been assigned to the D_1 and D_2 reaction centre subunits and six each to the CP47 and CP43 proteins (Rhee *et al.* 1998; Hankamer *et al.* 1999). The D_1–CP43 and D_2–CP47 helices are related by a pseudo twofold axis and show a structural organization similar to that of the transmembrane helices of PsaA and PsaB reaction centre proteins of photosystem I (PS I) (Hankamer *et al.* 1999; Schubert *et al.* 1998). The core complex contains no chlorophyll *b* and therefore no Lhcb proteins, including PsbS. It does, however, contain ten or more other single transmembrane proteins, including PsbE, F, H, I, K, L, X, T_c and W (Barber *et al.* 1997; Zheleva *et al.* 1998; Hankamer *et al.* 1999) and densities corresponding to these subunits have been revealed by electron crystallography (Barber & Kühlbrandt 1999; Hankamer *et al.* 1999). The PS II core complex seems to exist normally as a dimer, centred within a supercomplex (Boekema *et al.* 1995; Barber 1998; Nield *et al.* 2000). Attached to the lumenal surface of this dimer are the extrinsic proteins of the oxygen evolving complex (OEC) having apparent molecular masses of 33 kDa, 23 kDa and 17 kDa. These proteins do not ligate Mn but provide an environment for stabilizing the $(Mn)_4$ cluster and maintaining the appropriate level of Ca^{2+} and Cl for the water oxidase activity (Debus 1992). The regions flanking the central dimer are composed of Lhcb proteins and the question arises as to whether the PsbS protein is located in this region. In this paper we explore this possibility using an antibody raised specifically to the PsbS protein.

2. MATERIAL AND METHODS

(a) *Isolation of PS II*

PS II enriched membranes (BBY, after Berthold *et al.* (1981)) were isolated from market spinach as previously described (Hankamer *et al.* 1997). These were then solubilized using 25 mM β-D-dodecyl maltoside and subjected to sucrose density gradient centrifugation at 90 000 *g* overnight for 14 h at 4 °C. Samples were carefully taken from each band and subjected to 10–17% SDS–polyacrylamide gel electrophoresis.

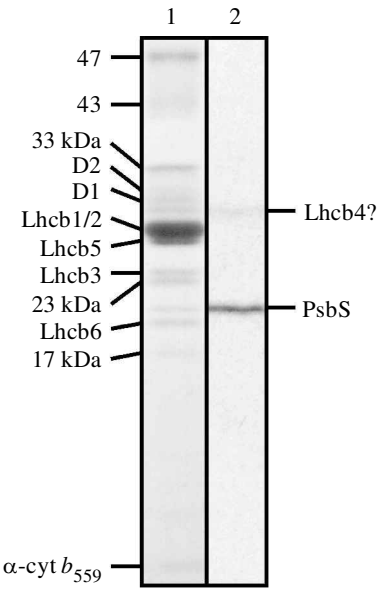

Figure 3. SDS–PAGE of PS II-enriched BBY membranes (1 μg chlorophyll) stained with Coomassie blue (lane one) compared with an immunoblot using an antibody raised to the PsbS protein (lane two).

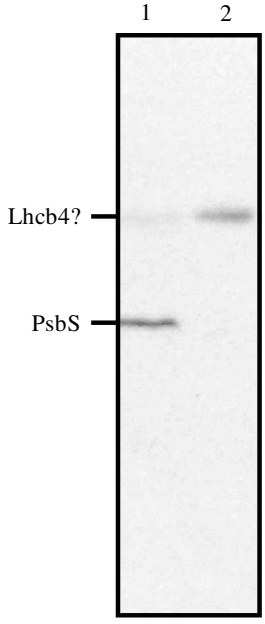

Figure 4. PsbS immunoblot of PS II-enriched BBY membranes (lane one) and the LHC II–PS II supercomplex, respectively (lane two). Both lanes loaded at 1 μg chlorophyll.

(b) *SDS–polyacrylamide gel electrophoresis and immunoblotting*

Analysis of fractions derived from sucrose density centrifugation was carried out using gradient SDS–PAGE (10–17% polyacrylamide) containing 6 M urea and the protein bands visualized using Coomassie R-250 staining. Immunoblotting was performed as described previously (Hankamer *et al.* 1997) using antibodies to the PsbS raised in rabbit against a purified protein isolated from spinach at a dilution of 1:1000 and to Lhcb6 as in Hankamer *et al.* (1997).

(c) *Electron microscopy and image processing*

Images of the LHC II–PS II supercomplex were obtained using a Philips CM200 FEG-equipped electron microscope operating at 200 kV (Philips Nederland, Eindhoven, The Netherlands). A Gatan liquid N_2 cryostage (Gatan Inc., Pleasanton, CA, USA) was used. Single particle analysis was conducted on images as described in Nield *et al.* (2000) and the data shown here give an update of the model presented in that publication.

3. RESULTS

Figure 1 shows a continuous sucrose density gradient with various chlorophyll-containing bands derived from solubilizing PS II enriched membranes with 25 mM β-D-dodecyl maltoside. As reported previously, fraction nine contains the LHC II–PS II supercomplex (Hankamer *et al.* 1997) and was originally used for negative stain electron microscopy (Boekema *et al.* 1995, 1998). More recently, a three-dimensional (3D) structure at *ca.* 2.4 Å resolution has been obtained for this supercomplex using single particle analyses of images obtained by cryoelectron microscopy in the absence of negative stain (Nield *et al.* 2000). Figure 2 shows a surface rendered oblique view of the 3D structure calculated by Nield *et al.* (2000). The supercomplex is a dimer of PS II having a molecular mass of *ca.* 1000 kDa and dimensions of 330 × 165 × 110 Å overall length, width, and height, respectively. The thickness of the supercomplex is *ca.* 60 Å at its edges, as expected for a membrane spanning region, and the protrusions on the lumenal surface extend to about 50 Å. These protrusions have been assigned to the extrinsic proteins of the OEC as indicated in figure 2. The supercomplex evolves oxygen at high rates and contains about 200 chlorophylls in total (i.e. 100 chlorophylls per reaction centre of which 75 are chlorophyll *a* and 25 chlorophyll *b* (Hankamer *et al.* 1997)). The central region of the complex contains the core dimer made up of D_1, D_2, CP47 and CP43 proteins as well as a number of other lower molecular weight proteins (Hankamer *et al.* 1997; Zheleva *et al.* 1998). According to immunoblotting analyses, the flanking 'tip' regions contain the chlorophyll *a*/*b* binding proteins Lhcb1, Lhcb2, Lhcb4 (CP29) and Lhcb5 (CP26) (Hankamer *et al.* 1997). The chlorophyll analysis is consistent with one copy each of CP29 and CP26 and one trimer of LHC II (composed of Lhcb1/Lhcb2) per reaction centre. Based on these stoichiometries and on electron crystallographic data (Kühlbrandt *et al.* 1994; Rhee *et al.* 1998; Hankamer *et al.* 1999) a model for the positioning of the Lhcb proteins within the supercomplex has been presented (Barber 1998; Barber *et al.* 1999).

Although a preliminary study had indicated that perhaps the PsbS protein was located in the LHC II–PS II supercomplex (Eshaghi *et al.* 1999), a re-evaluation was carried out on the supercomplex preparation used for electron microscopy and single particle analysis (i.e. fraction nine in figure 1). As figure 3 shows, the immunoblotting readily detected the PsbS protein in the PS II-enriched membranes (BBY). The strong band corresponds to an apparent molecular mass of *ca.* 22 kDa that is weakly observed in the Coomassie stained SDS–polyacrylamide gel. Also, the PsbS antibody showed a weak cross-reaction at *ca.* 29 kDa, which is presumably with one or more of the Lhcb proteins since they have

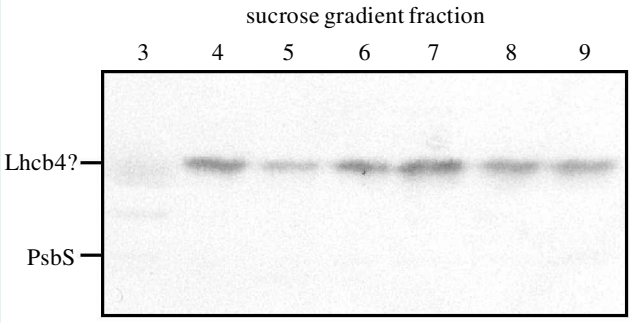

Figure 5. PsbS immunoblotting of fractions three to nine. The 29 kDa band is a cross-reaction with Lhcb proteins, most likely to be Lhcb4. Lanes were loaded at 1 μg chlorophyll.

some sequence homologies with the PsbS protein (Wedel *et al*. 1992; Kim *et al*. 1994). The positioning of this cross-reaction towards the high molecular weight side of the LHC II band suggests the likely candidate is Lhcb4 (CP29) (Hankamer *et al*. 1997). This weak band is therefore useful as an internal control, as shown in figure 4. In this immunoblot, PS II membranes are compared with the supercomplex isolated as fraction nine from the sucrose density gradient. The data clearly indicate that in the case of the supercomplex the cross-reaction at *ca*. 29 kDa occurs but there is no immunological detection of the PsbS protein at 22 kDa. We therefore conclude that the PsbS protein is not present in the LHC II–PS II supercomplex for which a 3D structure has been obtained (Nield *et al*. 2000).

The question therefore arises as to the location of the PsbS protein. In an attempt to answer this question immunoblotting analyses were conducted on all fractions of the sucrose density gradient. Initially, fractions three to nine were investigated. Figure 5 shows immunoblotting of these fractions where it can be seen that, with the exception of fraction three, anti-PsbS detected the 29 kDa secondary band but not the PsbS protein. Coomassie blue staining (not shown) indicated that fractions four to nine all contained PS II proteins indicative of various forms of PS II cores, while fraction three consisted of Lhcb proteins only. Nevertheless, fraction three did not contain significant levels of PsbS or the protein giving rise to the band at 29 kDa.

The above results suggest that the PsbS protein is easily solubilized and remains near the top of the sucrose density gradient. Indeed, a low level of the PsbS protein was detected in fraction three (see figure 5). Immunoblotting with anti-PsbS detected its presence in fraction two but not in the chlorophyll-free fraction one. The cross-reaction detected in fraction two is seen in figure 6, lane four, where it is compared with the anti-PsbS blot of BBY membranes at the same chlorophyll concentration (lane three). A distinct difference between lanes three and four is that the 29 kDa secondary band is clearly observed in the BBY membranes but not in fraction two. Coomassie staining (lanes one and two) also detected the PsbS protein in both BBY membranes and fraction two and emphasized that the latter contained other Lhcb proteins. It did not, however, have a distinguishable band at *ca*. 29 kDa as did the BBY membranes, which seems to be the origin of the secondary cross-reaction. We believe that this band is that of Lhcb4 (i.e. CP29).

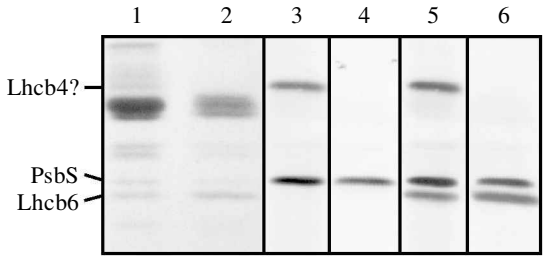

Figure 6. Lanes one and two, Coomassie stained gels of BBY membranes and fraction two, respectively. Lanes three and four, immunoblotting with anti-PsbS of BBY and fraction two, respectively. Lanes five and six, immunoblotting of the anti-PsbS blot with anti-Lhcb6. Chlorophyll loading per lane was 2 μg.

A protein band just below PsbS is clearly seen in lanes one and two of figure 6. By further immunoblotting the anti-PsbS blot (lanes three and four) with antibody raised to Lhcb6 (CP24) we obtained the result shown in lanes five and six of figure 6. Clearly, the lower band is Lhcb6 (CP24). This protein is therefore also present in fraction two along with the PsbS protein.

4. DISCUSSION

We conclude that the PsbS protein is not contained within the LHC II–PS II supercomplex for which a 3D structure has been obtained at 24 Å resolution. Indeed, a more recent, unpublished structure of the spinach LHC II–PS II supercomplex gives an improved distribution of protein density in the flanking regions occupied by the Lhcb proteins. Figure 7*a* shows a cross-section through the new map with figure 7*b* being the same but with an overlay of the transmembrane helices of the dimeric core complex and of the Lhcb proteins known to be present. The position of the dimeric core and its transmembrane helices is known with some certainty (Hankamer *et al*. 1999; Nield *et al*. 2000), as is the structure of the LHC II trimer (Kühlbrandt *et al*. 1994). The position of the trimer within the supercomplex was somewhat of an approximation in earlier models (Barber *et al*. 1999) but the new data shown in figure 7 allow us to position it with a little more confidence, given the improved resolution of features in the flanking region. Assuming CP29 and CP26 have structures very similar to a LHC II monomer, then they can also be accommodated within the model as shown in figure 7. Given that the PsbS protein is predicted to have four transmembrane helices and would be an extension of the LHC II structure (see figure 7*c*), it is clear that there is not sufficient space within the LHC II–PS II supercomplex to accommodate it. This is therefore consistent with the immunoblotting analysis.

The fact that the majority of the PsbS protein is found in fraction two at the top of the sucrose density gradient is consistent with the original work of Ljungberg *et al*. (1984) who found that this protein immunoprecipitated from detergent-solubilized PS II membranes using antibodies raised against the extrinsic 33 and 23 kDa OEC proteins. In our case OEC proteins were found in the non-chlorophyll-containing fraction one (data not shown). Fraction two, however, did contain some chlorophyll binding Lhcb protein and in particular Lhcb6 (CP24).

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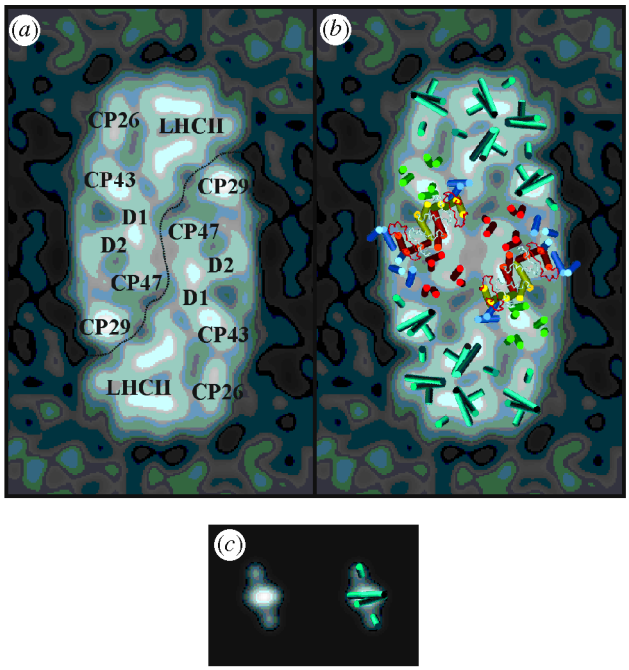

Figure 7. (*a*) A projection of the LHC II–PS II supercomplex of spinach showing density in the membrane region towards the stromal side (improved model of that shown in fig. 3d of Nield *et al*. 2000). (*b*) Superimposition of the helical organization of the known subunits of the LHC II–PS II supercomplex based on models derived from electron crystallography (Kühlbrandt *et al*. 1994; Rhee *et al*. 1998; Hankamer *et al*. 1999). (*c*) A calculated model of the projection of the four transmembrane helices of PsbS derived from the structure of LHC II by symmetrization of helix II about helices I and III filtered down to 20 Å.

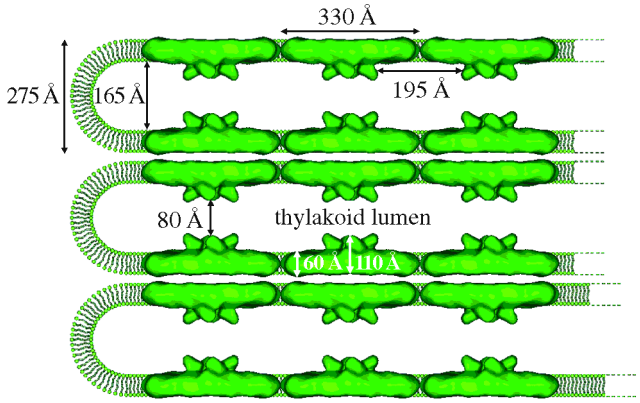

Figure 8. A diagrammatic representation of the granal region of the thylakoid membrane of higher plants showing how the LHC II–PS II supercomplex could be organized in regions devoid of interconnecting LHC II trimers: that is equivalent to the arrays observed by freeze-etching (Seibert *et al*. 1987). The model does not show the interconnecting chlorophyll binding proteins LHC II, CP24 and PsbS and does not include other protein complexes, e.g. the cytochrome b_6f complex.

We believe that these chlorophyll *a*/*b* binding proteins and many of those in fraction three are located in regions that interconnect the LHC II–PS II supercomplex. Indeed, the recent work of Boekema *et al*. (1998, 1999) indicates that the LHC II–PS II supercomplex shown in figure 7 is interconnected by trimers of LHC II with CP24, possibly acting as a linker (Bassi & Dainese 1992). Given that on average there are about 250 chlorophylls per PS II reaction centre, this would mean that for every supercomplex there are about four to five trimers of LHC II. We therefore assume that the PsbS protein is located within the additional pool of LHC II along with CP24, which would be in line with the finding that selective extraction of the PsbS protein leads to efficient separation of the chlorophyll *a*/*b* light-harvesting complex II from the PS II reaction centre core (Kim *et al*. 1994). Digestion studies with trypsin revealed that the LHC II might even shield the N-terminus of PsbS against protease attack (Kim *et al*. 1994). The detergent β-D-dodecyl maltoside seems to be especially effective in removing PsbS from PS II (Harrer *et al*. 1998). The ease of removal of the PsbS protein from PS II does not necessarily mean that it is located at some distance from the reaction centre core. For example, in the case of CP24 it has been postulated that it is positioned close to CP29 and therefore to CP47 (Harrer *et al*. 1998). Our findings therefore do not rule out the possibility that like CP24, CP26 and CP29, PsbS forms a 'linker' between LHC II trimers and the reaction centre core. In this way it could act, via the xanthophyll cycle mechanism, as a strategic quenching centre.

Under some circumstances the LHC II–PS II supercomplexes seem to form ordered arrays in the granal membrane as observed by freeze-etching (Seibert *et al*. 1987). A model for such an organization of the LHC II–PS II supercomplexes within the grana is shown in figure 8. Although this model does not incorporate the other 'linking' LHC II, CP proteins or PsbS, and ignores the possibility of the presence of other types of complexes (e.g. the cytochrome b_6f complex), it does give an indication how PS II complexes may be organized in the granal stacks and how energy transfer processes could occur, not only along the membrane plane but also between adjacent membranes. Indeed, we observed that isolated LHC II–PS II supercomplexes are readily attracted by their stromal faces, forming structures similar to those shown in figure 8.

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REFERENCES

Andersson, B. & Barber, J. 1996 Mechanisms of photodamage and protein degradation during photoinhibition of photosystem two. In *Advances in photosynthetic research series*: *environmental stress and the environment* (ed. N. R. Baker), pp. 101–121. Dordrecht, The Netherlands: Kluwer.

Barber, J. 1995 Molecular basis of the vulnerability of photosystem II to damage by light. *Aust. J. Plant Physiol.* **22**, 201–208.

Barber, J. 1998 Photosystem two. *Biochim. Biophys. Acta* **1365**, 269–277.

Barber, J. & Andersson, B. 1992 Too much of a good thing: light can be bad for photosynthesis. *Trends Biochem. Sci.* **17**, 61–66.

Barber, J. & Kühlbrandt, W. 1999 Photosystem two. *Curr. Opin. Struct. Biol.* **9**, 469–475.

Barber, J., Nield, J., Morris, E. P., Zheleva, D. & Hankamer, B. 1997 The structure, function and dynamics of photosystem II. *Physiol. Plant.* **100**, 817–827.

Barber, J., Nield, J., Morris, E. P. & Hankamer, B. 1999 Subunit positioning in photosystem II revisited. *Trends Biochem. Sci.* **278**, 43–45.

Bassi, R. & Dainese, P. 1992 A supramolecular light-harvesting complex from chloroplast photosystem II membranes. *Eur. J. Biochem.* **204**, 317–326.

Berthold, D. A., Babcock, G. T. & Yocum, C. F. 1981 A highly resolved, oxygen-evolving PS II preparation from spinach thylakoid membranes—electron-paramagnetic resonance and electron transport properties. *FEBS Lett.* **134**, 231–234.

Boekema, E. J., Hankamer, B., Bald, D., Kruip, J., Nield, J., Boonstra, A. F., Barber, J. & Rögner, M. 1995 Supramolecular structure of the photosystem II complex from green plants and cyanobacteria. *Proc. Natl Acad. Sci. USA* **92**, 175–179.

Boekema, E. J., Van Roon, H. & Dekker, J. P. 1998 Specific association of photosystem II and light-harvesting complex II in partially solubilized photosystem II membranes. *FEBS Lett.* **424**, 95–98.

Boekema, E. J., Van Roon, H., Calkoen, F., Bassi, R. & Dekker, J. P. 1999 Multiple types of association of photosystem II and its light-harvesting antenna in partially solubilized photosystem II membranes. *Biochemistry* **38**, 2223–2239.

Debus, R. J. 1992 The manganese and calcium ions of photosynthetic oxygen evolution. *Biochim. Biophys. Acta* **1102**, 269–352.

Demmig-Adams, B. 1990 Carotenoids and photoprotection in plants: a role for the xanthophylls-zeaxanthin. *Biochim. Biophys. Acta* **1020**, 1–24.

Diner, B. A. & Babcock, G. T. 1996 Structure, dynamics and energy conversion efficiency in photosystem II. In *Advances in photosynthesis: the light reactions* (ed. D. R. Ort & C. F. Yocum), pp. 213–247. Dordrecht, The Netherlands: Kluwer.

Dolganov, N. A. M., Bhaya, D. & Grossman, A. R. 1995 Cyanobacterial protein with similarity to the chlorophyll *a*/*b* binding proteins of higher plants: evolution and regulation. *Proc. Natl Acad. Sci. USA* **92**, 636–640.

Eshaghi, S., Andersson, B. & Barber, J. 1999 Isolation of a highly active PS II–LHC II supercomplex from thylakoid membranes by a direct method. *FEBS Lett.* **446**, 23–26.

Funk, C. & Vermaas, W. 1999 A cyanobacterial gene family coding for single-helix proteins resembling part of the light-harvesting proteins from higher plants. *Biochemistry* **38**, 9397–9404.

Funk, C., Schröder, W. P., Green, B. R., Renger, G. & Andersson, B. 1994 The intrinsic 22 kDa protein is a chlorophyll-binding subunit of photosystem II. *FEBS Lett.* **342**, 261–266.

Funk, C., Schröder, W. P., Napiwotzki, A., Tjus, S. E., Renger, G. & Andersson, B. 1995*a* The PS II-S protein of higher plants: a new type of pigment-binding protein. *Biochemistry* **34**, 11 133–11 141.

Funk, C., Adamska, I., Green, B. R., Andersson, B. & Renger, G. 1995*b* The nuclear-encoded chlorophyll-binding photosystem II-S protein is stable in the absence of pigments. *J. Biol. Chem.* **270**, 30 141–30 147.

Green, B. R. & Kühlbrandt, W. 1995 Sequence conservation of the light harvesting and stress–response proteins in relation to the three dimensional molecular structure of LHC II. *Photosynth. Res.* **44**, 139–148.

Green, B. R. & Pichersky, E. 1994 Hypothesis for the evolution of three-helix Chl *a*/*b* and Chl *a*/*c* light harvesting proteins from two-helix and four-helix ancestors. *Photosynth. Res.* **39**, 149–162.

Hankamer, B., Nield, J., Zheleva, D., Boekema, E. J., Jansson, S. & Barber, J. 1997 Isolation and biochemical characterization of monomeric and dimeric photosystem II complexes from spinach and their relevance to the organization of photosystem II *in vivo*. *Eur. J. Biochem.* **243**, 422–429.

Hankamer, B., Morris, E. P. & Barber, J. 1999 Revealing the structure of the oxygen evolving core dimer of photosystem II by cryoelectron crystallography. *Nature Struct. Biol.* **6**, 560–564.

Harrer, R., Bassi, R., Testi, M. G. & Schaefer, C. 1998 Nearest-neighbour analysis of a photosystem II complex from *Marchantia polymorpha* L. (liverwort), which contains reaction center and antenna proteins. *Eur. J. Biochem.* **255**, 196–205.

Horton, P., Ruban, A. V., Rees, D., Pascal, A. A., Noctor, A. & Young, A. J. 1991 Control of the light-harvesting function of chloroplast membranes by aggregation of the LHC II chlorophyll–protein complex. *FEBS Lett.* **291**, 1–4.

Horton, P., Ruban, A. V. & Walters, R. G. 1996 Regulation of light harvesting in green plants. *A. Rev. Plant Mol. Biol.* **47**, 655–684.

Jansson, S. 1994 The light-harvesting chlorophyll *a*/*b*-binding proteins. *Biochim. Biophys. Acta* **1184**, 1–19.

Kim, S., Pichersky, R. & Yocum, C. F. 1994 Topological studies of spinach 22 kDa protein of photosystem II. *Biochim. Biophys. Acta* **1188**, 339–348.

Kühlbrandt, W., Wang, D. N. & Fujiyoshi, Y. 1994 Atomic model of plant light-harvesting complex. *Nature* **350**, 614–621.

Li, X.-P., Bjorkman, S., Shih, C., Grossman, A., Rosenquist, M., Jansson, S. & Niyogi, K. 2000 A pigment-binding protein essential for regulation of photosynthetic light harvesting. *Nature* **403**, 391–395.

Ljungberg, U., Akerlund, H.-E., Larsson, C. & Andersson, B. 1984 Identification of polypeptides associated with the 23 and 33 kDa proteins of photosynthetic oxygen evolution. *Biochim. Biophys. Acta* **767**, 145–152.

Nield, J., Orlova, E., Morris, E., Gowen, B., Van Heel, M. & Barber, J. 2000 3D map of the plant photosystem two supercomplex obtained by cryoelectron microscopy and single particle analysis. *Nature Struct. Biol.* **7**, 44–47.

Nixon, P. J. & Diner, B. A. 1992 Aspartate 170 of the photosystem II reaction center polypeptide D1 is involved in the assembly of the oxygen-evolving manganese cluster. *Biochemistry* **31**, 942–948.

Park, Y.-I., Chow, W. S., Anderson, J. M. & Hurry, V. M. 1996 Differential susceptibility of photosystem II to light stress in light-acclimated pea leaves depends on the capacity for photochemical and non-radiative dissipation of light. *Plant Science* **115**, 137–149.

Rhee, K.-H., Morris, E. P., Zheleva, D., Hankamer, B., Kühlbrandt, W. & Barber, J. 1997 Two-dimensional structure of plant photosystem II at 8 Å resolution. *Nature* **389**, 522–526.

Rhee, K.-H., Morris, E. P., Barber, J. & Kühlbrandt, W. 1998 Three-dimensional structure of photosystem II reaction center at 8Å resolution. *Nature* **396**, 283–286.

Schubert, W.-B., Klukas, O., Saenger, W., Witt, H. T., Fromme, P. & Krauss, N. 1998 A common ancestor for oxygen and anoxygenic photosynthetic systems—a comparison based on the structural model of photosystem I. *J. Mol. Biol.* **280**, 297–314.

Seibert, M., De Wit, M. & Staehelin, L. A. 1987 Structural localisation of the oxygen evolving apparatus to multimeric (tetrameric) particles on the lumenal surface of freeze-etched photosynthetic membranes. *J. Cell Biol.* **105**, 2257–2265.

Sharma, J., Panico, M., Shipton, C. A., Nilsson, F., Morris, H. R. & Barber, J. 1997 Primary structure characterisation of the PS II D1 and D2 subunits. *J. Biol. Chem.* **272**, 33 158–33 166.

Telfer, A., Bishop, S. M., Phillips, D. & Barber, J. 1994 Isolated photosynthetic reaction center of photosystem II as a sensitizer for the formation of singlet oxygen: detection and quantum yield determination using a chemical trapping technique. *J. Biol. Chem.* **269**, 13 244–13 253.

Wedel, N., Klein, R., Ljungberg, U., Andersson, B. & Herrmann, R. G. 1992 The single copy gene PsbS codes for a phylogenetically intriguing 22 kDa polypeptide of photosystem II. *FEBS Lett.* **314**, 61–66.

Zheleva, D., Sharma, J., Panico, M., Morris, H. R. & Barber, J. 1998 Isolation and characterization of monomeric and dimeric CP47–RC PS II complexes. *J. Biol. Chem.* **273**, 16 122–16 127.

Discussion

E. Hideg (*Institute of Plant Biology, Biological Research Centre, Szeged, Hungary*). What kind of experimental evidence supports the localization of singlet oxygen production on D_1 protein? Do structural data support the possibility of localized triplet production in PS II reaction centre?

J. Barber. Of the four densities that we attribute to chlorophyll *a* in our 3D map, it seems that the one expected to be ligated to D_1 His198 is closest to D_1 Tyr161, known as Yz. For this reason we assume that this is the P_{680} chlorophyll and thus is the main site for singlet oxygen production via the triplet state. This would perhaps explain why the D_1 protein is more vulnerable to attack by singlet oxygen and other oxidative processes than the other proteins of PS II. Indeed using mass spectrometry we have located the main sites of oxidative damage (formation of oxyamino acids) to be in the D_1 protein and particularly in the region (D_1 Pro173 to D_1 Met214) where P_{680} is located (Sharma *et al.* 1997).

U. Heber (*Department of Botany, Würzburg University, Germany*). My question relates to D_1 protein turnover and photodamage. Sometimes it is quite cold in Britain and even more so in Siberia. Protein turnover is temperature dependent. As far as I know photosystem II remains intact in pine trees in the wintertime. How is D_1 stabilized in the cold?

J. Barber. We have shown that under normal *in vivo* conditions the removal of damaged D_1 protein occurs only when a newly synthesized D_1 protein is available. That is, in the presence of chloramphenicol, which blocks protein synthesis, the removal of the damaged D_1 protein is significantly slowed despite severe photoinhibition (Komenda & Barber 1995). Therefore it seems reasonable that when low temperatures significantly slow protein synthesis, the damaged D_1 protein remains intact within the photoinhibited PS II reaction centre. To my mind, this seems a sensible strategy avoiding the unnecessary, and energetically costly, disassembly and degradation of the complete PS II complex when replacement D_1 protein is not available.

K. Asada (*Department of Biotechnology, Fukuyama University, Japan*). How about the function of cytochrome b_{559} in the protection from photo excess stress?

J. Barber. There is increasing evidence that the high potential form of cytochrome b_{559} (Cyt b_{559}) protects PS II by acting as a secondary electron donor to P^+_{680}· but it is generally agreed that this is a one-off donation and there is no rapid cyclic electron flow involved. The electron donation probably occurs by the following pathway:

$$\text{Cyt}b_{559} \rightarrow \text{d Chl}_z \rightarrow \beta\text{-car} \rightarrow P^+_{680};$$

where Chl_z is a chlorophyll molecule coordinated to His118 located towards the lumenal end of the B transmembrane helix of the D_1 protein and β-car is a β-carotene located in the D_1 protein 15–20 Å from P^+_{680}· (Telfer *et al.* 1991). If P_{680} is oxidized again by excess photons then Chl_z becomes a long-lived oxidized species. This accumulation of Chl^+_z would also be facilitated if cytochrome b_{559} converted to its low potential form. Unlike P^+_{680}·, Chl^+_z is likely to have a relatively low potential so that is harmless. Brudvig and colleagues (Stewart & Brudvig 1998) have shown that Chl^+_z is a fluorescence quencher and have therefore suggested that this oxidized species acts as a site for photoprotection against excess excitations.

Based on our structural work, it is possible that the two unassigned helices located at the N-terminus of the D_1 protein in our structure and coloured blue (see figure 7*b*) could be the α- and β-subunits of Cyt b_{559}. This would then place the haem of the cytochrome at about 17–23 Å from Chl_z.

J. F. Allen (*Department of Plant Biochemistry, Lund University, Sweden*). At these resolutions how do you distinguish between different single-helix proteins in the complex? I am particularly interested in the position, interactions and functions of PsbH.

J. Barber. We are absolutely certain of the location of the 12 transmembrane helices of CP43 and CP47 (coloured green and red, respectively, in figure 7*b*) and know their order by analogy with the corresponding helices of the PS I reaction centre, for which there is now a high resolution structure. Although we can identify the ten transmembrane helices of the reaction centre we cannot be absolutely sure which five helices to assign to the D_1 and D_2 proteins. Based on a number of observations we favour the five helices adjacent on the CP43 side as being those of the D_1 protein (i.e. the five helices coloured yellow in figure 7*b*). In addition to these 22 helices we have identified at least ten further transmembrane helices in the PS II core complex (Hankamer *et al.* 1999; B. Hankamer, E. P. Morris and J. Barber, unpublished data). One of these helices belongs to PsbH and, although not yet confirmed, we believe this protein to be located at the interface between the two monomers within the dimer where it may play a role in stabilizing the dimeric configuration by interacting in its phosphorylated or non-phosphorylated state with the thylakoid lipid phosphatidylglycerol which has the usual trans-hexadecanoic fatty acid (Kruse *et al.* 2000).

Additional References

Hankamer, B., Morris, E. P. & Barber, J. 1999 Revealing the structure of the oxygen evolving core dimer of photosystem II by cryoelectron crystallography. *Nature Struct. Biol.* **6**, 560–564.

Komenda, J. & Barber, J. 1995 Comparison of *psbO* and *psbH* deletion mutants of *Synechocystis* PCC 6803 indicate that degradation of D1 protein is regulated by the Q_B site and is dependent on protein synthesis. *Biochemistry* **34**, 9625–9631.

Kruse, O., Hankamer, B., Konczak, C., Gerle, C., Morris, E., Radunz, A., Schmid, G. H. & Barber, J. 2000 Phosphatidyl glycerol is involved in the dimerisation of photosystem II. *J. Biol. Chem.* **275**, 6509–6514.

Sharma, J., Panico, M., Shipton, C. A., Nilsson, F., Morris, H. R. & Barber, J. 1997 Primary structure characterisation of the PS II D1 and D2 subunits. *J. Biol. Chem.* **272**, 33 158–33 166.

Stewart, D. H. & Brudvig, G. W. 1998 Cytochrome b559 of photosystem II. *Biochim. Biophys. Acta* **1367**, 63–87.

Telfer, A., De Las Rivas, J. & Barber, J. 1991 Beta-carotene within the isolated photosystem II reaction centre: photooxidation and irreversible bleaching of this chromophore by oxidised P_{680}. *Biochim. Biophys. Acta* **1060**, 106–114.

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