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Minireview

Engineering the chloroplast encoded proteins of Chlamydomonas

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Abstract

Over a decade ago (1988), John Boynton and colleagues successfully transformed the chloroplast genome of Chlamydomonas for the first time by complementation of a chloroplast deletion mutant. Since the first demonstration of chloroplast transformation the function and structure of many chloroplast encoded subunits of the photosynthetic apparatus has been characterized by site-directed mutagenesis. With the completion of the sequencing of the Chlamydomonas chloroplast genome the genetic tools are now in hand to characterize structure–function relationships for each of the chloroplast-encoded proteins of the photosynthetic apparatus.

Introduction

The fact that the first successful transformation of the chloroplast genome was accomplished in Chlamydomonas was not surprising. Chlamydomonas has been a model organism for the study of things 'chloroplastic' since the 1950s (reviewed in Harris 1989). Photosynthetic mutants (acetate +) can be easily rescued in Chlamydomonas since the alga could be grown as a facultative heterotroph. Chlamydomonas also synthesizes chlorophyll in the dark and assembles intact photosynthetic complexes, a trait that has proven valuable for the study of light-sensitive mutants. In addition, Chlamydomonas' dominant haploid generation, its 'yeast-like' culturing properties, and its uniparental mode of chloroplast inheritance facilitate the rapid isolation and genetic dissection of the inheritance patterns of photosynthetic mutants.

By the 1970s it was evident that gene exchange by homologous recombination was operational in the chloroplast genome of Chlamydomonas. Jean-David Rochaix (2002; see Figure 1 for his photograph) has provided a historical minireview of the three genomes of Chlamydomonas. Mapping studies of crosses between strains having divergent gene sequences in the inverted repeat regions of the chloroplast genome suggested that the conserved sequence identity of the multi-copy genes in this region was probably maintained by an effective DNA recombination system. Treatment of Chlamydomonas with chemical reagents known to induce SOS-type recombinatorial repair responses in bacteria were shown to accelerate rates of chloroplast DNA intermolecular recombination and the generation of exceptional chloroplast genotypes (combinations of plus- and minus-type chloroplast genomes) during matings (Wurtz et al. 1977).

Chlamydomonas has proven to be an ideal organism for site-directed mutagenesis studies for other reasons as well (see Rochaix et al. 1998). Unlike higher plants, Chlamydomonas has a single chloroplast per cell with 100 or fewer genomes. Higher plant cells may have as many as 100 chloroplasts per cell with each chloroplast having approximately 100 genomes. The selection of a unique chloroplast transformation event in higher plants is clearly a daunting task

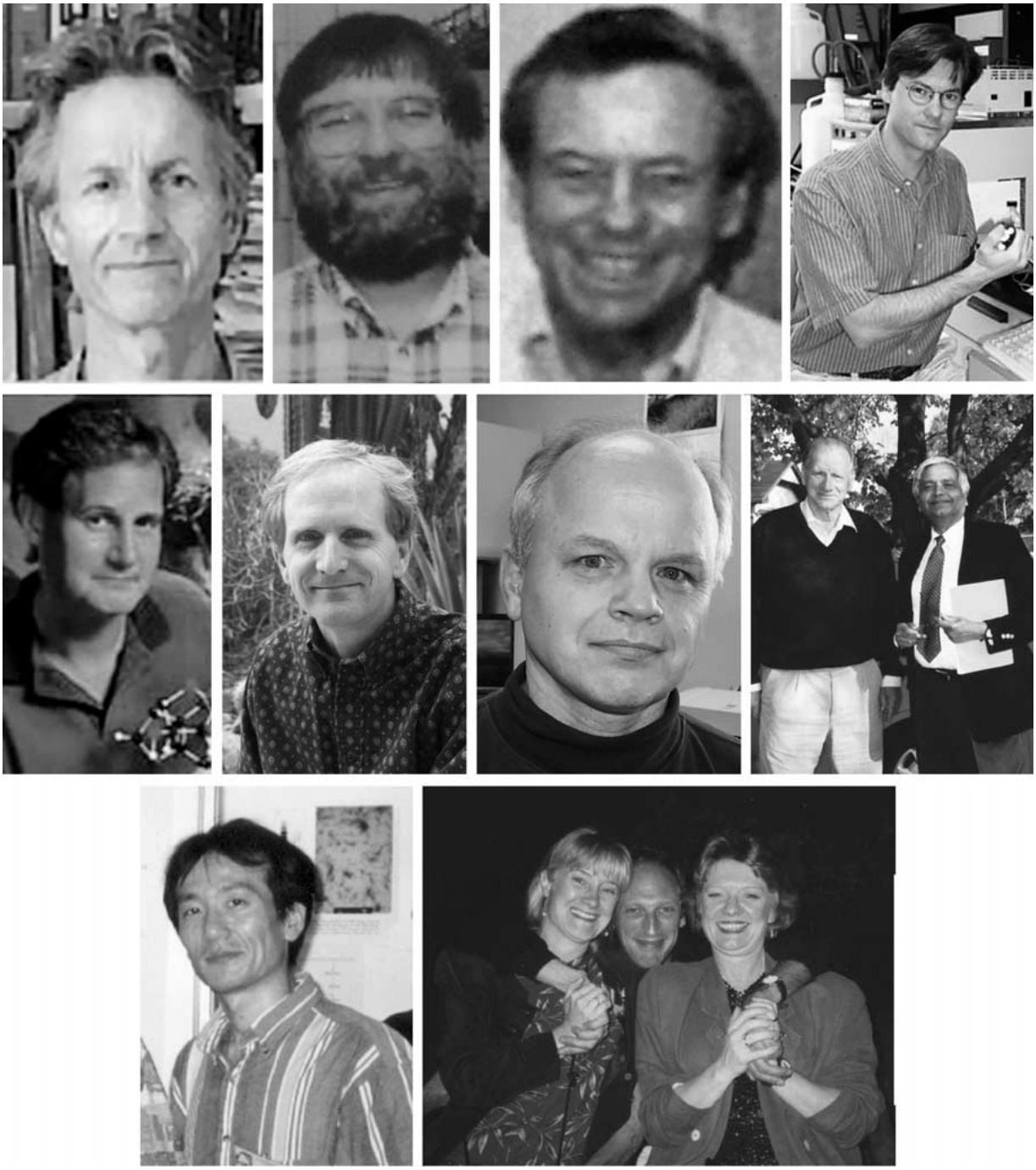

Figure 1. Early pioneers involved in the development of site-directed mutagenesis of chloroplast encoded proteins. *Top row* (*from left to right*): Jean-David Rochaix, John Boynton, Nicholas Gillham and Kevin Redding; *Middle row* (*from left to right*): Wayne Frasch, Andrew Webber, Richard Sayre and Achim Trebst (with Govindjee); *Bottom row* (*from left to right*): Jun Minagawa and Francis-André Wollman [with Susan Golden (left) and Christa Critchley (right)].

given its multi-cellularity and made even more difficult given the large number of chloroplast genomes per plant cell.

John E. Boynton and Nick Gillham and their coworkers reported in 1988, the first stable transformation of the chloroplast genome in Chlamydomonas

(Boynton et al. 1988; see Figure 1 for photographs of Boynton and Gillham). They complemented an *atpB* deletion mutant with a wild-type *atpB* gene. The chloroplast *atpB* gene was shot into Chlamydomonas by particle gun mediated bombardment. The integrated *atpB* gene restored photoautotrophic growth to the acetate requiring deletion mutant and integrated into genomic regions that were homologous to DNA sequences present on the transforming plasmid. This early work demonstrated the potential of chloroplast transformation for engineering the genome and proteins of the photosynthetic apparatus by site-directed mutagenesis.

The first site-directed mutation engineered into a chloroplast gene was targeted to the *psbA* gene of Chlamydomonas (Roffey and Sayre 1990; Roffey et al. 1991; see Figure 1 for a photograph of one of the authors (Sayre)). We had recently learned of the first chloroplast transformations by John Boynton and colleagues at the International Conference on the Cell and Molecular Biology of Chlamydomonas held at Cold Spring Harbor, New York. It was Robin's assignment as a new graduate student to generate the first engineered chloroplast encoded Photosystem II protein, the D1 protein of the photosynthetic reaction center. The D1 protein is encoded by the *psbA* gene. Using a 3.0 kbp subclone of the *psbA* gene (7.0 kbp), in which amino acid residue D1-H195 had been converted to a tyrosine, Robin generated chloroplast transformants by particle gun bombardment using wild-type Chlamydomonas cells. The D1-H195 residue was predicted to be located near the chlorophyll special pair binding site (D1-H198) based on the analogous bacterial reaction center structural models and thought to modulate the redox potential of the primary electron donor. A tyrosine residue was substituted for the histidine at position 195 based on the hypothesis that a tyrosine residue could serve as a redox sensitive, EPR-detectable probe of the primary donor. The D1-H195Y transformant was generated by co-transformation of the Chlamydomonas chloroplast genome with two plasmids. One plasmid contained the mutated *psbA* gene fragment and the second plasmid contained a mutant chloroplast 16S rRNA gene which conferred spectinomycin resistance. A small fragment of the *psbA* gene was used since it had been demonstrated that the intact 7 kbp *psbA* gene was unstable in *E. coli*. Chloroplast transformants were primarily selected on the basis of spectinomycin resistance and then for the diagnostic RFLP (restriction fragment length polymorpism) site introduced by site-directed mutagenesis. Since, the integration of each transgene (*psbA* and *16SrRNA)* is an independent event it was expected that only a small fraction of the spectinomycin resistant colonies would also have the *psbA* transgene integrated. The observed frequency of co-transformation was 5%, thus 1 in 20 colonies screened by polymerase chain reaction (PCR) and restriction enzyme digest for the diagnostic *psbA* RFLP site was positive, a very workable number. Since recombination between the host genome and the introduced plasmid is a rare event, all initial transformants were shown to be heteroplasmic for the introduced gene and had to be driven to homoplasmy (elimination of wild-type gene copies) via multiple rounds ($\geq$3) of sub-cloning and screening for the diagnostic RFLP marker and loss of wild-type RFLP patterns. The time required to secure a homoplasmic transgenic line (from the time of the initial transformation event) was typically two to three months. Latter, additional D1-H195 mutants were generated. In collaboration with Dave Kramer and Govindjee (at Urbana, Illinois; see Figure 1 for a photograph of Govindjee), Robin Roffey and one of us (RTS) analyzed the $P680^+$ reduction kinetics of the D1-H195D mutant (potentially negatively charged) and showed that they were 100-fold slower than wild type. Further, the reduction in electron transfer rates in the mutant was attributed to a shift in the E_M of either $Tyr_Z/Tyr_Z^\bullet$ or $P680/P680^+$ (Roffey et al. 1994a, b). Unfortunately, there was no evidence of oxidation of the D1-H195Y residue.

Substantial improvements in the selection of chloroplast transformants were achieved with the introduction of several new tools including, antibiotic resistance genes linked to the chloroplast gene of interest and the use of intronless chloroplast genes targeted for gene replacement in deletion mutants (Goldschmidt-Clermont 1991; Johanningmeier and Heiss 1993; Minagawa and Crofts 1994; Minagawa et al. 1996; see Figure 1 for a photograph of Minagawa). These strategies reduced the screening time needed for the isolation for mutants by a factor of two and eliminated the need to screen for homoplasmic co-transformants.

In the following sections we highlight some of the more notable photosynthetic phenotypes generated by site-directed mutagenesis of the Chlamydomonas chloroplast genome and discuss the contributions of various investigators to the elucidation of the structure and function of the photosynthetic apparatus. The Chlamydomonas chloroplast mutants discussed here

are not all inclusive. For recent reviews, see Rochaix et al. (1998).

Photosystem II

Early choices of residues to target for site-specific mutagenesis were guided by models of the folding topology of the D1 and D2 proteins. These models were based on sequence and topology similarities between the Photosystem (PS) II D1 and D2 proteins and bacterial reaction center L- and M-subunits. Later PS II structural models developed by Svensson et al. (1990) and by Xiong et al. (1996, 1998a) helped further define structure–function relationships in the PS II complex and assist in directed mutagenesis studies.

Some of the earliest site-directed mutants generated in Chlamydomonas were *psb*A mutants (D1-N266T/I259S and D1-N266T/S264A/I259S) that conferred herbicide resistance (Przbilla et al. 1991; see Figure 1 for a photograph of one of the co-authors of Przbilla et al., Achim Trebst). These mutants were selected on the basis of metrubuzine resistance (10^{-5} M) eliminating the need for the use of some independent selectable marker for identification of the chloroplast transformants. Later, herbicide resistant or supersensitive *psbA* mutants located at residues D1-A251 and D1-A250 were generated (Lardans et al. 1997; Johanningmeier et al. 2000). The D1-A251 and A250 residues are located near the Q_B binding site. Replacement of the D1-251 alanine with polar residues including, Arg, Asp, Gln, Glu and His resulted in non-photosynthetic phenotypes. In contrast, non-polar substitutions of the D1-A251 residue were capable of photosynthetic growth. Several of the D1-A251 mutants accumulated a truncated, 24–25 kDa N-terminal form of the D1 protein that turned over rapidly. These results were in contrast to those obtained with identical cyanobacterial mutants. In cyanobacteria no D1-A251 mutants had non-photosynthetic phenotypes. These results were among the first that indicated that there were often phenotypic differences between identical cyanobacterial and chloroplastic PS II mutants.

One of the first PS II residues targeted for site-directed mutagenesis *in vitro* was the D1-H190 residue (Roffey et al. 1994a, b). The D1-H190 residue was predicted from modeling studies to have pseudo-C2 symmetry with the D2-H189 residue. Both residues were predicted to be located in a lumenal extrinsic alpha-helical domain known as the CD loop. The CD loops of the D1 and D2 proteins had been predicted to be located near the redox active tyrosines Z and D, respectively. Early electron nuclear double resonance (ENDOR) and extended X-ray absorption fine structure (EXAFS) studies of the tetra-Mn complex also suggested that a histidine residue may be involved in coordination of the Mn atom(s) of the water oxidizing complex. The D1-H190F mutant was unable to evolve oxygen and was missing the functional Mn of the water-oxidizing complex. The mutant was capable, however, of charge separation in the presence of artificial electron donors and could flash-accumulate a high chlorophyll fluorescence state in the presence of DCMU (3′(3,4-dichlorophenol)-1′1-dimetylurea). Electron paramagnetic resonance (EPR) analyses indicated that the yield of Signal II_{fast} attributed to $Y_Z^\bullet$ formation was only 10% of wild-type thylakoids although the D1 content of thylakoids was identical to wild type. Importantly, the hyperfine structure of the $Y_Z^\bullet$ EPR signal was unchanged relative to wild type (Roffey et al. 1994b). This was in contrast to the modified hyperfine structure of Signal II_{slow} ($Y_D^\bullet$) observed in cyanobacterial D2-H189 mutants attributed to hydrogen bonding interactions between D2-H189 and D2-Y160 residues. These results suggested that the D1-H190 residue did not hydrogen bond to $Y_Z^\bullet$ unlike the D2-H189 residue. Again, in collaboration with David Kramer and Govindjee, Roffey and one of us (RTS) showed that the D1-H190F mutant was unable to form the A_T thermoluminescence band that had been attributed to charge recombination between Q_A^- and $Y_Z^\bullet$ (Kramer et al. 1994). [For a historical review on thermoluminescence, see Vass (2003).] While it was originally suggested that the loss of the A_T thermoluminescence band could be attributed to loss of a redox active histidine, it is now apparent that the D1-H190 mutants may cause gross structural changes in the PS II reaction center complex. This interpretation is based on the recent structural evidence that indicates the D1-H190 residue is located some distance from $Y_Z^\bullet$.

One attractive model for the function of the D1-H190 residue, that was consistent with all of the phenotypic data, was a proposal by the late Gerald T. Babcock (see his photographs in Renger 2003) that the D1-H190 residue functioned as a proton acceptor from Y_Z during its oxidation (Bloomberg et al. 1997). This model for D1-H190 function was a central component of the metallo-radical model for water oxidation that attempted to account for the observed simultaneous oxidation and de-protonation of Y_Z and the subsequent reduction and protonation of $Y_Z^\bullet$ by the

tetra-Mn complex. If, however, the D1-H190 residue is located many angstroms from Y_Z then some other residue must function as the proton acceptor from Y_Z. The identity of this residue remains to be determined.

A common theme for targeted site-directed mutations in the PS II as well as PS I complex has been the choice of symmetry-related histidine residues. It was known from the bacterial photosynthetic reaction center structure that 2 symmetry-related histidines were involved in coordination of the chlorophylls of the special pair, the chlorophyll monomers and the non-heme iron. Following the isolation of intact PS II reaction centers it became apparent that the PS II reaction center complex had two extra chlorophylls in addition to the four predicted from structural similarities to the bacterial photosynthetic reaction center. [For a history of the isolation of the PS II reaction center, see Satoh (2003).] Schelvis et al. (1994) had predicted that these additional chlorophylls were located 30 Å from the chlorophyll special pair or P680 based on analyses of chlorophyll fluorescence decay kinetics using isolated PS II reaction centers. Analyses of the D1 and D2 protein sequences indicated there was a pair of symmetry related histidine residues located near the middle of the B-transmembrane spans of the D1 and D2 proteins located at residues 118 and 117, respectively (Hutchison and Sayre 1995). These residues are located approximately 30 Å from the chlorophyll special pair. Furthermore, the corresponding residues in the bacterial L- and M-subunits are not conserved and their side chains are incompatible with coordination of the chlorophylls.

The coordination of the additional PS II reaction center chlorophylls by the D1-H118 and D2-H117 residues has been confirmed by the recent PS II crystal structures (Zouni et al. 2001). (For a history of the crystallization of PS II, see Horst T. Witt, this issue) In addition to their proposed role in energy transfer to the chlorophyll special pair, at least one of the peripheral accessory chlorophylls (Chl_Z) has been proposed to function in a low quantum yield electron transfer loop around PS II involved in its photo-protection (Koulougliotis et al. 1994). The Chl_Z cycle facilitates charge recombination between Q_A^- and $P680^+$ reducing photoinhibition and turnover of the PS II complex. According to the model, Chl_Z is inefficiently oxidized by $P680^+$ and re-reduced by cytochrome b_{559} (Thompson and Brudvig 1988). The PS II carotenoid is thought to facilitate electron transfer from Chl_Z to the primary donor. To determine if the additional PS II chlorophylls were coordinated by these residues and to identify which of the two peripheral accessory chlorophylls was involved in the Chl_Z mediated electron transfer cycle a series of site directed mutations was engineered. The generation of the D1-H118 mutants was greatly facilitated by the development of an intronless *psbA* gene construct linked to a bacterial *aadA* gene conferring spectinomycin resistance and flanked on the 5′ and 3′ ends of the construct by regions of chloroplast DNA homologous to the *psbA* region of the chloroplast genome (Minagawa et al. 1996; Ruffle et al. 2001). This plasmid eliminated the instability issues associated with using the intron-containing *psbA* gene, and provided a direct mechanism for selection (antibiotic resistance) of transformants in *psbA* deletion mutants.

All of the D2-H117 mutants generated were capable of oxygen evolution, albeit at reduced rates relative to wild type. In contrast, only the D1-H118Q mutants were able to form a functional PS II complex capable of charge separation. Characterization of the D1-H118Q and D2-H118Q mutant phenotypes was carried out by three very able postdocs in the lab (at Ohio State University), Ron Hutchison, Stewart Ruffle and Jun Wang. Consistent with its predicted involvement in energy transfer, it was observed that the 30 ps chlorophyll fluorescence decay lifetime component was the only chlorophyll fluorescence decay lifetime component altered (shifted to 10 ps) in PS II reaction centers of D2-H117 mutants (Johnston et al. 2000). Subsequently, the identity of the chlorophyll participating in the Chl_Z cycle was determined. The Chl_Z cation can be detected by EPR and when photoaccumulated at cryogenic temperatures quenches chlorophyll fluorescence. The chlorophyll resonance Raman spectra of Chl_Z had been shown to be altered in D1-H118 but not D2-H118 mutants in cyanobacteria suggesting that the redox active Chl_Z was coordinated by the D1-H118 residue (Stewart et al. 1998). Recently, the location of redox active Chl_Z has been called into question based on the PS II crystal structure models that indicated that Cyt b_{559} heme, the immediate electron donor to Chl_Z^+, is located near the peripheral accessory chlorophyll coordinated by the D2-H117 residue (Zouni et al. 2001; Kamiya and Shen 2003). Analyses of D1-H118Q and D2-H117Q mutants in Chlamydomonas, however, indicated that the Chl_Z^+ EPR spectrum and Chl_Z-dependent quenching of chlorophyll fluorescence were altered only in D2-H117Q mutants and not D1-H118Q mutants (Wang et al. 2002). Interestingly, both the D1-H118Q and D2-H117Q mutants have reduced sensitivity to

photoinhibition, most likely associated with a reduced ability to form charge-separated states (Ruffle et al. 2001).

A major difference between PS II and the quinone type bacterial reaction center has been is that bicarbonate ions are required for PS II, but not for the functioning of bacterial reaction center (see Blubaugh and Govindjee 1988; Govindjee and van Rensen 1993). Bicarbonate ions play important roles on both the acceptor and the donor sides of PS II. For historical minireviews, see Stemler (2002) and van Rensen (2002). Jin Xiong, then in Govindjee's laboratory, established a crucial role of D1-R-257 in this bicarbonate effect through site-directed mutagenesis in *Chlamydomonas reinhardtii* (Xiong et al. 1998b).

New information is also emerging from *psbA* mutagenesis studies on the control of electron transfer along the active branch of electron transfer pathway in PS II. Mutagenesis of the D1-E130 residue, predicted to hydrogen bond to the ring-V carbonyl of the active branch pheophytin, alters the quantum efficiency of charge separation on the active branch pathway (Dorlet et al. 2001). [For a historical account of the first measurements on the charge separation in isolated PS II reaction centers, see Seibert and Wasielewski (2003).] The elimination of the hydrogen bond in D1-E130L mutants blocks charge transfer, while residues having intermediate hydrogen bonding strengths have intermediate rates of oxygen evolution. Further studies are currently underway to resolve the issues that control the directionality of electron transfer in PS II.

Photosystem I

Nelson and Ben-Shem (2002) have provided a historical background on the past and the future of the Photosystem (PS) I reaction center, whereas Petra Fromme and Paul Mathis (this issue) have discussed the structure and function of PS I. Targeted mutagenesis of the PS I complex has progressed rapidly in the last few years, particularly after the release of a high-resolution PS I crystal structure. Prior to the elucidation of the PS I crystal structure, however, mutagenesis studies in PS I were hampered by the lack of a well-resolved PS I structural model. One of the earliest successes in PS I mutagenesis studies was the identification of the P700 ligands (Webber et al. 1996; see Figure 1 for a photograph of Webber). Targeted mutagenesis of the PsaA-H676 and PsaB-H656 residues located in transmembrane span X was shown to alter both the midpoint potential of P700 as well as the ENDOR signal attributed to $P700^+$. Systematic mutagenesis of all the paired histidines in the last six transmembrane spans of the PsaA and psaB proteins, by Redding et al. (1998; see Figure 1 for a photograph of Redding), confirmed the location of the P700 ligands identified by Webber et al. (1996).

Perhaps one of the more exciting contributions of site-directed mutagenesis to the elucidation of photosynthetic electron transfer processes has been the demonstration that charge separation in PS I occurs by either of two parallel electron transfer pathways from P700 to the F_X iron–sulfur cluster. Both forward electron transfers from A_1^- to F_X and charge recombination from A_1^- to $P700^+$ have biphasic kinetics. This characteristic had been attributed to a low equilibrium constant between A_1 and F_X leading to fast equilibration between A_1 and F_X and slow decay of the quasi-equilibrium state associated with electron transfer between F_X and F_A/F_B. An alternate explanation for the biphasic kinetics is that there are two active electron transfer branches that reduce F_X. These two pathways presumably differ in the kinetics of electron transfer from the quinone bound by the PsaA or PsaB proteins and F_X. To test this hypothesis Kevin Redding's lab (Guergova-Kuras et al. 2001) mutagenized two symmetry-related and conserved tryptophan residues (psaA-W693F and psaB-W673F) that π–π stack with the phylloquinones bound by the PsaA and PsaB subunits. They demonstrated that one mutation (psaB-W673F) affected only the fast kinetic component of F_X reduction while the other mutation (psaA-W693F) affected only the slow kinetic component of F_X reduction. They suggested that the mutations altered the distance, midpoint potential or reorganization energies of the two phylliquinones, which in turn lead to a change in the rate of electron transfer. These results strongly support the hypothesis that electron transfer in PS I can proceed down either branch of the two parallel electron transfer pathways.

Cytochrome b_6f complex

Günter Hauska provides a historical account of the isolation of Cytochrome b_6f complex in this issue. Further, William Cramer provides a discussion of its structure, also in this issue. The potential for mutagenesis studies of the cytochrome b_6f complex in Chlamydomonas are just now being realized. Significantly, the cytochrome b_6f complex is dispensable

in Chlamydomonas when grown heterotrophically, allowing for the isolation of otherwise lethal mutants. Some of the earliest mutagenesis studies focused on the structure of the Q_0-binding site. The Q_0 plastoquinol-binding site of the cytochrome b_6f complex includes contributions from several protein subunits including cytochrome b_6 and subunit IV. Mutagenesis of the conserved lumenal PEWY sequence in subunit IV to PWYE blocked photosynthetic growth and resulted in the loss of plastoquinol binding at the Q_0 site (Zito et al. 1999; see Figure 1 for a photograph of one of the co-authors of Zito et al., Francis-André Wollman). Interestingly chlorophyll fluorescence quenching associated with state I–II transitions did not occur in these mutants indicating they were locked in state-I transition. This affect was associated with a poorly phosphorylated light harvesting complex (LHC)-II complex demonstrating that plastiquinol binding to the Q_0 site was required for activation of the LHCII kinase. [See Allen (2002) for a history of the mechanism of the 'state-changes.']

ATP synthase

For a history of the discoveries in photophosphorylation, see André T. Jagendorf (2002). There have been only a few chloroplast encoded, site-directed mutants of the ATPase complex generated in Chlamydomonas. Wayne Frasch and colleagues have identified residues of the F_1-β subunit involved in coordination of the magnesium atom of Mg-ATP (Hu et al. 1996; see Figure 1 for a photograph of one of the co-authors of Hu et al., Wayne Frasch). The β-E197 and β-D262 residues were demonstrated to provide Mg ligands for Mg-ATP during the conversion of the low-affinity site-3 conformation to the high-affinity site-1 conformation. Mutagenesis of the residues β-E197 and β-D262 residues to non-acidic residues eliminated the carboxylate Mg-ligands and substantially impaired photosynthetic growth and ATP synthesis. The mutations also caused large changes in the ^{51}V hyperfine tensors of vanadate–nucleotide bound to site 1 but not to vanadate–nucleotide complexes bound site 3 consistent with alterations in the coordination chemistry of vanadate and by inference magnesium.

Concluding remarks

We have often been told by investigators using Chlamydomonas for the first time that the Chlamydomonas scientific community is among the most approachable and helpful scientific communities they have encountered. This culture of collaboration has fostered much advancement in chloroplast transformation technology and has facilitated interactions between molecular biologists and biophysicists. One of us (RTS) has certainly been helped by the mentoring provided in his career by Peter Homann, who first introduced him to using algae for photosynthetic studies and taught him to think more critically; by George Cheniae, in whose lab he did the first PS II Chlamydomonas work; and by Lawrence Bogorad in whose lab he learned molecular biology and had the freedom to explore new ideas. The collegiality of the Chlamydomonas scientific community and the unique biological properties of this organism, including the recent completion of the genome project, insure that Chlamydomonas will remain atop the hierarchy of model photosynthetic eukaryotes well into the future.

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