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Minireview

Engine of life and big bang of evolution: a personal perspective

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Abstract

Photosystem II (PS II) is the engine for essentially all life on our planet and its beginning 2.5 billion years ago was the 'big bang of evolution.' It produces reducing equivalents for making organic compounds on an enormous scale and at the same time provides us with an oxygenic atmosphere and protection against UV radiation (in the form of the ozone layer). In 1967, when I began my career in photosynthesis research, little was known about PS II. The Z-scheme had been formulated [Hill and Bendall (1960) Nature 186: 136–137] and Boardman and Anderson [(1964) Nature 203: 166–167] had isolated PS II as a discrete biochemical entity. PS II was known not only to be the source of oxygen but of variable chlorophyll fluorescence [Duysens and Sweers (1963) In: Studies on Microalgae and Photosynthetic Bacteria, pp. 353–372. University of Tokyo Press, Tokyo] and delayed chlorophyll fluorescence [Arnold and Davidson (1954) J Gen Physiol 37: 677–684]. P680 had just been discovered [Döring et al. (1967) Z Naturforsch 22b: 639–644]. No wonder the 'black box of PS II' was described at that time by Bessel Kok and George Cheniae [Current Topics in Bioenergetics 1: 1–47 (1966)] as the '*inner sanctum of photosynthesis*.' What a change in our level of understanding of PS II since then! The contributions of many talented scientists have unraveled the mechanisms and structural basis of PS II function and we are now very close to revealing the molecular details of the remarkable and thermodynamically demanding reaction which it catalyzes, namely the splitting of water into its elemental constituents. It has been a privilege to be involved in this journey.

Abbreviations: A23187 – antibiotic ionophore which facilitates Mg^{2+}/H^{+} exchange across membranes; Chl – chlorophyll; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EM – electron microscopy; EPR – electron paramagnetic resonance; LHCII – light-harvesting complex of PS II; NPQ – nonphotochemical quenching (of chlorophyll fluorescence); PS I – Photosystem I; PS II – Photosystem II

Getting to know my way around Photosystem II (PS II)

Delayed chlorophyll fluorescence

In 1967, I went to work in Lou Duysens' laboratory in the University of Leiden (The Netherlands) as a post-doc. I knew little or nothing about photosynthesis but something about membrane physiology and biophysics. It was not surprising therefore that I found myself collaborating with Bert Kraan on a project which was prompted by an intriguing observation of Berger Mayne and Rod Clayton (1966). They had shown that by subjecting chloroplast thylakoids to an acid–base shift, similar to that used by André Jagendorf and E. Uribe (1966) to induce ATP synthesis in the dark, brought about acceleration in the emission of delayed chlorophyll (Chl) fluorescence (also called delayed light emission) from PS II. This acid–base induced luminescence was clearly a membrane-controlled phenomenon and it therefore seemed to me that a similar stimulation of delayed fluorescence should occur if an electrical gradient of correct polarity was induced rather than a pH gradient. This thinking, of course, was triggered not only by my PhD training in membrane biophysics, under the

supervision of Professor Jack Dainty, but also by the concepts Peter Mitchell was vigorously advocating at that time (Mitchell 1966). My idea was to induce the membrane potential by creating a salt gradient across the chloroplast thylakoid membrane. At about the same time a similar approach was used by Baz Jackson and Tony Crofts (1969) to induce carotenoid band shifts in the bacterial chromatophore membrane. Given that the thylakoid membrane would be expected to show differential ionic permeabilities, then a diffusion potential would be created. Indeed the first experiments using KCl pulses resulted in the anticipated stimulation of delayed light emission. The size of the salt induced signal increased if valinomycin was present or when the potassium salt consisted of a large, poorly diffusible anion, such as benzoate. Clearly it was the generation of membrane potential with inside (lumen) positive that lowered the activation energy for delayed light emission; $L\alpha \exp(E-\Delta\Psi)$ where E is the activation energy necessary to lift the electron from a metastable state to the Chl singlet, $\Delta\Psi$ is the membrane potential and L is the intensity of the delayed light. The results obtained in Leiden were published (Barber and Kraan 1970) and after joining the faculty at Imperial College in 1968, I continued to exploit the phenomenon. I began by estimating the size of the light-induced membrane potential generated across the thylakoid membrane of higher-plant chloroplasts and to calculate the relative and absolute permeabilities of a number of ionic species used to create the diffusion potential (Barber 1972a, b). Perhaps more importantly, my analysis of salt-induced luminescence implied that the species involved in the back reactions giving rise to the emission of light were positioned on opposite sides of the membrane and therefore vectorial movement of charge occurred in line with Mitchell's principles. This view was shared with Colin Wraight and Antony (Tony) Crofts (1971) who also recognized that the intensity of millisecond delayed light reflected the size of the high energy state across the thylakoid membrane and vectorial nature of the recombination process. William (Bill) Arnold (Arnold and Azzi 1971) discovered 'electroluminescence'; Kenneth (Ken) Sauer (Ellenson and Sauer 1976) and Hans van Gorkom (de Grooth and van Gorkom 1981) studied the effects of electrical gradients on delayed light emission. However, the usefulness of delayed light emission as a tool to study the electron transfer and thermodynamics of PS II was brought to the forefront by Bill Rutherford, Govindjee and Yorinao Inoue (1984) who investigated, in parallel, different phases of delayed light emission and thermoluminescence. Although originally discovered by Arnold and Sherwood (1957), it was this pioneering work that was the basis for the correlation of different thermoluminescence bands with different recombination processes. From this analysis emerged a technique which has provided much information about charge storage in PS II, as reviewed in Imre Vass and Inoue (1992); see also the historical perspective by Vass (2003).

Prompt chlorophyll fluorescence

It had been shown by Arnold and colleagues (Strehler and Arnold 1951; Arnold and Davidson 1954) that delayed fluorescence had the same spectrum as Chl prompt fluorescence, therefore originating from the singlet state of Chl *a*. It was also known from the pioneering work of Louis N.M. (Lou) Duysens (Duysens and Sweers 1963) and others (reviewed by Govindjee and Papageorgiou 1971; for an historical account, see Govindjee 1995) that the majority of the prompt Chl fluorescence from higher plants and algae originated from PS II and that its yield varied with the redox state of the PS II reaction center (RC). When the RC was open the yield was low (F_o), and rose to a maximum (F_m) as the RC closed. As shown by Hans Kautsky (Kautsky et al. 1960); Jean Lavorel (1959); Govindjee (Munday and Govindjee 1969) and others, in the case of intact cells this fluorescence rise could be complex, reflecting the secondary electron transfer events into and out of the PS II RC, as elegantly analyzed by Shmuel Malkin and Bessel Kok (1966). Also the kinetics of the fluorescence rise from F_o (the initial) to F_m (maximal) included the possibility of energy transfer between adjacent PS II units as first studied by Pierre Joliot and Anne Joliot (1964), see also Joliot and Joliot (2003). Even after reaching the F_m level, subsequent changes in fluorescence yield occurred due to other phenomena associated with membrane gradients (Δ pH; Wraight and Crofts 1970) or state-transitions (Bonaventura and Myers 1969); see also Allen (2002). It was therefore important that my quantitative analyses of delayed fluorescence took into account changes in fluorescence yield (Hipkins and Barber 1974; Barber et al. 1977b). Because of this I became aware of the large changes in the F_m level that could be induced with isolated thylakoid membranes by changing the salt levels of the suspension medium. In a medium of low ionic strength (typically 5 mM NaCl) the F_m level was relatively low but addition of, for example, 5 mM $MgCl_2$ resulted

in a large increase in fluorescence yield giving an F_m/F_o ratio more typical of that observed *in vivo*. The Mg^{2+}-induced increase in the F_m level had been observed and studied initially by Peter Homann (1969) and Norio Murata (1969) and later by others (e.g., Mohanty et al. 1973).

It was therefore a natural extension of my studies of delayed luminescence to explore this Mg^{2+}-induced phenomenon. Together with Alison Telfer, John Mills and Jenny Nicolson, we found that the 'high *in vivo* F_m' level was maintained in isolated intact chloroplasts even when suspended in low salt medium (Barber et al. 1974b; Mills and Barber 1975). Only when the isolated intact chloroplasts were osmotically shocked did the F_m drop to the 'low salt' level. Addition of 5 mM $MgCl_2$ restored the F_m level to that observed with the intact chloroplasts. Moreover, when the intact chloroplasts were illuminated with actinic light, the F_m level slowly declined with time, an effect reminiscent of that observed in intact leaves and algal cells as originally described by Kautsky et al. (1960). Addition of uncouplers restored the F_m level to its original high value. We therefore interpreted most of this slow decrease in F_m as being due to the build up of a Δ pH across the thylakoid membrane. This phenomenon has subsequently been termed 'nonphotochemical quenching' or NPQ. My work in this area was paralleled by that of G. Heinrich Krause (1974) who later, with Claudie Vernotte and Jean-Marie Briantais, went on to publish a definitive paper which essentially established the relationship between Chl *a* fluorescence changes and proton gradient during the slow decay of fluorescence transient (Briantais et al. 1979; Krause et al. 1982).

At about that time I became aware of the work of Lester Packer (Murakami and Packer 1971) and others who showed that under low salt conditions isolated thylakoids become unstacked and that the addition of 5 mM Mg^{2+} induced stacking to form granal/stromal regions. We therefore concluded that within the intact chloroplasts there was sufficient Mg^{2+} to maintain thylakoid membrane stacking and the high F_m level. We also concluded that it was likely that in the intact chloroplast, Mg^{2+} acted as the counterion for H^+-pumping as emphasized by the action of ionophore A23187 (Barber et al. 1974c). Much of my thinking on this subject was presented in Chapter 3 of 'The Intact Chloroplast' which was the first volume of a series entitled Topics in Photosynthesis (Barber 1976).

First thoughts about PS II structure

The apparent correlation between the F_m level and conformational state of the thylakoids was reinforced by freeze-fracture studies which indicated that various particles attributed to Photosystem I (PS I), PS II, light harvesting and the ATP synthase CF_0–CF_1 complexes, were randomized under low salt conditions but partitioned into stacked and unstacked regions of the membrane on the addition of 5 mM $MgCl_2$ (Ojakian and Satir 1974; Staehelin 1976). Moreover these changes could also be induced by other divalent cations and by higher levels of monovalent cations such as Na^+ and K^+. Using Chl fluorescence I decided, with the help of John Mills, Andrew Love, Geoff Searle, Fred Chow, Herb Nakatani and Barry Rubin, to make a thorough analysis of these cation-induced effects and concluded that they controlled the structure of the thylakoid membrane by electrostatically screening surface electrical charges (Barber et al. 1977a; Barber and Searle 1978, 1979; Barber and Chow 1979; Chow and Barber 1980; Nakatani and Barber 1980; Rubin and Barber 1980) and thus changing the balance between coulombic repulsive forces and electrodynamic attractive forces (e.g., long-range van der Waals) (Barber 1980b, 1982).

It was the in-depth analyses and associated experiments which led me to suggest that the two photosystems were laterally separated, with PS II located in the granal regions and PS I in the stromal lamellae (Barber 1980a, b; see discussions in Barber 1979 and associated recorded discussions; also see Anderson and Andersson 1981 and Barber et al. 1981); a similar idea had emerged earlier from the PhD thesis of Bertil Andersson (1978). Bertil Andersson together with Jan Anderson, obtained biochemical evidence to support the concept (1980). For further historical perspectives on this topic, see Anderson (2002) and Albertsson (2003). Thus lowering of the F_m fluorescence level when thylakoid membranes were unstacked could be understood in terms of the intermixing of the two photosystems, resulting in an increase in excitation energy transfer from PS II to PS I. The idea of lateral heterogeneity was, however, not totally new at that time. Roderick (Rod) Park and P.V. (Raj) Sane (1971) and Staehelin et al. (1976) have reviewed the early literature. Further, based on the studies of separation of PS I and PS II by nondetergent methods (Sane et al. 1970), Park and Sane (1971) proposed a model in which PS I was located in the stroma lamellae, granal margins and end membranes, whereas both PS I and PS II were

located in appressed membranes in grana (partitions). It was the dogma at that time that PS I and PS II were stoichiometrically related in the grana and in this way were responsible for noncyclic electron flow. Such a relationship was, for example, the basis of Warren Butler's 'tripartite model' where he proposed that the two photosystems were excitonically coupled. At the Ciba Meeting in London (1979) those present did not challenge this concept and therefore my ideas presented at this meeting seemed to go nowhere. However the idea of the 'extreme' lateral separation of pigment systems is now well accepted: PS II is only in the appressed grana region, but PS I is in the stroma lamellae, the end membranes and margins of grana (for one of the current models, see Albertsson 2001). For an historical account of the chloroplast structure, see Staehelin (2003).

The concept of lateral separation of PS II and PS I in higher plant chloroplasts is the basis for biochemical and structural studies of PS II heralded by the work of Berthold, Babcock and Yocum (1981) who described a simple procedure to isolate a PS II-enriched stacked membrane fraction using Triton X-100 (known as BBYs).

The era of PS II biochemistry

The BBY preparation has had enormous implications both for biochemical and biophysical measurements. It provided, for example, the starting point for isolating the PS II RC consisting of the D1 and D2 proteins and the α- and β-subunits of cytochrome *b*559 by Nanba and Satoh (1987); see also Satoh (2003). My colleagues and I were able to reproduce their method (Barber et al. 1987; Marder et al. 1987) and later modified it so as to increase the stability of the isolated complex (Chapman et al. 1988). At about the same time, Seibert et al. (1988) described the substitution of lauryl maltoside for Triton to improve the stability of the RC. Further, McTavish et al. (1989) and Crystal et al. (1989) provided methods to scrub oxygen out of the system to stabilize the RCs for accurate experimental use in the presence of light. The low Chl level bound to this stabilized preparation, which we determined to be 6 Chls per 2 pheophytin (Gounaris et al. 1990), at last provided an experimental system for investigating the kinetics of primary charge separation in PS II using femto- and pico-second spectroscopy. In this respect I had a very productive collaboration with Professor Lord Porter and colleagues and PhD students, especially David Klug, Paula Booth, Linda Giorgi, Gary Hastings, Steve Merry and James Durrant (summarized in Klug et al. 1998). At about the same time, experiments at the Argonne National laboratory, using RC preparations, prepared by Seibert and coworkers, were conducted by Mike Wasielewski, Govindjee and Mike Seibert and their coworkers (Wasielewski et al. 1989; summarized in Seibert and Wasielewski 2003). The overlap in the absorption of the chlorins within the PS II RC gave rise to the delocalization of excited states and contrasted with the primary photochemistry of bacterial RCs (Durrant et al. 1995). The work also showed that the P680 triplet state was long lived (1 ms) under anaerobic conditions with no quenching by the β-carotene molecules present in the RC (Durrant et al. 1990). When oxygen was present the lifetime of the P680 triplet shortened to 20 μs and singlet oxygen was formed (Telfer et al. 1994). I believe that it is this inherent and unique property of PS II that gives rise to its vulnerability to photoinduced damage manifested by the rapid turnover of the D1 protein (see Barber 1994). In PS II it is not possible to place the β-carotene sufficiently close to the high-potential chlorophylls of P680 for triplet–triplet transfer and at the same time avoid their photo-oxidation. Therefore, the generation of highly reactive singlet oxygen is not prevented by rapid transfer of the chlorophyll triplet to a nearby carotenoid.

Although the isolated D1/D2/Cyt *b*559 complex proved to be a useful preparation that once and for all established the functional homologies between the D1 and D2 proteins and the L and M subunits of the bacterial RC, it also had many shortcomings. It did not retain true Q_A and Q_B activities, the nonheme iron was not present and the EPR signals due to the formation of Tyr_Z and Tyr_D radicals were not detected. Therefore it was unlikely that such a stripped down complex could carry out full PS II function; namely water–plastoquinone oxidoreductase activity. I therefore turned my attentions to larger, fully functional PS II complexes with the view to determine their structures.

Structure of PS II

Monomer or dimer?

With considerable input from my PhD student Ben Hankamer, we developed protocols to isolate oxygen evolving PS II complex from spinach. Ben found mild digestion procedures produced dimeric as well

as monomeric core complexes with molecular mass of about 500 and 250 kDa, respectively (Hankamer et al. 1997b). At that time I was not convinced that the dimeric form was of physiological significance although others had suggested PS II was dimeric (Seibert et al. 1987; Bassi et al. 1989; Peter and Thornber 1991; Lyon et al. 1993; Seibert 1995). However, it was the work of Jon Nield, another PhD student, who together with Ben isolated a large dimeric supercomplex that contained in addition to the core PS II proteins, the light harvesting complex (LHCII), CP29 and CP26, which persuaded me that the dimeric state was physiologically relevant. The first structural information of our PS II core dimer and the LHCII–PS II supercomplex was obtained by electron microscopy (EM) through collaboration with Egbert Boekema and Mathias Rögner (Boekema et al. 1995). This work clearly showed how the core dimer was centrally placed within the supercomplex and in some cases we could see particles where the flanking LHCII, CP29 and CP26 proteins had been lost from one side (Figure 1). The idea that PS II was dimeric had been advocated and accepted for cyanobacteria without controversy. However, the earlier EM work of Andreas Holzenberg and Robert C. Ford (Holzenberg et al. 1993) on PS II structure of higher plants had been interpreted as monomeric. Thus for some time the monomer–dimer issue became controversial as discussed in our 1997 review (Hankamer et al. 1997a).

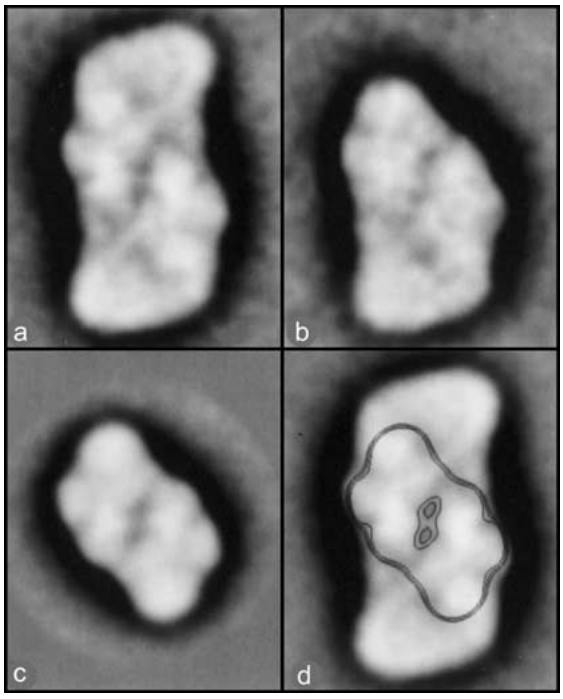

Figure 1. Averaged images of the top views of PS II particles isolated from spinach observed in the electron microscope after negative staining. (a) Complete LHCII–PS II supercomplex having dimensions of approximately 29×12 nm. (b) A top view of the LHCII–PS II supercomplex missing a set of Cab-proteins (LHCII, CP29 and CP26) on one side of the core dimer. (c) PS II core dimer having dimensions of approximately 17×10 nm. (d) Complete LHCII–PS II supercomplex with an outline of the core dimer. (Modified from Boekema et al. 1995.)

CP47–RC complex

The biochemical protocols established by Ben Hankamer and Jon Nield paved the way for our subsequent structural studies. In the absence of good 3-D crystals, I decided to exploit high resolution EM both through single particle analyses and electron crystallography. Our first success was to grow 2-D crystals of PS II subcore complexes containing the D1, D2 and CP47 proteins as well as low molecular weight proteins including the α- and β-subunits of cytochrome *b*559 (CP47–RC complex). At about the same time, Nakazato et al. (1996) also produced 2-D crystals of PS II. However, we did not have direct access to a high-voltage electron microscope with a low-temperature stage required to obtain structures with good resolution. Thus, I collaborated with Werner Kühlbrandt at the European Molecular Biology Laboratory (EMBL) in Heidelberg (Germany). He had just taken on a new PhD student, Kyong-He Rhee from Korea, who would conduct the electron cryomicroscopy and subsequent image processing. At Imperial College, my colleague Ed Morris, could use our low-voltage electron microscope to check the quality of the 2-D crystals. The collaboration worked well and in 1997 we obtained an 8 Å projection map of PS II (Rhee et al. 1997). One year later the 3-D map was calculated and for the first time we were able to visualize directly the organization of the transmembrane helices in PS II (Rhee et al. 1998). Ten helices were readily identified as those of the D1 and D2 proteins since they were organized in a manner very similar to those of the L and M subunits. Six further transmembrane helices were assigned to CP47 which were arranged in a circular manner very much like the six N-terminal helices of the PS I RC proteins, PsaA and PsaB which had been determined by X-ray crystallography to 4 Å by the Berlin group (Krauß et al. 1996). This structural similarity

established that there is a common evolutionary origin for PS I and PS II RC (Rhee et al. 1998); the same idea was also proposed by Schubert et al. (1998) at about the same time. We assigned other densities to seven further transmembrane helices and to the porphyrin head groups of Chls and pheophytin molecules. According to these assignments CP47 bound 14 Chl molecules and more importantly the chlorins bound within the D1 and D2 proteins were arranged some what like those in the bacterial RC. However, our work established that there was no real 'special pair' and that the four chlorophylls on the donor side were equally spaced based on center to center distances (Figure 2). This important conclusion was confirmed three years later by the X-ray studies of Zouni et al. (2001) (see Figure 2b) and again by Kamiya and Shen (2003). However, a comparison of Figures 2a and b does show some differences in the orientation of accessory chlorophylls. Our work, and that of the X-ray crystallographers, has opened up the debate as to 'where and what is P680?' (see Barber 2001). From recent work (Dekker and van Grondelle 2000; Prokhorenko and Holzwarth 2000; Diner et al. 2001) it seems likely that PS II contrasts with the purple bacterial RC in that primary charge separation originates from an 'accessory' chlorophyll in PS II and that 'hole' migration occurs as indicated in Figure 3 (Barber and Archer 2001; Barter et al. 2003).

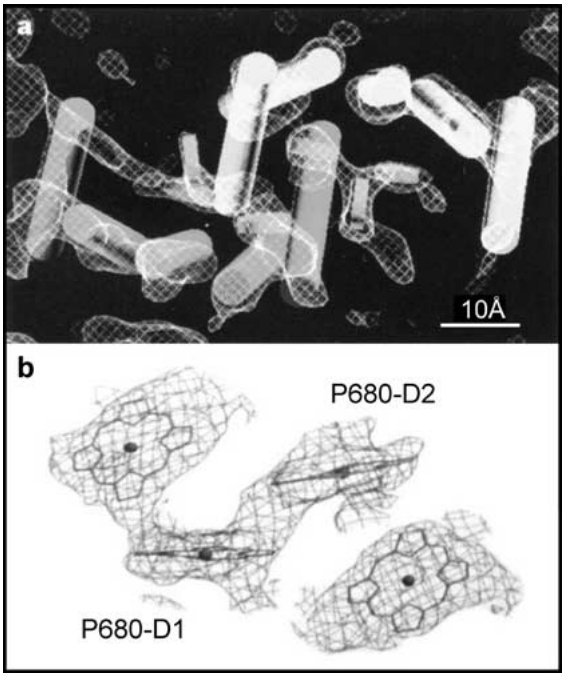

Figure 2. (a) Positioning of the four 'core' chlorophylls and two pheophytins within the D1 and D2 transmembrane helices showing the absence of a special pair in PS II. The data was obtained by electron crystallography and published in Rhee et al. (1998). (b) Positioning of the four 'core' chlorophylls of P680 as determined by X-ray crystallography confirming the absence of a special pair in PS II (Zouni et al. 2001) where P680-D1 is the Chl ligated to D1His198 and P680-D2 is the Chl ligated to D2His197.

With hind sight there were two other aspects of our analysis of the electron density map of the CP47–RC complex which we should have recognized. We identified in Rhee et al. (1998), but did not report, that two helices in our structure were likely to be the subunits of cytochrome *b*559 (Cyt *b*559) because there was sufficient density between them to accommodate a heme group. Kyong-He Rhee, however, did include this assignment in her PhD thesis (Rhee 1998) which was confirmed by X-ray structure analysis (Zouni et al. 2001). The other assignment relates to the two extra Chls of the D1 and D2 proteins which were predicted to be ligated to their B-helices at His118 and later were visualized in the X-ray structures (Zouni et al. 2001; Kamiya and Shen 2003). We observed the density for these two extra Chls but incorrectly interpreted them as possible transmembrane helices. Therefore the CP47–RC complex contains five transmembrane helices of low molecular weight subunits, not seven as originally reported. From N-terminal analyses and mass spectrometry we concluded that these five low molecular weight proteins are the α- and β-subunits of Cyt *b*559, PsbI, $PsbT_C$ and PsbW (Zheleva et al. 1998).

RC core dimer

Although the 3-D analysis of the CP47–RC subcomplex had been highly successful, I was frustrated by the fact that it was not a fully functional complex able to evolve oxygen. Thus with Ben Hankamer I took up the challenge to grow good 2-D crystals of the PS II core dimer. Using reconstitution procedures we were able to obtain rather good 2-D crystals which initially provided a low resolution 3-D model after analyzing negatively stained samples (Morris et al. 1997). With the arrival of a Phillips CM200 electron microscope at Imperial College with a field emission gun and cryo facilities, we were able to exploit further the potential of the 2-D crystals. Ed Morris conducted the cryo-EM and by 1999 we had obtained a projection map of the spinach core dimer at about 9 Å (Hankamer et al. 1999). This was an important step since it established that CP43 was definitely located on the opposite

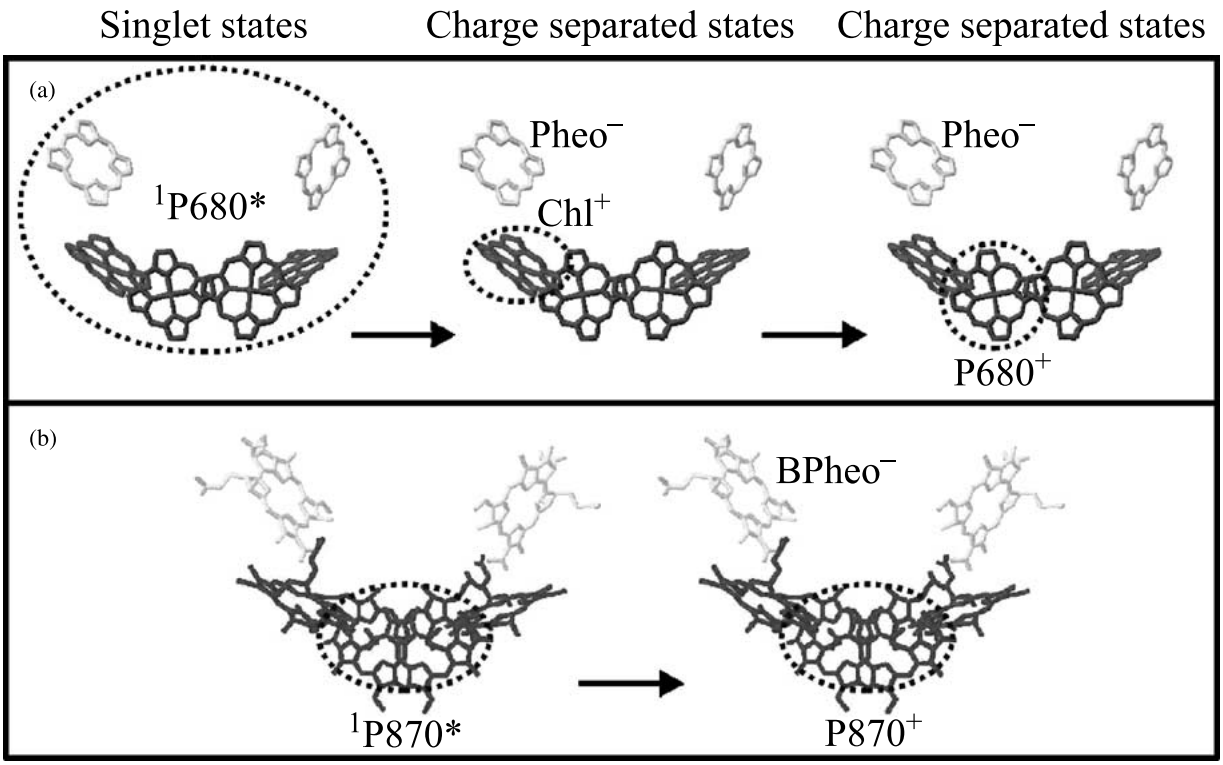

Figure 3. Schemes for primary charge separation in PS II (a) and purple bacteria (e.g., *Rhodobacter sphaeroides*), (b), emphasizing the distinct difference between the two systems. In the case of PS II the initial oxidant Chl^+ is thought to be the accessory chlorophyll bound to the D1 branch of the reaction centre while $P680^+$ is assumed to be the chlorophyll ligated to D1His198 (taken from Barber and Archer 2001).

side of the D1/D2 proteins to CP47 and also revealed densities, which were not present in the earlier CP47–RC map. From the very beginning I assumed that D1 was adjacent to CP43 based on the idea that CP43 becomes dislodged from PS II to allow the turnover of the D1 protein (Barbato et al. 1992). The X-ray models have since verified this positioning.

From 1999 onwards we worked hard to obtain a 3-D structure of the spinach PS II core dimer by merging data obtained from tilted crystals. Although by the summer of 2000 we had made good progress our work was somewhat over shadowed by the report at a Gordon Conference of a 3-D model of PS II at 3.8 Å. This model was calculated from X-ray diffraction data collected from crystals of PS II cores isolated from the thermophilic cyanobacterium *Synechococcus elongatus* (Zouni et al. 2001; see Witt, this issue). It was with some satisfaction that I noted that their model was consistent with our work. Although the Berlin group initially thought they were crystallizing a monomer (Zouni et al. 2000) it was clear that their PS II complex was dimeric and very similar to that described by us for spinach. Moreover their assignment of the transmembrane helices of D1, D2, CP43 and CP47 was also consistent with ours. They also observed that P680 was not a special pair and that CP47 bound 14 Chls. Of course the X-ray structure surpassed our efforts in that it was at a higher resolution and readily located the metal centers in the complex, especially the density of the Mn cluster. On returning to London from the Gordon Conference, I decided to complete our 3-D structure based on electron crystallography and publish it, which we did in 2001 (Hankamer et al. 2001a). This 3-D structure shown in Figure 4 remains the only higher plant model of PS II at a level of resolution sufficient to identify transmembrane helices. As shown in Figure 5, it is remarkably similar to the cyanobacterial PS II but there seems to be differences in the number and positioning of some of the low molecular weight proteins (Hankamer et al. 2001b; Barber 2002, 2003). At present, the resolution of the X-ray structures of Zouni et al. (2001) and Kamiya and Shen (2003) are not sufficient to assign the single transmembrane

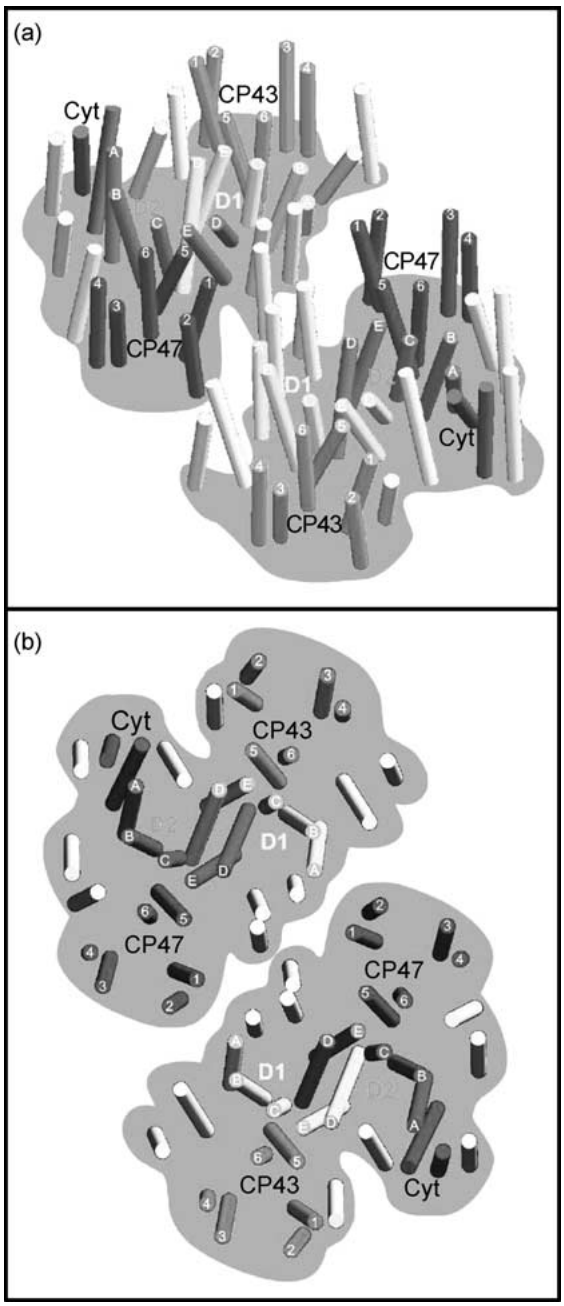

Figure 4. 3-D structural model of the PS II core dimer of spinach derived by electron crystallography (Hankamer et al. 2001a, b) showing transmembrane helices in (a) oblique view and (b) top view from the lumenal side.

helices to specific gene products and therefore the labeling shown in Figure 5 is tentative. Nevertheless, the two structures raise the question as to exactly what happens when some genes encoding the low molecular weight proteins have been inactivated. For example, together with Steve Mayes and Zhi-Hong Zhang, we had inactivated the genes encoding for PsbH, PsbK, PsbN as well as PsbO in *Synechocystis* 6803 (Mayes et al. 1991, 1993; Zhang et al. 1993). Others had inactivated other genes, for example *psb*I (Ikeuchi et al. 1995; Kunster et al. 1995). In all these cases PS II complex assembled and the mutants grew photoautotrophically. I ask myself, what plugs the 'hole' left by the absent protein?

The success of our work with higher plant PS II gives credit to those who pioneered electron crystallography as a technique for studying the structure of membrane proteins, particularly Richard Henderson and Nigel Unwin (1975). However the technique is limited and ultimately cannot compete with X-ray diffraction analyses of high ordered 3-D crystals. Thus the way forward is to obtain a high-resolution structure of PS II by X-ray crystallography. We need to reach a resolution sufficient to gain information about:

(i) Positioning and identification of side chains so as to elucidate the nature of the ligands that coordinate the cofactors, particularly the Mn-cluster, and explore the relationship between their redox properties and protein environment.
(ii) The nature of the base close to Tyrosine Z (Yz or TyrZ) of the D1 protein in order to understand the role of this redox active amino acid in water oxidation. Is this base the D1His190 residue as proposed by site-directed mutagenesis (Debus 2001) and molecular modeling (e.g., Svensson et al. 1996)?
(iii) The distance between the water substrate molecules and TyrZ in order to assess whether the hydrogen-atom abstraction model of Jerry Babcock is feasible [Hoganson and Babcock (1997); see also Renger (2003)].
(iv) Factors that give rise to the high and low potential forms of cytochrome *b*559.
(v) The nature of the photoinduced damage of D1 protein and the mechanisms of its selective degradation and replacement (see Adir et al. 2003).

Ultimately we would hope to obtain a structure at sufficient resolution to identify substrate water molecules and to obtain X-ray diffraction data for PS II in its various S-state configurations with the view of fully elucidating the chemistry of water oxidation.

Supercomplexes

Despite the obvious importance of X-ray crystallography, EM will continue to give information about

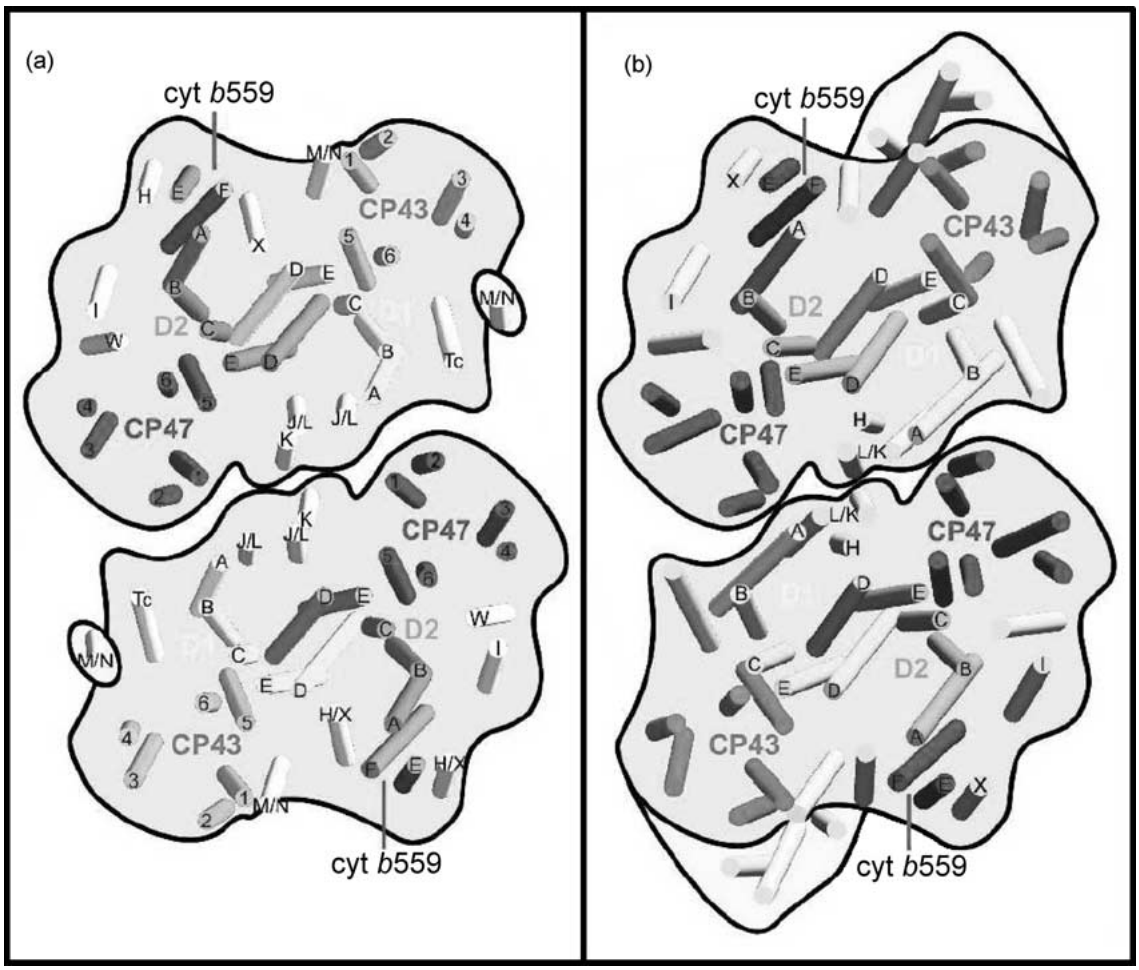

Figure 5. A comparison of the transmembrane helices in the core dimer of (a) spinach (Hankamer et al. 2001a) and (b) *S. elongatus* (Zouni et al. 2001). The helix organization of the D1 protein, D2, CP43, CP47, Cyt *b*559 and nine further helices of the small subunits seem to be identical in the two models. The labeling of small subunits has been made, tentatively, for spinach (Hankamer et al. 2001b) and for *S. elongatus* (Zouni et al. 2001).

the structural properties of PS II. It can provide a structural framework in which to incorporate high resolution X-ray data and thus explore the organization of macromolecular systems. With the help of Jon Nield in particular, I focused on elucidating the 3-D structure of the LHC II–PS II supercomplex that he, with Ben Hankamer, had isolated and biochemically characterized during his PhD studies. Today we have a 17 Å 3-D model determined by cryo-EM (i.e., in the absence of electron dense stain) and used it to investigate how LHC II trimers may interact with the RC core dimer (Nield et al. 2000a, b) (see Figure 6) as well as gain a glimpse of the organization of the extrinsic proteins of the oxygen evolving complex (OEC) attached to the lumenal surface of plant PS II (Nield et al. 2002). Kamiya and Shen (2003) have identified the three extrinsic subunits of cyanobacteria and most of the loop structures of CP43 and CP47 in their 3.7 Å structure of PS II.

The EM approach has also allowed us to discover that PS I can form a supercomplex with a PS II-like protein. During his PhD studies, Tom Bibby was looking for a modified form of PS II, which may form when cyanobacteria experience iron deficiency. Using *Synechocystis* Pasteur Culture Collection (PCC) 6803 I had hypothesized that the product of the iron-stress-induced *isi*A gene could replace CP43 in normal PS II so as to convert it from a water oxidase to a

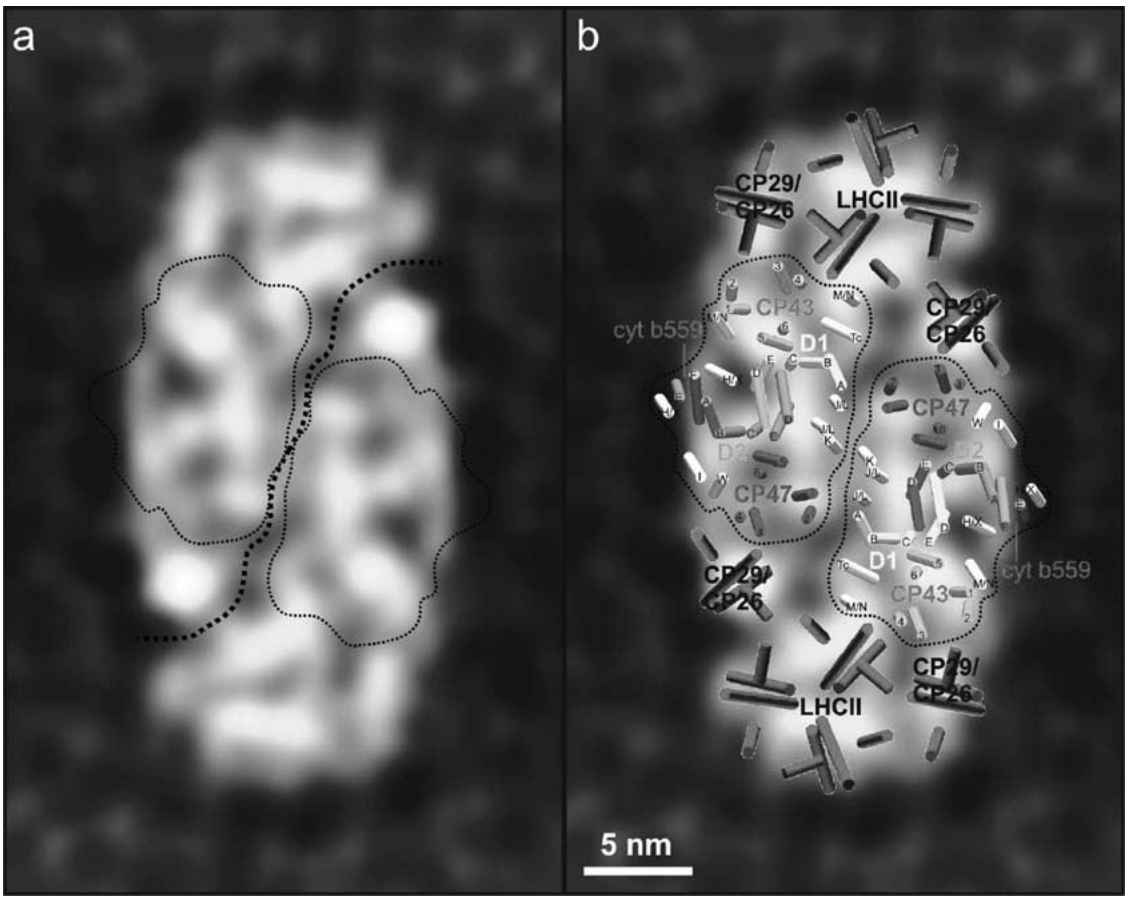

Figure 6. The relationship between the transmembrane helices of the central PS II core dimer and those of the outer antenna system of the LHCII–PS II supercomplex of spinach based on a combination of electron crystallographic and single particle analyses. (a) 2-D top-view projection derived from a 17 Å 3-D map of the LHCII–PS II supercomplex obtained by cryo-EM showing the transmembrane domain (Nield et al. 2000c). The large dotted line separates the two monomers that make up the supercomplex and the fine dotted lines outline the central position of the core dimer. (b) Helices of the spinach core dimer from Figure 4a and the antenna Cab proteins, Lhcb1,2,4 (CP29) and 5 (CP26), based on the structure of the LHCII (Kühlbrandt et al. 1994), incorporated into the projection shown in (a) along the lines of previous modeling, but with modifications (Nield et al. 2000a, b; Hankamer et al. 2001a, b). Of special note is that according to the modeling, a group of two low molecular weight subunits (tentatively labeled T_C and M/N) adjacent to helix B of the D1 protein seems to form a structural link to the LHCII trimer. In contrast *S. elongatus* has only one transmembrane helix assigned to this region (see Figure 5b).

plastocyanin oxidase. The *isi*A gene product is very similar to CP43 except it lacks the large loop joining transmembrane helices 5 and 6. For this reason it is often called CP43′. Was it therefore possible that CP43′ could replace CP43 and prevent the binding of OEC proteins? If so then perhaps $P680^+$ would be reduced by plastocyanin and facilitate cyclic electron flow similar to that which occurs in purple photosynthetic bacteria. Such a cycle would generate a Δ pH across the thylakoid membrane and help the organism to survive under iron stress conditions when the level of PS I drops significantly due to poor supply of iron. Tom Bibby did not find this hypothetical, modified form of PS II but his efforts resulted in the discovery of a supercomplex with 18 subunits of CP43′, forming a light-harvesting antenna ring around the central trimeric PS I RC (Bibby et al. 2001a, b). At about the same time a similar structure was found in a species of *Synechococcus* (Boekema et al. 2001), also under iron-deficiency. Seeing this beautiful structure under the microscope was very exciting, an excitement which was enhanced when we showed that a very similar PS I light-harvesting antenna ring could be found in *Prochlorococcus marinus* SS120 (Bibby

et al. 2001c). *Prochlorococcus* is probably the most abundant photosynthetic organism in the oceans and, like other prochlorophytes, contains a CP43′-like protein which binds chlorophyll *b* as well as chlorophyll *a* (known as Pcb proteins). We are continuing to combine EM, single-particle analysis and biochemical methodologies to study supercomplexes of PS I and PS II in a wide range of organisms which use the CP43-like proteins as light harvesting systems either under iron-rich or iron-deficient conditions (Bibby et al. 2003a, b). Iron deficiency, of course, is common in the natural environment and therefore the new structures we are looking at are physiologically important and abundant.

A giant step forward

Since writing the above text, my PS II journey has taken one giant step forward. In collaboration with my colleagues at Imperial College, London, So Iwata, Tina Iverson, Kristina Ferreira and Karim Maghlaoui, I have been lucky enough to obtain a complete and highly refined structure of PS II at a resolution sufficient to map over 2600 side chains and determine the position and orientations of 36 Chls and 7 carotenoids. In so doing we have revealed the details of the protein environments of the redox-active cofactors including the ligands for the metal center responsible for water oxidation. All but one of the low molecular weight subunits have now been assigned (Figure 7a). The details of this work have recently been published (Ferreira et al. 2004). Suffice to say that there are many facets of PS II function, which can now be addressed against a structural background, that hitherto was not possible. Here it seems appropriate that I should address the specific points that I raised in the section 'RC core dimer' before obtaining this new structure of PS II.

We have concluded that the metal center is composed of a cubane-like Mn_3CaO_4 cluster linked to a fourth Mn ion by a mono-μ-oxo bridge (Figure 7b). The three Mn ions of this cubane-like structure have protein ligands including, surprisingly, one provided by the large extrinsic loop of CP43 (D1 Asp342, D1 His332, D1 Glu189 and CP43 Glu354). Water oxidation probably occurs at the fourth Mn ion having two protein ligands (D1 Glu333 and D1 Asp170). The Ca^{2+} within the cubane-like structure has three μ-oxo ligands but no direct protein ligands although D1 Ala344 comes close to it. Many of these protein ligands have been predicted by a wide range of excellent mutational studies reviewed by Rick Debus (2001) and Bruce Diner (2001). The proposed structure for the metal center is compatible with a mechanism of water oxidation in which a highly electrophilic oxo or oxyl radical, formed at the active Mn ion, undergoes nucleophilic attack by the oxygen of the second substrate water molecule contained within the coordination sphere of the Ca^{2+} along the lines of that suggested by Per Siegbahn (2002), Gary Brudvig (Vrettos et al. 2001) and Vincent Pecoraro et al. (1998). The structure we have proposed for the metal center is attractive because of its robustness and tolerance to ligand exchange. However, future studies are required to improve the resolution of our model and to investigate any structural changes that occur in the higher S-states.

Our structure is of sufficient reliability to conclude that D1 His190 is in hydrogen bonding distance to TyrZ as would be required for the latter to be oxidized by $P680^+$. The adjacent D1 Glu189 is not hydrogen bonded to D1 His190 but forms a protein ligand for one of the Mn in the cubane. There is no indication that water substrate protons would exit the active site via TyrZ as proposed by Babcock and colleagues (Hoganson and Babcock 1997) although the distance between the substrate water molecules in the active center and Tyr_Z is sufficiently short for hydrogen bonding interactions. We have identified a potential proton channel leading from the active site involving a number of polar residues but this channel is almost on the opposite side to the electron transfer pathway and involves D1 Asp61, D1 Glu65, D2 Glu312 and D2 Lys317. It is possible, however, that Tyr_Z could act as a proton acceptor at the S_4 to S_0 step of the S-state cycle and in this way aid deprotonation of the second substrate water molecule associated with Ca^{2+}.

Of the many other details of the structure none of them help to explain the remaining points which I raised in the section 'RC core dimer', namely the factors which give rise to the high-and low-potential forms of Cyt *b*559 and to the mechanism of D1 protein turnover. Perhaps future studies on PS II complexes, which have been photoinhibited prior to crystallization, will help to resolve these more dynamic aspects of PS II. It is of note, however, that the PsbO protein bridges the two monomers within the dimer and could play a role in D1 protein turnover by detaching and inducing monomerization.

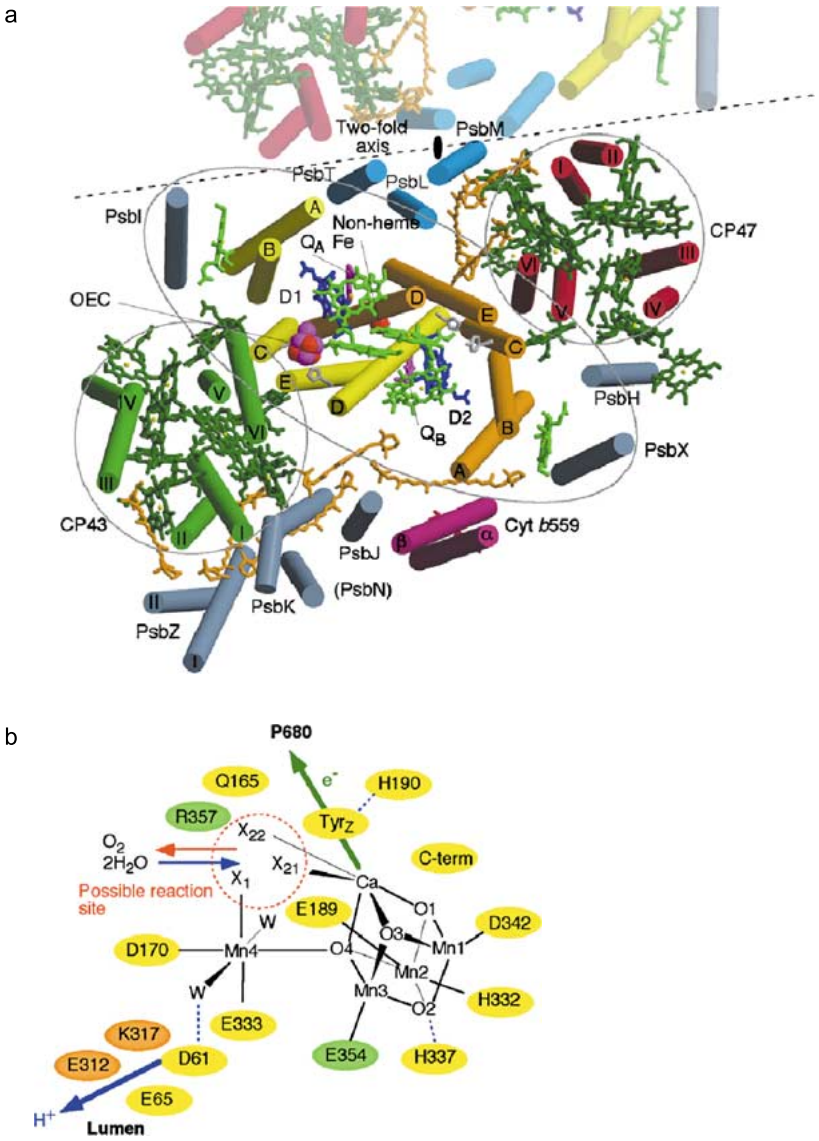

Figure 7. (a) View of the PS II monomer along the membrane normal from the lumenal side showing the assignment of the various subunits. One low molecular weight transmembrane helix is tentatively labeled as PsbN. A part of the other monomer in the dimer is shown to emphasize the region of monomer/monomer interaction along the dotted line. Also shown are the assigned 36 chlorophylls (6 binding to D1/D2, 16 to CP47 and 14 to CP43) and 7 carotenoids. The pseudo two-fold axis is perpendicular to the membrane plane passing through the non-heme Fe relates the transmembrane helices of the D1/D2 heterodimer, the low molecular weight subunits, PsbI and PsbX, and CP43 and CP47, as emphasized by the black circles around these subunits. The metal cluster of the oxygen evolving center is shown as red (oxygen atoms), magenta (Mn ions) and cyan (Ca^{2+}) balls and is located on the D1 side of the complex. Helices are represented as cylinders with D1 in yellow, D2 in orange, CP47 in red, CP43 in green, cyt b559 in wine red, PsbL, PsbM and PsbT in medium blue, and PsbH, PsbI, PsbJ, PsbK, PsbX, PsbZ and the putative PsbN in grey. The extrinsic proteins are PsbO in blue, PsbU in magenta and PsbV in cyan. Chlorophylls of the D1/D2 reaction center are light green, pheophytins are blue, chlorophylls of the antenna complexes are dark green, β-carotenes are in orange, hemes are in red, non-heme Fe is red, Q_A and Q_B are magenta. (b) A diagrammatic representation of the cubane-like Mn_3CaO_4 cluster bridged to a fourth Mn ion by a μ-oxo bridge indicating the various protein ligands where yellow represents D1 residues, orange D2 residues, and green CP43 residues. The positioning of the Mn ions and Ca^{2+} are based on anomalous diffraction data and Fourier difference calculations. The cubane-like structure provides three oxo-ligands for the Ca^{2+} and three of the four Mn ions (Mn1, Mn2 and Mn3). The fourth Mn (Mn4) is probably bound to the high affinity site and is actively involved in water oxidation chemistry by binding one of the substrate water molecules. The other water substrate molecule probably binds to the calcium as indicated. The ligand denoted X could be chloride. H^+/water channel leading from and to the catalytic center is identified and is on the opposite side of the metal cluster to that of the electron transfer pathway to P680. The overall figure is based on the X-ray structure of Ferreira et al. (2004) and (b) is a modification of the scheme given in this paper to emphasize the possible sites for substrate water binding. For a color version of this figure, see color section in the front of the issue.

Final comments

There is no question that our progress in understanding the structure of PS II has taken a dramatic step forward over the past few years and it has been a privilege to have been involved in some of this progress. I am sure that X-ray crystallography will soon provide us with the level of detail needed to fully elucidate the mechanisms of the 'engine of life.' Its appearance about 2.5 billion years ago was indeed the 'big bang' of evolution but how did it evolve from a purple bacterial-like RC? (For a discussion on evolution, see Olson and Blankenship, this issue.) No doubt new microorganisms will be found which will help us to fill the gap between the anoxygenic and oxygenic systems. Is there a PS II-like RC complex which catalyzes light driven cyclic electron flow involving the cytochrome b_6f complex? Does, for example, the 'CP43′/D1/D2/CP47' complex exist as we hypothesized when Tom Bibby started his PhD project? If so, does it act as a plastocyanin/Cyt c_6 oxidase? Dan Arnon was very keen about the idea of a plastocyanin driven cyclic pathway around PS II (Arnon and Tang 1989) which was the motivation behind the experiments I conducted with him when I visited Berkeley in 1990 for a 3-month study leave (Arnon and Barber 1990). Clearly such a cycle cannot occur when the Mn-cluster and extrinsic proteins are present and $P680^+$ is shielded from any electron donors other than water. But how did the Mn-cluster and its associated 'protein shield' evolve? My own recent exploration of various types of oxyphotobacteria has opened my eyes to the level of diversity that exists in the natural environment. Who would have guessed, for example, that a Chl *d*-containing RC existed as in the case of that recently discovered in *Acaryochloris marina* (Miyashita et al. 1996). Instead of having a P700 composed of Chl *a* it has P740, composed of Chl *d*, as its primary donor (Hu et al. 1998) and it remains an open question whether Chl *d* acts as a primary donor in PS II in this organism. If it does, then this will stimulate a serious debate about the thermodynamics of water oxidation because the redox energy available will be less than that provided by Chl *a*. Then there has been the discovery that the aerobic purple photosynthetic bacterium *Acidiphilium rubrum* uses Zn-bacteriochlorophyll instead of Mg-bacteriochlorophyll (Wakoo et al. 1996). It seems to me that there are many more exciting discoveries to be made as we cast our net more broadly for new experimental systems.

Along with these new discoveries will come the complete understanding of the chemistry of water oxidation which, with the new structural information, is now in sight.

Acknowledgments

My PS II journey would not have been possible if I had not been continuously funded by the UK research councils (Science Research Council, Agriculture and Food Research Council, and Biotechnology and Biological Sciences Research Council) and without the input of dedicated and talented PhD students, post-doctoral associates and academic visitors to my laboratory at Imperial College as well as numerous collaborations with colleagues in other laboratories. It is impossible to name the extensive list of those who have contributed but I particularly wish to acknowledge my very long collaboration with Alison Telfer (Figure 8) who joined my group as a post-doc in 1972 and who has survived 31 years of dealing with me. I also wish to give special recognition to Ben Hankamer, Jon Nield, Ed Morris and Tom Bibby (Figure 9) who, along with Egbert Boekema, Werner Kühlbrandt, Tina Iverson and So Iwata, have played an important role in my recent structural studies. There have been, of course, many excellent graduate students, Bill Varley, Mike Hipkins, John Mills, Peter Nixon, Julian Whitelegge, William Newell, Bob Ford, Herb Nakatani, Roy Mansfield, Steve Mayes, Cathy Shipton, Katie Cook, Paula Booth, James Durrant to name just a few. My more recent graduate students

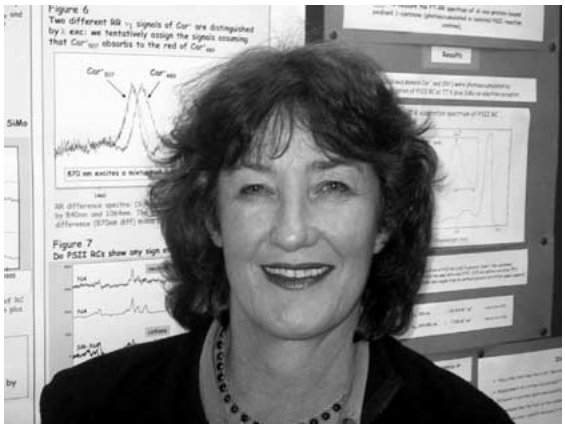

Figure 8. Alison Telfer, a long-time associate, photographed in 2003.

Figure 9. A photograph taken in my laboratory at Imperial College, London in 2002. *Left to right*: Ed Morris, Ben Hankamer, the author, Jon Nield and Tom Bibby.

Figure 10. A photograph of Professor Dan Arnon taken in Palma de Mallorca when he lectured on the Advanced Course in Photosynthesis entitled Trends in Photosynthesis Research in September 1990, which resulted in his last publication (Arnon 1992).

in the final stages of their studies are James Duncan, Kristina Ferreira, James Berry and Karim Maghlaoui. These and other PhD students, post-docs and visitors have all contributed to the vitality of my laboratory and my research career and I thank and applaud them all.

During my academic career I never managed to take a full year sabbatical but of the short study leaves I took my most memorable was spent in Berkeley at the University of California, Berkeley, in 1999, when I enjoyed endless hours discussing science and the meaning of life with Dan Arnon (Figure 10) as well as

Figure 11. A photograph of Professor Lord Porter OM, Nobel Laureate, with whom the author collaborated over a period of 30 years. Perhaps the most classical study of this collaboration was the first ever time-resolving of energy transfer in a photosynthetic light-harvesting system, namely the transfer of energy from phycobiliproteins to PS II in the red alga *Porphyridium cruentum* (Porter et al. 1978). (Also see Brody 2002; and Mimuro 2002.)

exploring ideas about plastocyanin as a donor to PS II. The Royal Institution also offered me an additional environment to conduct research where George Porter (Figure 11) provided inspiration and friendship and gave me the opportunity to investigate the reactions of PS II using ultrafast spectroscopy.

This paper was read by four reviewers, and edited by Govindjee. I thank them all for many suggestions that has improved my presentation.

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