Chapter 4

The D1 and D2 Core Proteins

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Summary

In 1977, Chua and Gillham (J Cell Biology 74: 441–452) reported for the first time the existence of two chloroplast-encoded proteins within the thylakoid membrane of the green alga *Chlamydomonas reinhardtii*, which they termed D-1 and D-2. The D1 and D2 proteins are now recognized as the Photosystem II reaction center polypeptides with a key role in binding all of the co-factors involved in photosynthetic water oxidation. In this chapter we summarize some of the biochemical and mutagenesis data that has been instrumental in shaping this view of the D1 and D2 proteins.

I. Introduction

Although the Photosystem II (PS II) complex — i.e., water-plastoquinone oxidoreductase, is composed of over twenty-five subunits, the important light-induced electron transfer reactions occur within a heterodimer composed of the D1 and D2 polypeptides (D1/D2). Recent structural studies have now identified the positions of the Chl and Pheo pigments within D1/D2 and, importantly, the location of the Mn-Ca metal cluster which catalyses photosynthetic water oxidation (Zouni et al., 2001; Kamiya and Shen, 2003; Biesiadka et al., 2004; Ferreira et al., 2004). Perhaps one of the most reassuring aspects of the structural studies has been how much had been correctly predicted in advance. In this chapter, we describe the background that led to the emergence of D1 and D2 as the PS II reaction center (RC) subunits, plus the key mutagenesis and biochemical experiments that identified the likely binding sites for the various pigments and redox-active components within the D1 and D2 heterodimer.

Abbreviations: BRC – reaction center from purple non-sulfur photosynthetic bacteria; B_A/B_B – PS II chlorophylls in similar position to the two accessory BChls of the BRC; BChl – bacteriochlorophyll; BPheo – bacteriopheophytin; C-terminal – carboxyl-terminal; Chl – chlorophyll; Chl Z_{D1} –peripheral Chl in the PS II RC, ligated by D1-His118; Chl Z_{D2} – peripheral Chl in the PS II RC, ligated by D2-His117; Cyt – cytochrome; ENDOR – electron nuclear double resonance; EPR – electron paramagnetic resonance; ESEEM – electron spin-echo envelope modulation; FTIR – Fourier transform infrared ; P – special pair of BChl in the BRC; P680 – historical term for the primary electron donor within PS II; P_A/P_B – chlorophylls in PS II in similar position to the special pair of BChl of BRC; $Pheo_A/Pheo_B$ – pheophytin *a* molecules in PS II in similar position to the BPheo molecules of the BRC; Q_A – primary quinone electron acceptor; Q_B – secondary quinone electron acceptor; RC – reaction center; S_2 – Mn cluster that has accumulated two of the four oxidizing equivalents needed for water oxidation; SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Y_D – redox-active tyrosine, D2-Tyr160; Y_Z – redox-active tyrosine, D1-Tyr161, which acts as the immediate oxidant of the Mn cluster

II. Identification of the D1 and D2 Proteins

A. Terminology

The D1 and D2 nomenclature was first used in studies aimed at identifying those thylakoid proteins that were synthesized by the chloroplast of the green alga *Chlamydomonas reinhardtii* (Chua and Gillham, 1977). Cells were pulse-labeled with [^{14}C]-acetate in the presence of an inhibitor of cytoplasmic protein synthesis, and the radiolabeled thylakoid proteins separated by denaturing polyacrylamide gel electrophoresis. Two broad areas of radioactivity were identified by autoradiography and termed 'diffuse band-1' (D-1) and 'diffuse band-2' (D-2). In these experiments, D-1 possessed an apparent molecular mass of about 34 kDa and D-2 appeared to be 30 kDa. Although D-1 and D-2 might have been composed of a number of different co-migrating polypeptides, the notation D1 and D2 (sometimes D_1 and D_2) has been subsequently used to describe the *psbA* and *psbD* gene products, respectively, in all oxygenic photosynthetic organisms.

B. Linking the psbA Gene to Its Gene Product in the Thylakoid Membrane

1. The Rapidly-Turning-Over 32-kDa Protein

Early studies on the synthesis of thylakoid membrane proteins in isolated chloroplasts of higher plants revealed that a major product was a protein of apparent molecular mass 32 kDa (Bottomley et al., 1974; Eaglesham and Ellis, 1974). Given that it did not seem to correspond to an abundant polypeptide, this particular protein appeared to be undergoing rapid synthesis and degradation within the membrane — hence its name. The gene encoding this protein, now termed *psbA*, was mapped onto the chloroplast genome by a number of different groups and the first sequences, from spinach and tobacco, were published in 1982 (Zurawski et al., 1982).

2. The 32-kDa Herbicide-Binding Protein

A large number of herbicides block photosynthetic electron flow on the acceptor side of PS II. Through the use of the radiolabeled photoaffinity herbicide, azido-[^{14}C]-atrazine, Pfister and co-workers showed that a 32-kDa protein was a target (Pfister et al., 1981). By proteolytic fingerprinting this particular tagged protein and the 32-kDa rapidly-turning-over protein, it was concluded that they were one and the same (Steinback et al., 1981). Confirmation that *psbA* encoded the herbicide-binding protein came with the identification of a mutation, Ser264Gly, in the *psbA* gene product of a spontaneous atrazine-resistant biotype of *Amaranthus hybridus* (Hirschberg and McIntosh, 1983). This same mutation has now been found in a variety of herbicide-resistant photosynthetic organisms (for a review, see Oettmeier, 1999).

3. The Q_B-Binding Protein

Several lines of evidence (for a review, see Kyle, 1985) have indicated that the binding site for herbicides in PS II was coincident with, or near to, the binding site for the secondary quinone electron acceptor, Q_B, which takes part in the binary gate mechanism for the transmission of electrons into the plastoquinone pool (Bouges-Bocquet, 1973; Velthuys and Amesz 1974). These included the influence of the Q_B redox state on the affinity of PS II for herbicides (Velthuys, 1981), EPR studies (Rutherford et al., 1984), inhibition of herbicide binding using plastoquinone analogues (Vermaas et al., 1983; Oettmeier et al., 1984) and slowing of Q_B reduction in atrazine-resistant plants (Bowes et al., 1980). Together these data led to the idea that D1, encoded by *psbA*, was the Q_B-binding protein of PS II, as well as a target for several classes of herbicide (for a review, see Oettmeier, 1999).

C. The Link Between the psbD Gene and Its Gene Product

The *psbD* gene was located first on the chloroplast genome of *C. reinhardtii* using a combination of antibodies raised against proteins in the D2 region of denaturing gels and an *E. coli*-based transcription-translation of cloned chloroplast DNA fragments (Rochaix, 1981). The sequence was first published in 1984 (Rochaix et al., 1984) and later in an amended form (Erickson et al., 1986). Using this gene, *psbD* was subsequently isolated from a variety of chloroplasts (Alt et al., 1984; Holschuh et al., 1984; Rasmussen et al., 1984) and cyanobacterial sources (Williams and Chisholm, 1987). The identification of the *psbD* gene product in thylakoids was initially uncertain, especially for higher plants, because of the lack of specific antibodies. For *C. reinhardtii,* D2 was identified indirectly on the basis of radioactive labeling of thylakoid proteins and concluded to be a component of the PS II core complex (Delepelaire, 1984). Confirmation that the *psbA* and *psbD* gene products were indeed components of the PS II complex from higher plants came with the use of specific antibodies raised to the gene products expressed in *E. coli* (Nixon et al., 1986).

III. The Primary Structures of D1 and D2

D1 is synthesized as a precursor protein in higher plants (Grebanier et al., 1978) with, unusually, a carboxyl-terminal (C-terminal) extension (Marder et al., 1984). With the apparent exception of *Euglena gracilis* (Karabin et al., 1984) and some species of dinoflagellates (Yamamoto et al., 2001), the presence of a C-terminal extension for D1 appears ubiquitous. Protein sequencing of mature D1 from spinach (M Takahashi et al., 1988; Y Takahashi et al., 1990), the green alga *C. reinhardtii* (B. A. Diner, personal communication) and the cyanobacterium *Synechocystis* 6803 (Nixon et al., 1992a) has confirmed that in all cases the C-terminal residue of mature D1 is Ala344 so that 9, 8 and 16 amino-acid residues are removed,

respectively, from their precursor molecules. In contrast, D2 is not C-terminally processed (Y. Takahashi et al., 1990).

In spinach, the initiating N-formylmethionine residue of both D1 and D2 is removed leaving Thr2 at the N-terminus, which is acetylated. These Thr residues can also be phosphorylated in both D1 and D2 (Michel et al., 1988). The physiological significance for D1 and D2 phosphorylation in higher plants remains unclear although evidence points to a role in regulating their degradation (Koivuniemi et al., 1995). D1 has also been suggested to be palmitoylated in higher plants (Mattoo and Edelman, 1987) but the site of attachment and physiological importance remain obscure. For cyanobacteria, D1 and D2 do not appear to be phosphorylated, whereas in the green alga *C. reinhardtii* there are reports that D2, but not D1, is phosphorylated (Delepelaire, 1983, 1984; de Vitry et al., 1991). Bands assigned to the phosphorylated (D2.1) and non-phosphorylated forms (D2.2) of D2 have been resolved by SDS-PAGE conducted in the presence of 8 M urea (Delepelaire, 1983, 1984). The phosphorylation site in D2.1 has yet to be identified.

The molecular masses for D1 and D2, determined by mass spectrometry, are approximately 38.0 kDa and 39.5 kDa, respectively, as predicted from the gene sequences (Sharma et al., 1997). However, upon SDS-PAGE analysis they usually migrate with apparent sizes of between 30–34 kDa (Satoh et al., 1983), depending on the electrophoretic conditions, most notably the concentration of urea in the gel. In the absence of urea, D1 migrates as two immunodetectable bands (at 34 and 30 kDa), the faster of which is likely to be a more compact structural 'conformer' (Taylor et al., 1988). D2 migrates between these two D1 bands (Nixon et al., 1986; Sayre et al., 1986). Inclusion of urea at concentrations greater than 6M causes the two D1 bands to collapse to a single band of apparent size 30 kDa, so that D2 now migrates slower than D1. Before the availability of specific antisera, the aberrant electrophoretic migration of D1 and D2 together with their poor staining by Coomassie blue, led to much confusion concerning their identification. It is even possible that the radiolabeled bands first assigned to D1 and D2 in non-urea containing polyacrylamide gels (Chua and Gillham, 1977) actually represent the two different electrophoretic conformers of D1 (Nixon, 1988).

IV. Identification of the D1 and D2 Proteins as the Photosystem II Reaction Center Subunits

A. Interpretations of Sequence Similarities

DNA sequencing studies revealed early on that the *psbA* and *psbD* gene products shared some sequence similarity and possessed similar hydropathy profiles, suggestive of an evolutionary relatedness (Rochaix et al., 1984). While the overall sequence identity between the two was relatively unimpressive (27% in *C. reinhardtii*), there were regions of high sequence identity approaching 60% (Rochaix et al., 1984). In concurrent work, it was realized that the L and M subunits of the RC of purple non-sulfur photosynthetic bacteria (hereafter called the BRC) also showed significant regions of sequence similarity to D1 and D2 (Williams et al., 1983, 1984; Youvan et al., 1984). In the absence of detailed structural information, the significance of these similarities was unclear. Based on what was known about D1 and PS II at the time, it was suggested that the areas of similarity were related to a function in quinone binding (Hearst and Sauer, 1984; Rochaix et al., 1984).

A major step in understanding the structure of PS II came paradoxically with the elucidation of the structure of the BRC from *Rhodopseudomonas viridis* (Deisenhofer et al., 1985). The previously observed areas of strong sequence similarity between D1/D2 and the L/M subunits were in regions of the BRC involved in binding the special pair of BChl molecules, and not quinone as first thought. Other key residues such as the four His residues involved in ligating the non-heme iron on the acceptor side of the BRC were also found in D1 and D2. Consequently it was quickly realized from these studies that D1 and D2 were most likely the RC subunits of PS II (Michel and Deisenhofer, 1986) fulfilling roles analogous to L/M of the BRC. Other key residues in L/M were conserved in D1/D2 so that folding models could be drawn for D1/D2 in the absence of direct experimental data (Trebst, 1986; Barber, 1987a; Michel and Deisenhofer, 1988). At that time the dogma in the literature, based on a variety of biochemical evidence, was that CP47, encoded by *psbB*, was the PS II RC subunit (Camm and Green, 1983; Nakatani et al., 1984; de Vitry et al., 1984; Satoh, 1986). However with hindsight it is now clear that D1 and D2 were either present in the active PS II preparations but

had escaped detection or that inappropriate assays for PS II RC activity were used.

B. Isolation of Photosystem II Reaction Center Complexes

The first direct experimental support for the D1/D2 RC model of PS II came with the isolation by Nanba and Satoh (1987) from spinach of the so-called PS II RC complex, sometimes referred to as the 'D1/D2/Cyt b_{559}' complex. Although this preparation did not contain Mn, lacked a fully functional secondary electron donor, Y_Z, and had lost the two quinone electron acceptors, Q_A and Q_B, it still retained the ability to perform light-induced charge separation indicative of the formation of the primary radical pair, $P680^+$ $Pheo^-$ (Danielius et al., 1987; Y. Takahashi et al., 1987). As the complex lacked CP47, this result suggested that D1 and D2 bound P680 and the redox-active Pheo. Other features to support the presence of P680 and the Pheo electron acceptor within this complex were the abilities to generate a spin-polarized chlorophyll triplet state (Rutherford et al., 1981; Okamura et al., 1987) associated with charge recombination of the radical pair and to photoaccumulate either reduced Pheo in the presence of dithionite (Nanba and Satoh, 1987), or oxidized chlorophyll, possibly $P680^+$, upon addition of silicomolybdate (Barber et al., 1987). For a review of the isolated PS II RC see Satoh (1993).

Analysis of the PS II RC complex by SDS-PAGE and Coomassie-blue staining revealed two bands with approximate sizes of 30 kDa plus a further fainter staining band at about 60 kDa. The CP47 and CP43 apopolypeptides were both undetectable (Nanba and Satoh, 1987). Vital immunochemical experiments later confirmed the two 30-kDa bands as the D1 and D2 subunits and the 60-kDa band as either a mixture of D1 and D2 homodimers (Satoh et al., 1987) or a D1/D2 heterodimer (Marder et al., 1987). Also present within this preparation were the low molecular mass α and β subunits of cytochrome b_{559}, which could be detected spectroscopically (Nanba and Satoh, 1987), and the PsbI subunit, also encoded by the chloroplast genome (Ikeuchi and Inoue, 1988a; Webber et al., 1989). The nuclear-encoded PsbW subunit was found later in some preparations of the D1/D2/Cyt b_{559} complex (Lorković et al., 1995), but its absence in other preparations suggests a location on the periphery of the D1/D2/Cyt b_{559} complex (Alizadeh et al., 1999). Important confirmation that D1 and D2 bound the Chl and Pheo molecules of the RC came with the isolation and analysis of a D1/D2 complex depleted of Cyt b_{559} and PsbI (Tang et al., 1990).

D1/D2/Cyt b_{559} complexes have now been isolated from *C. reinhardtii* (Alizadeh et al., 1995), and *Synechocystis* 6803 (Giorgi et al., 1996), so it is clear that the subunit structure of the PS II RC is conserved across the whole range of oxygenic organisms. Radio-labeling studies (Alizadeh et al., 1999) and determination of the ratio of amino acids (Satoh, 1993) have further indicated that the five subunits are present in equimolar amounts.

C. Folding Models of the D1/D2 Heterodimer

Based on hydropathy plots, D1 was originally proposed to consist of seven transmembrane helices (Rao et al., 1983), rather than the five predicted by Michel and Deisenhofer (1986). Experiments involving the binding of site-specific D1 antibodies to inside-out and right-side-out thylakoid vesicles, and trypsin accessibility to D1, provided early support for a five transmembrane helix model for D1 (Sayre et al., 1986).

In the absence of detailed structural information, a number of computer-based models were developed for the D1/D2 heterodimer using the structure of the BRC as a template (Svensson et al., 1990; Ruffle et al., 1992; Svensson et al., 1996; Xiong et al., 1998). In all models there are two branches, A and B, of chlorins spanning the membrane (Fig. 1). Based on the nomenclature developed for the BRC, the 2 Chls analogous to the special pair of BChl molecules are designated in this chapter as P_A and P_B, the 2 Chls analogous to the accessory BChl molecules are designated B_A and B_B (these Chls are termed Chl_{D1} and Chl_{D2} in Chapters 19–21), and the 2 pheophytins are designated $Pheo_A$ and $Pheo_B$. Midway between the primary (Q_A) and secondary (Q_B) quinone electron acceptors is a non-heme iron atom (Fe). Two additional chlorophylls (Chl Z_{D1} and Chl Z_{D2}) found in PS II, but not the BRC, are included in Fig. 1 (Xiong et al., 1998).

Superficially the computer-generated models are very similar, although there are significant differences with regard to the amino-acid residues lining the co-factor binding sites (reviewed in Xiong et al., 1998). Nevertheless these models are consistent with early structural data obtained by EPR such as the orientations of $Pheo_A$ and Q_A (Dorlet et al., 2000) and distances estimated from the spin-lattice relaxation of paragmagnetic species: 39.5 ± 2.5 Å for Fe(II)-

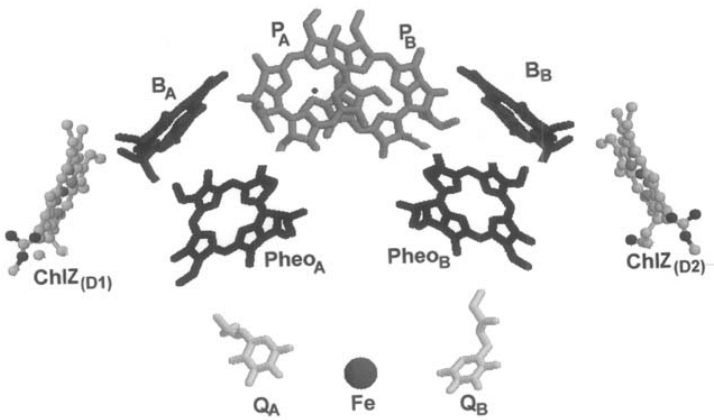

Fig. 1. Nomenclature and model of the transmembrane arrangement of the pigment, quinone and non-heme cofactors within the D1/D2 heterodimer, based on a close analogy to the BRC. The P_A/P_B and B_A/B_B Chl molecules within PS II occupy similar (but not identical) positions to the special-pair and accessory BChl molecules, respectively, of the BRC. $Pheo_A$ and $Pheo_B$ are equivalent to the two BPheo molecules of the BRC. Q_A is the primary quinone electron acceptor, Q_B the secondary quinone electron acceptor, and Fe the non-heme iron atom. In the BRC, electron transfer proceeds down the 'A' branch from P_A/P_B via B_A to $BPheo_A$ then Q_A and finally to Q_B. In PS II there are two extra Chls termed Chl $Z_{(D1)}$ and Chl $Z_{(D2)}$, ligated by D1-His118 and D2-His117, respectively.

Chl Z^+ (Koulougliotis et al., 1994), 37 ± 5 Å for both Fe(II)-$Y_D^{\bullet}$ and Fe(II)-$Y_Z^{\bullet}$ (Koulougliotis et al., 1995) and 20 ± 4.2 Å for Fe(II)-$Pheo_A^-$ (Deligiannakis and Rutherford, 1996). The close structural similarity between the acceptor sides of PS II and the BRC, suggested by these and other early studies, has now been conclusively shown in recent X-ray crystallographic studies (Zouni et al., 2001; Kamiya and Shen, 2003; Biasiadka et al., 2004; Ferreira et al., 2004).

Figure 2 displays folding models for D1 and D2 based on those of Svensson et al. (1996). Highlighted are key residues predicted from sequence comparisons to be involved in binding various cofactors within PS II. Unfortunately the lack of sequence similarity between D1/D2 and L/M in the extrinsic loops connecting the transmembrane helices has limited the ability of the computer-based structural models to provide clear guidance as the structure of the lumenal regions and hence the possible site of the Mn cluster.

V. Mutagenesis of the D1 and D2 Proteins

Two model organisms have been widely used for the mutagenesis of D1 and D2: the cyanobacterium *Synechocystis* sp. PCC 6803 (reviewed by Nixon et al., 1992b; Vermaas, 1993; Debus 2001; Diner 2001) and the green alga *Chlamydomonas reinhardtii* (reviewed by Ruffle and Sayre, 1998). The ability of a D1 protein from a chloroplast to be successfully integrated into a cyanobacterial PS II complex, without loss of O_2-evolving activity (Nixon et al., 1991), suggests that the mechanism of water oxidation by PS II is highly conserved throughout nature. In general, mutagenesis experiments have provided strong support for the general arrangement of pigments shown in Fig. 1 and have provided crucial information on the location of the redox-active components within PS II including the Mn cluster. In the following sections we will discuss some of the key mutants. Unless indicated otherwise, the numbering of residues comes from the *Synechocystis* sequences. For further details, see Chapters 9 (Diner and Britt) and 11 (Debus).

A. Mutations Affecting the Donor Side

1. Tyrosines Y_Z and Y_D

The first demonstration of the power and usefulness of mutagenesis studies for PS II came with the identification of the tyrosine electron donors, Y_Z and Y_D. In the early literature these electron donors are sometimes called Z and D, respectively. Z lies on the electron transfer pathway connecting the Mn cluster to $P680^+$ whereas D is on a side-path. In their oxidized states they give rise to EPR signals of similar shape, the so-called Signal II, but show different kinetics

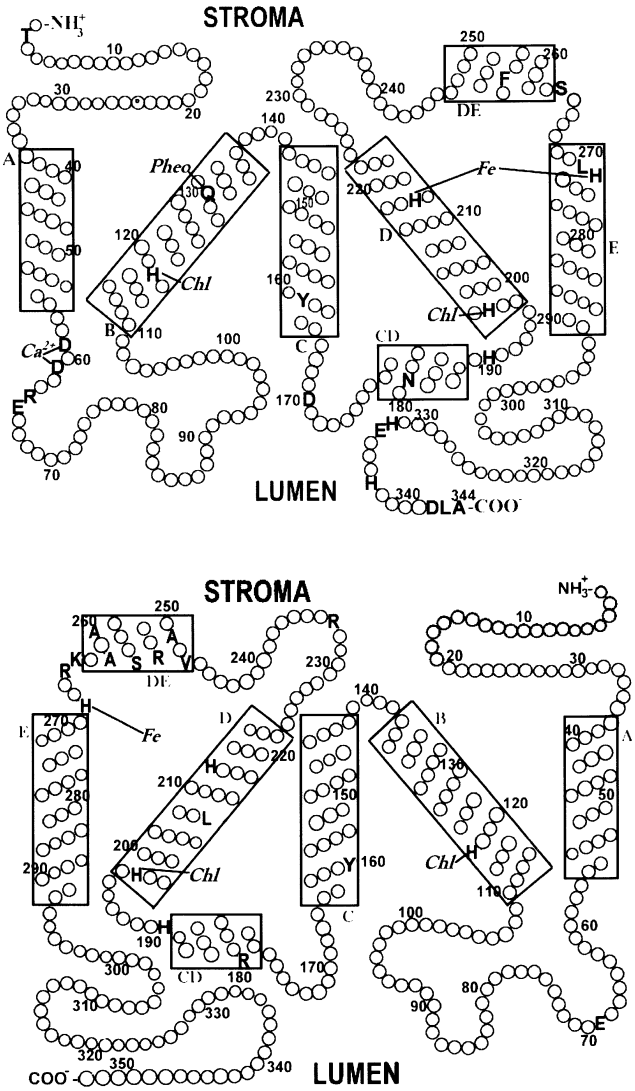

Fig. 2. Folding models for the D1 (top) and D2 (bottom) subunitsof *Synechocystis* 6803 based on ones drawn by Svensson et al. (1996). Each subunit consists of five (A-E) transmembrane helices and two (CD and DE) helices that lie parallel to the membrane. Amino-acid residues that are discussed in the text are indicated by the one-letter code. Possible amino-acid ligands to P_A, P_B, Chl Z_{D1}, Chl $Z_{D2,}$ $Pheo_A$, $Pheo_B$, and Fe are indicated.

of formation and decay: oxidation of Z gives rise to Signal II_{vf} (very fast) in intact PS II or Signal II_f (fast) in samples lacking a Mn cluster (Blankenship et al., 1975). Signal II_s (slow) (Babcock and Sauer, 1973) is an extremely stable signal and is associated with oxidation of D. On the basis of the hyperfine structure of Signal II and its simulation using model compounds, together with other arguments, early work suggested that Z might be a plastosemiquinone cation (PQH_2^+) (O'Malley et al., 1984). However, PS II complexes with fully functional Z and Q_A only contain sufficient extractable plastoquinone to

account for the single Q_A molecule (de Vitry et al., 1986). A resolution to the chemical identity of D, and by extrapolation Z, came with a beautiful series of experiments by Barry and Babcock in which they showed that Signal II_s became narrower when PS II incorporated deuterated tyrosine but not deuterated plastoquinone, so implicating tyrosine as the origin of D (Barry and Babcock, 1987). A similar analysis of isolated PS II complexes containing deuterated tyrosine later confirmed Z to be also a tyrosine residue (Boerner and Barry, 1993). Radio-iodination experiments in which $^{125}I^-$ is activated by PS II-mediated oxidation prior to modification of protein side chains, suggested that D1 and D2 contained Z and D, respectively (Y. Takahashi et al., 1986; Ikeuchi and Inoue, 1987). Folding models predicted the presence of two symmetrically placed Tyr residues, D1-Tyr161 and D2-Tyr160, in the region of iodination (Ikeuchi and Inoue, 1988b; Y. Takahashi and Satoh, 1989). Mutation of D2-Tyr160 to Phe in *Synechocystis* 6803 caused loss of the dark stable Signal II associated with D (Debus et al., 1988a; Vermaas et al., 1988) but still allowed assembly of an O_2-evolving PS II complex. Mutation in *Synechocystis* 6803 of the symmetrical tyrosine in D1 to either Phe (Debus et al., 1988b; Metz et al., 1989), or Trp, His, Cys and Met (Nixon et al., 1992b), all produced strains that were incapable of photosynthetic water oxidation. Analysis of manganese-depleted core complexes isolated from these mutants by EPR and optical spectroscopy further demonstrated that the lesion in electron transfer was at the level of donor Z (Metz et al., 1989). So far no other residue appears to be oxidized when placed at this position. Together these data led to the attribution of D1-Tyr161 and D2-Tyr160 as donors Y_Z and Y_D, respectively. It is now recognized that when oxidized, Y_Z and Y_D form the neutral radical species, $Y_Z^{\bullet}$ and $Y_D^{\bullet}$, and not the cationic species sometimes cited in the literature (reviewed by Diner, 2001; Chapter 9, Dinner and Britt).

2. D1-His190 and D2-His189

A variety of EPR evidence has indicated that $Y_Z^{\bullet}$ and $Y_D^{\bullet}$ are H-bonded (reviewed by Diner, 2001). ENDOR (Tang et al., 1993, Campbell et al., 1997), high-field EPR (Un et al., 1996) and FTIR (Hienerwadel et al., 1997) experiments, using a D2-His189 mutant of *Synechocystis* 6803, strongly support the presence of a H-bond between $Y_D^{\bullet}$ and D2-His189. The situation regarding the role of D1-His190 appeared more complicated with ^{15}N pulsed-ENDOR not favoring a direct H-bond between $Y_Z^{\bullet}$ and D1-His190 (Campbell, 1999). These experimental results are consistent with several of the computer-generated models, which have suggested that D2-His189 might H-bond to the phenolic oxygen of $Y_D^{\bullet}$, but that D1-His190 is too far away to H-bond to $Y_Z^{\bullet}$ (reviewed by Xiong et al., 1998).

All mutants created at D1-His190 in *Synechocystis* 6803 (Diner et al., 1991a; Nixon et al., 1992b; Nixon and Diner, 1994; Chu et al., 1995a; Diner and Nixon, 1998; Hays et al., 1998; 1999) and *C. reinhardtii* (Roffey et al., 1994) are unable to evolve O_2 apart from Lys and Arg substitutions at approximately 13% of wild type rates (Chu et al., 1995a; Hays et al., 1998). The oxidation of Y_Z is dramatically slowed by greater than 200-fold in mutants such as D1-His190Gln and Asp (Diner et al., 1991a; Diner and Nixon, 1998; Hays et al., 1998) but can be partially restored through the addition of small organic bases such as imidazole (Hays et al., 1998). Similarly the rate of re-reduction of $Y_Z^{\bullet}$ in manganese-depleted PS II complexes, through charge recombination, is also much slower and likewise can be accelerated through addition of small bases (Hays et al., 1998). Together these data support a model in which D1-His190 acts as a general acid/base catalyzing the protonation/abstraction of the phenolic proton of Y_Z during its proton-coupled reduction/oxidation (Hayes et al., 1998). Unless Y_Z is able to be deprotonated, its oxidation is blocked. There is still debate as to whether D1-His190 forms a direct H-bond to Y_Z or is linked indirectly through other proton-carriers. See Chapter 9, Diner and Britt, for a more detailed account.

3. Mutants Affecting the Binding and Function of the Manganese Cluster

Based on folding models for D1 and D2, residues exposed to the lumen have been subjected to site-directed mutagenesis (for reviews, see Diner, 2001; and Debus, 2001). The chief targets for mutagenesis were carboxylates and histidine residues, both of which are excellent candidates for ligating metal ions. Some early support for a role for His in ligating the cluster came from chemical modification experiments (Tamura et al., 1989). Pulsed EPR experiments have since unambiguously confirmed this proposal (Tang et al., 1994). The participation of histidine and carboxylate residues in Mn binding in PS II has also been suggested on the basis of non-competitive inhi-

bition experiments between exogenous Mn^{2+} and the artificial electron donor to PS II, diphenylcarbazide (Seibert et al., 1989; Preston and Seibert, 1991).

One of the surprises of the brute-force mutagenesis approach was how difficult it was to knock out the assembly of a functional Mn cluster (Pakrasi and Vermaas 1992; Nixon et al., 1992b). In part this may reflect the ability of alternate ligands such as water and chloride to substitute for a missing proteinaceous ligand. Nevertheless, this observation strengthened the significance of residues at which mutation specifically blocked Mn assembly.

a. The LF-1 Mutant of Scenedesmus obliquus

One of the first and most important mutants affecting the assembly of the Mn cluster in PS II was the LF-1 (low fluorescence –1) mutant of *Scenedesmus obliquus*, which was generated through X-ray mutagenesis (Metz and Bishop, 1980). Originally identified in chlorophyll-fluorescence based screens as low-fluorescent, the LF-1 was consistent with an impairment on the donor side of PS II. Biochemical studies indicated that the PS II could accumulate and retain electron transfer activity across the membrane but was unable to assemble a functional Mn cluster, with only 1–2 Mn bound to each PS II center (Metz and Bishop, 1980; Metz et al., 1980). A polypeptide of apparent molecular mass 34 kDa in the wild type showed a shift to an apparent molecular mass of 36 kDa in LF-1. No other differences in the electrophoretograms were detected, so the 34-kDa subunit was suggested to be involved in binding Mn (Metz et al., 1980). Originally thought to be a modified form of the 33-kDa extrinsic protein involved in stabilizing of the Mn cluster (Bishop, 1983) or possibly D2 (Metz and Seibert, 1984), the 36-kDa polypeptide is now known to be precursor D1 (Metz et al., 1986; Taylor et al., 1988; Diner et al., 1988). The phenotype of LF-1 results from a single base deletion and a consequent frameshift in the coding region for CtpA, the D1 processing protease (Trost et al., 1997), which is needed to remove the C-terminal extension. That the inability to assemble the Mn cluster was due to a block in D1 maturation, rather than some other target for the processing protease, was confirmed through the construction of the D1-S345P mutant of *Synechocystis* 6803 which was unable to process precursor D1 because of a modified cleavage site. The phenotype of this mutant was essentially identical to that of LF-1 (Nixon et al., 1992a).

Overall the LF-1 mutant provided the first mutagenesis evidence to indicate a role for D1 in the assembly of the Mn cluster, not just in Q_B binding. However, it still remains unclear to what extent the C-terminal extension causes indirect structural perturbations on the lumenal side of PS II.

b. D1-Asp170

This was the first amino acid residue to be identified as important for assembly of a functional Mn cluster (Nixon and Diner, 1990). Mutants created at this residue in either *Synechocystis* 6803 (Nixon and Diner, 1992; Boerner et al., 1992) or *C. reinhardtii* (Whitelegge et al., 1995) were to varying degrees able to accumulate active PS II. Retention of a residue that was capable of ligation to a metal allowed the assembly of a cluster whereas substitution with a non-ligand such as Ala showed no O_2 evolution but was still able to assemble an otherwise functional PS II complex with regard to electron transfer from Y_Z to Q_B (Nixon and Diner, 1992). Mutations at Asp170 also affected the ability of detergent-solubilized PS II complexes, with the Mn cluster removed, to bind and oxidize Mn^{2+} to Mn^{3+} at a high-affinity site within PS II (Diner and Nixon, 1992; Nixon and Diner, 1992). This high-affinity site, implicated first by EPR (Hoganson et al., 1989), is probably identical to the binding site for the first Mn ion in the natural light-driven process of photoactivation that assembles the functional Mn cluster (Radmer and Cheniae, 1977). More recent evidence to support the idea that Asp170 acts as a ligand to the Mn (III) has come from parallel mode EPR measurements (Campbell et al., 2000). The structural model of PS II from Imperial College London has now confirmed a role for Asp170 in ligating one of the Mn ions of the intact cluster (Ferreira et al., 2004). Somewhat surprisingly mutants D1-Asp170Val, Leu and Ile are able to assemble a functional cluster in a fraction of the centers despite the presence of a non-ligand at this position (Chu et al., 1995a). Presumably an alternative ligand is operating in these mutants.

c. His332, Glu333, His337, Asp342 in the Carboxyl-Terminal Region of D1

Mutation of a number of residues in the C-terminal region of D1 specifically compromised the ability to assemble a functional Mn cluster (Nixon et al., 1992a; Nixon and Diner, 1994; Chu et al., 1995b). In the case

of some, but not all, of the mutants created at D1-His332, D1-Glu333 and D1-Asp342 O_2 evolution is blocked (Nixon et al., 1992b; Nixon and Diner, 1994; Chu et al., 1995b), consistent with their roles as Mn ligands (Ferreira et al., 2004). Those substitutions that allow assembly of a Mn cluster have the potential to act as ligands. Many of the mutants constructed in this region of D1, such as at D1-His337, allow O_2 evolution but the activity is readily photoinhibited (Nixon et al., 1992b; Chu et al., 1995b). The most detailed analysis so far of mutants in this region of D1 has come from an ESEEM study of PS II complexes isolated from a D1-His332Glu mutant of *Synechocystis* 6803, in which the Mn cluster assembles but is unable to pass beyond the S_2 state (Debus et al., 2001). Since this residue is now known to be a ligand to the Mn cluster (Ferreira et al., 2004), these results highlight the importance of appropriate ligation for controlling the redox properties of the cluster. Of importance was the finding that the high-affinity Mn^{2+}-binding site characterized in isolated PS II complexes is largely preserved in C-terminal mutants lacking a functional cluster (Nixon et al., 1992a,b; Nixon and Diner, 1994). This feature distinguishes the C-terminal D1 mutants from those at D1-Asp170.

d. The Carboxyl-Terminal Residue at Ala-344

In some of the first mutagenesis studies on D1 in *Synechocystis* 6803, the effect of deleting progressively larger portions of the C-terminus was examined (Nixon et al., 1992a). Removal of the C-terminal extension, so that only the mature form of D1 was synthesized, did not block assembly of functional PS II. This important finding, which holds also for the green alga *C. reinhardtii* (Lers et al., 1992; Schrader and Johanningmeier, 1992), showed that the C-terminal extension was not required for integration of D1 into the thylakoid membrane, a possibility that had been suggested in early studies on D1 maturation (Marder et al., 1984). Removal of an additional residue, leaving D1-Leu343 as the C-terminal residue, allowed the assembly of PS II complexes competent in the oxidation of Y_Z but blocked the formation of an active Mn cluster. Deletion of additional residues further reduced the ability to accumulate PS II centers, all of which were inactive for O_2 evolution (Nixon et al., 1992a). These results therefore highlighted the functional importance of the C-terminal residue for the Mn cluster. A number of residues (Gly, Met, Ser, Val, Tyr, Lys) could substitute for Ala-344 without blocking totally the assembly of the Mn cluster. Therefore it was suggested that it was the free carboxyl group that was important for functionality (Nixon et al., 1992a).

e. Other Residues Affecting the Manganese Cluster

A number of D1 mutants have been characterized that retain oxygen-evolving ability but show modified properties. Amongst these are mutants created at D1-Asp59, D1-Asp61 and D1-Glu65, in the lumenal loop connecting helices A and B. These residues had been speculated for some time to be involved in binding metal ions, either Mn or Ca^{2+}. A role for D1-Asp59 and D1-Asp61 in binding Ca^{2+} was given impetus with the finding that photoautotrophic growth of mutants constructed at these sites was sensitive to the depletion of Ca^{2+} from the growth medium (Nixon and Diner, 1994; Chu et al., 1995a). More detailed analyses of mutants at these positions are consistent with an important role for these residues in stabilizing intermediates formed during the assembly of the Mn cluster, possibly through the binding of a calcium ion, although this has yet to be probed directly (Qian et al., 1999). In addition, mutants at these residues show slower production of O_2 indicative of a slower cycling through the higher S-state transitions (Hundelt et al., 1998; Qian et al., 1999).

Besides D1-Asp59 and D1-Asp61, the photoautotrophic growth of mutants at D1-Glu333 and D1-Asp342 in the C-terminal region are also sensitive to the depletion of Ca^{2+} (Nixon and Diner, 1994; Chu et al., 1995a) or chloride (Nixon and Diner, 1994) from the growth medium. These phenotypes are also observed with many D2 mutants (Keilty et al., 2001) and mutants lacking extrinsic proteins of PS II (summarized in Li and Burnap, 2001). Ca^{2+} and chloride play a role in the function of the Mn cluster, suggesting direct or indirect effects of all these mutations on their normal binding within PS II or in the case of chloride, a possible role as a replacement ligand to the cluster. One indirect effect of mutating these, and other residues such as D1-Arg64, might be to prevent correct binding of the extrinsic proteins to the lumenal side of PS II (Li and Burnap, 2001).

Another feature of many D1 mutants, including mutants constructed at the C-terminus of D1, as well as the D2-Glu69Gln mutant (Vermaas et al., 1990), is the enhanced sensitivity to light-induced inactivation of O_2 evolution by mutant cells. There are many

possible causes for such a phenotype including the damaging effects of relatively long-lived highly oxidizing species within PS II, the production of reactive oxygen species at the modified Mn clusters (Chu et al., 1995b) and effects on the D1 repair cycle (Dalla Chiesa et al., 1996). Thus enhanced sensitivity to photoinhibition is in itself not compelling evidence for a role for that residue in binding Mn. For more details see Chapter 11, Debus.

f. Model for Location and Assembly of the Manganese Cluster Based on Mutagenesis Data

On the basis of the mutagenesis data, Diner et al., (1991a) proposed a simple structural model for the location of the Mn cluster within D1 (Fig. 3). In order to bring all the crucial residues identified by mutagenesis into a compact cluster that could ligate the Mn cluster, the C-terminal region of D1 had to be folded back so that it was present close to D1-Asp170. The location of Asp170 only 9 residues away from D1-Tyr161, the oxidant of the cluster, also suggested that the Mn cluster was located close to Y_Z; indeed estimates from computer modeling studies placed Asp170 6–7 Å from Y_Z (e.g., Svenson et al., 1996). At the time of the proposal of the model in Fig. 3, EPR data suggested that the Mn cluster was greater than 10 Å from Y_Z (Hoganson and Babcock, 1988) and, in other work, possibly up to 32 Å away (Un et al., 1994). Such distances led to the exclusion of Asp170 as a ligand in early computer models (Svensson et al., 1990). However more recent EPR analyses have indicated a much smaller distance (7–9 Å) between the Mn cluster and Y_Z (Dorlet et al., 1998; Peloquin et al., 1998; Lakshmi et al., 1999). The proposed location of the Mn cluster in Fig. 3 also agrees very well with the structural models determined by X-ray crystallography (Kamiya and Shen, 2003; Biesiadka et al., 2004; Ferreira et al., 2004).

The mutagenesis data have also allowed a model to be proposed for the coupling of D1 processing to the assembly of the Mn cluster (Diner et al., 1991a; Nixon et al., 1992a). In this model, the first Mn ion is proposed to bind at a high-affinity site of which D1-Asp170 is an important component. It is this Mn ion that is oxidized first by PS II in photoactivation. In order to complete the assembly of the cluster, removal of the C-terminal extension is required. At the same time this cleavage reaction would reveal

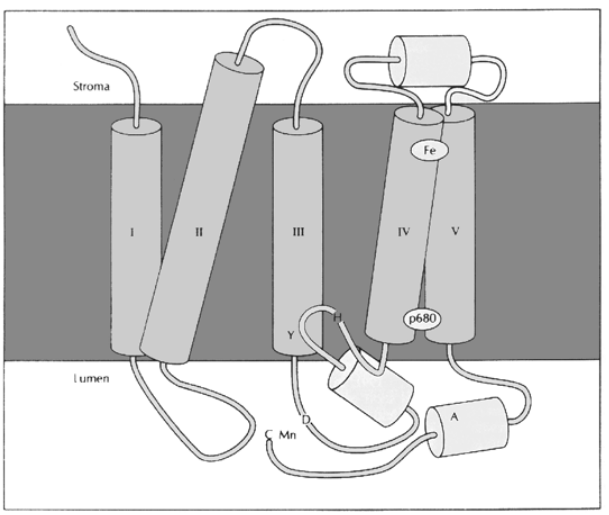

Fig. 3. The folding model of D1 proposed by Diner et al. (1991a), showing the location of the Mn cluster (Mn) within PS II based on the analysis of site-directed mutants of *Synechocystis* 6803. The five transmembrane helices of D1 are labeled here as I-V. The cluster was proposed to be coordinated by D1-Asp170 (D) and by residues within the C-terminal region of D1, including the C-terminus itself (C). Also shown are Yz (Y), the oxidant of the Mn cluster, which is thought to be close to D1-H190 (H), as well as P680 (p680) and the non-haem iron (Fe). A possible amphiphilic helix (helix A) was suggested to bring the C-terminal residues towards D1-Asp170 (D). Reprinted with permission from Elsevier Science.

the carboxy terminus at D1-Ala344, which might act as a ligand to the cluster, perhaps following a substantial conformational change to bring the C-terminus close to D1-Asp170. The involvement of the free C-terminus in ligating Mn (or Ca^{2+}) explains nicely why processing is required for assembly of a functional Mn cluster.

The presence of the C-terminal extension provides a selective advantage to cells growing in mixed culture compared to those without (Ivleva et al. 2000). However it remains unclear what advantage the extension conveys. One possibility is that removal of the extension is coupled to the stepwise assembly of the most stable and active cluster. In its absence, there might be an increased chance that inappropriate ligands are used so that although active clusters might still assemble, there is a greater likelihood of forming poorer functioning clusters with a greater propensity for causing damage to PS II. Alternatively, the C-terminal extension might play a role in optimizing the assembly of PS II in the PS II repair cycle. While the model in Fig. 3 emphasized an exclusive role for D1 in binding the Mn cluster, it was always recognized that other PS II subunits might participate, as eventually revealed in the structural model from Imperial College (Ferreira et al., 2004).

4. D1-His198 and D2-His197

The histidines that coordinate the two members of the special pair found in the L and M subunits of the BRC are conserved in PS II at D1-His198 and D2-His197. This naturally led to a model in which these latter residues also ligated the chlorophyll molecules, P_A and P_B, respectively, (Fig. 1) in a broadly similar way to that found in the BRC, although there had to be some structural distortion to explain the absence of strong excitonic coupling between the two Chls (Durrant et al., 1995). However, the presence of P_A and P_B in PS II was not immediately accepted because of the significant redox and spectral differences observed between PS II and the BRC. For instance, because $P680^+$ is much more oxidizing than the bacterial equivalent, van Gorkom and Schelvis (1993) pointed out that P_B as well as B_B might not be retained in PS II to avoid unwanted oxidation of Chl. Also models were suggested in which P_A was rotated from perpendicular to the membrane plane to 30 degrees (Noguchi et al., 1993) so as to accommodate EPR data on the orientation of the chlorophyll molecule carrying the $P680^T$ state (van Mieghem et al., 1991).

Early mutagenesis studies indicated that D1-His198 and D2-His197 were indeed important since replacement by leucine (Nixon et al., 1992b) and tyrosine (Vermaas et al., 1987), respectively, led to loss of PS II from the membrane. More recently a number of mutations have been made at these positions that allowed assembly of functional PS II complexes. These mutants have subsequently proved to be an invaluable resource in addressing questions regarding the nature of P680 (Diner et al., 2001) and the energetics of primary charge separation (Merry et al., 1998).

In the most detailed work so far on these mutants, Diner et al. (2001) have applied optical and EPR spectroscopies to examine the $P680^+$ and $P680^T$ states in core complexes isolated from mutants of *Synechocystis* 6803 constructed at either D1-His198 or D2-His197. Mutation of D1-His198 caused both shifts in the $P680^+$–P680-absorbance difference spectrum and appreciable modulation of the $P680^+$/P680 redox couple. Mutation of D2-His197 had less dramatic effects on these parameters. The triplet-minus-singlet optical difference spectrum for P680 was in contrast unchanged in all mutants. These results therefore support a model in which following charge separation the chlorophyll cation (usually called $P680^+$) is stabilized primarily on P_A (ligated by D1-His198) and to a lesser degree (approx 20%) on P_B (ligated by D2-His197). Results obtained with the above mutants and other data in the literature also support the idea that charge separation is initiated mainly from the singlet excited state of the accessory chlorophyll, B_A*, and that the P680 triplet state is stabilized at low temperature mainly on B_A and not P_A (Noguchi et al., 1998; Sarcina et al., 1998). At first sight, such a scheme for PS II primary photochemistry is in sharp contrast with that for the BRC where the BChl singlet, cation and triplet states are located on the special pair of BChls (P). However, a major rethinking of this dogma suggests that there are multiple pathways for stable charge separation in the BRC including charge separation from B_A* not just P* (van Brederode and van Grondelle, 1999; Dekker and van Grondelle, 2000). PS II differs from the BRC in that the excited singlet states are much closer in energy in the former resulting in extensive delocalization of the excited state of the RC within a multimer of pigments rather than localized to the special pair (Durrant et al., 1995). At low temperature (4K) the excited state energy in PS II is exclusively localized on B_A implying that charge separation at

this temperature must be occurring from B_A^* (Diner et al., 2001; Diner and Rappaport, 2002).

5. D1-His118 and D2-His117

Initial measurements of the pigment stoichiometry of the D1/D2/Cyt b_{559} complex isolated by Nanba and Satoh (1987) suggested a ratio of about 5 Chl per 2 Pheo molecules. For the BRC, there are 4 BChl molecules and 2 BPheo. Hence it was anticipated by analogy that the pigment stoichiometry for the D1/D2/Cyt b_{559} complex would in fact be 4 Chl/2 Pheo (Barber, 1987b). However, following suggestions that this was so (e.g., Barber et al., 1987; Aured et al., 1994; Chang et al., 1994; Pueyo et al., 1995), a consensus has now emerged in favor of a ratio of 6 Chl per 2 Pheo for the most stable form of the PS II RC (Gounaris et al., 1990; Kobayashi et al., 1990; Eijckelhoff and Dekker, 1995). Thus, the D1/D2/Cyt b_{559} complex contains 2 additional Chls compared with the BRC. Important confirmation that it was the D1/D2 heterodimer that bound these 2 extra Chl came with the isolation of a D1/D2 complex (Tang et al., 1990).

The possible ligands for these two 'extra' Chl molecules focused on two symmetrically related His residues, D1-His118 and D2-His117, not conserved in the BRC, which are predicted to lie in the second transmembrane helix of D1 and D2, respectively (Michel and Deisenhofer, 1988). Computer modeling studies placed these Chls on the exterior of the D1/D2 heterodimer in a position to interact with the neighboring CP47 and CP43 subunits and to act possibly as linker Chls connecting the pigments in CP47 and CP43 to those involved in primary photochemistry in D1 and D2 (Ruffle et al., 1992). Measurements of energy transfer within the isolated PS II RC indeed suggested two populations of pigments: A peripheral pool, which shows a relatively slow rate of energy transfer to P680 (Schelvis et al., 1994), possibly bound to D1-His118 and D2-His117, and an inner core of pigments that shows rapid equilibration of excitation energy (Durrant et al., 1992).

Mutation of D2-His117 to residues that could potentially coordinate Chl (Cys, Met, Asn and Thr) still allowed photoautotrophic growth in *Synechocystis* 6803, whereas other substitutions impaired accumulation of PS II to varying degrees (Pakrasi and Vermaas, 1992; Lince and Vermaas, 1998; Stewart et al., 1998). Fluorescence studies on the D2-His118Thr mutant suggested that energy transfer into the PS II RC was slowed, consistent with a role in binding Chl (Lince and Vermaas, 1998; Vasil'ev and Bruce, 2000). A comparison by EPR, near-infrared absorbance and resonance Raman spectroscopies of PS II complexes isolated from *Synechocystis* 6803 mutants D2-His117Gln and its counterpart, D1-His118Gln, (Stewart et al., 1998) led to the conclusion that D1-His118, and not D2-His117, was the axial ligand to Chl Z, a photooxidizable Chl molecule that had been identified earlier in PS II from higher plants (de Paula et al., 1985). The possible physiological role of Chl Z in protection form photoinhibition and its mode of oxidation and reduction are described in detail in Chapter 15 (Faller and Rutherford) of this volume. Based on symmetry arguments Stewart et al (1998) further suggested that D2-His117 also acted as an axial ligand to Chl but that this Chl, termed Chl D, was redox-inactive. The Chl Z and Chl D nomenclature is an extension of that used to describe the two redox-active tyrosines in PS II–Y_Z and Y_D.

The PS II structure determined to a resolution of 3.8 Å by Zouni and co-workers confirmed the presence of two Chls, termed Chl Z_{D1} and Chl Z_{D2}, within the predicted vicinity of D1-His118 and D2-His117, respectively (Zouni et al., 2001). One of the interesting features of this PS II structure was the finding that Cyt b_{559} was located on the D2 side of the complex (Zouni et al., 2001). This meant that Cyt b_{559}, suggested initially to be the direct reductant of Chl Z^+ (Thompson and Brudvig, 1988) was in fact closer to Chl D than Chl Z. Had Chl Z been misassigned so that D2-His117 rather than D1-His118 was the true ligand? The possibility that the Chl bound to D2-His117 could be redox-active has received recent experimental support from studies of D2-His117Asn/Gln and D1-His118Gln mutants of *C. reinhardtii* (Ruffle et al., 2001; Wang et al., 2002). The EPR spectrum assigned to Chl Z^+ obtained from PS II RCs isolated from the D2-His117Asn mutant was broadened compared to the wild type and the D1-His118Gln mutant, which both showed similar spectra. This broadening has been interpreted in terms of a possible rotation of the Chl (Wang et al., 2002). Mutation of both D1-His118 and D2-His117 in *C. reinhardtii* altered energy transfer into the PS II RC consistent with modification to peripheral Chls of the RC. These results are in apparent contradiction to the conclusions made by Stewart et al. (1998). A possible reconciliation of these data has recently emerged from comparative studies on the identity of the photooxidizable accessory Chls in PS II from *Synechocystis* 6803 and spinach (Tracewell et al., 2001).

For *Synechocystis* 6803 it appears that only Chl Z (Chl Z_{D1}) is photooxidized whereas in spinach, and hence possibly *C. reinhardtii*, both Chl Z and Chl D (Chl Z_{D2}) can be photooxidized. This contrasts with early studies where only one of the accessory Chls was thought to be photooxidizable in the plant system (de Paula et al., 1985). Given the current uncertainty it seems prudent to adopt the nomenclature Chl Z_{D1} and Chl Z_{D2} to describe these two Chl species rather than Chl Z and Chl D.

B. Mutations Affecting the Acceptor Side

1. The Pheophytins

It has long been established that Pheo is reduced during primary charge separation in PS II (Klimov et al., 1977). Pigment analysis of the PS II RC supported the presence of 2 Pheo per RC. Based on the analogy with the BRC the two Pheo would be on separate branches of co-factors in the RC, with the Pheo bound to D1 on the active branch. Sequence comparisons indicated that the C9-keto group of each Pheo would be positioned so that $Pheo_A$ could form a H-bond to residue 130 of D1 and $Pheo_B$ to residue 130 of D2 (Michel and Deisenhofer, 1988). Interestingly the residue at D1-130 is not totally conserved within PS II–for the majority of organisms it is a glutamic acid whereas for *Synechocystis* 6803 it is a glutamine. Early ENDOR (Lubitz et al., 1989), resonance Raman (Moenne-Loccoz et al., 1990) and FTIR experiments (Nabedryk et al., 1990) all suggested the presence of a H-bond to the photoactive Pheo in plants. Confirmation that residue 130 of D1 plays an important role in the optical properties of a redox-active Pheo came with the analysis of PS II RCs isolated from mutants D1-Gln130Glu and D1-Gln130Leu constructed in *Synechocystis* 6803 (Giorgi et al., 1996). The Q_x transition was shifted from 541.5 nm in the wild type to 540 nm in the D1-Gln130Leu mutant and to 544nm in the D1-Gln130Glu mutant. In plants, where residue D1-130 is naturally a Glu, the Q_x transition is already at 544 nm. The degree of the red shift in the mutants correlates with the probable strength of the H-bond to this residue, a similar behavior to that found in the equivalent mutants in the BRC at L-104 (Bylina et al., 1988). Since only 1 of the 2 Pheo in the PS II RC is photoactive (Nanba and Satoh, 1987), these data also provided compelling evidence to support the assumption that it was the D1-bound Pheo that was redox-active and that electron proceeded predominantly down the A-branch as in the BRC. This conclusion has been recently reinforced from analysis of PS II RCs in which the D1-bound Pheo was replaced by a 13-OH-Pheo derivative (Germano et al 2001). FTIR analysis of mutant *Synechocystis* 6803 PS II RCs (P. J. Nixon and J. Breton, unpublished) have confirmed the presence of a H-bond between residue 130 and the C9-keto group. This conclusion is also supported by a recent high field EPR study of PS II RCs isolated from D1-Glu130His, Gln and Leu mutants of *C. reinhardtii* (Dorlet et al., 2001). Overall these data provided important experimental support to the idea that the location and orientation of the redox-active Pheo is conserved between PS II and the BRC.

Besides identifying the location of pigment molecules within PS II through the effects of mutation on the optical, redox or vibrational properties of the molecule, it is possible to create mutants in which a different type of pigment molecule is inserted into the site. In BRCs, when an imidazole ligand is supplied in the correct region of the BPheo pocket, a BChl is inserted instead, presumably because the central Mg^{2+} is stabilized (Kirmaier et al., 1991). Based on sequence comparisons, the analogous mutants have now been constructed in PS II. The D2-Leu209His mutant of *Synechocystis* 6803 (DA Force and BA Diner, unpublished data) replaces the redox-active Pheo with a Chl, the result of which is that primary charge separation occurs with a reduced quantum yield. Mutant D1-Leu210His stabilizes insertion of a Chl for the redox-inactive Pheo (DA Force and BA Diner, unpublished data). This latter mutant is very important, as it has provided the first mutagenesis data in favor of a strong structural similarity between PS II and the BRC with regard to the redox-inactive Pheo molecule. A strong similarity between PS II and the BRC in terms of the orientation, not just the location, of the two Pheo molecules, is also suggested from recent spectroscopic experiments on isolated PS II RCs containing a 13-OH-Pheo derivative (Germano et al., 2001, 2002).

2. The Accessory Chlorophylls B_A and B_B

The His residues that act as axial ligands to the accessory BChls in the BRC are not conserved in D1 and D2 (Michel and Deisenhofer, 1988). This feature raised the possibility of quite dramatic differences to the BRC in this part of PS II. The identification of accessory molecules in the first structures of PS II

was vital evidence that this was not the case (Rhee et al., 1998). Presumably there are alternative ligands to these Chl in PS II such as water molecules, for which there is precedence (Hofmann et al., 1996), the protein backbone or perhaps a different type of amino-acid side chain. Inspection of the CD helices in D1 and D2 has identified two potential ligands: D2-Arg180 and D1-Asn181. Mutation of D2-Arg180 in *Synechocystis* 6803 has not provided definitive evidence concerning a role as a Chl ligand although there were clear effects on Y_D (Manna et al., 1998).

3. The Iron-Quinone Complex

A variety of early biophysical evidence suggested a close analogy between PS II and the BRC with regard the acceptor side of the complex (reviewed by Diner et al., 1991b). Both contain a tightly bound quinone, Q_A, which acts as a 1-electron carrier (Q_A/Q_A^-) and a second quinone, Q_B, which is reduced by Q_A^- to first the semiquinone anion, Q_B^-, and, following a second turnover of the RC, to the quinol. Following protonation the quinol, Q_BH_2, is released from the Q_B-binding site (Bouges-Bocquet, 1973; Velthuys and Amesz, 1974; Chapter 8, Petrouleas and Crofts). For the BRC, the binding sites for Q_A and Q_B are located in the M and L subunits, respectively. By analogy the equivalent sites in PS II would be in D2 and D1. For the BRC, the non-heme iron located between Q_A and Q_B is ligated by 4 His residues plus a glutamate, at position 232 in the M subunit of *Rhodopseudomonas viridis* (Deisenhofer et al., 1985). In PS II there is no obvious homologue to this Glu residue. Instead FTIR experiments indicate that bicarbonate acts as a bidentate ligand to the iron (Hienerwadel and Berthomieu, 1995). The bicarbonate ligand can be displaced competitively by a collection of ligands which include NO and carboxylate anions, resulting in a modest slowing of the reaction $Q_A^- Q_B \rightarrow Q_AQ_B^-$ and a marked slowing of the reaction $Q_A^- Q_B^- \rightarrow Q_AQ_BH_2$ (Diner and Petrouleas, 1990; Petrouleas et al., 1994). These observations and earlier bicarbonate depletion experiments have led to the conclusion that bicarbonate is involved in protonation reactions coupled to the formation of Q_B^- (Eaton-Rye and Govindjee, 1988) and of Q_BH^- (van Rensen et al., 1988) (reviewed in Diner et al., 1991b). Extensive mutagenesis experiments have also been directed at identifying residues that might be important for bicarbonate binding. Mutation of D2-Lys264 and D2-Arg265, which lie close to D2-His268 impair bicarbonate binding (Diner et al., 1991a). Two other residues, D2-Arg233 and D2-Arg251, have a weaker effect on bicarbonate binding (Cao et al., 1991).

Photosystem II differs from BRCs in that the non-heme iron is redox-active. First identified as Q_{400}, the iron shows reversible oxidation/reduction with a reduction potential of 400 mV at pH 7 (Diner and Petrouleas, 1987). The 4 His residues that ligate the iron in the BRC are conserved in D1 and D2 at D1-His215, D1-His272, D2-His214 and D2-His268. The effect of mutating these residues in PS II has not been fully explored although initial reports suggest, perhaps surprisingly, that replacement of D2-His268 by Gln (Vermaas et al., 1994) and of D1-His215 by Leu (Nixon et al., 1992b) leads to the accumulation of PS II centers but with a much lower quantum yield of Q_A reduction.

Computer generated models for PS II have identified a number of D2 residues that could line the Q_A-binding pocket, including D2-Ala249, Ser254, Ala258, Ala260 and His268 (e.g., Ruffle et al., 1992). Mutagenesis studies have now confirmed the importance of these residues for PS II function (Vermaas et al., 1994; Ermakova-Gerdes and Vermaas, 1998; Ermakova-Gerdes et al., 2001). An interesting property of these mutants, such as the D2-Val247Met, Ala249Thr double mutant (Ermakova-Gerdes and Vermaas, 1998) and the D2-His268Gln mutant (Vermaas et al., 1994) is the apparent ease of loss of Q_A from its binding site and the ability to reconstitute with artificial quinones. D2-Trp253 has attracted attention because it is in an analogous position to residue M-Trp250 of *Rp. viridis*, which lies between Q_A and $BPheo_A$, and which is required for retention of Q_A (Coleman and Youvan, 1990). In the D2-Trp253Phe mutant, Q_A^- is destabilized, consistent with a close location for this residue (Vermaas, 1993).

Analysis by ESEEM of PS II centers isolated from wild type *Synechocystis* 6803 and mutant D2-Ala260Gly, in which ^{15}N had been incorporated, has provided overwhelming experimental support for a weak H-bond between an oxygen of Q_A and the peptide nitrogen of D2-Ala260 (Peloquin et al., 1999). Such an interaction is predicted from structural models based on the BRC (Diner et al., 1991b). Another, stronger H-bond exists between Q_A^- and an unidentified peptide nitrogen. However, evidence for a predicted H-bond between Q_A^- and D2-His215, thought to ligate the non-heme iron, based on structural homology with the BRCs, is not apparent in the ESEEM spectra (Peloquin et al., 1999; but see

Deligiannakis et al., 1999). The inability to see this interaction does not necessarily mean that it does not exist, as the magnetic coupling may be extremely weak and may depend on the pH and the coordination of the non-heme iron. Thus, while there is significant structural conservation between the Q_A-binding sites of PS II and the BRC, the strength of the H-bonds to Q_A^- is likely to depend on subtle differences in the position of the H-bonding residues and on the interaction of D2-His215 with the non-heme iron.

The Q_B-binding pocket within PS II is formed from residues present in transmembrane helices D and E of D1 and the stromal DE loop connecting them (Fig. 2). The predicted DE loop in D1 is 17 amino-acid residues longer than that found in the L subunit. Mutagenesis has indicated that PS II can tolerate dramatic changes in the length and sequence of this loop although there are deleterious effects on Q_B function (Kless et al., 1994; Nixon et al., 1995).

Sequence alignments between D1 and the L subunit indicate only 9 out of 77 residues to be identical in the region from D1-Phe206 to D1-Gly282. Of the conserved residues, residue D1-His215 is the equivalent to L-His190 of *Rp. viridis*, which H-bonds to Q_B and ligates the non-heme iron. D1-Ser264 appears to be homologous to L-Ser223 of the BRC, since mutation of both gives rise to herbicide resistance (Oettmeier, 1999). L-Ser223 plays a role in the protonation of Q_B^- to Q_BH (Paddock et al., 1990) and might be involved in stabilizing the binding of Q_BH^- in the Q_B site (Lancaster and Michel, 1997). Replacement of D1-Ser264 by Gly reduces the affinity for Q_B but has little effect on the protonation of the quinol in PS II (Taoka and Crofts, 1990) indicating other pathways for protonation, such as via D1-His252, which was suggested early on to have a role in the protonation of Q_B^- and Q_BH^- (H. Robinson and A.R. Crofts, personal communication). In support of this view, a recent report (Lupínková et al., 2002) shows a dramatic effect of mutating this site on the rate of oxidation of Q_A^-. Other residues identified by mutagenesis as important for Q_B binding are D1-Phe255 and D1-Leu271 (Ohad and Hirschberg, 1992). For further details see Chapter 8, Petrouleas and Crofts.

PS II is a known target for a number of different classes of herbicides, including the triazines and ureas. Herbicide resistance is afforded by mutation of any one of 16 amino-acid residues within D1, in a region extending from D1-Phe211 to D1-Leu275 (reviewed by Oettmeier, 1998). According to the structural models for D1, these mutations cluster within a defined region of D1 (Erickson et al., 1989). The binding pocket for the herbicides in PS II overlaps with that for Q_B so that many, but not all, herbicide-resistant mutants also affect the binding of Q_B and modulate its redox properties (Ohad and Hirschberg, 1992).

VI. Concluding Remarks

The study of D1 and D2 mutants has proved to be a remarkably successful approach to probe the link between PS II structure and function. Not only has it provided ample evidence to support the close analogies between PS II and the BRC with respect to the electron acceptor sides of both complexes, but it has also identified several residues in D1, which could not have been predicted by comparison to BRCs, that play key roles in photosynthetic water-oxidation. The importance of D1-Asp170 for assembly of the Mn cluster led to the suggestion that the Mn cluster was close to the CD loop of D1 (Nixon and Diner, 1992). Mutagenesis experiments also identified residues within the C-terminal region of D1 that were important for assembly of the Mn cluster. The emerging structural models of PS II have confirmed that these regions of D1 are indeed involved in ligating the Mn cluster (Zouni et al., 2001; Kamiya and Shen, 2003; Biesiadka et al., 2004; Ferreira et al., 2004). Now that a detailed structural model for PS II is available, with side-chain information (Ferreira et al., 2004), there will be tremendous interest in the construction and analysis of new mutants (and possibly the reassessment of previously constructed ones). To this end, it would be highly beneficial to develop a thermophilic cyanobacterium, such as *Thermosynechococcus elongatus*, as a suitable model system to combine mutagenesis and spectroscopic experiments with structural studies.

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