Genetic Manipulation of Photosystem Two Polypeptides in

*Chlamydomonas reinhardtii*

A thesis submitted for the degree of

Doctor of Philosophy

by

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Abstract

Photosystem two (PSII) catalyses the light-driven oxidation of water to molecular oxygen and protons in oxygenic photosynthesis. In order to elucidate the structure and function of PSII a multidisciplinary approach has been underway over recent years which has included biochemical, biophysical, molecular, genetic and crystallographic studies of PSII from a number of different organisms. The molecular approach can provide an insight into the function of particular polypeptides and their amino acid residues by carrying out deletions of polypeptides or altering specific amino acids thought to be of importance in the function of PSII.

Chlamydomonas reinhardtii is amenable to genetic manipulation and is a model system for the study of PSII. Site-directed mutants have been created in an attempt to elucidate the role of amino acid residues important in the mechanism of water oxidation. Unfortunately, biophysical analysis of PSII in C. reinhardtii has been hindered by the poor range of highly active PSII preparations compared to those available from higher plants. This thesis describes the construction of a novel, stable mutant of C. reinhardtii in which the PsbH polypeptide, a component of PSII, is tagged with six histidine residues at the carboxyl terminal end. This tag facilitates a rapid PSII purification procedure using nickel column affinity chromatography. The C. reinhardtii PSII is free from Photosystem I (PSI) contamination and is suitable for biophysical background allowing detailed studies of PSII by techniques that were previously difficult or impossible to utilise. The construction of several PSII mutants created in the his-tagged background are described and their analysis presented. Both the D1 and D2 polypeptides were mutated allowing us to investigate the mechanism of water oxidation.

In addition to analysis of PSII site-directed mutants further attempts at epitope tagging the C. reinhardtii PsbH polypeptide are presented. Epitope tagging has become a standard method which enables rapid and effective characterisation, purification and in vivo localisation of the protein products of tagged genes. This work presents attempts at tagging the PsbH polypeptide with both six and ten histidine residues, a haemagglutinin tag and also with the mature pea plastocyanin sequence.
For Mum, Dad and Chris
Acknowledgements

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<td>μE</td>
<td>microEinstein</td>
</tr>
<tr>
<td>A₀</td>
<td>Chl α, PSI primary electron acceptor</td>
</tr>
<tr>
<td>A₁</td>
<td>a quinone, PSI primary electron acceptor</td>
</tr>
<tr>
<td>AMPS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BBY</td>
<td>higher plant thylakoid preparations (Berthold et al., 1981)</td>
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<tr>
<td>bp</td>
<td>base pairs (kb: kilobase pairs)</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumen</td>
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<td>Chl a/b</td>
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<td>2’deoxycytidine 5’triphosphate</td>
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<td>HEPES</td>
<td>N-[2-Hydroxyethyl]piperazine-N’[2-ethanesulfonic acid]</td>
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### Abbreviations

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<td>Immobilised metal affinity chromatography</td>
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<td>NADPH</td>
<td>reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NTA</td>
<td>nitrilotriacetic acid</td>
</tr>
<tr>
<td>OD&lt;sub&gt;750&lt;/sub&gt;</td>
<td>optical density at 750nm</td>
</tr>
<tr>
<td>OEE</td>
<td>oxygen evolution enhancer</td>
</tr>
<tr>
<td>P</td>
<td>pigment molecules</td>
</tr>
<tr>
<td>P680</td>
<td>primary electron donor in PSII</td>
</tr>
<tr>
<td>P700</td>
<td>primary electron donor in PSI</td>
</tr>
<tr>
<td>P870</td>
<td>the primary electron donor in the bacterial RC</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PC</td>
<td>plastocyanin</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>Pheo</td>
<td>pheophytin</td>
</tr>
<tr>
<td>PSI</td>
<td>photosystem one</td>
</tr>
<tr>
<td>PSII</td>
<td>photosystem two</td>
</tr>
<tr>
<td>QA</td>
<td>plastoquinone-9, PSII electron acceptor A</td>
</tr>
<tr>
<td>QB</td>
<td>plastoquinone-9, PSII electron acceptor B</td>
</tr>
<tr>
<td>RC</td>
<td>reaction centre</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>Definition</td>
</tr>
<tr>
<td>---------------</td>
<td>------------</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<td>sodium dodecyl sulphate</td>
</tr>
<tr>
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<td>saline sodium citrate</td>
</tr>
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<td>tris-acetate EDTA</td>
</tr>
<tr>
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<td>tris-acetate phosphate</td>
</tr>
<tr>
<td>TE</td>
<td>tris-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethlenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminoethane</td>
</tr>
<tr>
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<td>ultraviolet</td>
</tr>
<tr>
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<td>volume for volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight for volume</td>
</tr>
<tr>
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<td>water oxidising complex</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
<tr>
<td>ycf</td>
<td>hypothetical chloroplast open reading frame</td>
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<tr>
<td>Y_D</td>
<td>tyrosine 160 on D2, a tyrosine electron donor in PSII</td>
</tr>
<tr>
<td>Y_Z</td>
<td>tyrosine 161 on D1, a tyrosine electron donor in PSII</td>
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</table>
CHAPTER 1

Introduction
1.1 Introduction

The process of photosynthesis utilises solar energy to convert carbon, in the form of atmospheric CO$_2$, to an organic form. As such photosynthesis provides the ultimate energy source for almost all organisms on the planet today and it is responsible for Earth’s oxidising atmosphere. The trapping and utilisation of sunlight carried out by plants, algae and bacteria can be summarised by the following equation:

$$2H_2A + CO_2 \rightarrow [CH_2O] + H_2O + 2A;$$

where, $2H_2A$ is an oxidisable substrate and $[CH_2O]$ represents stored organic matter (van Niel, 1962). Many photosynthetic organisms utilise water as the oxidisable substrate and oxygen is evolved as a by-product. As such the above equation can be rewritten as follows:

$$2H_2O + CO_2 \rightarrow [CH_2O] + H_2O + O_2$$

The organisms that carry out this “oxygenic” photosynthesis include plants, algae and cyanobacteria. Those that do not utilise water include the purple sulphur bacteria, the purple non-sulphur bacteria, green bacteria.

When photosynthetic cyanobacteria first harnessed water as a substrate to drive the photosynthetic reaction they were able to dominate their environment due to the vast abundance of water. As the by-product of this reaction, oxygen, became more and more prevalent, the Earth’s atmosphere changed from anaerobic to aerobic. Existing organisms had to adapt to their new environment or they did not survive. This change in atmosphere was a main factor in allowing the evolution of higher organisms. The oxygenic atmosphere, by allowing ozone formation, also provided a protective barrier against the harmful ultra-violet rays of the sun.
1.2 The Photosynthetic Reactions

Photosynthesis is a series of reactions ultimately yielding carbon compounds that can be utilised by the organism or stored for future use. The complex reactions can be simplified into what have historically been termed the “light” and “dark” reactions (Mathews et al., 2000); more correctly they are now referred to as the “light-requiring” and “stromal” reactions (Figure 1.1). The light reactions involve the absorption of light required for a series of electron transfer steps that ultimately lead to water oxidation (in oxygenic photosynthesis) and the production of ATP and NADPH. In the dark reactions ATP and NADPH are used to fix CO₂ to give organic carbon.

In oxygenic photosynthetic organisms the conversion of light energy takes place in thylakoid membranes. In eukaryotes these membranes are located within the chloroplast while in cyanobacteria they are found in the cell cytosol. In higher plants and some algae, the thylakoids form a continuous folded membrane network composed of single, non-appressed membranes and stacks of tightly paired or appressed membranes (Anderson & Andersson, 1982). The outer thylakoid surface faces the chloroplast stroma, where the proteins required for the fixation and reduction of CO₂ are located. The stroma is separated from the cell cytoplasm by a double membrane termed the chloroplast envelope. The thylakoid membranes enclose a continuous space referred to as the thylakoid lumen (Anderson & Andersson, 1988).

A number of membrane-bound complexes constitute the photosynthetic apparatus, illustrated in Figure 1.2. They are the electron transfer complexes, Photosystem I (PSI) and Photosystem II (PSII), cytochrome b₆f (cyt b₆f), light-harvesting pigment complexes and the ATP synthase. Together these complexes produce ATP and NADPH required for the conversion of CO₂ to organic carbon.
Figure 1.1 The two sub-processes of oxygenic photosynthesis. The overall process of photosynthesis is divided into light and dark (or stromal) reactions. The light reactions, which require visible light as an energy source, provide reducing power (in the form of NADPH), ATP and O₂. The NADPH and ATP drive the dark (non-light requiring) reactions, fixing CO₂ into carbohydrate.
Figure 1.2 Schematic drawing showing the major protein complexes of the thylakoid membrane. These complexes act as the photosynthetic machinery responsible for light absorption and electron and proton transfer. The reactions of the thylakoid membrane drive the C3 photosynthetic carbon cycle that takes place in the chloroplast stroma. Light-driven linear electron flow results in a build up of protons in the lumen used to drive ATP formation by ATP synthase. Energy derived from absorbed light is also stored by the formation of NADPH.

Key: PSII, photosystem II; PSI, photosystem I; PQ and PQH2, plastoquinone and reduced plastoquinone; cyt, cytochrome; FeS, Rieske iron–sulphur protein; PC, plastocyanin; Fd, ferredoxin; FNR, ferredoxin-NADP reductase.
1.3 Photosynthetic Reaction Centres

Photosynthetic organisms all contain supra-molecular membrane-bound complexes known as photosystems. These photosystems contain light-absorbing pigments and a series of electron transport components, such as cytochromes and quinones. A special pair of pigment molecules (P) can be activated by light energy to an excited state (P*). In this state the reduction potential of the pigment molecule is large enough to allow transfer of an electron to a nearby acceptor molecule. This transfer is extremely fast but a back reaction is possible. To prevent this inefficient recombination, the electron is transferred to a secondary electron acceptor, ultimately reaching the terminal electron acceptor along an electron transport chain. The initial electron donor molecules (P+) are re-reduced and the electron transfer chain is essentially reset, ready for a second light activation event. This electron transport within the reaction centre (RC) is directional such that it gives rise to a negative charge on one side of the membrane and a positive charge on the opposite side.

Photosynthetic RC complexes are not identical. Biochemical and biophysical surveys of photosynthetic complexes from a variety of bacterial and eukaryotic organisms have shown that there are two different RC types (reviewed by Blankenship, 1992; Golbeck, 1993). Members of each RC type share some degree of protein structural similarity (Rhee et al., 1998) and the primary charge separation event in both types is the transfer of an electron from an excited chlorophyll to a nearby, chlorophyll-based electron carrier and subsequently to a quinone-based secondary electron acceptor. The two RC types can be readily distinguished by the identity of their first stable electron acceptor. They are known as the quinone-reducing (Type II) and iron-sulphur-reducing (Type I) photosynthetic RC types. Despite the basic similarity of the RC types they do differ in that each has unique protein subunits, redox active intermediates and sources of electrons for the reduction of the oxidised primary donor.
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The Type II RC is found in purple sulphur bacteria and in PSII. The co-factors that carry out the photochemistry are a special pair of chlorophyll molecules (bacteriochlorophyll in photosynthetic bacteria) which donate an electron to a pheophytin molecule (a chlorophyll lacking the magnesium co-factor). This is followed by the electron acceptors, quinones Q_A and Q_B. A non-haem iron atom is present between these two quinones on an axis of symmetry through the RC. The redox components are co-ordinated by a heterodimer composed of protein subunits L and M in bacteria and D1 and D2 in PSII. The similarity between PSII and the purple sulphur bacteria led to the proposal that D1 is descended from subunit L and D2 from M, but genetic analysis shows that D1 and D2 are more closely related to one another than to their counterparts in the bacterial RC. Evolutionary analysis suggests the bacterial RC and PSII are related through a common ancestral homodimer (Nugent, 1996). Although the purple sulphur bacteria contain Type II RCs they are unable to oxidise water. PSII is able to oxidise water, firstly, due to the high mid-point potential (E_m) of P680 (the primary electron donor in PSII). At 1.15V (Nugent, 1996) this redox potential is more than double that of P870 (the primary electron donor in the bacterial RC) and is high enough to be used to oxidise water. Secondly, the PSII complex has evolved a system for charge accumulation needed for the four-electron chemistry of water splitting (Section 1.5).

The Type I RC is typified by PSI and contains iron-sulphur centres. Green sulphur bacteria of the genus Chlorobiaceae and Gram-positive Heliobacteria also contain Type I RCs. The primary electron donor of PSI is a special pair of chlorophylls followed by the primary electron acceptor, A_0, a Chl a molecule and the secondary acceptor A_1, a quinone. After A_1 the electron passes to the first of the iron-sulphur centres, F_X, and this co-factor subsequently transfers the electron to the other iron-sulphur centres F_A and F_B. The electron transport chain co-factors are co-ordinated by a heterodimer formed by the PsaA and PsaB proteins - each having 11
membrane spanning helices (for a review of electron transport in PSI see Nugent 1996).

Structural data indicate a close evolutionary link not only between the bacterial RC and PSII (as noted above) but also between these RCs and PSI (Rhee et al., 1998). Figure 1.3 shows a diagrammatic comparison of these RCs indicating the structural similarities. The data suggest that the 11-helix-containing proteins of PSI, PsaA and PsaB, have evolved from the genetic fusion of a six-helix CP47/43-like protein with a five-helix-containing RC protein as seen in PSII. In support of this concept, phylogenetic relationships between photosynthetic bacteria indicate that the Chloroflexaceae, which have a RC similar to that of purple sulphur bacteria, are the most ancient photosynthetic organisms on earth (Mathis, 1990), suggesting PSI evolved from a PSII-type RC.
Figure 1.3 Comparison of the types of reaction centre (RC) found in photosynthetic organisms.

A - bacterial RC. The L and M subunits, which bind the pigments active in charge separation (red), are related by local near two-fold symmetry. The RC is surrounded by a ring of light-harvesting proteins (LH1).

B - photosystem II. The D1 and D2 proteins are structurally and functionally homologous to the L and M subunits of the bacterial reaction centre and hold the active pigments in a similar configuration. Light energy is collected by LHCII (in plants) and channelled into the reaction centre by the core antenna proteins, CP43 and CP47, which are positioned at either side of the D1–D2 heterodimer.

C - photosystem I. The PsaA and PsaB proteins form a heterodimer. PsaA and PsaB each consist of a reaction centre system equivalent to D1 or D2, and a core antenna equivalent to CP43 or CP47.
With the exception of the cyanobacteria, all prokaryotes have either a Type-I or a Type-II RC but not both. The eukaryotic photosynthetic organisms and cyanobacteria have both types of RC (PSI and PSII) and they can oxidise water. The PSII complex carries out the light-dependent oxidation of water and reduction of plastoquinone while PSI complex carries out the light-dependent oxidation of a soluble copper (plastocyanin) or haem (cytochrome $c_6$)-containing protein and the reduction of an FeS protein (ferredoxin) which in turn reduces NADP$^+$. The two photosystems (PSI and PSII) function in series according to the "Z-scheme" postulated by Hill and Bendall (1961). This scheme is shown in Figure 1.4. Electrons are transferred from water to NADP$^+$, via the cyt $b_6f$ complex, while releasing or transporting protons across a membrane to drive ATP synthesis, ultimately facilitating the fixation of carbon. As Figure 1.4 shows, the two photosystems co-operate in series. Light excitation of the primary electron donor of PSII, P680, results in electron transfer from an excited state of P680 to a pheophytin molecule which in turn reduces a bound plastoquinone molecule, $Q_A$. $Q_A$ reduces a second quinone $Q_B$ and electrons from $Q_B$ are transferred to plastocyanin (PC) via the cyt $b_6f$ complex. On the donor side of PSII, the chlorophyll cation radical, P680$,^+$, oxidises a tyrosine residue $Y_Z$ (Debus et al., 1988a,b). $Y_Z$, in turn, oxidises a cluster of four manganese (Mn) atoms. It is widely accepted that this metal centre is the catalytic site of water oxidation. It is proposed that the Mn-cluster accumulates the four oxidising equivalents that are necessary to release O$_2$ from two molecules of water (Nugent, 2001 and papers therein).

Cyt $b_6f$ consists of cytochrome $f$ and two $b$-type cyttochromes, a 17 kDa polypeptide (subunit IV), a Rieske iron-sulphur centre and four small subunits (Hope, 1993; Cramer et al., 1994). The complex accepts two electrons from PSII, one electron reduces the iron-sulphur centre which, in turn reduces PC via cyt$f$, the other electron reduces cyt$b_6$ (low potential) and participates in a quinone-dependent cycle,
involving both types of cyt\textsubscript{b\textsubscript{6}}, to pump protons as described by Mitchell’s Q cycle (Mitchell et al., 1975). Electrons from PC are transferred to PSI. Light absorption by PSI results in the transfer of an electron from an excited state of P700 to NADP\textsuperscript{+} via secondary electron acceptors, iron-sulphur centres F\textsubscript{X}, F\textsubscript{A} and F\textsubscript{B}, and then to ferrodoxin-NADP\textsuperscript{+} oxidoreductase via ferredoxin, forming NADPH (reviewed in Andersson & Franzen, 1992; Nugent, 1996).

The transfer of electrons from water to NADP\textsuperscript{+} and the concurrent pumping of H\textsuperscript{+} from the stroma to the intra-thylakoid space generates a proton gradient and a membrane potential which is used to drive ATP synthesis at the ATP synthase (Mitchell, 1979). Cyclic electron flow around PSI, via cyt b\textsubscript{0}f, also contributes to the proton gradient across the membrane. This can be used to drive ATP synthesis in so-called cyclic photophosphorylation. This anaerobic process involves PSI only and the electrons are transferred in a closed circuit. It is thought to be an important mechanism by which bacterial cells and plants can produce extra ATP when exposed to stress conditions such as high temperature or drought (Bendall & Manasse, 1995).
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1.6 Photosystem Photosystem I

NADPH

0.4

NADP Photon

Figure 1.4 The two light reactions of the "Z-scheme" of photosynthesis. The electron transfer through PSI and PSII via cyt b6f is shown.

Adapted from Mathews et al., 2000
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In higher plants there is a distinct distribution of complexes between stacked and non-stacked regions of the thylakoid membrane (Andersson & Anderson 1980; Anderson & Andersson, 1982). In particular, the two photosystems are physically separated such that PSI is confined to the non-stacked regions, which are in direct contact with the surrounding stroma. In contrast, PSII is mainly located within the stacked grana thylakoids (Figure 1.5). In both PSI and PSII the electron-accepting side of the complex is located towards the stromal side of the thylakoid while the donor side is towards the lumen. This results in electron transfer across the membrane and translocation of protons from the stroma to the lumen.

Light energy reaches the photosystems by way of the light-harvesting complexes (LHCs). These complexes are organised to collect and deliver light energy by means of excitation transfer to the RCs. In plants and green algae the light-harvesting complexes are integral membrane proteins, which typically bind Chl \(a\), Chl \(b\) and carotenoids that are used to efficiently harvest the spectrum of light energy that drives photosynthesis (Kuhlbrandt et al., 1994). In plants and green algae, both LHCI (associated with PSI) and LHCII (associated with PSII) are composed of a family of related chlorophyll-binding proteins encoded by a nuclear gene family known as \(lhcA\) or \(lhcB\). The transfer of energy from the LHCs to the RC occurs by one of two methods. The first method, known as resonance energy transfer, is intermolecular and depends on the overlap between the fluorescence emission spectrum of the donor molecule and the excitation spectrum of the acceptor. The second method takes place over shorter distances such that interactions can occur between molecular orbitals so that excitation energy can effectively be shared between two molecules in a process known as delocalised exciton coupling. These methods allow rapid transfer of energy between pigments towards the RC avoiding the loss of energy by fluorescence or conversion to heat.
Figure 1.5 Distribution of multiprotein complexes in the thylakoid membrane. PSI is located mainly in the nonstacked regions while PSII is primarily in stacked regions. The cyt $b_{6}f$ complex is found in both stacked and unstacked regions while the ATP synthase complex is mainly in the unstacked regions.
1.4 The Photosystem II Reaction Centre

1.4.1 Introduction

PSII is unique in that not only can it perform light-driven electron transport but it can also oxidise water, bringing about the release of electrons and protons with simultaneous release of oxygen. Despite this important role, our knowledge of PSII was very limited up until about 25 years ago: since then progress has been quite dramatic. Very recently, crystals of PSII have been produced that are of a high enough quality to provide structural analysis of this RC (Zouni et al., 2000).

PSII can be described as a water-plastoquinone oxidoreductase. It is a multisubunit complex comprising of about 25 polypeptides. At the heart of PSII is a heterodimer of two related proteins termed D1 and D2. This heterodimer binds the co-factors involved in PSII electron transport (Figure 1.4); these co-factors are shown in Figure 1.6. The primary electron donor is P680. The structure and/or environment of P680 must be different to other RC primary donors, which have much lower $E_m$ values, and it is apparent from comparative spectroscopic analyses of the *Rhodopseudomonas viridis* primary donor and P680 that they are dissimilar. In the purple bacterial RC the chlorophyll special pair is known to be a dimer, but structural data show that the chlorophylls in PSII are further apart than those in the purple bacterial RC (Rhee et al., 1998), and it has been suggested that P680 has properties of both a chlorophyll monomer and a chlorophyll dimer (reviewed in van Gorkom & Schelvis, 1993). The general consensus appears to favour P680 as a weakly interacting chlorophyll dimer or multimer (Durrant et al., 1995).
Figure 1.6 Diagrammatic view of the PSII co-factors. The view is shown in the plane of the membrane. The P680 special pair is shown edge towards the viewer with an accessory chlorophyll either side. Above these are the plastoquinones, QA and QB, either side of a circle representing the non-haem iron. Bicarbonate is shown bound at or near the non-haem iron. Near to P680 are the redox active tyrosines YZ and YD, with two Mn dimers close to YZ.
Upon excitation by light, P680 donates an electron to the primary electron acceptor, pheophytin, in the picosecond time scale. Biochemical analysis indicates that there are at least two pheophytins per P680 in PSII RC preparations (Nanba & Satoh, 1987) but, only the pheophytin associated with D1 participates in electron transfer. The position of the pheophytin in PSII has been modelled, both by Ruffle et al., 1992 and Svensson et al., 1996, to be homologous to that in the bacterial RC. Following reduction of pheophytin, electrons are passed to the quinone, QA. QB then accepts two electrons and two protons and is released from its binding site into the lipid matrix of the membrane. QA and QB are both plastoquinone-9 molecules, although their protein environment causes them to have different properties. QA is a single electron acceptor and stabilises the charge-separated state (P680+/QA). Under high light conditions QA can be doubly reduced initiating photoinhibition or the turnover of the D1 protein (Keren et al., 1995). In contrast, QB becomes doubly reduced and protonated. In this state, QBH2, it becomes dissociated from PSII and is replaced by another plastoquinone-9 molecule. The structure of the QA and QB binding sites have been modelled on the basis of the R. viridis photosynthetic reaction centre crystal structure (Ruffle, et al., 1992).

The oxidised P680+ molecule is re-reduced by water via a tyrosine residue on D1 termed YZ. In oxygen-evolving centres the radical can be detected by electron paramagnetic resonance (EPR) spectroscopy in illuminated samples but rapidly decays in the dark at room temperature. Its spectrum is known as signal II_{very fast} (Babcock et al., 1989) and is very difficult to see. In Mn-depleted centres this signal is slowed (signal II_{fast}) and, hence, is easier to detect and analyse. A spectrally similar radical can be generated giving rise to a dark stable radical denoted EPR signal II_{slow} (Babcock et al., 1989). This alternate donor, YD, does not participate directly in steady state electron transfer from the water oxidising complex (WOC) to P680+ and has a markedly lower redox potential than YZ. Site-
Chapter 1 - Introduction

directed mutagenesis showed the two redox active radicals, $Y_Z$ and $Y_D$, to be $D1-Y161$ (Debus 1988b; Metz et al., 1989) and $D2-Y160$ (Debus et al., 1988a; Vermaas et al., 1988; Metz et al., 1989), respectively.

PSII must catalytically extract the electrons from water and it is generally accepted that this takes place via a cluster of four Mn atoms within the WOC. The four-electron gate on the donor (lumenal) side of PSII contrasts with the two-electron process on the acceptor (stromal) side of PSII. Four turnovers of primary charge separation are required to create the four oxidising equivalents required for the conversion of two molecules of water to oxygen (Section 1.5).

1.4.2 PSII polypeptides

The genes encoding PSII proteins are known as psb genes. About 25 polypeptides are known to make up PSII, but it is likely that more are involved which have yet to be assigned. The polypeptides known to compose PSII are summarised in Table 1.1. PSII has the same function in all oxygenic organisms and the polypeptide composition is similar, but some differences are apparent. These include the presence of the PsbQ and PsbP proteins in plants and green algae to create the correct environment on the lumen side of the membrane, while in cyanobacteria the polypeptides PsbU and cytochrome c-550 (PsbV) create the required protein environment (Table 1.1). In addition, the extent of post-translational modifications such as phosphorylation, acetylation and palmitoylation can also vary between organisms. Details of the PSII proteins of particular relevance in this research are given in subsequent sections.
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<table>
<thead>
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<th>Common Name</th>
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<td>38</td>
<td>C</td>
</tr>
<tr>
<td>psbB</td>
<td>CP47</td>
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**Table 1.1** Photosystem II subunits. The table shows the gene (found exclusively in higher plants and algae or cyanobacteria), gene location (C = chloroplast genome, N = nuclear genome) in eukaryotes, the mature protein size and common name. Proteins are termed either PsbA or PSII-A etc. but many have a common name.

* psbY gives only one protein product in cyanobacteria and non-green algae, where it is plastid-encoded. psbY gives two mature polypeptides in green algae and plants.

(Adapted from Barber et al., 1997)
1.4.2.1 The D1 and D2 polypeptides

The D1 and D2 proteins that form the photochemical core of PSII are encoded by *psbA* and *psbD* respectively. They are hydrophobic proteins each with five transmembrane spanning helices. The D1/D2 heterodimer binds most of the electron transport chain co-factors. These include the Chl *a* molecules of P680, the primary acceptor, pheophytin, the plastoquinones Q*<sub>A</sub>* and Q*<sub>B</sub>* and the non-haem-iron. Four additional chlorophylls (Chl Z and accessory chlorophylls), β-carotene and the non-photochemical pheophytin are also bound. The D1 and D2 polypeptides also provide most of the ligands for the Mn and calcium ions in the WOC. It has also been shown that these core polypeptides bind other PSII polypeptides such as the 33 kDa protein. Although a crystal structure of PSII is available (Zouni *et al.*, 2000) the resolution is not sufficient to allow identification of co-factor binding sites. Comparison of the amino acid sequences of the L and M proteins of the bacterial RC with those of the D1 and D2 proteins, coupled with the bacterial RC crystal structure (Michel & Diesenhoffer, 1988), has allowed the putative identification of binding sites for a number of these co-factors and extrinsic proteins. In addition, mutagenesis of possible residues has provided more evidence in support of, or against, these putative binding sites. Unfortunately, it is very difficult to identify ligands to the WOC because there are no analogous residues in the bacterial system.

In higher plants, but not in algae or cyanobacteria, the threonines near to the N-terminus of D1 and D2 can be reversibly phosphorylated, but the role of this is not clear (Michel *et al.*, 1988). The D1 protein is also involved in photoprotection of PSII. Turnover of PSI and PSII is regulated to prevent imbalances in light energy transfer and electron flow. PSII possesses several photoprotection mechanisms designed to protect it from the harmful effects of light which cause photoinhibition (Aro *et al.*, 1993; Barber, 1998). These mechanisms dissipate excess excitation energy, minimising photodamage. When the ability of the PSII electron donors to
reduce the highly oxidising P680$^+$ is exceeded, the lifetime of P680$^+$ increases. This leads to damage to the reaction centre. Once damaged D1 is degraded. This protein turns over more rapidly than any other protein in the thylakoid membrane (Mattoo et al., 1984), presumably to provide a supply of newly synthesised, undamaged PSII complexes.

1.4.2.2 The CP47 and CP43 polypeptides

CP47 and CP43 are integral membrane components of PSII. They are encoded by the \textit{psbB} and \textit{psbC} genes respectively. They are structurally related Chl \textit{a}-binding proteins that act as transducers of excitation energy from the light-harvesting proteins to the reaction centre core (Bricker & Ghanotakis, 1996). Each polypeptide is highly conserved (80-85% identity) between different organisms and binds either 12-14 or 20-25 Chl \textit{a} molecules (de Vitry et al., 1984; Tang & Satoh, 1984). Each protein has six membrane spanning helices and a large luminal loop between helices V and VI (Bricker, 1990). They contain a considerable number of histidine residues which have been proposed to bind chlorophylls (Vermaas et al., 1987; Bricker, 1990; Sayre & Wrobel-Boerner, 1994).

Deletion or inactivation mutants of the \textit{psbC} gene encoding CP43 are unable to evolve oxygen in either cyanobacteria or \textit{Chlamydomonas}. However, they do contain PSII complexes capable of charge separation and retain the CP47 protein (de Vitry et al., 1984; Vermaas et al., 1988; Rogner \textit{et al.}, 1991). The CP43 protein is not required for water oxidation as photoassembly of oxygen-evolving PSII can be obtained using CP47 RC preparations that lack CP43. In contrast, cyanobacterial and \textit{Chlamydomonas} mutants in \textit{psbB} do not accumulate a reaction centre complex and are incapable of charge separation (de Vitry \textit{et al.}, 1984; Monod \textit{et al.}, 1992). This suggests CP47 is more closely associated with the RC core than CP43. Recent structural assignments suggest that CP47 is adjacent to D2 and CP43 is near D1 (Hankamer \textit{et al.}, 1999; Barber \textit{et al.}, 2000). These polypeptides are important in stabilising the RC core and
modify quinone binding. CP47, especially the predicted hydrophilic loops on the lumen side of the membrane, is involved in stabilising the WOC (Gleiter et al., 1994). Site-directed mutagenesis of residues located on these large and small lumenally-exposed domains of CP47, in Synechocystis sp. PCC 6803, lead to losses in oxygen evolution (Eaton-Rye & Vermaas, 1991; Haag et al., 1993; Gleiter et al., 1994; Putnam-Evans & Bricker, 1994).

1.4.2.3 Cytochrome b<sub>559</sub>

Cyt b<sub>559</sub> is composed of two polypeptides (α and β) that are proposed to co-ordinate a haem via histidine axial ligands. The α and β subunits are encoded by the chloroplast genes psbE and psbF. The gene products are 82-86 and 38-44 amino acids in size, respectively. It is unclear, at this time, whether the structure of the cytochrome is heterodimeric or a combination of homodimers, although the presence of a heterodimer is favoured. Also in dispute is the number of cyt b<sub>559</sub>'s present per PSII. Buser and coworkers (1992) determined that there is only one cyt b<sub>559</sub> per PSII; this work argues against other observations which propose that the 1:1 ratio is due to the loss of cytochromes caused by over-solubilisation of PSII particles (van Leeuwen et al., 1991).

In higher plants and cyanobacteria psbE and psbF are co-transcribed (reviewed in Erickson & Rochaix, 1992). In contrast, the genes are separated in the Chlamydomonas genome (Alizadeh et al., 1994). Disruption of the psbE (Shukla et al., 1992) and psbF (Pakrasi et al., 1990) genes has been reported in the cyanobacterium Synechocystis sp. PCC 6803. The psbE gene has also been deleted in C. reinhardtii (Morais et al., 1998). These mutants are non-photosynthetic with barely detectable levels of the RC core proteins (D1 and D2).

The haems can exist in a variety of redox potential forms, including a high potential (approximately 350-400 mV) and low potential form (20-80
mV) (Cramer & Whitmarsh, 1977). The reasons for the different redox potential forms are unknown.

A number of roles for cyt $b_{559}$ have been proposed. However, it is thought that cyt $b_{559}$ is involved in cyclic electron flow around PSII, because it has been shown to be both photo-oxidised by P680$^+$ and photo-reduced by plastoquinol. This is likely to form part of the PSII defence against photoinhibition and/or oxygen radicals.

A detailed review of the relationship between the $\alpha$ and $\beta$ subunits of cyt $b_{559}$ was presented by Stewart and Brudvig, 1998.

1.4.2.4 The Psbl polypeptide

The Psbl protein is one of several small polypeptides present in PSII RC particles (Nanba & Satoh, 1987). Based on its gene sequence the Psbl protein is predicted to have 37 amino acids (4.8 kDa) and a single transmembrane $\alpha$ helical domain. The *Chlamydomonas*, cyanobacterial and higher plant Psbl proteins all share sequence similarity (reviewed by Erickson & Rochaix, 1992). Evidence suggests that Psbl does not bind any co-factor or pigment.

The deletion of the *psbl* gene has been reported in *C. reinhardtii* (Künstner *et al.*, 1995). These mutants were able to evolve oxygen, but the amount of D1 protein was reduced. The *psbl*-deficient lines were also capable of photoautotrophic growth in dim light, but were sensitive to high light. These data suggest that the Psbl polypeptide stabilises the PSII complex and may protect the complex from photoinhibition.

1.4.2.5 The extrinsic polypeptides PsbO, PsbP and PsbQ

The three polypeptides, commonly referred to as the PSII extrinsic proteins are encoded by the nuclear genes *psbO*, *psbP* and *psbQ*. They code for proteins 33 kDa, 23 kDa and 17 kDa in size, respectively, and are also termed the oxygen evolution enhancer (OEE) proteins. All three proteins
are present in photosynthetic eukaryotes but in cyanobacteria only PsbO is present (Section 1.4.2). These proteins bind to the lumenal or donor side of PSII (Andersson et al., 1984).

The PsbO protein is essential for water oxidation in oxygenic eukaryotes. The *C. reinhardtii* strain FUD44 lacks the transcript and gene product for *psbO* and cannot grow photoautotrophically or evolve oxygen (Mayfield et al., 1987). The mutant still has the other two extrinsic polypeptides associated with the donor side of PSII but it is not apparent whether they are bound to the membrane. In contrast, the PsbO protein is not as crucial in *Synechocystis* sp. PCC 6803 where the deletion of the *psbO* gene only reduces the rate of oxygen evolution (Burnap & Sherman, 1991). The deletion mutants are also more sensitive to photoinhibitory treatments than wild type but can grow photoautotrophically at reduced rates. When the cells are starved of calcium ions, however, photosynthetic growth is lost (Philbrick et al., 1991). In the mutant the WOC is less stable when calcium is absent.

Evidence suggests that the 33 kDa polypeptide is involved in stabilising the WOC. It is possible that the OEE1 co-ordinates some of the Mn of the WOC. However, biophysical measurements of the WOC indicate that the structure is essentially unchanged upon the removal of the 33 kDa protein. These observations include an EPR multi-line signal that is associated with the $S_2$ state of the WOC (Miller et al., 1987) as well as an X-ray absorption K-edge spectrum from the $S_1$ state (Cole et al., 1987).

In eukaryotes, high rates of oxygen evolution can only be observed when the 33, 23 and 17 kDa subunits are present, unless exogenous calcium and chloride ions are present (Åkerlund et al., 1982; Ghanotakis & Yocum, 1990; Debus, 1992). *C. reinhardtii* mutants lacking the 23 kDa protein have lower rates of oxygen evolution and a decreased abundance of PSII compared with wild type but they can grow photoautotrophically. However, efficient rates of oxygen evolution were obtained in this mutant in the
presence of exogenous chloride ions (Rova et al., 1994). Work by Rova and co-workers (1996) studying the process of photoactivation in a *Chlamydomonas* mutant strain that lacks the *psbP* gene product (FUD39) suggests that the 23 kDa protein is required on the donor side of PSII to sequester chloride ions during PSII assembly. It also appears that the 23 kDa protein is required for the binding of the 17 kDa subunit (Miyao & Murata, 1989). There have been no reports of PsbQ mutants in *C. reinhardtii* and the role of the PsbQ gene product is unknown.

### 1.4.2.6 Low mass PSII polypeptides

There are many small polypeptides in PSII, both chloroplast- and nuclear-encoded. These are listed earlier in Table 1.1. Many of these small polypeptides do not, despite investigations, have a specific role assigned to them with respect to PSII activity. For a detailed discussion of these small PSII polypeptides, see Barber et al. (1997).

### 1.4.2.7 The PsbH polypeptide

The PsbH, or 10 kDa polypeptide as it is known, is encoded by the *psbH* gene (Johnson & Schmidt, 1993; O'Connor et al., 1998). This gene is located on the chloroplast genome in the *psbB* operon. In *Chlamydomonas* the polypeptide is 88 amino acids in size and has an estimated molecular mass of 9.3 kDa. In higher plants the *psbH* gene encodes a smaller, 73 amino acid protein. The PsbH protein contains a single transmembrane $\alpha$ helix with a number of hydrophobic residues conserved between species. PsbH is phosphorylated, possibly at the second threonine (PsbH-T2) (Dedner et al., 1988), but the presence of an additional phosphorylation site is also a possibility (J. Nugent personal communication). The sequence of the *Synechocystis* sp. PCC 6803 *psbH* gene shows that this protein is truncated at the amino terminus and is consequently missing the PsbH-T2 phosphorylation site. Work by Race & Gourarris (1993) has suggested that
another unspecified residue may be phosphorylated in cyanobacteria. The role of the phosphorylated threonine residue at the second position in the polypeptide has also been investigated by site-directed mutagenesis in *Chlamydomonas* (Cheater *et al.*, 1995). Analysis of the mutant PsbH-T2A suggested that the phosphorylation of PsbH may not be critical to PSII function. This conclusion was reached when experiments showed the T2A mutant to behave as wild type (O’Connor *et al.*, 1998). More recently a second phosphorylation site has been identified using mass spectrometry in the *Arabidopsis thaliana* PsbH protein at T4 (Vener *et al.*, 2001). In contrast to phosphorylation at T2, modification of this residue is highly sensitive to the ambient light conditions, being rapidly dephosphorylated when plants are placed in darkness. This confirmation and identification of the second phosphorylation site in PsbH, and the demonstration that modification of the site is light-responsive *in vivo* reopens the debate on the role of phosphorylation in PsbH function.

There is as yet no confirmed function for PsbH. Evidence suggests that it may have a structural role in the assembly or the stability of the PSII complex. The *C. reinhardtii* psbH gene was disrupted using a spectinomycin resistance cassette (Ruffle *et al.*, 1995). The transformants were unable to grow photoautotrophically and did not evolve oxygen. The mutant cells lacking the PsbH polypeptide have no Y<sub>D</sub>* EPR signal, which is indicative of a functional PSII complex (Ruffle *et al.*, 1995). It has also been demonstrated that PsbH deletion mutants fail to accumulate the PSII core complex proteins (D1, D2, CP47 and CP43) in dark grown cells, suggesting that lack of PSII activity in these mutants is unrelated to photoinhibition (Summer *et al.*, 1997).

In contrast to *C. reinhardtii*, the PsbH protein is not required for functional PSII in cyanobacteria. In the cyanobacterium *Synechocystis sp. PCC 6803* psbH deletion mutants are able to grow photoautotrophically (Mayes *et al.*, 1993). However, the mutants are impaired in Q<sub>A</sub> to Q<sub>B</sub>
electron transport. Experimental evidence leads to the conclusion that in the absence of PsbH the Q_B site is adversely affected leading to an impaired electron flow from Q_A to Q_B. In their later studies, Komenda & Barber (1995) discovered that changes around Q_B affect the turnover of the D1 protein causing a defective repair/replacement process after D1 has been damaged. This along with other research (Sundby et al., 1989; Stys et al., 1995) led to a possible role for PsbH in protection from photoinhibition to be proposed.

1.4.3 Structure of PSII

Despite its importance the structure of PSII has yet to be determined, but in recent years progress has been made towards understanding the structure using X-ray crystallography electron microscopy and single particle analysis. PSII is complex with many subunits (Section 1.4.2), causing this work to be difficult but 3D structural details of both the RC and the overall structure of PSII are now being revealed (Rhee et al., 1998; Hankamer et al., 1999; Neild et al., 2000; Zouni et al., 2000). Data gained so far indicate that the assumptions made about the structure of PSII by comparison to the bacterial reaction centre (Michel & Deisenhoffer, 1988) are correct. Unfortunately, the structural resolution is not yet good enough to assign individual amino acids that act as ligands to co-factors or to obtain detailed information about the WOC.

Various forms of two-dimensional crystals have been reported (Holzenburg et al., 1993; Marr et al., 1996; Lyon et al., 1998; Hankamer et al., 1999). Two-dimensional crystals from CP47-RC subcores and PSII cores have yielded structures at sufficient resolution to reveal the secondary structure of the membrane region of PSII. The structure of the CP47-RC subcore complex was obtained by electron crystallography at 8Å (Rhee et al., 1988). Figure 1.7 shows the three-dimensional map of the monomeric PSII RC complex, from the side (a) and from the lumenal surface (b). The
five helices of the D1 and D2 proteins were assigned by comparison with the L and M subunits of the bacterial RC. The adjacent group of three pairs of helices were assigned to CP47 on the basis that this protein is closely associated with the D1/D2 heterodimer and has six transmembrane helices (Bricker, 1990). The remaining helices could not be accurately assigned.

The two-dimensional crystals of the CP47-RC were devoid of the WOC. Relatively recently, Hankamer and co-workers (1999) conducted cryoelectron crystallography on two-dimensional crystals of the PSII core complex that maintain the ability to oxidise water. This complex crystallises as a rotationally symmetrical dimer as shown in Figure 1.8a. Work by Barber and colleagues also led to the isolation of the LHCII-PSII supercore complex (Boekema et al., 1999a, b). This complex consists of the core complex plus minor Chl a/b binding proteins (CP29, CP26 and CP24) and some LHCII. These supercore complexes maintain their in vivo dimeric arrangement and have an intact WOC. Projection maps of this supercore-complex are shown in Figure 1.8 (Barber & Kühlbrandt, 1999). Progress towards elucidating the cyanobacterial PSII structure has been made using the more stable PSII found in the thermophilic cyanobacterium, *Synechococcus elongatus* (Kuhl et al., 1999; Zouni et al., 2000).

The structure of PSII has been probed in other ways such as by analysis of preparations with different polypeptide compositions. Detergents can be used to gradually strip polypeptides from PSII, revealing their approximate location in the complex. Many genes coding for PSII components have been deleted or disrupted in both cyanobacteria and green algae using molecular biological techniques. As described previously, this reveals information such as whether the protein is required for PSII assembly or a particular activity.
Figure 1.7 Three-dimensional map of the monomeric PSII RC complex.

**a** - side view with lumenal surface below, cylinders indicating the positions of the transmembrane helices.

**b** - view from the lumenal side, cylinders indicate the positions of the 23 transmembrane helices seen.

**Key** - D1, yellow; D2, orange; CP47, red; others, blue.
Figure 1.8 Three-dimensional map of the dimeric PSII complex.

a - Helix organization of major intrinsic proteins of the PSII. The helix organization of the D1 and D2 proteins (shown in red) and of CP47 (shown in green) derives from an 8-Å three-dimensional structure of the CP47-D1-D2 subcore complex. The six helices of CP43 (shown in green) have been superimposed - on the assumption that they are identical to those of CP47 and are related to them by a pseudo-twofold-symmetry axis that is shared with the D1 and D2 proteins.

b - Subunit positioning of major proteins of the LHCII-PSII supercomplex. The helices of CP47, CP43 and the D1-D2 heterodimer are positioned as in (a). The positions of the transmembrane and surface helices of the LHCII trimer, CP29 and CP26 are based on structural data and sequence homologies. Both (a) and (b) are viewed from the lumenal side.

c - Side view of the negatively stained LHCII-PSII supercomplex that identifies protrusions due to the 33-kDa and 23-kDa extrinsic proteins.
1.5 The Water Oxidising Complex

Although much is known about the function of PSII, there are still many unanswered questions about the molecular structure of the WOC and the details of its mechanism. Water is normally a very stable molecule due to the high mid-point potential of the $\text{H}_2\text{O}/\text{O}_2$ redox couple. In order to catalyse the oxidation of water PSII must act at a higher potential than any other known biological system, this is one of the factors that has led to such interest in this reaction.

Evidence to date suggests that the core of the WOC is a cluster of four Mn ions, with calcium and chloride as essential co-factors. It is positioned on the lumenal side of PSII and is protected by a number of extrinsic proteins. The WOC cycles through a series of redox states operating as a device for the accumulation of charge equivalents, which binds and oxidises the substrate water. After four photochemical charge separation events, molecular oxygen is released. This cycling of the WOC was first described by Kok (1970), and the five redox states were termed $S$-states (Figure 1.9). The dark-stable state is $S_1$. After a few minutes in the dark, about 75% of PSII is in $S_1$ and 25% in $S_0$. The $S_2$ and $S_3$ states are unstable with short half lives at room temperature, decaying back to $S_1$. The $S_4$ state has a lifetime of about 1.5-4.5 ms, spontaneously decaying to $S_0$. There is continued debate about which state or states bind substrate water, but it is accepted that oxygen is released in the transient $S_4$-state, $S_0$ is restored and the cycle is reset. Examination of the possible mechanisms show that oxidation of the WOC must be an almost electroneutral process with proton release during the $S$-state cycle providing the necessary charge balance.
Figure 1.9 The S-state cycle. The diagram shows possible Mn oxidation state changes for two of the Mn atoms of the WOC. The other two Mn atoms are proposed to remain in the same oxidation state. One proton and one electron can be released on each S state change but this will depend on the preparation and conditions, which influence the pKa values of amino acids and water oxidation intermediates.
The Mn valence states during the S-state cycle are not known. The two main schemes for the accumulation of oxidising equivalents have either Mn oxidised during each of the S-state transitions or Mn oxidation on some transitions with S₂ to S₃ not involving Mn oxidation. EPR spectroscopy has been used to probe the WOC and its turnover (Debus, 1992). Two EPR signals, termed the multi-line signal and the g = 4.1 signal, have been identified as arising from the S₂ state. The production of these EPR signals support a Mn valence change in one pair of Mn, to give a Mn³⁺/Mn⁴⁺ dimer on the S₁ to S₂ step. Further EPR signals from the S₀, S₁ and S₃ states have been discovered in recent years (Nugent, 2000). The properties of these have confirmed that the Mn cluster is oxidised on both the S₀ to S₁ and S₁ and S₂ transitions.

Investigations to date indicate that the intact complex contains two non-equivalent Mn dimers. One calcium and one chloride ion may be associated with one of the dimers, but more studies are required to confirm this. The roles played by calcium and chloride ions are unclear but the data is consistent with the view that they are required for the functional integrity of the complex. The data showing that Y₂ is close to the WOC has led to models suggesting how Y₂ and the Mn cluster interact in the mechanism of water oxidation (Hoganson & Babcock, 1997; Haumann & Junge, 1999; Limburg et al., 1999; Mulkidjanian, 1999; Nugent, 2001).

1.6 Histidine Tagging of PSII Proteins

In order to learn more about the structure and mechanism of PSII good quality, active PSII must be isolated. Several types of oxygen-evolving PSII core complex have been prepared from higher plants, for example the PSII-LHCII super-core complex (Eshaghi et al., 1999) and the PSII RC (Rhee et al., 1998) have been isolated from spinach. When the mechanism of PSII is investigated using site-directed mutagenesis both in cyanobacteria and green algae, it is important that the PSII is of a similar purity to that
isolated from higher plants to allow the effects of mutations to be analysed. PSII has been isolated from these species by detergent solubilisation of their thylakoids followed by ultracentrifugation and anion exchange and/or gel filtration chromatography (Shim et al., 1990; Bumann & Oesterhelt, 1994; Tang & Diner, 1994). In these core complex preparations, the structure and photochemical capabilities of the PSII RC are usually well-preserved, but the electron donor side of PSII, in particular the oxygen-evolving activity, differed from one preparation to another. Presumably this was due to different extents of damage to the WOC (see Debus, 1992 for a review).

Recently, histidine-tagging technology has been employed, thus facilitating the purification of PSII cores from C. reinhardtii (Sugiura et al., 1998, 1999), from Synechocystis sp. PCC 6803 (Bricker et al., 1998) and from S. elongatus (Sugiura & Inoue, 1999). The level to which the isolated PSII has been characterised varies both in techniques used and the depth of the investigation. Here a short review of these PSII cores is presented.

The first successful PSII purification using his-tagging technology was the isolation of C. reinhardtii PSII following the his-tagging of the C-terminus of the D2 polypeptide (Sugiura et al., 1998). The PSII complex could be isolated from tagged transformants using a simple metal-affinity chromatography separation of detergent-solubilised thylakoids. In this work six histidine residues were genetically attached to the N-terminus of the D1 protein or the C-terminus of the D2 protein. The N-terminus of D1 was chosen because it is known that the C-terminus is involved in binding essential co-factors. The C-terminus of D2 was chosen based on the successful purification of the bacterial RC by engineering a polyhistidine tag on the C-terminus of the M subunit and utilising nickel-nitritolriacetic acid (Ni-NTA) resin (Goldsmith & Boxer, 1996).

Following transformation with the tagged genes, transformants harbouring the D1 N-terminus tag did not grow photoautotrophically, indicating that the tag affected functional assembly of the PSII complex. In
contrast, the addition of a six-histidine tag on the C-terminus of D2 did not affect the stability of the Mn cluster, as shown by the normal growth, high oxygen-evolving capacity and normal thermoluminescence properties of the D2-tagged cells. Although the total yield of PSII on a chlorophyll basis was relatively low, the method was efficient and the isolated PSII was relatively pure. Subsequent work further corroborated that there was no detectable contamination by PSI or LHC proteins and that the his-tagging did not have a detrimental effect on PSII function. The authors concluded that this "His-tag strategy" could be widely applied for biochemical, biophysical and crystallographic studies of Chlamydomonas PSII.

Following the publication of the method used for purifying PSII from C. reinhardtii it was logical that similar methods should be used elsewhere. The cyanobacterium Synechocystis sp. PCC 6803 offers a number of useful properties for studying PSII. The organism can grow photoheterotrophically in the absence of PSII, allowing the study of otherwise lethal phenotypes. It is naturally transformable and, perhaps most importantly, its genome has been completely sequenced.

The isolation of PSII from cyanobacteria has proved difficult and time consuming using traditional methods, but the introduction of a his-tag onto the C-terminus of the CP47 protein of PSII in Synechocystis sp. PCC 6803 resulted in the isolation of a highly active and stable PSII complex (Bricker et al., 1998). In this preparation a cobalt, rather than nickel, column was used successfully to bind the his-tag and, hence, the PSII complex. This tagged mutant exhibited PSII characteristics very similar to the wild-type strain.

Although it was proposed that the PSII complexes isolated using his-tagging technology from C. reinhardtii and in Synechocystis sp. PCC 6803 would be suitable for further biochemical and biophysical analysis, Sugiura and Inoue (1999) proposed that the stability of the complexes would not be sufficient for many experimental techniques. In an attempt to overcome this
problem of stability they isolated a functional and highly stable PSII core complex using his-tagging technology. A six-histidine tag was genetically incorporated onto the C-terminus of the PSII-CP43 protein in the thermophilic cyanobacterium *S. elongatus*. The purified core complex is extremely stable, showing practically no change in its protein subunit composition during incubation at room temperature for as long as twenty-one days. This indicates the tight binding of the three extrinsic proteins and the absence of proteolytic enzymes. This work also reveals that the majority of the PSII core complex particles isolated are dimeric, suggesting the heterogeneity seen in many other preparations may be caused by prolonged contact with detergent. This is also supported by recent work in which the structure of PSII has been visualised and appears as a dimer in complex with the LHC proteins (Barber & Kühlbrandt, 1999 and references therein). The PSII core complex isolated shows absorption peaks, oxygen-evolving activity and Chl/RC ratio are all identical to those determined for PSII cores isolated from cyanobacteria using traditional methods (Tang & Diner, 1994).

EPR spectrophotometry has played an important role in studying the WOC, but there is very little literature on the characterisation of his-tagged PSII cores from *Synechocystis* and *Chlamydomonas*. In contrast, the his-tagged PSII core from *S. elongatus* was subject to an in-depth EPR investigation, looking at the $S_0$, $S_1$, $S_2$ and $S_3$-states (Boussac *et al.*, 2000). This work showed that the his-tag itself did not influence the EPR signals of the Mn cluster. Furthermore, the detection of signals previously unreported in cyanobacteria was reported. This very detailed analysis showed that the *S. elongatus* PSII cores are well-suited to EPR analysis and suggests that this preparation will prove suitable and useful for future spectroscopic, enzymological and, perhaps, crystallographic investigations.
1.7 *Chlamydomonas reinhardtii* as a model organism

1.7.1 Introduction

The genus *Chlamydomonas* comprises unicellular, biflagellate green algae and accounts for the largest of the 33 genera in the family Chlamydomonadaceae (Harris, 1989). *Chlamydomonas* species are found in a multitude of environments including marine and fresh waters, soil, and even snow. Several *Chlamydomonas* species have become important experimental organisms in fields such as cell and molecular biology, genetics, plant physiology and biotechnology due to its exceptional utility as a model organism.

The most popular species of *Chlamydomonas* to be used as a model organism has long been *C. reinhardtii*. The standard laboratory strains of *C. reinhardtii* all derive from a zygospore isolated from a soil sample collected in Massachusetts, USA in 1945. It has a simple sexual cycle, well-established genetics and is easy to culture. As a model organism for the study of photosynthesis its most useful attribute is its ability to grow either photoautotrophically or heterotrophically, using acetate as a carbon source. This property makes it possible to isolate viable, non-photosynthetic mutants, a property that is lacking in higher plant models such as *Arabidopsis*. The speed and ease with which such photosynthetic mutants can be created in *C. reinhardtii*, in comparison to higher plants is also advantageous. Compared with higher plants, photosynthetic research using *C. reinhardtii* also benefits from its ability to synthesise chlorophyll in the dark. Chlorophyll is a major component of the photosynthetic complexes and without it these complexes cannot assemble properly. Using *C. reinhardtii* it is possible to grow photosynthetic mutants in complete darkness that assemble mature chloroplasts.
1.7.2 Cell structure and life cycle

*C. reinhardtii* cells range from 8-22 μm in length and are ovate or spherical in shape (Figure 1.10b). The cell wall is complex, composed of glycoproteins and it has a high hydroxyproline content. Two flagella are present at the anterior. These terminate within the cell at a pair of basal bodies, connected to the nucleus by centrin fibres. The nucleus is partially surrounded by a large, cup-shaped chloroplast that takes up approximately 40% of the cell volume. A pyrenoid is present within the chloroplast, distal to the nucleus and starch grains surround the pyrenoid. An eyespot is located at the anterior of the chloroplast and numerous mitochondria are present (Figure 1.10a).

In common with most model organisms *C. reinhardtii* has a relatively short life cycle (Figure 1.11). *C. reinhardtii* is a heterothallic species with two mating types (*mt*<sup>+</sup> and *mt<sup>−</sup>*). Vegetative cells are haploid, and reproduce asexually by division into two, or sometimes small multiples of two, progeny cells. Gametogenesis does occur and can be induced in the laboratory by nitrogen deprivation and blue light. Gametes look like vegetative cells, but have differentiated mating structures near their apices. Diploid zygotes are formed by fusion of gametes, they possess a hard, impermeable wall that acts to protect the zygote. Under suitable conditions the zygospores germinate, at which point meiosis occurs yielding four haploid, vegetative cells - a “tetrad” (Harris, 1989). Nuclear genes are inherited in a Mendelian fashion and segregate 2:2 among tetrad products. Chloroplast genes are inherited uniparentally from the *mt<sup>+</sup>* parent in most zygotes, while mitochondrial genes are inherited uniparentally from the *mt<sup>−</sup>* parent. This cycle is easy to manipulate under controlled culture conditions and synchronised cell division can be achieved by growing cells in a cycle of 12 hours light:12 hours dark.
Figure 1.10 *Chlamydomonas reinhardtii.*
a - drawing of a *C. reinhardtii* cell (courtesy of Keith Roberts, John Innes Institute, Norwich)
b - Light micrograph of a *C. reinhardtii* cell (courtesy of Ola Sodiende)
Chapter 1 - Introduction

Gamete activation and release of cell walls involve mt- gametes and mt+ gametes. Flagellar adhesion between mt+ & mt- gametes initiates the process.

Gametogenesis occurs in N-free medium, leading to mt- vegetative cells and mt+ vegetative cells through asexual reproduction (mitosis).

Activation of mating structures and germination of mt+ fertilisation tubules are followed by fusion of mt+ fertilisation tubule with mt- mating structure, resulting in vegetative cells (1n), zygote (2n), and complete cell fusion.

**Figure 1.11** The life cycle of *Chlamydomonas reinhardtii*. (Adapted from the image at http://www.botany.duke.edu/chlamy/images/lifecycle.gif)
1.7.3 The chloroplast genome of C. reinhardtii

In contrast to higher plant cells that contain many chloroplasts, C. reinhardtii cells contain just one large chloroplast. The C. reinhardtii chloroplast is bounded by a double membrane and contains a thylakoid membrane very similar to that of higher plants. The organelle’s genome is 196 kb in size and contains approximately 120 genes (Gillham, 1978). About 80 copies of the genome are present per cell. These numerous copies are organised into between 8 and 10 DNA-protein complexes known as nucleoids (Kuriowa et al., 1981). The C. reinhardtii genome also contains a large (21 kb) inverted repeat (IR), which is a characteristic feature of chloroplast DNA. Unlike higher plants, which typically have a large and small single copy region, the C. reinhardtii IR separates two single copy regions of approximately equal size. The gene content of the C. reinhardtii chloroplast genome is remarkably similar to that of higher plants. This is in agreement with the general assumption that the chloroplasts of green algae and plants arose from a common bacterial ancestor (Blankenship, 1992). The sequencing of the C. reinhardtii chloroplast genome is near completion and the data available from the Chlamydomonas Genetic Centre, based at Duke University, North Carolina, USA.

1.7.4 Transformation of the C. reinhardtii genomes

Although the green alga C. reinhardtii is of little commercial importance it has proven to be of significant value in the development of algal transformation technology. The relative ease with which its genomes can be transformed has significantly enhanced the utility of this alga as a model organism. It should be noted that Chlamydomonas is the only organism in which transformation techniques have been developed for all three genomes: nuclear, mitochondrial and chloroplast.

Vegetative cells of C. reinhardtii possess a haploid nuclear genome of approximately 100 Mb. Transformation of the nuclear genome is possible
using a multitude of techniques including biolistics, electroporation, vortexing with silicon carbide whiskers or agitating with glass beads (recently reviewed in Kindle & Sodeinde, 1994; Stevens & Purton, 1997; Lumbreras & Purton, 1998a). The simplest method is the glass bead method (Kindle, 1990) but this requires the prior removal of the proteinaceous cell wall. A number of markers have been developed in order to select for transformed *Chlamydomonas* strains. The argininosuccinate lysase 7 (*ARG7*) gene (Debuchy et al., 1989) and the nitrate reductase 1 (*NIT1*) gene (Fernandez et al., 1989) can each be used as a selectable marker when co-transformed into cells with a gene for which there is no marker. The *Chlamydomonas* emetine resistance gene *CRY1* (Nelson et al., 1994) and the *Streptoalloteichus hindustanus* phleomycin resistance gene *ble* (Lumbreras et al., 1998b) can also be used as dominant selectable markers.

There is a low frequency of homologous recombination in the *Chlamydomonas* nuclear genome (Sodeinde & Kindle, 1993) and this has prevented the development of routine methods for gene targeting due to the random integration of DNA (Debuchy et al., 1989; Kindle et al., 1989). This property has proved advantageous, however in enabling random insertional mutagenesis which has led to isolation of genes encoding a wide variety of functions (reviewed in Kindle, 1998). The analysis of such mutants unfortunately can be complicated by the fact that the introduced DNA can cause large deletions (Tam et al., 1993).

The mitochondrial genome of *C. reinhardtii* consists of a 15.8 kb linear molecule with inverted repeats at its ends and is present in multiple copies per cell. The genome has been completely sequenced and contains 13 genes (Vahrenholz et al., 1993). These genes code for components of the respiratory enzyme complexes, tRNAs, tRNAs and a reverse transcriptase, possibly involved in DNA replication. Transformation of the mitochondrial genome in *C. reinhardtii* is not routine although there has been an example of transformation using the biollistic technique to rescue the *dum1* mutant to
respiratory competence (Randolph-Anderson et al., 1993).

Transformation of the *C. reinhardtii* chloroplast genome is achieved using the biolistic process to deliver DNA across the cell and chloroplast membranes (Boynton et al., 1988). This method gives high transformation rates, one reason being that the chloroplast makes a good target given that it takes up a large proportion of the total cell volume. The transforming DNA integrates into the chloroplast genome by homologous recombination (Newman et al., 1991). This allows precise modification of the chloroplast genes. Early transformation studies relied on the rescue of *C. reinhardtii* mutants that carried deletions in key photosynthetic genes. These mutants could be restored to photoautotrophic growth by transformation with the corresponding cloned, chloroplast gene. It is also possible to transform wild-type chloroplasts with genes carrying mutations that confer resistance to antibiotics or herbicide (Boynton & Gillham, 1993). Changes in cloned genes are then co-transformed with the marker DNA and selected for using the antibiotic or herbicide. The most commonly used selectable marker is based on the bacterial antibiotic resistance gene *aadA* which encodes an aminoglycoside 3'-adenyl transferase. Goldschmidt-Clermont (1991) constructed a chimeric marker comprising the *aadA* gene under the control of the promoter from *C. reinhardtii* *atpA* gene. Introduction of this marker into the chloroplast results in transformed cells resistant to spectinomycin and streptomycin. This so-called "*aadA* cassette" can be used to disrupt or modify genes using a single plasmid construct. In theory, any part of the chloroplast genome can be altered as the *aadA* cassette can be integrated at any site given that flanking regions of homology are provided for homologous recombination. Although the chloroplast contains many copies of its genome, homoplasmic transformants (in which all copies are identical) are obtained after several rounds of single colony isolations on selective media. An exception to this occurs when an essential gene is disrupted and the genome remains in a heteroplasmic state, in which copies
of the wild-type essential gene are retained along with mutated copies.

One problem with chloroplast transformation has been the lack of alternative selectable markers that are as easy to use as the \textit{aadA} cassette. The original form of the cassette allowed its use just once in any given transformation experiment and this has hindered \textit{C. reinhardtii} chloroplast transgenics. In order to overcome this, new versions of the \textit{aadA} cassette have been developed that allow its excision. This allows repeated rounds of transformation using the same marker and has been termed "recycling" (Fischer \textit{et al.}, 1996). This approach has proved successful for the generation of novel deletion mutants (Redding \textit{et al.}, 1998) but cannot be applied to existing \textit{aadA} transformants, hence additional markers similar to the \textit{aadA} cassette were required. One such marker has been developed by Bateman and Purton (2000). This dominant marker is based on the bacterial \textit{aphA-6} gene, which encodes an aminoglycoside phosphotransferase. This chimeric marker is under the control of the \textit{psbA} promoter. The so-called \textit{"aphA-6 cassette"} confers resistance to kanamycin or amikacin and can be used in the same way as the \textit{aadA} cassette. The availability of this marker makes serial transformation of the \textit{C. reinhardtii} chloroplast genome possible.
CHAPTER 2

Materials and Methods
2.1 Reagents, Chemicals and Enzymes

All chemicals used were of the highest grade available wherever possible, and where no supplier is mentioned, were purchased from Sigma Chemical Co. (Poole, UK). Restriction endonucleases and other DNA modifying enzymes and polymerases were obtained from New England Biolabs (Hitchin, UK), Promega (Southampton, UK), Stratagene (Cambridge, UK) and Boehringer Mannheim (Lewes, UK). Radiolabelled [$\alpha$-$^{32}$P] 2'-deoxycytidine 5'-triphosphate (dCTP) was purchased from Amersham (Buckinghamshire, UK). Water used to prepare solutions and media was purified using the Maxima Ultra-pure Water system (ELGA, Buckinghamshire, UK).

2.2 Higher Plant Material

2.2.1 Cultivation of pea seedlings

Peas (the cultivar Kelvedon Wonder) were soaked overnight in distilled water (dH$_2$O). Eighty grams of peas were scattered in seed trays 2/3 filled with multipurpose compost, these were covered with a layer of compost and watered well. Peas were allowed to germinate in low light for ~6 days then transferred to a greenhouse for ~8 days.

2.2.2 Isolation of PSII particles from higher plants

The method utilised for the isolation of PSII particles from pea seedlings is based on that detailed in Ford and Evans (1983). The preparation of thylakoid membranes and, subsequently, PSII particles was carried out exactly as given in Evans et al. (1995).

The method utilised for the isolation of PSII-enriched granal membranes from fresh market spinach is an adaptation of the “BBY” preparation detailed in Berthold et al. (1981) with minor modifications described in Rappaport et al. (1994). The membrane fragments were stored
in SMNC Buffer (0.3M sucrose, 25mM 2-[N-Morpholino]ethanesulfonic acid (MES) pH 6.5, 5mM MgCl$_2$, 10mM NaCl and 5mM CaCl$_2$).

2.2.3 Chlorophyll concentration measurements

Assays of chlorophyll concentration were carried out by adding an appropriate volume of sample to 10ml of 80% (v/v) acetone. This suspension was then mixed and filtered through Whatman 3MM paper. The absorbance of duplicate samples was then determined at 645, 647, 652, 653 and 664nm and an average absorbance calculated for each wavelength. The equations of Arnon (1949) and Porra et al. (1989) were then used to calculate chlorophyll concentrations.

2.3 Algal Material

2.3.1 C. reinhardtii strains

*C. reinhardtii* strain 2137 mating type + (mt+) (CC-1021), obtained from E. Harris at the *Chlamydomonas* Genetics Center at Duke University, California, was used as the wild type control for all experiments. Other strains utilised are detailed in Table 2.1.
Table 2.1 *C. reinhardtii* mutants used in this research

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>FuD Control</td>
<td>PSI minus, spec^®</td>
<td>J. Minagawa, RIKEN, Japan</td>
</tr>
<tr>
<td>dAA2-302</td>
<td>PSI minus, spec^®</td>
<td>J. Minagawa, RIKEN, Japan</td>
</tr>
<tr>
<td>D2-Y160F</td>
<td>PSI minus, spec^®; psbD site-directed mutant</td>
<td>J. Minagawa, RIKEN, Japan</td>
</tr>
<tr>
<td>D2-Y160F/H-his</td>
<td>PSI minus, spec^®; psbD site-directed mutant with a 6xhis tag at the C-terminal end of PsbH</td>
<td>J. Minagawa, RIKEN, Japan</td>
</tr>
<tr>
<td>D2-Y160F/D2-his</td>
<td>PSI minus, <em>spec^®</em> psbD site-directed mutant with a 6xhis tag at the on PsbD</td>
<td>J. Minagawa, RIKEN, Japan</td>
</tr>
<tr>
<td>D2-Y160F/B-his</td>
<td>PSI minus, spec^®; psbD site-directed mutant with a 6xhis tag on PsbB</td>
<td>J. Minagawa, RIKEN, Japan</td>
</tr>
<tr>
<td>D1-E189D</td>
<td>PSI minus, spec^®, psbA site-directed mutant</td>
<td>J. Minagawa, RIKEN, Japan</td>
</tr>
<tr>
<td>D1-E189L</td>
<td>PSI minus, spec^®, psbA site-directed mutant</td>
<td>J. Minagawa, RIKEN, Japan</td>
</tr>
<tr>
<td>D1-E189Q</td>
<td>PSI minus, spec^®, psbA site-directed mutant</td>
<td>J. Minagawa, RIKEN, Japan</td>
</tr>
<tr>
<td>FuD7 CC-741 mt+</td>
<td>PsbA minus mutant, deletions at both copies of psbA (Palmer et al., 1985)</td>
<td>E. Harris, <em>Chlamydomonas</em> Genetics Center, Duke University, USA.</td>
</tr>
<tr>
<td>ac-u-e: CC-1186 mt+</td>
<td>PsbA minus mutant, deletions at both copies of psbA (Palmer et al., 1985)</td>
<td>E. Harris, <em>Chlamydomonas</em> Genetics Center, Duke University, USA.</td>
</tr>
<tr>
<td>psbH null</td>
<td>PSII minus, spec^®</td>
<td>H. O’Connor, UCL, UK</td>
</tr>
<tr>
<td>H-his^®</td>
<td>As wild-type with a 6xhis tag at the C-terminal end of PsbH</td>
<td>this thesis</td>
</tr>
<tr>
<td>D1-W317F</td>
<td>psbA site-directed mutant, spec^®</td>
<td>this thesis</td>
</tr>
<tr>
<td>PsbA-null/ PsbH-null</td>
<td>Double deletion mutant, kanamycin^®</td>
<td>this thesis</td>
</tr>
<tr>
<td>H-his/ D1-W317F</td>
<td>psbA site-directed mutant, spec^® with a 6xhis tag at C-terminal end of PsbH</td>
<td>this thesis</td>
</tr>
<tr>
<td>Intronless psbA/ H-his^®</td>
<td>Double deletion mutant, kanamycin^®</td>
<td>this thesis</td>
</tr>
<tr>
<td>H-HA2-his^®</td>
<td>As wild-type with a 2xHA tag followed by a 6xhis tag at the C-terminal end of PsbH</td>
<td>this thesis</td>
</tr>
</tbody>
</table>

(*spec^® /spec^® - spectinomycin resistant/susceptible*)

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2.3.2 Growth and maintenance of C. reinhardtii strains

Media used to culture C. reinhardtii are detailed in Table 2.2. C. reinhardtii strains were maintained on Tris-acetate phosphate (TAP) media (Rochaix, 1988) solidified with 2% Bacto Agar (Difco, Detroit, USA) and incubated at 20°C under appropriate illumination, 0-45μE/m²/s. The cells were streaked to fresh plates every 3-4 weeks. Liquid cultures were grown in the appropriate medium in Erlenmeyer flasks in a New Brunswick (New Jersey, USA) G-25 illuminated orbital incubator at 25°C under the appropriate light conditions with aeration by shaking at 150rpm. Twenty-five millilitre starter cultures were inoculated from stock cultures and grown to stationary phase, then either harvested by centrifugation or an aliquot used as an inoculum for a larger volume of media. Where large volumes were required, cells were grown in 10L flasks with sterilised air (0.2μm nitrocellulose filters, Millipore, France) bubbled through them. C. reinhardtii media were supplemented with antibiotics where applicable as detailed in Table 2.3. Appropriate sterile technique was used at all stages.
Chapter 2 - Materials & Methods

Table 2.2 C. reinhardtii growth media

<table>
<thead>
<tr>
<th>For 1 litre</th>
<th>TAP Medium</th>
<th>HSM Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>972ml</td>
<td>924ml</td>
</tr>
<tr>
<td>Tris base</td>
<td>2.42g</td>
<td>-</td>
</tr>
<tr>
<td>4x Beijerinck salts*</td>
<td>25ml</td>
<td>25ml</td>
</tr>
<tr>
<td>1M (K)PO₄ (pH 7.0)°</td>
<td>1ml</td>
<td>-</td>
</tr>
<tr>
<td>2x PO₄ for HSM™</td>
<td>-</td>
<td>50ml</td>
</tr>
<tr>
<td>Trace elementsª</td>
<td>1ml</td>
<td>1ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>~1ml</td>
<td>-</td>
</tr>
</tbody>
</table>

References: Gorman and Levine (1965) Sueoka et al. (1967)

TAP = Tris Acetate Phosphate
HSM = High Salt Minimal

* 4x Beijerinck salts
16g NH₄Cl
2g CaCl₂
4g MgSO₄
dissolve in 1L distilled H₂O

° 1M (K)PO₄ pH 7.0
250ml 1M K₂PO₄
~170ml 1M KH₂PO₄ (titrate to pH 7.0)

™ 2x PO₄ for HSM
0.08M K₂HPO₄ (14.34g)
0.05M KH₂PO₄ (7.26g)
adjust to pH 6.9 with KOH, make up to 1L with distilled H₂O

ª Trace elements
i. Dissolve in 550ml distilled water in the order indicated below, then heat to 100°C
11.4g H₃BO₃
22g ZnSO₄ 7H₂O
5.06g MnCl₂ 4H₂O
4.99g FeSO₄ 7H₂O
1.61g CoCl₂ 6H₂O
1.57g CuSO₄ 4H₂O
1.1g (NH₄)₆Mo₇O₂₄ 4H₂O

ii. Dissolve 50g EDTA.Na₂ in 250ml H₂O by heating and add to the above solution. Reheat to 100°C. to 80-90°C and cool, adjust to pH 6.5-6.8 with KOH.

iii. Adjust to 1 litre. Incubate at room temperature for 2 weeks and allow rust coloured precipitate to form. The solution will change from green to purple.

iv. Filter through three layers of Whatman No. 1 paper under suction until the solution is clear. Store at 4°C.

From Rochaix (1988)
Table 2.3 Antibiotic concentrations in growth media

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>*Stock Concentration</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>100mg/ml in dH₂O</td>
<td>100µg/ml</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>50mg/ml in dH₂O</td>
<td>50µg/ml</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>15mg/ml in dH₂O</td>
<td>65µg/ml/100µg/ml</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>100mg/ml in dH₂O</td>
<td>100µg/ml</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>15mg/ml in ethanol</td>
<td>15µg/ml</td>
</tr>
</tbody>
</table>

*Stock solutions were sterilised with 0.22µm Millex-GP syringe-driven filter units (Millipore, France) and stored in aliquots at -20°C.

2.3.3 C. reinhardtii thylakoid membrane preparation

The method used to prepare thylakoid membranes from *C. reinhardtii* is a combined adaptation of the methods of Diner and Wollman (1980) and Shim *et al.* (1990). Details are given in O’Connor *et al.* (1998). Membrane samples were resuspended and stored in HEMNS Buffer (25mM N-[2-Hydroxyethyl]piperazine-N’[2-ethanesulfonic acid] (HEPES), 5mM EDTA, 5mM MgCl₂, 10mM NaCl, 330mM sucrose, pH 7.5) and frozen in liquid nitrogen for long-term storage.

2.3.4 C. reinhardtii PSII preparation

PSII from *C. reinhardtii* was purified from mutants containing a poly-histidine tag on one of the PSII polypeptides. Liquid cultures were harvested (Pellicon ultrafiltration with 0.22µm filter, Millipore), pelleted by centrifugation at 5000g for 10 min, washed once in Buffer A (25mM MES (pH 6.5), 100mM NaCl, 10% (v/v) glycerol, 10mM ascorbic acid) then resuspended in Buffer B (Buffer A containing 500µl protease inhibitors (Sigma)). The cells were broken in a cell disrupter (Constant Cell Disruption Systems, Warwickshire, UK) and the membranes collected by centrifugation at 31,000g for 30 min. The membrane pellet was resuspended...
in Buffer B. The PSII was solubilised at a concentration of 1mg Chl/ml with 25mM dodecyl maltoside (DM) in Buffer B using 10 slow plunges of a glass homogeniser. The solubilised PSII was centrifuged at 11,000g for 10 min to remove debris. The supernatant was added to washed nickel resin (Qiagen, West Sussex, UK) in Buffer C (25mM MES (pH 6.5), 100mM NaCl, 10% glycerol, 0.03% DM) plus protease inhibitors, 10mM ascorbic acid and 5mM imidazole. After 30 min, the slurry was poured into a chromatography column and washed with Buffer C until the eluate was clear. The PSII was eluted with Elution Buffer (40mM MES (pH 6.0), 100mM NaCl, 10% glycerol, 0.03% DM, 10mM ascorbic acid and 200mM imidazole). This was mixed with an equal volume of Polyethylene Glycol (PEG) Buffer (20mM MES (pH 6.3), 15mM NaCl, 20% (w/v) PEG 8000, 10mM ascorbic acid and 10mM EDTA.Na₂). The precipitate was pelleted by centrifugation at 11,000g for 10 min. The pellet was resuspended in 20mM MES (pH 6.3), 15mM NaCl, 10% PEG 8000, 10mM ascorbic acid and 10mM CaCl₂ and centrifuged again at 11,000g for 10 min. These steps decreased the imidazole concentration. The isolated PSII was finally resuspended in a small volume of 20mM MES (pH 6.3), 15mM NaCl, 333mM sucrose and 10mM CaCl₂. All stages were carried out in minimal light and at 4°C.

2.3.5 Chlorophyll concentration measurements

Chlorophyll assays were carried out on cell suspensions, membrane preparations and purified PSII according to the methods of Arnon (1949) and Porra et al. (1989). See Section 2.2.3 for further details.

2.3.6 Oxygen evolution measurements

Steady-state oxygen evolution studies were performed using a Clark type electrode. Measurements were recorded in the presence of the electron acceptors 2,6-dimethylbenzoquinone (DMBQ) (Aldrich Chemical Co.,
Dorset, UK) and potassium ferricyanide, each to a final concentration 1mM. Oxygen levels were measured for 1 min without illumination and for between 1 and 2 min under saturating light conditions. Cell measurements were recorded in TAP or HSM medium; thylakoid membrane measurements were recorded routinely in HEMNS buffer, pH 7.5 and PSII measurements were recorded in 20mM MES (pH 6.3), 15mM NaCl +/- 10mM CaCl₂.

2.3.7 Measurement of cell density

The cell density of *C. reinhardtii* in liquid culture was measured by two methods. Firstly, a 1ml sample of culture was removed and 10μl of tincture of iodine (0.25g iodine in 95% ethanol) added. Duplicate 1ml aliquots of cells were killed in this way and counted using a haemocytometer (Weber Scientific International Ltd., Teddington, UK) on a Wild Heerbrugg (Switzerland) microscope at x400 magnification. The average count was multiplied by 10⁴ to give cell density/ml. Secondly, the optical density at 750nm (OD₇₅₀) was measured at regular intervals until cultures reached stationary phase. The growth pattern was described by the log value of the optical density against time (Nield, 1997).

2.3.8 Total cell protein extract from *C. reinhardtii*

Total cell protein was extracted from *C. reinhardtii* using a simple lysis method. Cells were grown to mid-log phase and the cell density was measured at 750nm. Twenty millilitres of culture were centrifuged at 4000rpm for 5 min (MSE Mistral 100). Pellets were resuspended in 0.8M Tris-HCl, pH 8.3, 0.2M sorbitol. Prior to loading onto SDS gels 11μl of 10% SDS and 1μl of β-mercaptoethanol were added to 100μl of bacterial culture and incubated for 1 min at 100°C. The sample was microcentrifuged for 1 min at 13000rpm and then loaded onto the SDS gel.
2.4 Bacterial Strains

2.4.1 E. coli strains

The E. coli strains used and their genotypes are listed in Table 2.4.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>supE44ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA</td>
</tr>
<tr>
<td>JM109</td>
<td>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ[lac-proAB] F[traD36 proAB lacI^Δ lacZΔM15]</td>
</tr>
</tbody>
</table>

Table 2.4 E. coli strains From Brown (1991)

2.4.2 Growth and maintenance of bacterial strains

Media used to culture E. coli are detailed in Table 2.5. Working strains were maintained by growing overnight at 37°C on Luria Bertani (LB) media solidified with 2% granulated agar (Difco), supplemented with the appropriate antibiotic if necessary. Plates were stored at 4°C. Long term storage of E. coli cultures at -70°C was in the form of frozen glycerol stocks. Bacteria in liquid cultures were grown by inoculating a suitable volume of LB media, supplemented with antibiotics where appropriate, with a single colony and incubated at 37°C for 3-16 h with shaking.

Table 2.5 E. coli growth medium

<table>
<thead>
<tr>
<th>For 1 litre</th>
<th>Lauria Bertani Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bactotryptone</td>
<td>10g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>to 1 litre</td>
</tr>
</tbody>
</table>

From Sambrook et al., 1989

Media were supplemented with antibiotics, as detailed in Table 2.3.
2.5 DNA Preparation

2.5.1 Purification of DNA from *C. reinhardtii*

Preparation of total DNA from *C. reinhardtii* was carried out using the DNeasy Tissue Kit (Qiagen). The tissue kit gives satisfactory amounts of DNA from *C. reinhardtii* using a slightly altered method outlined here. A 20ml stationary phase culture was pelleted by centrifugation (MSE Mistral 100) at 16060g for 3 min. The pellet was resuspended in a small amount of supernatant retained and microcentrifuged (MSE Microcentaur) for 1 min at 16060g. The protocol provided with the kit was then followed.

2.5.2 Isolation of plasmid DNA

Small-scale, crude preparations of plasmid DNA were performed using a modified version of the SDS-alkaline lysis method of Birnboim and Doly (1979), as detailed in Sambrook *et al.* (1989).

Small-scale plasmid DNA preparations for sequencing analysis, transformation etc. were performed using a Qiagen Miniprep Spin Kit according to the manufacturer’s guidelines (Qiagen). This is an adaptation of the alkaline lysis method of Birnboim and Doly (1979).

Larger-scale preparations of plasmid DNA was performed using a Qiagen Maxiprep Kit according to the manufacturer’s guidelines (Qiagen).

2.6 Recombinant DNA Techniques

2.6.1 Restriction enzyme digestion

Restriction enzyme digests were set up according to the manufacturer’s guidelines. Analytical digestion of plasmid DNA (0.25-2.5μg) was performed using 5-10 units of enzyme followed by incubation at the required temperature for 1-2 h. *C. reinhardtii* DNA was digested with 20-30 units of restriction enzyme for 2-3 h (or overnight) to ensure complete digestion. Digests were run on a 1% agarose gel to ensure that the reaction was successful.
2.6.2 Agarose gel electrophoresis of DNA

Agarose gels were made by melting 0.8 - 2% agarose in TAE Buffer (40mM Tris acetate, 10mM EDTA.Na₂, pH 8.0). Known molecular-weight standards were either purchased and used according to the manufacturer's guidelines, or obtained by digesting λ DNA with *Ava*I and *Bgl*II (λ A/B) and a 250ng aliquot applied to each gel. Sizes of markers are given in Table 2.6. Samples were mixed in the appropriate ratio with 6x Sample Loading Buffer (0.1M EDTA.Na, pH 8.0, 40% glycerol, 0.01% SDS and 0.01% bromophenol blue). The electrophoresis was carried out in a Hoefer (Newcastle-under-Lyme, UK) 10cm cooled-minigel apparatus in TAE buffer at 75-100V for 1-2 h or in a Hybaid (Middlesex, UK) 30cm maxigel apparatus at 50V overnight. The gel was then stained in 0.01% ethidium bromide for 20 min and rinsed in dH₂O, allowing visualisation of DNA bands using a UV light transilluminator (UVP GDS 7500, UVP Inc., California, USA). A record of each gel was kept using a UVP Gel Documentation System. Concentration of DNA was estimated by comparing with markers of known concentration on an agarose gel.
Table 2.6 DNA molecular weight markers

<table>
<thead>
<tr>
<th>Lambda A/B</th>
<th>MBI Fermentas 1Kb Ladder</th>
<th>MBI Fermentas 100bp Ladder Plus</th>
<th>NEB 100bp Ladder</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 677 bp</td>
<td>10 000 bp</td>
<td>3 000 bp</td>
<td>1 517 bp</td>
</tr>
<tr>
<td>8 614</td>
<td>8 000</td>
<td>2 000</td>
<td>1 200</td>
</tr>
<tr>
<td>5 462</td>
<td>6 000</td>
<td>1 500</td>
<td>1 000</td>
</tr>
<tr>
<td>4 305</td>
<td>5 000</td>
<td>1 200</td>
<td>900</td>
</tr>
<tr>
<td>3 730</td>
<td>4 000</td>
<td>1 031</td>
<td>800</td>
</tr>
<tr>
<td>2 392</td>
<td>3 500</td>
<td>900</td>
<td>700</td>
</tr>
<tr>
<td>2 213</td>
<td>3 000</td>
<td>800</td>
<td>600</td>
</tr>
<tr>
<td>1 881</td>
<td>2 500</td>
<td>700</td>
<td>517/500</td>
</tr>
<tr>
<td>1 602</td>
<td>2 000</td>
<td>600</td>
<td>400</td>
</tr>
<tr>
<td>1 426</td>
<td>1 500</td>
<td>500</td>
<td>300</td>
</tr>
<tr>
<td>1 074</td>
<td>1 000</td>
<td>400</td>
<td>200</td>
</tr>
<tr>
<td>540</td>
<td>750</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td>415</td>
<td>500</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>111</td>
<td>250</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

2.6.3 Recovering DNA from agarose gels

A slice containing the required DNA was removed from an ethidium bromide-stained agarose gel. DNA was recovered using the Qiaquick Gel Extraction Kit (Qiagen) following the manufacturer’s instructions. The DNA was eluted from the column in 30 or 50μl of 10mM Tris-Cl, pH 8.0.

2.6.4 Construction of recombinant plasmids

Recombinant plasmids were prepared by ligating DNA fragments into compatible restriction sites within parental vectors. Plasmids used or created in this work are detailed in Table 2.7.
### Table 2.7 Plasmids used in this thesis

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>p72B</td>
<td>pUC8 with the <em>C. reinhardtii</em> Eco19 chloroplast genome fragment cloned into the EcoRI site</td>
</tr>
<tr>
<td>p72B-SH</td>
<td>p72B with a 2.4 kb fragment removed using SmaI and HpaI</td>
</tr>
<tr>
<td>pH-his₆</td>
<td>As p72B with a 6 x his-tag at the C-terminal end of PsbH</td>
</tr>
<tr>
<td>pH-his₁₀</td>
<td>As pH-his₆ with a tag of ten histidines</td>
</tr>
<tr>
<td>pH-plastocyanin</td>
<td>As pH-his₆ but with the mature pea plastocyanin sequence inserted between the C-terminal end of PsbH and the 6 x his-tag</td>
</tr>
<tr>
<td>pH-HA₂his₆</td>
<td>As pH-his₆ but with a haemagglutinin tag inserted between the C-terminal end of PsbH and the 6 x his-tag</td>
</tr>
<tr>
<td>pBA153</td>
<td>Intronless PsbA plasmid (Minagawa &amp; Crofts, 1994)</td>
</tr>
<tr>
<td>pBA157</td>
<td>As pBA153 with an <em>aadA</em> cassette inserted</td>
</tr>
<tr>
<td>pBA153.W317Fspecᵦ</td>
<td>As pBA153 with the site-directed mutation D1 - W317F</td>
</tr>
<tr>
<td>p483-aadA-483</td>
<td>Plasmid containing the <em>aadA</em> cassette</td>
</tr>
<tr>
<td>p72BKmᵦ</td>
<td>A PsbH – null mutant where the <em>aphA</em>-6 cassette is used to disrupt PsbH (Bateman &amp; Purton, 2000)</td>
</tr>
</tbody>
</table>

Vectors cut with a single restriction enzyme were treated with calf intestinal alkaline phosphatase (Boehringer-Manheim) to prevent religation of parental plasmids and were subsequently run on an agarose gel and the vector fragment recovered as in Section 2.6.3. Those cut with two incompatible enzymes or with a blunt-cutting enzyme were directly run on an agarose gel and the required fragment recovered.
Ligation reactions were performed using 1:1 or 1:3 Molar ratios of vector:insert as detailed in Table 2.8.

**Table 2.8 Ligation reaction details**

<table>
<thead>
<tr>
<th>Vector:Insert ratio</th>
<th>Sticky End Ligation</th>
<th>Blunt End Ligation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:1</td>
<td>1:3</td>
</tr>
<tr>
<td>dH₂O</td>
<td>6µl</td>
<td>4µl</td>
</tr>
<tr>
<td>10x buffer</td>
<td>1µl</td>
<td>1µl</td>
</tr>
<tr>
<td>Digested vector</td>
<td>1µl</td>
<td>1µl</td>
</tr>
<tr>
<td>DNA insert</td>
<td>1µl</td>
<td>1µl</td>
</tr>
<tr>
<td>T4 DNA ligase (GIBCO)</td>
<td>1µl</td>
<td>1µl</td>
</tr>
</tbody>
</table>

Reaction Conditions: 16°C overnight / 37°C for 1 h

2.6.5 Transformation of E. coli

Recombinant plasmids were introduced into competent *E. coli* cells (DH5α or JM109; see Table 2.4) by a transformation method derived from that of Cohen *et al.* (1972) detailed in Tomely (1996). One alteration to this method was the use of 100mM MgCl₂ as opposed to CaCl₂ to resuspend the bacterial pellet in the first instance. Competent cells produced using this simple MgCl₂/CaCl₂ method were generally left for 16 h at 4°C prior to use. Typically 5µl of the ligation reaction was added to 100µl of competent cells and this mixture was incubated on ice for at least 30 min prior to being heat-shocked at 42°C for 1 min. After cooling briefly on ice, 1ml of LB medium was added to the cells which were incubated at 37°C for 1 h to allow expression of plasmid-encoded antibiotic resistance markers. Ten percent of the transformed cell suspension was then plated on selective media, with the remaining 90% being pelleted and resuspended in a small volume of supernatant and again plated. Plates were incubated at 37°C for
12-16 h. If the simple selective colour test “blue:white selection”, based on the α-complementation of the amino-terminal fragment of the β-galactosidase gene (lacZ) with its C-terminus, could be used the medium was supplemented with isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal) (Sambrook et al., 1989). Recombinants were transferred to 5-10ml of LB medium supplemented with the appropriate antibiotic(s) and grown up as in Section 2.4.2 for SDS-alkaline lysis analysis.

Controls were included as part of each transformation experiment. These were a “cells only” control when transforming parental plasmid DNA or, when transforming a ligation reaction, control reactions lacking vector or insert DNA were used.

2.6.6 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify DNA from both plasmids and genomic DNA. The template DNA was purified as detailed above. Primers were designed to be complementary to the sequences flanking the region of interest and ordered from MWG Biotech (Milton Keynes, UK). The primers used in this thesis are given in Table 2.9. A final concentration of 1μM of each primer was used in each reaction. The enzyme of choice for all PCR experiments was Vent DNA polymerase (New England Biolabs, Hitchin, UK) and 2 units were added to each 50μl reaction. The reaction buffer supplied with the enzyme was diluted as appropriate and supplemented with MgSO₄ (0-4μM final concentration) and with all four dNTPs (Ultrapure dNTP Set, Amersham, New Jersey, USA) to give 0.2mM of each in the reaction tube.

A programmable thermal cycler (Techne, Cambridge, UK) was used for the amplification process. An initial denaturation step of 3 min at 95°C was followed by 25-30 cycles of 1 min denaturation at 95°C, 1-2 min at appropriate annealing temperature and 1-3 min polymerisation at 72°C. A
final extension time of 5 min at 72°C completed the reaction. A fifth of the reaction volume was then run on a 1% agarose gel to analyse the products.

Occasionally a “Hot Start” was required for certain reactions. In this case, the reactants were separated from the polymerase using a wax plug. This prevented the reaction from proceeding until all reactants were at high temperature. This made the conditions more stringent, preventing false priming events.

Controls were included as part of each PCR experiment. These included reactions lacking template DNA or Vent DNA polymerase.

**Table 2.9 Primers used in this research**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRH10R</td>
<td>5'-gggctagctta(gtg)_{16}caattgagaaactttagc-3'</td>
</tr>
<tr>
<td>NRHD1</td>
<td>5'-aaagctggcagttctgaagg-3'</td>
</tr>
<tr>
<td>NRPC1</td>
<td>5'-gcaatcaatgggtttgagg-3'</td>
</tr>
<tr>
<td>NRPC2</td>
<td>5'-gcaggtccgactctagagg-3'</td>
</tr>
<tr>
<td>P615</td>
<td>5'-ctacatgggctggtgagg-3'</td>
</tr>
<tr>
<td>D1-W317FR</td>
<td>5'-gtgtctgaagtttagtacacag-3'</td>
</tr>
<tr>
<td>P2296R</td>
<td>5'-cagagccatggcatcttcac-3'</td>
</tr>
<tr>
<td>A944</td>
<td>5' cgaaggttacctcgg 3'</td>
</tr>
<tr>
<td>D373</td>
<td>5'-aatattacgtaacgatga-3'</td>
</tr>
<tr>
<td>D1483R</td>
<td>5'-tatattagaggcttacca-3'</td>
</tr>
<tr>
<td>D790</td>
<td>5'-ggttcagctctctgctcag-3'</td>
</tr>
<tr>
<td>NRH1</td>
<td>5'-aaagctggcagttctgaagg-3'</td>
</tr>
<tr>
<td>NRH6R</td>
<td>5'gggctagcttagtggtgttggtgtgtgtaattgagaaactttagc-3'</td>
</tr>
<tr>
<td>NRH2P</td>
<td>5'-tcaattatggcaacagg-3'</td>
</tr>
<tr>
<td>NRpsbHi3'</td>
<td>5'-cccgagatccagaaaaagtgaagctattaacg-3'</td>
</tr>
</tbody>
</table>
2.6.7 PCR purification

The required PCR product was purified, when necessary, using the QIAquick PCR Purification Kit (Qiagen) following the manufacturer’s instructions. The DNA was eluted from the column in 30 or 50μl of 10mM Tris-Cl, pH 8.0.

2.7 DNA Sequencing

An automated DNA sequencing service was provided by the UCL Biology Department. Sequencing was performed on an Applied Biosystems Incorporated Prism 377 DNA Sequencer or 310 Genetic Analyzer (Perkin Elmer Applied Biosystems Incorporated,). Plasmids were prepared as given in Section 2.5.2 and were submitted at a concentration of 500ng/5μl. The concentration of PCR products varied from 60ng-1000ng/5μl depending on length. Primers were submitted at 3.2pmol/μl.

Sequence data were analysed using EditSeq (Microsoft Windows 3.85 DNASTar, Inc., California, USA).

2.8 Southern Analysis

2.8.1 Transfer of DNA onto nylon membranes

The protocol used was based on that found in Sambrook et al. (1989). The DNA samples were digested with restriction enzymes as detailed in Section 2.6.1. DNA fragments were separated by agarose gel electrophoresis and a photograph was taken (Section 2.6.2). The DNA was denatured with 1.5M NaCl, 0.5M NaOH for 30 min and neutralised with 1M Tris (pH 8.0), 1.5M NaCl for 2x30 min. Constant agitation was applied. The gel was rinsed with dH2O then the DNA was transferred onto Hybond-N (Amersham, Buckinghamshire, UK) nylon membranes according to the method of Southern (1975) using 20x SSC (3M NaCl, 0.3M sodium citrate). Transfer was allowed to take place overnight following which the
membrane was baked for 2 h at 80°C to immobilise the DNA in a Whatman 3MM paper envelope. Membranes were stored in the 3MM paper envelope at room temperature.

2.8.2 Radiolabelling DNA probes

DNA fragments to be used as probes were labelled using a Prime-It II Random Primer Labelling Kit (Stratagene, Cambridge, UK) according to the manufacturer’s guidelines. This method is derived from Feinberg and Vogelstein (1984).

2.8.3 Hybridisation of labelled probes to Southern blots

Membranes prepared as in Section 2.7.1 were washed with 2% (v/v) HCl for 5 min then rinsed with dH₂O. The membranes were then incubated in Pre-hybridisation Solution [5x Denhardt’s Reagent, 5x SSC, 0.5% (w/v) SDS, sheared salmon sperm DNA 0.1mg/ml (where 50x Denhardt’s is 1% (w/v) BSA-Fraction V, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) Ficoll Type 400 )] in Hybaid (Middlesex, UK) bottles in a Hybaid dual hybridisation oven at 65°C for 4 h. The radiolabelled probe was prepared as in Section 2.7.2 and was added to fresh pre-hybridisation solution. The membrane was incubated in this solution overnight as above. Following hybridization, the membranes were washed sequentially in 2x SSC, 0.1% (w/v) SDS for 30 min at 65°C and 1x SSC, 0.1% (w/v) SDS for 30 min at 65°C. Membranes were heat-sealed in plastic and hybridised probes were detected by autoradiography at -70°C using Kodak BioMax MS-I Film (Sigma, Dorset, UK) in intensifying cassettes (Genetic Research Instruments, Essex, UK).
2.9 Gel Electrophoresis and Western Analysis of PSII Polypeptides

2.9.1 Tris-glycine SDS-PAGE

Standard SDS-PAGE was carried out according to the method of Laemmli (1970). Gels were prepared as described in Table 2.10 and were allowed a minimum of 4 h to polymerise. *C. reinhardtii* thylakoids and PSII or *P. sativum* PSII were solubilised in a sample buffer purchased from Sigma (Laemmli, 2x concentrate, sample buffer; Sigma) for 15 min at 80°C. Solubilised samples were microfuged at 13000rpm for 30 s then loaded onto the gel. Electrophoresis was carried out in Running Buffer (25mM Tris Base, 192mM glycine, 0.1% (w/v) SDS) at a constant voltage of 100-150V.

<table>
<thead>
<tr>
<th>Table 2.10 Constituents of SDS-PAGE gels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total 100ml</strong></td>
</tr>
<tr>
<td><em>40% polyacrylamide (acrylamide:bisacrylamide 37:1)</em></td>
</tr>
<tr>
<td>Tris-Base</td>
</tr>
<tr>
<td>SDS</td>
</tr>
<tr>
<td>pH with HCl and adjust to 100ml</td>
</tr>
</tbody>
</table>

For chemical polymerisation of gels

| Tetrathemylethylene diamine (TEMED) | 100µl | 100µl |
| 10% Ammonium persulphate (AMPS) | 1ml | 1ml |

*The percentage of acrylamide used in the resolving gel was altered according to the molecular weight of the polypeptide of interest; 15% was used routinely in this work.

After removal of the stacking gel, the resolving gels were stained with Coomassie Blue Stain (0.5% (w/v) Coomassie Brilliant Blue R-250, 45% (v/v) ethanol, 10% (v/v) glacial acetic acid) and destained overnight.
with **Destain Solution** (25% (v/v) methanol, 7% (v/v) glacial acetic acid). Stained gels were fixed with 10% (v/v) glycerol and stored in 5% (v/v) glacial acetic acid in heat-sealed plastic bags.

Protein markers utilised are shown in Table 2.11

### Table 2.11 Molecular weights (kDa) of protein markers used

<table>
<thead>
<tr>
<th>BioRad -Ultra-low molecular weight prestained markers</th>
<th>BioRad - Low molecular weight prestained markers</th>
<th>Sigma – Low range protein markers</th>
<th>Amersham - Rainbow molecular weight markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 500</td>
<td>103 000</td>
<td>66 000</td>
<td>250 000</td>
</tr>
<tr>
<td>14 400</td>
<td>77 000</td>
<td>45 000</td>
<td>160 000</td>
</tr>
<tr>
<td>10 600</td>
<td>50 000</td>
<td>36 000</td>
<td>105 000</td>
</tr>
<tr>
<td>8 500</td>
<td>34 300</td>
<td>29 000</td>
<td>75 000</td>
</tr>
<tr>
<td>6 200</td>
<td>28 800</td>
<td>24 000</td>
<td>50 000</td>
</tr>
<tr>
<td>3 500</td>
<td>20 700</td>
<td>20 000</td>
<td>35 000</td>
</tr>
<tr>
<td>2 500</td>
<td></td>
<td>14 200</td>
<td>30 000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 500</td>
<td>25 000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 000</td>
</tr>
</tbody>
</table>

### 2.9.2 Tris-glycine gradient LiDS-PAGE

In order to attain better resolution of PSII complex polypeptides the standard procedure of Laemmli (1970) was modified according to Ikeuchi and Inoue (1988). A 16-22% gradient gel was prepared as detailed in Table 2.12 and was allowed a minimum of 4 h to polymerise. Samples were prepared as above (Section 2.9.1) in **DTT Sample Buffer** (62.5mM Tris-HCl (pH 6.8), 100mM dithiothreitol, 50% (v/v) glycerol, 5% (w/v) LiDS, 0.025% (w/v) bromophenol blue) and were loaded onto the gradient gel. Electrophoresis was carried out in **Running Buffer** (25mM Tris-HCl (pH 8.3), 192mM glycine, 0.1% (w/v) LiDS) at a constant voltage of 100-150V.
Table 2.12 Constituents of a 16-22% LiDS-PAGE gradient gel

<table>
<thead>
<tr>
<th>Total 100ml</th>
<th>5% Stacking Gel</th>
<th>16% Resolving Gel</th>
<th>22% Resolving Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>*40% polyacrylamide (acrylamide:bisacrylamide 37:1)</td>
<td>12.5ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>*50% polyacrylamide (acrylamide:bisacrylamide 49:1)</td>
<td>-</td>
<td>32ml</td>
<td>44ml</td>
</tr>
<tr>
<td>Urea</td>
<td>-</td>
<td>45g</td>
<td>45g</td>
</tr>
<tr>
<td>Tris Base</td>
<td>1.82g</td>
<td>7.26g</td>
<td>7.26g</td>
</tr>
<tr>
<td>LiDS (SDS - Lithium Salt)</td>
<td>0.1g</td>
<td>0.1g</td>
<td>0.1g</td>
</tr>
<tr>
<td>pH with HCl and adjust to 100ml</td>
<td>pH 6.8</td>
<td>pH 8.9</td>
<td>pH 9.5</td>
</tr>
</tbody>
</table>

For chemical polymerisation of gels

<table>
<thead>
<tr>
<th>Tetramethylethylenediamine (TEMED)</th>
<th>50μl</th>
<th>30μl</th>
<th>30μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% (w/v) Ammonium persulphate (AMPS)</td>
<td>1.25ml</td>
<td>210μl</td>
<td>210μl</td>
</tr>
</tbody>
</table>

2.9.3 Western blotting and detection of proteins

Protein samples separated according to molecular weight by electrophoresis, as described above in Section 2.9.1 or 2.9.2 were transferred to Hybond-ECL (Amersham) nitrocellulose membranes by the method of Towbin et al. (1979) in a Bio-Rad wet transfer blotting system. Protein transfer was carried out in precooled Transfer Buffer (25mM Tris-HCl (pH 8.3), 192mM glycine, 10%(v/v) methanol) at a constant current of 0.5Amps at 4°C for approximately 2 h.

Preparation and probing of the membrane harbouring the proteins was carried out according to the ECL Western Blotting Analysis System instructions using the phosphate buffer system. Filters were probed using polyclonal antibodies as detailed in Table 2.13. The secondary antibody used for all blots was anti-rabbit IgG, horseradish peroxidase-linked whole antibody (from donkey) (Amersham). This antibody was diluted 1:5000 prior to use.
Immunodetection was carried out using the ECL chemiluminescence method (Amersham), following the manufacturer’s instructions.

**Table 2.13** Polyclonal antibodies used for polypeptide detection

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Detail</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>C-terminal</td>
<td>Gift from P. Nixon</td>
<td>Nixon <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>D2</td>
<td>C-terminal 12 amino acid residues</td>
<td>Gift from P. Nixon</td>
<td>Nixon <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>CP43</td>
<td>whole molecule, <em>C. reinhardtii</em> P6 polypeptide</td>
<td>Gift from N-H. Chua, via P. Nixon</td>
<td></td>
</tr>
<tr>
<td>33 kDa extrinsic protein</td>
<td>whole molecule, pea</td>
<td>Gift from P. Nixon</td>
<td>Chapman <em>et al.</em>, 1989</td>
</tr>
<tr>
<td>cyt b&lt;sub&gt;559&lt;/sub&gt; α subunit</td>
<td>N-terminal</td>
<td>Gift from P. Nixon</td>
<td>Morais, 1998</td>
</tr>
<tr>
<td>23 kDa</td>
<td>-</td>
<td>Gift from P. Nixon</td>
<td>Chapman <em>et al.</em>, 1989</td>
</tr>
<tr>
<td>PsaA</td>
<td>-</td>
<td>Gift from K. Redding</td>
<td></td>
</tr>
<tr>
<td>PsbW</td>
<td>whole protein, <em>C. reinhardtii</em></td>
<td>This laboratory</td>
<td>Bishop <em>et al.</em>, In preparation</td>
</tr>
</tbody>
</table>

2.10 Chloroplast Transformation of *Chlamydomonas reinhardtii*

The *C. reinhardtii* chloroplast genome was transformed by a method based on that reported by Boynton and co-workers (1988). Cells to be transformed were grown to mid-log phase (2-4x10<sup>6</sup> cells/ml) in TAP medium, as detailed in Section 2.3.2. Cell density was calculated using a haemocytometer as described in Section 2.3.7. Ten to twenty millilitres of culture were then transferred to a 20ml sterile Sterilin (Bibby Sterilin Ltd., Staffordshire, UK) tube and centrifuged at 4000rpm for 5 min (MSE Mistral 100). The supernatant was poured off and the pellet resuspended in 0.5ml of fresh medium. Four millilitres of 0.5% soft agar was added to the cells and this was used to overlay a selective plate prepared as described in Section 2.3.2. Transformation, using plasmids carrying specific (altered) genes, was carried out using the PDS-1000/He Biolistic Particle Delivery System (Biorad, California, USA) exactly as described in Hallahan *et al.* (1995).
Transformants were taken through at least three rounds of single colony isolations to obtain homoplasmic lines prior to further analysis.

In cases where the selective antibiotic was kanamycin, an altered method was used as described by Bateman and Purton (2000). In this case cells were spread onto sterile nylon filters (Gelman, Michigan, USA) on non-selective media and transformed. After recovery (24 h), the cell covered filters were transferred to selective plates.

Every transformation experiment included two control plates, one where the cells were not bombarded to indicate the levels of spontaneous transformants, and one where the cells were bombarded in the same way as above except that dH2O was used in place of DNA.

2.11 Electron Paramagnetic Resonance Spectroscopy

2.11.1 EPR sample preparation

0.3-0.4ml samples (pea PSII, C. reinhardtii thylakoids or PSII) were placed in calibrated quartz electron paramagnetic resonance (EPR) tubes. They were given a brief illumination to turn over the PSII reaction centre and restore YD+ lost on storage. Further procedures were carried out in the dark or under a dim green light. Samples were dark-adapted for ~3 h on ice. Precise details of sample preparations, additions and spectroscopy conditions are described in individual experiments.

When highly concentrated suspensions of whole cells were used for EPR analysis of PSII reaction centres, 200ml of cells were grown to 2-4 x 10^6 cells/ml. A chlorophyll concentration assay was then performed (Section 2.3.5). The cells were then pelleted by centrifugation in a Sorval (Cincinatti, USA) GSA rotor at 3000g for 10 min and the cells resuspended in 20 mM Tris-HCl, 5 mM EDTA.Na2, pH 8.0 buffer (approximately 30ml). This step was then repeated twice and then the cells were finally resuspended to a concentration of 2-3mg/ml chlorophyll. Approximately 0.3ml of sample was then injected into an EPR tube and treated as above.
2.11.2 EPR measurements

EPR analysis was performed using a Jeol RE1X X-Band spectrometer (Massachusetts, USA) with an Oxford Instrument (Oxford, UK) liquid helium cryostat. Spectra were recorded and manipulated using a Dell microcomputer running ASYST data acquisition and manipulation programs. No filtering, smoothing, fitting or background subtractions were used. Difference spectra were obtained only from subtraction of spectra from the same sample. The vertical scale in figures showing first-derivative EPR spectra is arbitrary, with spectra at the same instrument gain unless stated in the figure legend.

2.12 Measurement of the Reduction of P680*

2.12.1 Sample preparation

*C. reinhardtii* PSII (Section 2.3.4) and spinach PSII (Section 2.2.2) were diluted in SMNC Buffer (0.3M sucrose, 25mM MES pH 6.5, 5mM MgCl₂, 10mM NaCl and 5mM CaCl₂) to final concentrations of 20µg/ml (*C. reinhardtii* PSII) or 80µg/ml (spinach PSII) of chlorophyll to which 0.015% (w/v) of DM (*C. reinhardtii* PSII) or Triton X-100 (spinach PSII) was added to reduce light scattering due to large aggregates. The samples (250µl) were placed in a 4x4mm fluorescence cuvette. Immediately prior to the measurements, 100µM DCBQ was added.

2.12.2 Equipment and measurements

The equipment is described in detail in Schilstra *et al.* (1998). Measurements were the average of a given number of excitations, detailed on individual figures. Global and autocorrelation analyses were carried out as described in Schilstra *et al.* (1998), and miss-parameters were estimated using the equations derived by Lavorel (1976).
2.13 Thermoluminescence Measurements

*C. reinhardtii* cells were cultured in TAP at 22-23°C, at a light intensity of 2μE/m²/s. The cells were harvested by gentle filtration with Whatman glass microfibre circles (GF/C, 25mm diameter) to give 50μg *Chl/circle*. Thermoluminescence was measured with apparatus as described in Mayes *et al.* (1993). After a 3 min dark adaptation at 20°C, the samples were excited with a single saturating flash, provided by a 1539-A Xenon flash lamp, at 5°C (without DCMU) or at -2°C (with DCMU). This was followed by fast cooling to -40°C, from where slow heating with a rate of 20°C/min was initiated, and thermoluminescence was detected. Where indicated, 10μM DCMU was added in the dark at the beginning of the 3 min dark adaptation.

2.14 Fluorescence Measurements

Fluorescence induction was recorded up to 3 s in the presence or absence of 10μM DCMU. Flash-induced chlorophyll relaxation was measured by a double-modulation fluorimeter (PSI instruments, Brno, Czech Republic). The relaxation kinetics of fluorescence after the actinic flash (100%, 20μs) was monitored by measuring flashes (100%, 2.5μs) in the 150μs to 100 s time range with a 34 ms time resolution. The signal-to-noise ratio was improved by accumulating 20 traces measured at 20 s intervals on the same sample.
CHAPTER 3

The Redox Active Tyrosines in PSII. Analysis of the
Chlamydomonas reinhardtii Mutants: D2-Y160F,
D1-E189D, D1-E189L and D1-E189Q
3.1 Introduction

The D1 and D2 polypeptides are intrinsic membrane components of the PSII reaction centre (Section 1.4.2.1). The D1 and D2 heterodimer is responsible for binding nearly all of the redox co-factors of this photosystem. With the construction of psbA and psbD deletion host strains of *Synechocystis* 6803 and *C. reinhardtii* it became possible, using site-directed mutagenesis, to probe the ligand co-ordination sites within D1 and D2. Such analysis has provided information regarding the residues that co-ordinate the Mn-cluster which, coupled to an electron donor, is thought to be the catalytic site of water oxidation (for a review see Diner, 2001).

P680$^+$ is reduced by an intermediate electron donor, tyrosine 161 (*C. reinhardtii* numbering) located on D1, referred to as $Y_Z$. $Y_Z$ in turn is reduced by the WOC (Nugent, 2001). A second redox active tyrosine, $Y_D$ is located on the D2 polypeptide and is residue 160. $Y_Z^*$ and $Y_D^*$ can be observed by EPR as signals of similar line shape. Oxidation of a tyrosine releases the phenoxyl proton to produce the neutral radical, evidence suggests that this deprotonation occurs via the residue D1-H190 to which $Y_Z$ may be functionally coupled (Hays, *et al.*, 1998; Mamedov *et al.*, 1998). Recently, investigations suggest that $Y_Z$ may be directly involved in water oxidation, rather than just an electron transfer intermediate (Force *et al.*, 1997; Hoganson & Babcock, 1997; Candeieas *et al.*, 1998). $Y_Z$ is oxidised by P680$^+$ on the nanosecond timescale, but the exact rate is S-state dependent. $Y_Z^*$ reduction by the WOC is also S-state dependent, with the reduction of $Y_Z^*$, during the S$_3$ to S$_4$ transition, being the rate limiting step in water oxidation. The distance from $Y_Z$ to the Mn-cluster is estimated to be about 8Å (Hallahan, *et al.*, 1992; MacLachlan, *et al.*, 1993, 1994; Lakshmi, *et al.*, 1999).

$Y_D$ can also be oxidised by P680$^+$. It appears not to participate directly in steady state electron transfer from the water oxidizing complex to P680$^+$ and has a markedly lower redox potential than $Y_Z$. It is relatively stable in the oxidised state and is able to slowly undergo redox reactions with the WOC.
During dark adaptation, the WOC slowly relaxes to the $S_1$ state. This relaxation involves advancement from $S_0$ to $S_1$ via electron donation to $Y_D$ or by deactivation of $S_2$ or $S_3$. It has been suggested that $Y_D$ is involved in the photoactivation of the WOC and it may be required to oxidise Mn in the $S_{0p}$-state, maintaining the integrity of the Mn-cluster. $Y_D$ is about 35Å from $Y_Z$ (Haumann et al., 1999) and about the same distance from the manganese cluster (Debus, 1992). It is probably hydrogen bonded through its phenolic proton to a histidine residue, D2-His190 (Svensson, et al., 1990; Tang, et al., 1993; Kim et al., 1997). The protein environments around $Y_Z$ and $Y_D$ are different, $Y_D$ being located in a hydrophobic pocket and $Y_Z$ in a hydrophilic environment more accessible to solvent (Ruffle et al., 1992; Tang et al., 1996).

In this chapter I present analysis of a *C. reinhardtii* D2-Y160F mutant. Initial attempts to study this mutant using spectroscopic techniques were relatively unsuccessful. PSII cannot be isolated efficiently from *C. reinhardtii* using preparations similar to those used to prepare higher plant PSII (Section 1.6 and Section 4.1). The original D2-Y160F mutant was created in a PSI-deficient background (PSI) in the hope that complications due to the presence of PSI would be avoided. Unfortunately, this step did not improve the *C. reinhardtii* thylakoid membrane samples sufficiently to allow relevant spectroscopic analysis. This led to the isolation of PSII using histidine tagging technology as detailed in Chapter 4 and subsequently the analysis of PSII isolated in this way from a his-tagged D2-Y160F mutant.

In addition, a brief analysis of *C. reinhardtii* D1-E189 mutants is also presented. This work is included because it has been proposed that the residue D1-E189 is important in creating the correct environment around $Y_Z$ allowing it to participate in the mechanism of water oxidation. At least seventeen mutations have now been constructed at D1-E189 in *Synechocystis* (Chu et al., 1995 a&b; Svensson et al., 1998; Debus et al., 2000). The influence of the D1-E189 mutations on the assembly or stability
of the WOC is minor, therefore the residue is not believed to ligate the Mn-cluster (Chu et al., 1995a, b; Debus et al., 2000). EPR and electron transfer properties of PSII isolated from these mutants are consistent with D1-E189 participating in a proposed network of hydrogen bonds that modulates the properties of both YZ and the Mn-cluster (Debus et al., 2000).

3.2 Construction and Characterisation of the D2-Y160F/PSI' Mutant

3.2.1 Construction and sequencing of the D2-Y160F/PSI' mutant

The D2-Y160F/PSI' C. reinhardtii mutant and the PSI' control strain were kind gifts from Jun Minagawa, RIKEN, Japan. The mutation was created in plasmid pBD303. This plasmid has an insertional inactivation site at the psaA2 intron and incorporates the psbD gene. The site-directed mutation D2-Y160F was incorporated by PCR to yield the plasmid pBD303:Y160F. This plasmid was used to transform the host CC-2137 using the biolistic technique as described in Section 2.10.

The D2-Y160F mutant was sequenced over the psbD gene region to ensure that the mutants had not reverted to wild type. PCR was used to amplify the psbD gene from total genomic DNA isolated from wild-type, PSI' and the D2-Y160F strains. The PCR experiments were carried out using two primers, shown below, which flanked the psbD gene.

**Forward Primer**

D373 - 5'-aaatttaacgtaacgatga-3'

**Reverse Primer**

D1483R - 5'-tatattagacggttacca-3'
The reaction conditions were as given in Section 2.6.6, including 30 cycles with an annealing temperature of 50°C for 2 min. This combination of primers produced a fragment of ~1100 bp for all strains (Figure 3.1).

In order to verify that the Y to F mutation was present on the $psbD$ gene the PCR products were sequenced with primer D790 (Section 2.7).

**Sequencing Primer**

D790 - 5' - ggtttcatgcttcgtagcag-3'

The resulting sequence is given in Table 3.1, the Y to F change is shown in red.

<table>
<thead>
<tr>
<th>Sequencing analysis of the D2-PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>gct gta ttc gtt tca gta ttc cta att ttc</td>
</tr>
<tr>
<td>A  V  F  V  S  V  F  L  I  F  160</td>
</tr>
<tr>
<td>cca tta ggt caa tca ggt tgg ttc ttt gca</td>
</tr>
<tr>
<td>P  L  G  Q  S  G  W  F  F  A  170</td>
</tr>
<tr>
<td>cct aqt ttc ggt gta gct gct atc ttc cgt</td>
</tr>
<tr>
<td>P  S  F  G  V  A  A  I  F  R  180</td>
</tr>
<tr>
<td>ttc att tta ttc ttc caa ggn ttc cac aac</td>
</tr>
<tr>
<td>F  I  L  F  F  Q  G  F  H  N  190</td>
</tr>
<tr>
<td>tgg aca ctt aac cca ttc cac atg atg ggt</td>
</tr>
<tr>
<td>W  T  L  N  P  F  H  M  M  G  200</td>
</tr>
<tr>
<td>gtt gct ggt gtt tta ggt gat gct tta tta</td>
</tr>
<tr>
<td>V  A  G  V  L  G  A  A  L  L  210</td>
</tr>
<tr>
<td>tgt gct att cac agg tgt tac tgt . . . .</td>
</tr>
<tr>
<td>C  A  I  H  G  A  T  V  . . . . 218</td>
</tr>
</tbody>
</table>

**Table 3.1** Sequencing analysis of the D2 PCR product showing the Y to F change in the D2-Y160F/PSI' mutant. The amino acid numbering is from the start of the $psbD$ gene. Only an extract of the sequence is presented, showing the Y to F change.
Chapter 3 – Results

Forward Primer - D373
Reverse Primer - D1483R

Figure 3.1 PCR analysis of the D2-Y160F strain. The gel photograph shows wild-type, PSI and D2-Y160F PCR products when the given primers, flanking the psbD gene, are used.

1 = wild-type (cc-1021)
2 = PSI
3 = D2-Y160F No.1
4 = D2-Y160F No.2
M = markers

1.1kb
3.2.2 Growth analysis of transformants

The phenotype of the D2-Y160F mutant was examined by comparing growth in the light on TAP, TAP supplemented with spectinomycin and HSM (Figure 3.2). The D2-Y160F mutant is unable to grow photoautotrophically, requiring acetate as a carbon source; this is expected as the mutant is in a PSI background. The presence of the *aadA* cassette is apparent by the ability of the mutant to grow on TAP supplemented with spectinomycin. The spot tests also show the mutant and the PSI control strain to be sensitive to high light intensities.

Growth curves were carried out as in Section 2.3.7 in liquid TAP. These growth curves (Figure 3.3) show that the mutant does not grow to the same cell intensity as wild-type but a “normal” growth pattern is observed, similar to that for the PSI-deficient control.
Figure 3.2 Spot tests showing the growth characteristics of D2-Y160F. Fifteen microlitre aliquots of cells from liquid culture were spotted onto solid media and grown under moderate light, low light or in the dark. These spot tests show the growth phenotype of the D2-Y160F strain compared to the wild-type and PSI' strains. The mutant is unable to grow photoautotrophically and is sensitive to moderate light but will grow in low light. It is resistant to spectinomycin.

Light levels: moderate, 45 \( \mu \) Em\(^{-2}\)s\(^{-1}\); low, 5 \( \mu \) Em\(^{-2}\)s\(^{-1}\).
Figure 3.3 D2-Y160F transformant growth curves. This graph shows the growth pattern of the D2-Y160F mutant compared with wild-type and a PSI strain. The growth pattern is similar to wild-type in both the PSI-control and the D2-Y160F/PSI' mutant, but these strains do not grow to the same cell intensity as wild-type.
3.2.3 Oxygen evolution

In order to assess the effect of the Y to F change on the oxygen evolving capacity of *C. reinhardtii*, cells or membranes were subjected to steady-state oxygen evolution analysis as described in Section 2.3.6. Measurements were coupled to the electron acceptors DMBQ and K$_3$Fe(CN)$_6$. The oxygen evolution rates obtained are shown below (Table 3.2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>$O_2$ Evolution Rate ($\mu$molO$_2$/mg Chl*/hr)</th>
<th>Chl a / Chl b ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type cells</td>
<td>264 ± 18</td>
<td>2.6</td>
</tr>
<tr>
<td>PSI’ cells</td>
<td>0 – 80</td>
<td>2</td>
</tr>
<tr>
<td>D2-Y160F/PSI’ cells</td>
<td>85 ± 35</td>
<td>1.8</td>
</tr>
<tr>
<td>Wild type membranes</td>
<td>210 ± 20</td>
<td>2.7</td>
</tr>
<tr>
<td>PSI’ membranes</td>
<td>0 - 100</td>
<td>1.9</td>
</tr>
<tr>
<td>D2-Y160F/PSI’ F membranes</td>
<td>100 ± 40</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*Chlorophyll assay by the method of Porra et al., (1989)*

Table 3.2 Table showing the oxygen evolution characteristics of wild-type, PSI’ and D2-Y160F cells and membranes. Each value for cells is the average (± standard error) from three different cell cultures. Each value for membranes is the average (± standard error) of three or more individual membrane preparations.

3.2.4 EPR measurements

Using EPR spectroscopy it is possible to establish the PSI and PSII content of mutants or preparations by quantifying the characteristic spectra from their electron transfer components as described in Section 4.4.3. In this work EPR analysis has confirmed the mutant to be PSI deficient as the P700 signal, indicative of the presence of PSI, is not seen upon illumination. This technique also confirms that $Y_D$ is missing from the mutant strain D2-Y160F/PSI’. Figure 3.4 shows the spectra from untreated D2-Y160F/PSI’
and PSI thylakoid membranes measured in the dark. In the PSI membranes a $Y_D$ signal is present, this signal is not present in the D2-Y160F spectrum.

Figure 3.5 shows the EPR signal derived from $Y_Z$. We were able to trap and view the $Y_Z$ signal using Mn-depleted D2-Y160F/PSI thylakoid membranes. Mn-depletion was achieved in these membranes by hydroxylamine treatment. To trap the rapidly decaying $Y_Z^*$ radical, EPR tubes containing Mn-depleted membranes (Debus, 1992 and references therein) with 3 mM potassium ferricyanide as an acceptor were frozen under illumination as follows: the EPR samples were held above an unsilvered dewar containing a 230 K dry ice/ethanol bath and were illuminated for 15 seconds with saturating light provided by a 1kW lamp (Flectalux, Model No. GLS 1008). Samples were illuminated for a further 10 seconds while immersed in the 230 K bath, and then the light was turned off (Tommos, 1997). The samples were frozen and stored in liquid nitrogen until use.
Figure 3.4 Continuous wave EPR spectra of untreated, dark adapted D2-Y160F/PSI\textsuperscript{1} thylakoids from C. reinhardtii mutant. A clear Y\textgreek{Q} signal can be seen in the PSI\textsuperscript{1} spectrum, this is absent it the D2-Y160F spectrum as expected.

Experimental conditions:- microwave power, 10\textsuperscript{-2}mW, modulation amplitude, 0.2mT, microwave frequency, 9.055 GHz, temp., 15K, 3 scans per spectra.
Figure 3.5 Continuous wave EPR spectra of Mn-depleted D2-Y160F/PSI- C. reinhardtii thylakoid membranes. Mn-depleted D2-Y160F/PSI- membranes were treated as follows: (i) 230K illumination (red) and (ii) 230K illumination followed by thawing and refreezing in the dark (green). The difference spectrum following these treatments is shown in blue. This spectrum shows the signal from the redox active tyrosine Y$_2$. A P700 chlorophyll signal is not observed in the D2-Y160F/PSI- spectra indicating the absence of PSI.

The sample contained 4.25mg Chl/ml and 3μM K$_3$Fe(CN)$_6$. The transient Y$_2$ signal was trapped by illumination for 15 s at RT followed by 10 s illumination while the sample was lowered in an ethanol/dry ice bath at 230K. The light was switched off and the sample frozen in N$_2$ (liq.).

Experimental conditions:- as Figure 3.4
3.2.5 Limitations of this mutant

Using thylakoids of *C. reinhardtii* strains lacking PSI we were able to visualise the $Y_D$ and $Y_Z$ EPR signals from PSII. Unfortunately, this method of studying PSII still proved to be difficult when compared to the relative ease of PSII analysis from higher plants. An important PSII signal is the $S_2$ multi-line signal (Nugent, 2001 and references therein). When we attempted to visualize this signal in the PSI control thylakoid membranes or, indeed in the D2-Y160F membranes, we were unable to detect it even at chlorophyll levels, and therefore PSII levels, much greater than that required to see the multi-line signal in higher plant preparations. This may be due to different conditions being required to detect the *C. reinhardtii* multi-line signal or it could be a significant different between algal and higher plant PSII. Regrettably, this finding hampered plans of using the PSI background for the studying PSII mutants in *C. reinhardtii* and a method of purifying PSII seemed the only alternative. Our approach was the use of metal affinity chromatography to purify a his-tagged PSII complex as described in Chapter 4.

3.3 Construction of the Mutation in a His-tagged Background

The successful isolation of PSII using a his-tag on the C-terminal end of the D2 polypeptide of *C. reinhardtii* by Sugiura and co-workers (1998) has facilitated more in-depth analysis of PSII from this alga (Section 1.6). Once again, through our collaboration with Jun Minagawa we obtained the D2-Y160F mutant in a D2-his-tagged background allowing the isolation of the mutagenised PSII. However, after some initial characterisation and preliminary experiments, the instability of the D2-Y160F/D2-his$_6$ strain was observed. These findings were subsequently confirmed by experiments in Japan. After initial transformation, oxygen evolution rates are quite normal, approximately 150$\mu$molO$_2$/mg Chl/hr (Jun Minagawa - personal communication). However, after sub-culturing a number of times a much
lower oxygen evolving capacity is observed. Subsequently, the Y to F change was made in a H-his\textsubscript{6} strain, with a C-terminal histidine tag on the PSII protein PsbH, developed in this laboratory (Chapter 4) and a B-his strain, with a C-terminal histidine tag on the PSII protein PsbB, developed by Minagawa and co-workers (personal communication). This work concentrates mainly on the D2-Y160F mutant with a C-terminal his tag on psbH. This strain (D2-Y160F/H-his\textsubscript{6}) was created by transforming the H-his\textsubscript{6} strain (Chapter 4 of this thesis) with a plasmid containing the Y to F change on the psbD gene along with the aadA cassette using the biolistic technique (Section 2.10). Transformants were selected for on media containing spectinomycin, they were taken through rounds of single colony isolation until confirmed to be homoplasmic by Southern blottting (Section 2.8 and 4.3.1).

3.4 Characterisation of the D2-Y160F Tagged mutants

3.4.1 Growth analysis of transformants

The phenotypes of the D2-Y160F tagged mutants were examined by comparing growth in moderate light (45μE/m\textsuperscript{2}/s), low light (5μE/m\textsuperscript{2}/s) and in the dark on TAP, and HSM (Figure 3.6). The H-his and B-his tagged mutants grow as wild-type (Figure 3.6 a,b,c & d). The non-tagged mutant and the D2 tagged mutant do not grow photoautotrophically as they each lack PSI (d). The D2-tagged mutant is more sensitive to light compared to the untagged mutant (b) - suggesting that the tag is in some way detrimental to photosynthetic activity and, therefore, explaining why the mutant is unstable.
Figure 3.6 Spot tests showing the growth characteristics of his-tagged D2-Y160F mutants. Fifteen microlitre aliquots of cells from liquid culture were spotted onto solid media and grown under moderate light, low light or in the dark. These spot tests show the growth phenotype of the D2-Y160F/PSI' strain compared to the wild-type and H-his₆ strains. H-his/D2-Y160F and B-his/D2-Y160F mutants grow as wild-type under these conditions. Non-tagged D2-Y160F/PSI' and the D2-his/Y160F/PSI' do not grow photoautotrophically because they do not contain PSI. D2-his/Y160F/PSI' is more sensitive to light than the untagged mutant and the mutants tagged on alternative polypeptides.

Light levels: moderate, \(45 \mu \text{Em}^{-2} \text{s}^{-1}\); low, \(5 \mu \text{Em}^{-2} \text{s}^{-1}\).
3.4.2 Oxygen evolution

In order to assess the effect of the mutation and/or the his-tag on rates of oxygen evolution transformant cells were subjected to steady state oxygen evolution analysis as described in Section 3.2.3, these are presented in Table 3.3. The measurements indicate that the removal of PSI negatively affect oxygen evolving capacity (Table 3.2 & 3.3) and changes the Chl \( a/b \) ratio. The rates measured also show the variability in rates gained when the his-tag was incorporated on the D2 polypeptide. When the his-tag is incorporated at the C-terminus of PsbH or PsbB rates of oxygen evolution of cells are comparable with the wild-type and the H-his strain. Continued analysis with the H-his\(_6\) and D2-Y160F/H-his\(_6\) strains suggests they are quite stable.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( O_2 ) Evolution Rate ( \mu )molO(_2)/mg Chl(^*)/hr</th>
<th>Chl ( a / Chl ) b ratio</th>
<th>Cells</th>
<th>PSII</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>264 ±18</td>
<td></td>
<td>2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-his(_6)</td>
<td>272 ± 6</td>
<td>3700 ± 1000</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2-Y160F/PSI(_1)</td>
<td>85 ± 35</td>
<td></td>
<td>1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2-Y160F/D2-his(_6)/PSI(_1)</td>
<td>0 -115</td>
<td>680 ± 110</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2-Y160F/H-his(_6)</td>
<td>280 ± 10</td>
<td>800 ± 55</td>
<td>2.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2-Y160F/B-his(_6)</td>
<td>272 ± 15</td>
<td></td>
<td>2.14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\*Chlorophyll assay by the method of Porra et al., (1989)

Table 3.3 The oxygen evolution characteristics of wild-type, H-his\(_6\) and D2-Y160F mutant cells and PSII samples. Each value is from measurements in triplicate from three different cell cultures of an average (± standard error) of 3 different PSII preparations.
3.4.3 EPR measurements

The suitability of *C. reinhardtii* PSII isolated by his-tagging technology is shown in Chapter 4 of this thesis. However, it has been suggested that the PSII from this alga is too labile to carry out routine spectroscopic analysis successfully (Sugiura & Inoue, 1999). Figure 3.7 shows spectra from H-his\textsubscript{6} (essentially wild-type) and D2-Y160F/H-his\textsubscript{6} PSII samples. Samples were illuminated at 200K and the spectra recorded. Each sample was then stored at 77K for one week, the resulting spectrum was recorded then the sample was re-illuminated in the EPR cavity and a third spectrum recorded. After initial illumination the signal from Y\textsubscript{D} is seen in the wild-type (H-his\textsubscript{6}) sample but not in the D2-Y160F/H-his\textsubscript{6} PSII, as expected. The signal seen in D2-Y160F/H-his\textsubscript{6} PSII spectra is likely to be from oxidised carotenoid or the accessory chlorophyll, Chl Z\textsuperscript{+}. After storage of these samples for one week this signal and the signal from Y\textsubscript{D} are significantly reduced. However, following illumination both signals can be seen again. These spectra confirm the absence of the redox active tyrosine, Y\textsubscript{D}, in the D2-Y160F/H-his\textsubscript{6} PSII.
Figure 3.7 Continuous wave EPR spectra PSII from H-his$_6$ (wild-type) and D2-Y160F/H-his$_6$ C. reinhardtii strains. Samples were illuminated at 200K, stored at RT for 1 week then re-illuminated in the cavity at 10K. The Y$_D$ signal is clearly seen in the wild-type spectrum but is not present in the spectrum for D2-Y160F. The signal in the D2-Y160F spectrum is probably Chl $Z^-$ or oxidised carotenoid.

The spectra are not corrected for Chl content, H-his$_6$ PSII = 2.9mg/ml (a/b 4.2) and D2-Y160F PSII = 1.6mg/ml (a/b 9.8). Experimental conditions:- microwave power, 0.001 mW, temp., 10K, modulation amplitude, 0.2mT.
Figure 3.8 shows a broader EPR scan of H-his$_6$ and D2-Y160F/H-his$_6$ PSII samples. In spectra a and b the S$_2$ EPR signals can be seen, the Mn multi-line (labelled 3) and the g = 4.1 (labelled 1) are present in both H-his$_6$ (a) and D2-Y160F/H-his$_6$ (b) PSII. Although the S2 EPR spectra are present in *C. reinhardtii* PSII samples the spectra are not identical to those obtained from higher plant samples (see Section 4.4.3). In spectra a, b and c some cyt $b_{559}$ is oxidised by the illumination, this signal is labelled 2. Unfortunately, the spectra presented here were recorded under slightly different conditions (see Figure 3.8 legend) therefore direct comparisons of the signal sizes cannot be made. Several other measurements did indicate that the multi-line signal from the D2-Y160F/H-his$_6$ PSII was reduced to about 50% that of wild-type, which appears to be in agreement with the reduced oxygen evolving rates in the isolated PSII from both D2-Y160F/H-his$_6$ and D2-Y160F/D2-his (Table 3.3). The presence of the S2 state signals and the cyt $b_{559}$ signal show that we do have PSII present in the D2-Y160F/H-his$_6$ sample. These initial measurements indicate that this type of PSII preparation will be suitable for spectroscopic analysis.
Chapter 3 – Results

Figure 3.8 Continuous wave EPR spectra PSII from H-his₆ (wild-type) and D2-Y160F/H-his₆ C. reinhardtii strains. Traces a and b show the S₂ multi-line signal (3) and the g = 4.1 signal (1). The oxidised cyt b₅₅₉ signal (2) is also clearly seen. Traces a & b are the difference spectra of a dark-adapted sample and this sample following 200K illumination. The S₂-EPR signals in wild-type and D2-Y160F are comparable in shape, however the signal size is reduced. Trace c shows the cyt b₅₅₉ signal, this trace is the difference spectrum of a dark-adapted sample and this sample following 77K illumination.

Experimental details:-
(a) H-his₆ PSII = 2.9mg/ml Chl, a/b 7.9, microwave power, 10mW, temp., 6.4K
(b) D2-Y160F PSII = 1.24 mg/ml Chl, a/b >10 (this spectrum has been multiplied by 1.5) microwave power, 20mW, temp., 5.5K
(c) D2-Y160F PSII = 1.85 mg/ml Chl, a/b 3.7 microwave power, 20mW, Temp, 9.5K.
3.5 Kinetic Analysis of the His-tagged Mutant

The evidence from basic characterisation of his-tagged mutants suggests that in some cases the tag can disrupt the protein structure enough to affect function. This appears to be what has happened in the case of the his-tag on the C-terminal end of D2. Although initial observations suggest that the his-tag on the C-terminus of PsbH is not detrimental to function, characterisation of the kinetic properties of the electron donor and acceptor sides of the modified PSII are needed to further corroborate the initial findings.

3.5.1 Thermoluminescence studies

Thermoluminescence is a burst of light emission at characteristic temperatures caused by charge recombination between electron donors and acceptors when pre-illuminated PSII RC's are rapidly cooled and then rewarmed gradually in darkness (Hall and Rao, 1995). The thermoluminescence measurements presented here were carried out in collaboration with Imre Vass (Institute of Plant Biology, Szeged, Hungary). Figure 3.9 shows the single, flash-induced thermoluminescence curves for H-his$_6$, D2-Y160F/H-his$_6$, PSI control and D2-Y160F/PSI$^+$ cells. The cells were prepared and treated as described in Section 2.13. When excited with a single flash H-his$_6$ cells show a thermoluminescence band peaking at 32-34°C (Figure 3.9a). This band peak is assigned to the B thermoluminescence band originating from charge recombination between S$_2$ and Q$_B^-$. When the electron transfer between Q$_A$ and Q$_B$ was blocked by 10 μM DCMU prior to excitation, the emission temperature downshifted to 15-16°C, indicative of conversion of the B-band to the Q-band, which originates from and S$_2$Q$_A^-$ charge recombination. These data also show that the H-his$_6$ cells behave as expected for wild-type (not shown, O’Connor et al., 1998).

In contrast, the peak positions of the main thermoluminescence
bands in the mutant D2-Y160F/H-his$_6$ (Figure 3.9c) are slightly shifted towards higher temperatures: 35-37°C for the B-band and 21-22°C for the Q-band. This shows that the $S_2Q_A^-$ and $S_2Q_B^-$ charge pairs are more stabilised in the mutant than in the H-his$_6$ control.

The thermoluminescence curves for the PSI control and D2-Y160F cells were the same (Figures 3.9c & d). In the absence of DCMU, the main peak is shifted downwards to 20-22°C but the Q-band is similar to that in H-his$_6$ (and, therefore wild-type) at 16-18°C. The peak temperatures in the presence and absence of DCMU are quite close, indicating that in the mutants lacking PSI the PQ-pool is reduced, thus a single exciting flash can create stable $Q_A^-$. 
Figure 3.9 Single flash induced thermoluminescence curves for H-his$_6$ (a), PSI' (b), D2-Y160F/H-his$_6$ (c) and D2-Y160F/PSI' (d) C. reinhardtii strains. Measurements were taken in the absence (solid line) and in the presence (dashed line) of DCMU.
3.5.2 Fluorescence studies

To obtain information regarding possible perturbations of the acceptor side of the PSII complex, kinetic measurements of fluorescence induction and relaxation were measured both in the presence and absence of DCMU, these measurements were again carried out in collaboration with Imre Vass. Fluorescence induction curves provide information about electron transport in PSII. Such curves allow the variable fluorescence, $F_v$ and maximum fluorescence, $F_m$ to be measured. It has been shown that the photochemical yield of PSII is equal to the ratio $F_v:F_m$ and it has been verified experimentally that this ratio is proportional to the rate of photosynthesis measured as $O_2$ evolution or $CO_2$ assimilation (Hall & Rao, 1995). Fluorescence relaxation curves can be described by components: two exponential components (fast and middle phase) and one component with second order kinetics (slow phase). Monitoring these phases gives information on the re-oxidation of $Q_A$ in centres which contain bound $Q_b$, any centres in which the $Q_B$ binding site is empty at the time of the actinic flash and the re-oxidation of $Q_A$ through charge recombination with the $S_2$ state of the WOC.

Measurements were carried out as described in Section 2.14. $F_v:F_m$ ratios are shown in Table 3.4.

<table>
<thead>
<tr>
<th>C. reinhardtii strain</th>
<th>$F_v:F_m +$ DCMU</th>
<th>$F_v:F_m -$ DCMU</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-his$_6$ cells</td>
<td>0.69</td>
<td>0.74</td>
</tr>
<tr>
<td>D2-Y160F/H-his$_6$ cells</td>
<td>0.49</td>
<td>0.41</td>
</tr>
<tr>
<td>PSI$^-$ cells</td>
<td>0.56</td>
<td>0.63</td>
</tr>
<tr>
<td>D2-Y160F/PSI$^-$ cells</td>
<td>0.18</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Table 3.4 The $F_v:F_m$ ratios of C. reinhardtii cells.
The flash induction curve for H-his$_6$ cells (Figure 3.10) is well structured and shows all the characteristic phases of intact cells, as expected from a wild-type strain. In contrast, the fluorescence induction curve for D2-Y160F/H-his$_6$ cells (Figure 3.10) has a small $F_v:F_m$ value both with and without DCMU (Table 3.4). In PSI cells the $F_0$ level is much higher (about 4-5 times) than in H-his$_6$ cells. The fluorescence rise ($F_m$) is not followed by a decline phase showing that in this mutant the PQ-pool cannot be reoxidised. A similar pattern is seen in D2-Y160F/PSI cells however, the $F_v:F_m$ ratio is smaller (Table 3.4).

In H-his$_6$ cells the half-time of the fast phase is $\approx 0.4$ ms (amp. $\approx 60\%$). This reflects $Q_A^-$ re-oxidation in the centres which contain bound $Q_B^*$. The half-time of the middle phase is $\approx 90$ ms (amp. $\approx 17\%$) and arises from the centres where the $Q_B$ site is empty at the time of the actinic flash. The slow phase with $\approx 3$ s half-time (amp. $\approx 23\%$) reflects $Q_A^-$ re-oxidation through charge recombination with the $S_2$ state of the WOC. The presence of DCMU almost completely abolishes the first two phases such that $Q_A^-\text{re-oxidation occurs almost only via charge recombination with the }S_2\text{ state. The flash induced relaxation kinetics are similar to H-his}_6\text{ cells in the other mutants (Figure 3.11).}$

These data firstly indicate that the his-tag on the C-terminus of PsbH appears not to be detrimental to PSII function. In addition, the reduced $F_v:F_m$ ratio in D2-Y160F/H-his$_6$ cells suggests a reduction in photosynthetic activity as indicated by the reduced oxygen evolving rate of this mutant (Table 3.2 and 3.3).
**Figure 3.10** Fluorescence induction curves for H-his$_6$ (a), PSI$^-$ (b), D2-Y160F/H-his$_6$ (c) and D2-Y160F/PSI$^-$ (d) *C. reinhardtii* strains. Measurements were taken in the absence (solid line) and in the presence (dashed line) of DCMU.
Figure 3.11 Fluorescence relaxation curves for H-his₆ (a), PSI⁻ (b), D2-Y160F/H-his₆ (c) and D2-Y160F/PSI⁻ (d) *C. reinhardtii* strains. Measurements were taken in the absence (closed symbols) and in the presence (open symbols) of DCMU. Curves are normalised to the same initial amplitude.
3.5.3 P680$^+$ reduction kinetics

P680$^+$ is reduced by YZ in a multiphasic reaction that occurs over a timescale from 20 ns to 30 µs (Schilstra et al., 1998). The reduction kinetics have contributions from both oxygen-evolving particles and particles whose S-state cycle is disrupted or inhibited. In particles without a functional WOC the P680$^+$ reduction kinetics are significantly slower than in fully functional particles. The P680$^+$ lifetime in tris-treated BBY’s (i.e. having a non-functional WOC) is about 10-15 µs (Conjeaud & Mathis, 1980), while in untreated BBY’s the dominant kinetic components are 20-200 ns, however there are S-state dependent components whose life-times are in the microsecond timescale. Studies on the kinetics of P680$^+$ reduction suggest a role for proton coupled electron transfer processes, in which proton/hydrogen transfer is required to complete the reduction of P680$^+$ through an alteration in the equilibrium between YZ/YZ$^+$ and P680/P680$^+$ (Schilstra et al., 1998). In this work, the P680$^+$ reduction kinetic measurements were carried out in collaboration with Maria Schilstra and Chris Jeans at Imperial College London, as described in Section 2.12. The kinetics were measured for PSII particles isolated via histidine tagging technology (Chapter 4) from a YD-less C. reinhardtii strain (further indicated as D2-Y160F/his6 PSII) and a control C. reinhardtii strain (H-his6 PSII). Spinach PSII (S-PSII) was isolated as in Section 2.2.2 and acted as an overall control because the reduction kinetics have been well-characterised in this sample type (Schilstra et al., 1998). Data collected allowed a comparison of the three samples.

It has been suggested that YD may be involved in the photoactivation process, donating electrons to P680$^+$ to minimise possible photodamage caused by this oxidant in the absence of a functioning Mn cluster (Magnuson et al., 1999). It has also been proposed to be involved in stabilising the intact cluster through its reaction with the S0 state, in which Mn is thought to be more weakly bound (Nugent, 1987). Our results show
that $Y_D$ is not essential for assembly of the Mn cluster, and that normal S-state cycling occurs in $Y_D$-less samples.

Figure 3.12 shows the overall $P680^+$ reduction kinetics in the three preparations. S-PSII and H-his$_6$ PSII reduction kinetics are essentially identical showing that H-his$_6$ PSII is very similar to BBY higher plant preparations; the slight difference between the kinetics may be due to small differences in the fraction of particles lacking the oxygen-evolving complex. Importantly, the kinetics in the D2-Y160F/his$_6$ PSII are indistinguishable from those in the H-his$_6$ PSII for the first few microseconds, after this the microsecond reduction kinetics, previously shown to be proton/hydrogen transfer limited (Schilstra et al., 1998), in the mutant are slowed compared to both the other preparations. In summary, the average kinetics of $P680^+$ reduction in PSII from essentially wild-type $C. reinhardtii$ are shown to be comparable to those of PSII from spinach (Figure 3.12). The average kinetics in D2-Y160F/his$_6$ PSII are slowed after the first few microseconds.

In order to eliminate any possible contributions from damaged particles the deviations from the average $P680^+$ reduction kinetics due to S-state cycling over twenty one consecutive flashes were analysed (Figure 3.13). The data show that the period-4 oscillatory kinetics are almost identical in all three particles, as are the miss factors: 12% for BBYs, 10% for H-his$_6$ PSII and 13% for D2-Y160F/his$_6$ PSII. This normal oscillatory nature of the kinetics of this mutant indicates that the S-state cycling of the Mn-cluster is unaffected by either the presence of the his-tag or the absence of $Y_D$. 

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Figure 3.12 Average P680+ reduction kinetics in wild-type PSII and Y_D-less PSII from *Chlamydomonas* compared with BBYs.

a. Kinetics in all three preparations on a 40 μs timescale. The upper line represents D2-Y160F/His6 PSII, the lower line represents H-his PSII. P680+ reduction kinetics in BBYs (○) are shown at one point per microsecond for clarity. Curves are the average of 100 excitations, collected at 20 ns/channel.

b. Close up of the first 10 μs of (a). For clarity, the BBY data (○) are shown at one point per 300 ns.
**Figure 3.13** Oscillations in the kinetics of P680⁺ reduction in BBYs (■), H-his₅ PSII (○) and D2-Y160F/his₅ PSII (▲). Difference from mean data on a 2.5 µs timescale were fitted to a sum of three exponentials and an offset. Lifetimes were 20 ns, 130 ns and 600 ns. Amplitudes are plotted here.
The simplest interpretation of the results presented here is that the replacement of Y_D with phenylalanine perturbs the normal proton transfer pathways which occur during P680^+ reduction. The electron transfers which are not rate limited by proton transfer seem largely unaffected, since the kinetics in D2-Y160F/his_6 PSII before 2-3 μs are virtually indistinguishable from those of H-his_6 PSII. The results suggest that Y_D affects the proton/hydrogen transfers which accompany the relaxation phases of P680^+ reduction. This does not necessarily mean that Y_D is directly involved in a proton transfer pathway, but merely that its electrostatic or structural contribution is required for normal proton/hydrogen transfer. Previous work on a Y_D-less mutant of Synechocystis showed that the structure or redox properties of Y_Z were altered (Boerner et al., 1993), so this kind of ‘action at a distance’ is not unprecedented. Therefore, it is quite likely that Y_D may affect normal proton transfer pathways in PSII by virtue of the fact that its phenolic proton moves during its redox reactions, causing pK shifts of nearby side chains.

In conclusion, we have shown that the loss of Y_D has no effect on the oscillatory nature of P680^+ reduction, but that it has affected the proton/hydrogen transfer coupled electron transfers which occur with a microsecond rate constant. We see no evidence for a change in the free energy gap between P680 and Y_Z which implies that, at least for the sub-microsecond phases, the presence of Y_D^ox does not affect the redox potential of P680/P680^+. The most likely cause of the altered microsecond kinetics in the mutant are shifts in the pKs of residues in a hydrogen bonded network in and around the Y_D site. Such shifts could easily come about due to the absence of the phenolic proton of Y_D, which is known to be closely associated with Y_D in both its oxidised and reduced states.
3.6 D1-E189 Mutant Analysis

3.6.1 Construction of the D1-E189 mutants

The three E189 mutants, E189D, E189Q and E189L were a kind gift of Jun Minagawa. The construction of the site-directed change in these mutants is detailed in Svensson et al. (1998). In order to study these mutants using EPR spectroscopy they were subsequently created in a PSI background (Section 3.1).

3.6.2 Biochemical analysis

The growth characteristics, Chl \( a:b \) and oxygen evolving capacity of the mutants were measured. Figure 3.14 shows that the L and Q mutants are both sensitive to light, they are spectinomycin resistant as expected. The two mutants shown were unable to grow photoautotrophically on HSM, however this is expected as the mutant strains are lacking PSI. In initial analysis of the site-directed mutants by Svensson et al. (1998) the mutants were produced in a wild-type background hence they could be tested for photoautotrophic growth relating directly to the mutation. The E189L and E189Q could both grow photoautotrophically while E189D was unable to grow photoautotrophically.
**Figure 3.14** Spot tests showing the growth characteristics of D1-E189L and Q. Fifteen microlitre aliquots of cells from liquid culture were spotted onto solid media and grown under moderate light, low light or in the dark. These spot tests show the growth phenotype of the mutant strains compared to the wild-type and PSI strains. The mutants are unable to grow photoautotrophically and are sensitive to moderate light.

Light levels: moderate, 45 μEm^{-2}s^{-1}; low, 5 μEm^{-2}s^{-1}.
The oxygen evolving capacity and Chl $a:b$ ratios for the mutants in a PSI$^-$ background are presented in Table 3.5. All the mutants show reduced rates of oxygen evolution compared to wild-type. The Chl $a:b$ ratios are also reduced.

<table>
<thead>
<tr>
<th>Sample</th>
<th>O$_2$ Evolution Rate $\mu$molO$_2$/mg Chl$^+$/hr</th>
<th>Chl $a$ / Chl $b$ ratio$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type cells</td>
<td>264 ± 18</td>
<td>2.6</td>
</tr>
<tr>
<td>PSI$^-$ cells</td>
<td>0 - 80</td>
<td>2</td>
</tr>
<tr>
<td>D1-E189D</td>
<td>0</td>
<td>1.6</td>
</tr>
<tr>
<td>D1-E189L</td>
<td>20 ± 10</td>
<td>1.8</td>
</tr>
<tr>
<td>D1-E189Q</td>
<td>0</td>
<td>1.7</td>
</tr>
</tbody>
</table>

$^*$Chlorophyll assay by the method of Porra et al., (1989)

**Table 3.5** Table showing the oxygen evolution characteristics of wild-type, PSI$^-$ and D-E189 mutant cells. Each value is the average ($\pm$ standard error) from three different cell cultures.

### 3.6.3 Thermoluminescence and fluorescence studies

Thermoluminescence curves were measured as given in Section 2.13 and fluorescence measurements were carried out as described in Section 2.14 in collaboration with Imre Vass. Figure 3.15 shows the single, flash-induced thermoluminescence curves for PSI$^-$ control, D1-E189L/PSI$^-$ and D1-E189Q/PSI$^-$ cells. When excited with a single flash the main thermoluminescence band in the mutant D1-E189L/PSI$^-$ (Figure 3.15c) consists of two components: one with a peak position at $\approx 19^\circ$C from charge recombination of $S_2Q_A^-$ and another one with a peak position at $\approx 40-45^\circ$C. The detected signals in the D1-E189Q/PSI$^-$ cells (Figure 3.16b) are extremely small suggesting very little photosynthetic activity.
Figure 3.15 Single flash induced thermoluminescence curves for PSI (a), D1-E189Q (b) and D1-E189L (c) *C. reinhardtii* strains. Measurements were taken in the absence (solid line) and in the presence (dashed line) of DCMU.
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Fluorescence induction and relaxation properties in the D1-E189L/PSI cells are very similar to those of the PSI control (Figure 3.16a&c and Figure 3.17a&c). In the presence of DCMU, the fluorescence reaches its maximum slightly slower than in the PSI control, which indicates that the effect of DCMU is not complete. In the presence of DCMU, the fluorescence relaxation curves are different suggesting that some of the centres the DCMU cannot bind to the Qb site in E189L/PSI cells. The signals in the D1-E189Q/PSI cells (Figure 3.16a&b and Figure 3.17a&b) are extremely small suggesting very little photosynthetic activity.
Figure 3.16 Fluorescence induction curves for PSI', D1-E189Q and D1-E189L C. reinhardtii strains. Measurements were taken in the absence (solid line) and in the presence (dashed line) of DCMU.
Figure 3.17 Fluorescence relaxation curves for PSI (a), D1-E189Q (b) and D1-E189L (c) C. reinhardtii strains. Measurements were taken in the absence (closed symbols) and in the presence (open symbols) of DCMU. Curves are normalised to the same initial amplitude, except for D1-E189Q because the signal amplitude from it is negligible.
3.7 Discussion

The mechanism of photosynthetic water oxidation is still very much under debate. In this work I have attempted to use site-directed mutagenesis of *C. reinhardtii* to gain more information about the reactions that lead to water splitting. I set out to study site-directed mutants spectroscopically using EPR and hoped to carry out subsequent analysis using ENDOR. It had already been demonstrated that the study of PSII from *C. reinhardtii* was not as straightforward as studying higher plant PSII due to difficulties in isolating PSII from this alga (Shim *et al.*, 1990). Simple detergent treatment cannot be used because the thylakoid membranes do not stack to the same extent in *C. reinhardtii* as in higher plants, therefore a distribution of photosystems is not seen (Section 1.3; Figure 1.5). In order to use EPR spectroscopy to study PSII the absence of PSI is required due to PSI signals that mask those of PSII. Our initial strategy to overcome this problem was to create the *C. reinhardtii* site-directed mutants in a PSI-deficient background. This strategy successfully removed the PSI signals, and some of the PSII EPR signals could be seen (Section 3.2.4), however a number of PSII signals routinely manipulated to study PSII function (e.g. Mn multiline) could not be seen. Unfortunately, it appeared that this technique would not be suitable in allowing an in-depth analysis of PSII function in these mutants and an alternative strategy was required. The strategy we used was to isolate PSII using histidine tagging technology as detailed in Chapter 4 and to subsequently construct PSII site-directed mutants in this his-tagged background (this Chapter and Chapter 6). The mutants studied in this chapter were a D2-Y160F mutant and D1-E189 mutants.

The D2-Y160F mutant lacks the redox active tyrosine Y$_D$. By studying this mutant we hoped to gain an understanding of Y$_D$'s role in water oxidation and also obtain further information about the redox active tyrosine Y$_Z$ in the absence of the complicating EPR signals arising from Y$_D$. The creation of this mutant in a his-tagged background allowed it to be
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studied by EPR. Initially the mutation was created in a background in which the PSII D2 polypeptide was tagged. Initial characterisation of this mutant showed it to be unstable (Section 3.3). This finding led us to create the mutation in a his-tagged background in which PsbH was tagged (Chapter 4). This mutant has been shown to be stable and suitable for spectroscopic analysis.

The kinetic data shown here implies that the his-tag on PsbH does not affect PSII function and also suggests that the D2-Y160F change causes a reduction in photosynthetic activity as corroborated by the reduced oxygen evolving rate of this mutant. The P680+ reduction kinetics presented here show that in oxygen evolving D2-Y160F/his6 PSII particles, the nanosecond phases of Y to P680+ electron transfer are not altered, however the loss of YD affects the proton/hydrogen coupled electron transfers which occur with a microsecond rate constant. The most likely cause of these altered microsecond kinetics are shifts in the pKs of residues in a hydrogen bonded network in and around the YD site. The simplest interpretation of the results is that YD is required for normal proton/hydrogen transfer in PSII.

On the basis of modelling studies (Svensson et al., 1991; Ruffle et al., 1992; Svensson et al., 1996) the D1-E189 residue has been proposed to be a possible ligand to the Mn cluster. The mutants D1-E189D, L and Q were looked at in this work, however analysis was hindered by the difficulties in studying C. reinhardtii PSII highlighted above. Subsequent in-depth investigation of D1-E189 mutants in Synechocystis sp. 6803 by Debus and co-workers (2000) has provided further information about the role of this amino acid in the mechanism of water oxidation. In this work the E to Q, L, R, K and I mutations support photoautotrophic growth. In mutations that abolish photoautotrophic growth the redox properties of the Mn cluster and YZ are altered and the magnetic properties of the Mn cluster are perturbed. In contrast, the initial characterisation of the three C.
reinhardtii mutants presented here suggest that changes to D1-E189 are more deleterious to PSII function in the alga than in Synechocystis. This may indicate slight differences in the detailed working of PSII in different photosynthetic species. The D1 E189D change abolishes oxygen evolution as in Synechocystis but the D1-E189Q in C. reinhardtii also abolishes oxygen evolution and rates in the D1-E189L mutant are greatly reduced compared to wild-type. The kinetic data confirms that there is little or no photosynthetic activity in the D1-E189Q mutant and reduced activity in D1-E189L. Further analysis of these mutants is required to provide more details regarding the role of D1-E189 in the mechanism of water oxidation but it is clear that it is required, perhaps as part of the network of hydrogen bonds that are involved in transferring the proton abstracted from YZ to the lumenal surface (Chu et al., 1995a & b).

This work has provided further information regarding the roles of amino acids involved in the mechanism of water oxidation. Now that a successful method of isolating PSII from C. reinhardtii has been established further analysis is required to obtain a better understanding of the effects of such mutations.
CHAPTER 4

A Rapid Purification of PSII from *Chlamydomonas reinhardtii* - using the His-tagged PsbH Polypeptide
4.1 Introduction

The study of photosynthesis requires a multidisciplinary approach including biochemical, biophysical, molecular, genetic and crystallographic studies. The green alga *C. reinhardtii* is used as a model organism to study photosynthesis (Section 1.7). It is amenable to genetic manipulation and photosynthetic mutants can be created quickly and easily in comparison with higher plants. Genetic manipulation, in terms of site-directed mutagenesis, allows single amino acids to be probed and the effects of such changes to be investigated.

The availability of such site-directed mutagenesis systems in *C. reinhardtii* provides the opportunity to make alterations of amino acids thought to be important in PSII function. One disadvantage of using *C. reinhardtii* to study PSII function is the difficulty with which active PSII can be isolated. In higher plants a number of preparations have been developed that allow PSII to be purified from thylakoid membranes (Berthold *et al.*, 1981; Eshaghi *et al.*, 1999; Ford & Evans, 1983). These methods all exploit the phenomenon that in higher plant thylakoids there is a physical separation of PSI and PSII under defined conditions (Section 1.3, Figure 1.5), with PSII located mainly in the granal stacks and PSI mainly in the unstacked, stromal regions (Andersson & Anderson, 1980). This distribution allows detergents to be used to solubilise the non-stacked thylakoids, hence PSII fragments can be recovered via centrifugation. Unfortunately, this stacking does not occur to any great extent in *C. reinhardtii* and without the ordered separation of the photosystems such a technique cannot be employed efficiently. A number of PSII preparations from *C. reinhardtii* have been developed (de Vitry *et al.*, 1991; Bumann & Osterhelt, 1994; Shim *et al.*, 1990) but these are generally lengthy procedures often resulting in labile PSII in small quantities that preclude the use of many standard investigative techniques. In recent years PSII preparations from *C. reinhardtii* have been developed using histidine
tagging followed by metal affinity chromatography. The first published was the tagging of the D2 polypeptide at its C-terminal end (Sugiura et al., 1998). This allowed the purification of a core PSII preparation.

Immobilised metal affinity chromatography (IMAC) can be used to purify proteins that are tagged with histidine (Section 1.6). Histidine residues form particularly stable co-ordination compounds with metal ions due to participation of imidazole side-chains in chelation. However, because histidines are relatively uncommon, most proteins do not exhibit high affinities for metal complexes.

In this work the chelating ligand used was nitrilotriacetic acid (NTA) (Qiagen). NTA occupies four of the six ligand binding sites in the co-ordination sphere of the nickel ion (Figure 4.1) leaving two sites free to interact with the his-tag. NTA binds the metal ions under a wide range of conditions hence, it is ideal for purification of a wide range of proteins.

This work describes the tagging of the *C. reinhardtii* PSII polypeptide PsbH and the rapid purification of PSII that retains very high levels of O₂ evolution. A hexa-histidine tag was engineered at the C-terminal end of the small PsbH polypeptide. PSII was successfully purified by binding this protein to nickel using nickel-NTA technology (Qiagen). This chapter explains the construction of the tagged *C. reinhardtii* strain, the development and optimisation of the purification technique, and subsequent analysis of PSII from *C. reinhardtii* that proved to be suitable for biophysical and optical analysis.
Figure 4.1 Interaction between residues of the his-tag and the nickel-NTA matrix. Two nickel ligand binding sites remain free to interact with the histidine residues. This figure shows two neighbouring histidines binding to the Ni-NTA, however two non-consecutive histidines may bind depending on protein structure and chain flexibility.
4.2 Construction of the His-tagged PsbH Transformant

4.2.1 Construction of the his-tagged psbH plasmid

In order to purify PSII using IMAC a C. reinhardtii transformant was created with a tag of six histidines at the C-terminal end of the chloroplast-encoded psbH gene. Plasmid p72B, containing the 4.4 kb *EcoRl* fragment (*Eco19*) of the *C. reinhardtii* chloroplast genome inserted into pUC8, was obtained from the Chlamydomonas Genetics Centre, Duke University, North Carolina, USA. This fragment contains the *psbH* gene (Figure 4.2a). This plasmid was manipulated to create the tagged version of the *psbH* gene. The procedure is summarised in Figure 4.2b. In order to eliminate unwanted *NheI* sites upstream of *psbH*, p72B was cut with *SmaI* and *HpaI* to produce plasmid p72B-SH that contains a unique *NheI* site 40 bases downstream of the *psbU* stop codon and a unique *BstXI* site within the coding sequence. This *BstXI-NheI* region was replaced using a PCR strategy in which the downstream primer extended PsbH by eight codons (encoding QLHHHHHHH). A unique *MfeI* site was also introduced into this modified region, to serve as a molecular marker for the tagged *psbH*. The primer sequences are shown below, the histidine tag is shown in red and the *NheI* and *MfeI* restriction sites are shown in green and blue respectively.

**Forward primer**

NRH1 - 5'-aaagctgctgtctctgaagg-3'

**Reverse primer**

NRH6R - 5'-gggctagctttagttgctcagggag-3'

The reaction conditions were as given in Section 2.6.6, but, after an initial denaturing step, two cycles were used with different annealing temperatures. The first step consisted of five cycles with an annealing temperature of 54°C for 1 min. The second step consisted of 25 cycles with an annealing temperature of 62°C for 1 min. A product of ~550 bp with a
BstXI site at the 5’ end and an NheI site at the 3’ end was cloned into the BstXI and NheI sites of p72B-SH to create pH-his6 (Figure 4.2b).

4.2.2 Sequencing of tagged psbH plasmid

The plasmid pH-his6 was sequenced (Section 2.7) to confirm the tag was present and in-frame with the psbH gene and that the unique restriction enzyme site, MfeI, was present. The sequencing primer H2P (5’ TCA ATT ATG GCA AC A GG 3’) was used to sequence through the psbH gene and subsequent tag. The resulting sequence is given in Table 4.1.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>psbH tagged sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH-his6</td>
<td>5’atg gca aca gga act tct aaa gct aaa cca M A T G T S K A K P *10</td>
</tr>
<tr>
<td>(*amino acid numbering from the psbH start codon)</td>
<td>tca aaa gta aat tca gac ttc caa gaa cct S K V N S D F Q E P 20</td>
</tr>
<tr>
<td></td>
<td>ggt tta gtt aca cca tta ggt act tta tta G L V T P L G T L L 30</td>
</tr>
<tr>
<td></td>
<td>cgt cca ctt aac tca gaa gca ggt aaaa gta R P L N S E A G K V 40</td>
</tr>
<tr>
<td></td>
<td>tta cca ggc tgg ggt aca act gtt tta atg L P G W G T T V L M 50</td>
</tr>
<tr>
<td></td>
<td>gct gta ttt atc ctt tta ttt gca gca ttc A V F I L L F A A P 60</td>
</tr>
<tr>
<td></td>
<td>tta tta atc att tta gaa att tac aac agt L L I I I E I Y N S 70</td>
</tr>
<tr>
<td></td>
<td>tct tta att tta gat gac gtt tct atg agt S L I I L D D V S M S 80</td>
</tr>
<tr>
<td></td>
<td>tgg gaa act tta gct aca gtt tct cca ttg W E T L A K V S Q L 90</td>
</tr>
<tr>
<td></td>
<td>cac cac cac cac cac cac taa H H H H H H * 96</td>
</tr>
</tbody>
</table>

Table 4.1 Sequence of the his-tagged psbH gene.
Figure 4.2a The Eco19 fragment of the *C. reinhardtii* chloroplast genome.

Figure 4.2b Schematic summary of the construction of pH-his\(_6\). This plasmid contains the *psbH* gene tagged at the C-terminal end with six histidines. p72B was cut with *SmaI* and *HpaI* to create p72B-SH. PCR was used to create a tag of six histidines at the 3’ end of the *psbH* gene. p72B-SH and the PCR product were each digested with *BstXI* and *NheI*, and the endogenous fragment of p72B-SH was replaced with the PCR fragment creating the plasmid pH-his\(_6\).
4.2.3 Transformation of C. reinhardtii using the his-tagged psbH gene

The plasmid, pH-his6, was introduced into C. reinhardtii using the biolistic technique and the transforming DNA entered the genome by homologous recombination. The target strain was a psbH disruption mutant, H-null opposite (O’Connor et al., 1998), in which psbH is disrupted with the spectinomycin resistance cassette, aadA. The H-null mutant lacks PSII and is unable to grow photoautotrophically, therefore, transformants were selected for by recovery of photoautotrophic growth under moderate light (~45μE/m²/s).

4.3 Characterisation of the His-tagged Transformant

Colonies arising from transformation of H-null with pH-his6 (Section 4.2.3) were picked and taken through three rounds of single colony isolations on the selective medium HSM, in order to obtain homoplasmic lines. Total DNA was purified from these transformants, H-null and CC-1021 by the method given in Section 2.5.1. The DNA was used to carry out Southern blotting, PCR and sequencing analysis. Growth characteristics and the oxygen-evolving capacity of the transformants were analysed. Fluorescence and thermoluminescence measurements were also undertaken, the details of which are presented in Chapter 3.

4.3.1 Southern analysis of transformants

Transformants were analysed by Southern blotting to confirm that the PsbH-disrupted strain, H-null, had been transformed with the tagged psbH gene. Total genomic DNA digested with EcoRI and doubly digested with EcoRI and MfeI (Section 2.6.1) was separated on a 0.8% TAE gel by electrophoresis at 40V. The DNA was transferred to a nylon membrane (Section 2.8.1) and hybridised to a probe specific for the psbH gene. Figure 4.3a shows the autoradiogram of this blot. The presence of a single hybridising band of approximately 4.4 kb for the transformant shows that
the psbH gene has correctly inserted into the H-null genome (Figure 4.3a&d). The blot also confirms the homoplasmicity of the transformant as there is no trace of the 6.4 kb hybridising band detected in H-null. When the DNA is digested with both EcoRI and MfeI the single hybridising band is reduced only in the transformant, to ~3.6 kb, this shows that the MfeI site is present in the transformant only (Figure 4.3a&d). As the unique restriction enzyme site is present, the tag is also very likely to be present. Wild-type and H-null DNA have been included as controls.
Genomic DNA was prepared from WT, H-null and a H-hiS transformant. DNA was digested with EcoRI or with EcoRI and MfeI. The filter was probed with the 250bp BstX/Avel fragment of psbH.

**Figure 4.3** Southern analysis of a putative H-hiS transformant. The Southern blot shows that the H-hiS transformant contains the psbH gene and the unique restriction enzyme site MfeI (a). Figures b,c and d show the expected sizes of fragments to which the probe hybridises in wild-type, H-null and transformant strains respectively.
4.3.2 PCR and sequencing analysis of transformants

PCR was used to amplify the \textit{psbH} gene from total genomic DNA isolated from wild-type, H-null and the H-his\textsubscript{6} transformant cells. The PCR fragments can be used as a quick alternative to a Southern blot to ascertain whether the \textit{psbH} gene has been restored. More importantly the PCR product can be sequenced to confirm the restoration of the gene and the incorporation of the his-tag.

The PCR experiments were carried out using two primers, shown below, which flanked the \textit{psbH} gene.

\textbf{Forward primer}

NRH2P - 5'-tcaattatggcaacagg-3'

\textbf{Reverse primer}

NRpsbHi3' - 5'-cccgggatccaaagaaaagtgagctattaacg-3'

The reaction conditions were as given in Section 2.6.6, including 20 cycles with an annealing temperature of 56°C for 1 min. This combination of primers produced a fragment \textasciitilde450 bp for the transformant and wild-type, while the H-null fragment was \textasciitilde2450 bp (Figure 4.4).

In order to verify that the his-tag was present on the \textit{psbH} gene, the PCR products from amplification of transformant DNA with primers NRH2P and NRpsbHi3' were sequenced with primer NRH2P (Section 2.7). Figure 4.5 shows an extract from the automated DNA sequencing and the his-tag can be seen in shaded boxes. This sequence shows the tag to be in-frame with the \textit{psbH} gene as expected.
Figure 4.4 PCR analysis of H-his₆ transformants. The gel photograph shows wild-type, H-null and H-his₆ transformant PCR products when the given primers, flanking the psbH gene, are used. The product of ~450bp for wild-type and the transformant indicates that the psbH gene is present. The product of ~2450bp for the H-null strain indicates the disruption of the psbH gene with the aadA cassette. Lane 4 is a control where the PCR reaction took place in the absence of template DNA.
Figure 4.5 Automated DNA sequence of the sense strand of the PCR-amplified \(psb\text{H-his}_{6}\) gene in the \(C.\ reinhardtii\) strain H-his\(_{6}\) (Figure 4.4). The six-histidine tag is seen at the C-terminal end in shaded boxes. The coding region is underlined.
4.3.3 Growth analysis of transformants

The phenotype of the H-his₆ transformant was examined by comparing growth in the light on TAP, TAP supplemented with spectinomycin, and HSM. Under these conditions the H-his₆ transformant grows as wild-type, this is shown in Figure 4.6a. The loss of the *aadA* cassette in the transformant is shown by its inability to grow on TAP supplemented with spectinomycin. The plates were incubated for the same period of time, showing the slower photoautotrophic growth of both wild-type and the H-his₆ transformant on HSM, compared to the faster mixotrophic growth on TAP.

Growth curves were carried out as in Section 2.3.7 in liquid TAP and HSM. These growth curves (Figure 4.6b, growth in TAP) show the growth of the transformant to be comparable to that of wild-type.
Figure 4.6a Spot tests showing the growth characteristics of H-his₆. Three 15μl aliquots (labelled A, B and C) of cells from liquid culture were spotted onto solid media and grown under moderate light. These spot tests show the growth phenotype of the H-his₆ transformant compared with the wild-type and H-null strains. The transformant behaves as wild-type under these conditions.

Figure 4.6b H-his₆ transformant growth curves. These measurements of growth in TAP media show that the growth pattern of H-his₆ is similar to wild-type.
4.3.4 Oxygen evolution

In order to assess whether the addition of the his-tag on the C-terminal end of PsbH had any detrimental effect on the oxygen-evolving activity of the PSII complex, transformant cells were subjected to steady-state oxygen evolution analysis as described in Section 2.3.6. Measurements were taken in the presence of the electron acceptors DMBQ and K$_3$Fe(CN)$_6$. The oxygen evolution rates obtained for wild-type and transformant cells are comparable (Table 3.2), indicating that the tag does not have a detrimental effect on the oxygen evolving capacity of PSII.

<table>
<thead>
<tr>
<th>Sample</th>
<th>O$_2$ evolution rate (μmolO$_2$/mg Chl$/^*$hr)</th>
<th>Chl a / Chl b ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type cells</td>
<td>264 ± 18</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>H-null (PSII)$^+$ cells</td>
<td>0</td>
<td>---</td>
</tr>
<tr>
<td>H-his$_6$ transformant cells</td>
<td>272 ± 6</td>
<td>2.5 ± 0.1</td>
</tr>
</tbody>
</table>

*Chlorophyll assay by the method of Porra et al. (1989)

**Table 4.2** The oxygen evolution characteristics of wild-type, H-null and H-his$_6$ transformant cells. Each value is from measurements in triplicate from three different cell cultures.

### 4.4 Purification of PSII from H-his$_6$ transformants

PSII from *C. reinhardtii* was purified according to the method given in Section 2.3.4. This method was optimised to reproducibly produce a highly active preparation suitable for biophysical analysis. The PSII isolated was analysed using a variety of methods to ensure that there was no contamination from other photosynthetic complexes such as PSI. Basic biochemical analysis was carried out and the polypeptide content analysed by SDS-PAGE and Western analysis. Such analysis of PSII isolated during the optimisation procedure showed that the alteration of conditions allowed complexes with differing polypeptide composition to be isolated.
4.4.1 Oxygen evolution

In order to assess activity, the isolated PSII was subjected to steady-state oxygen evolution analysis as described in Section 2.3.6. Measurements were taken in the presence of the electron acceptors DMBQ and K$_3$Fe(CN)$_6$. Measurements were routinely taken in the presence and absence of calcium, the presence of which increased the oxygen-evolving capacity. Table 4.3 shows that the rates of oxygen evolution are very high, indicating a highly active PSII preparation. The chlorophyll a:b ratio is also high which indicates that the preparation does not contain light harvesting complexes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>O$_2$ evolution rate $\mu$molO$_2$/mg Chl* / hr</th>
<th>Chl a / Chl b ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-his$_6$ PSII</td>
<td>3700 ± 1000</td>
<td>9.0 ± 1.1</td>
</tr>
</tbody>
</table>

*Chlorophyll assay by the method of Porra et al. (1989)

**Table 4.3** The oxygen evolution characteristics of isolated H-his$_6$ PSII. The value is from measurements in triplicate from three different preparations.

4.4.2 Polypeptide profile of H-his$_6$ PSII

The isolated PSII was run on polyacrylamide gels as given in Section 2.9. Initially, Coomassie-stained bands were tentatively identified by comparison with spinach PSII (Ikeuchi & Inoue, 1988; Eshaghi et al., 1999). The pea PSII polypeptides were identified by antibody binding (Figure 4.7). As a control for this polyacrylamide gel wild-type cells were treated exactly as the H-his$_6$ cells and were run through a Ni-NTA column as in Section 2.3.4, some non-specific binding did occur but this was minimal as can be seen in Figure 4.7 (lane 2). In order to more accurately assign polypeptides to a specific band Western analysis was carried out as given in Section 2.9.3. The results of this analysis are shown in Figure 4.8.
where antibody binding was detected by the ECL method (Section 2.9.3). The polypeptides D1, D2, CP43, OEE2, acyt b_{559} and PsbW (not shown, Bishop et al., submitted) were all identified in the PSII isolated. The 33 kDa protein was not identified using the antibody raised against the pea protein but the polypeptide is thought to be present given that the 23 kDa is identified and our knowledge of PSII shows that the 33 kDa is required for subsequent binding of the other extrinsic polypeptides (Miyao & Murata, 1983; Miyao & Murata, 1989). It should also be noted that the phosphorylated PsbH polypeptide has been identified in this PSII preparation using phosphothreonine antibodies (Olaf Kruse, personal communication, 2000) and the his-tagged PsbH has been tentatively identified on a polyacrylamide gel using a Pierce Gel Code System (Illinois, USA).

In order to confirm that the preparation was not contaminated with PSI, a Western blot loaded with both thylakoids and PSII was probed with an antibody raised against the intrinsic PSI polypeptide, PsaA. This blot, shown in Figure 4.9, confirms the absence of PSI in this PSII preparation, even when ten-fold more PSII was loaded onto the polyacrylamide gel than is routinely used for Western analysis.
Figure 4.7 Coomassie-stained SDS-PAGE gel showing putative PSII subunits in the *C.reinhardtii* H-his$_6$ PSII. Bands were tentatively identified by comparison with similar gels where pea PSII bands were detected with antibodies.
Figure 4.8 Western analysis of H-his₈ PSII. Antibodies against a small number of polypeptides were available. Analysis shows the D1, D2, CP43, 23kDa, cyt₇559 and PsbW (not shown) to be present in this preparation. Western analysis indicates that the key polypeptides involved in oxygen evolution and PSII RC reactions are present.
Figure 4.9 Western analysis of H-his$_6$ PSII showing the absence of PSI. This blot shows the detection of the PsaA polypeptide in $C.\ reinhardtii$ thylakoids and its absence in PSII isolated using the strain H-his$_6$. This blot confirms that the isolated PSII is free of PSI.
4.4.3 EPR spectroscopic measurements of H-his$_6$ PSII

The isolated PSII should be suitable for biophysical analysis. In order to confirm this suitability, some initial EPR measurements were made using the preparation. Using EPR spectroscopy it is also possible to establish the PSI and PSII content of mutants or preparations by quantifying the characteristic spectra from their electron transfer components; for example, the oxidised tyrosine Y$_D$ signal is indicative of PSII whereas the P700 signal is indicative of PSI. EPR can detect the presence of the photosystems even at low levels, and hence it is an extremely sensitive diagnostic tool. In Figure 4.10a the spectrum of Y$_D$ (a marker of PSII) is absent in the H-null thylakoids in the dark but is restored in the H-his$_6$ transformant thylakoids in the dark (Figure 4.10b). Upon illumination, the signal for the P700$^+$ chlorophyll (a marker of PSI) is present in both H-null and H-his$_6$ thylakoids (Figure 4.10a and b, illuminated). These spectra confirm that the H-null mutant lacks PSII but contains PSI, whereas the H-his$_6$ transformant contains both PSI and PSII. The purified H-his$_6$ PSII retains the Y$_D$ spectrum (Figure 4.10c, dark) but has lost the light-induced P700$^+$ signal. This shows that the H-his$_6$ PSII does not contain PSI. The small increase on illumination of the H-his$_6$ PSII (Figure 4.10c, illuminated) is partly due to oxidised Y$_D$ and partly to a PSII Chl$^+$ species (ChlZ) which can be distinguished from P700$^+$ by its broader line width. No signal from the PSI iron-sulphur centre A or B was detected by EPR (not shown) confirming that this small increase was not due to residual PSI.
Figure 4.10 EPR spectra showing the recovery of PSII in the H-his₆ transformant and the purified PSII from the nickel column chromatography. In each pair of spectra, the inner line in black is the dark spectrum, whereas the coloured line is the spectrum following illumination for 2.5 min at 12K. EPR conditions: microwave power 0.001mW, modulation amplitude 0.2mT, temperature 12K, microwave frequency 9.090GHz.
The H-his$_6$ PSII also allows detection of the Mn multiline signal that can be seen in the S2 state (Nugent, 2001 and references therein). This S2 EPR signal is detected easily in pea and spinach PSII; in the example for pea PSII, shown in Figure 4.11a, both the Mn multiline (labelled 3) and the $g = 4.1$ (labelled 1) forms of S2 signal are present. We were only able detect a very small multiline in *C. reinhardtii* thylakoids, but in H-his$_6$ PSII a Mn multiline very similar to that in higher plants is detected (Figure 4.11b). We see much less $g = 4.1$ signal, however, in the *C. reinhardtii* PSII sample shown. In Figure 4.11a and b some cyt $b_{559}$ is also oxidised by the 200K illumination (labelled 2). Spectrum C is that of a H-his$_6$ PSII sample prepared so that cyt $b_{559}$ was pre-oxidised and therefore unavailable as an electron donor. In this spectrum no cyt $b_{559}$ signal is seen, the Mn multiline was smaller in amplitude and the $g = 4.1$ signal was slightly increased (Figure 4.11c). This series of spectra also shows that in the isolated H-his$_6$ PSII the iron-semiquinone signal from QA (labelled 4) is more prominent than in pea PSII (Figure 4.11b). The $g = 1.9$ form of this signal is present in pea PSII (Figure 4.11) whereas *C. reinhardtii* PSII (4.11b & c) contains a sharper $g = 1.8$ form.
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Figure 4.11 EPR spectra showing the S\textsubscript{2} state of the Mn cluster of PSII in pea and C. reinhardtii. Each trace is the difference spectrum of a dark-adapted sample and this sample following 200K illumination. The spectrum for pea PSII (a) acts as a control/comparison for the C. reinhardtii spectra. In pea PSII both the Mn-multi-line (3) and the g = 4.1 (1) EPR signals can be seen. The oxidised cyt\textsubscript{b}\textsubscript{559} signal (2) is also clearly seen. In the C. reinhardtii spectra (b and c) a Mn-multi-line, similar to that seen in pea, is present. The g = 4.1 signal is much smaller in these spectra. Spectrum c is that of C. reinhardtii prepared so that the cyt\textsubscript{b}\textsubscript{559} is fully oxidised; hence, no signal is seen.

Concentrations of samples are as follows: Pea PSII 11mg/ml, H-his\textsubscript{6} PSII 3.2mg/ml, H-his\textsubscript{6} PSII with oxidised cyt\textsubscript{b}\textsubscript{559} 1.9mg/ml.

EPR conditions: microwave power 10mW, modulation amplitude 2mT, temperature 6.5K, microwave frequency 9.090GHz.
4.5 Discussion

PSII-enriched thylakoid membranes from higher plants, such as pea and spinach, are widely used for the biophysical analysis of the PSII complex. Methods to isolate such membranes are generally based on, or similar to, that described by Berthold et al. (1981). Given the lack of a high-resolution PSII structure until very recently (Zouni et al., 2000) such preparations have proved invaluable in investigating the mechanism of PSII. Unfortunately, higher plants are not as amenable to genetic engineering as the green alga *C. reinhardtii* is. One important advantage *C. reinhardtii* has over plants is its ability to grow on acetate, allowing it to dispense with photosynthetic carbon assimilation. This allows mutations to be created in the photosynthetic complexes that would be lethal in higher plants that do not possess this useful characteristic. Unfortunately, purification of *C. reinhardtii* PSII has proved difficult but metal affinity chromatography has now been used to purify PSII containing histidine-tagged polypeptides as discussed in Section 1.6.

In this work we used the PSII polypeptide PsbH as the site of the histidine tag in the hope that we could purify a more complete PSII complex than that of Sugiura et al. (1998), who tagged the central PSII polypeptide, D2 (PsbD). We expected to isolate a more complete PSII complex on the premise that PsbH is located towards the periphery of the PSII complex, possibly in the region where two PSII monomers join to form a dimer (Hankamer et al., 1999). The tagging of the PsbH polypeptide also allows its isolation for further studies which may shed light on the elusive function of this phosphoprotein, essential for PSII assembly/function. The choice of PsbH as the tagged peripheral protein allowed selection of tagged transformants without the use of a selectable marker, transformants were selected for by the recovery of photosynthetic growth (Section 4.2.3). This is advantageous as it facilitates subsequent mutations to be made in the histagged mutant using the limited selectable markers available (as shown in
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Chapter 6).

The procedure for purifying PSII from the H-his$_6$ transformant is described in Section 2.3.4. This procedure has been developed and optimised to reproducibly produce a highly active preparation for biophysical analysis. However, we believe our PSII yield is only about 10-15% that expected from the volume of cells on a chlorophyll basis. This low yield could be due to a number of factors. Firstly, the histidine tag may be partially or completely lost due to proteolysis. We attempted to overcome this by adding a cocktail of protease inhibitors (Sigma) at various points throughout the purification procedure. Moreover, further work was carried out to extend this his-tag to overcome the possibility of reduced binding capacity due to the reduced length of the tag. This extension is described in Chapter 5 of this thesis. It is also possible that PsbH is not present in all the solubilised PSII complexes: if so, the PSII isolated using the tag on PsbH represents only a single fraction of a mixture of solubilised PSII complexes with different polypeptide composition. Following from this argument is the possibility that there is a high percentage of PsbH no longer bound to PSII complexes; as such, many of the Ni$^{2+}$ binding sites are chelating just the polypeptide, not a complete complex. These ideas appear to be in-line with the fact that when the volume of Ni-NTA column used was increased the yield of PSII remained the same. Obviously, if the poor yield were only due to a lack of binding sites our yield would increase after this alteration.

A further possible factor that may have affected PSII yield was the presence of copper in the growth media of *C. reinhardtii* cells. Initial EPR analysis identified a large, contaminating copper signal not expected from isolated PSII. Copper is one of a number of metals that can chelate histidine residues (Arnold & Haymore, 1991) so it was thought likely that this signal was due to copper being bound to some of the his-tags. Only two histidine residues are required to bind to the NI-NTA column; up to four sites may remain free to bind copper that is present in *C. reinhardtii* growth media.
TAP (Rochaix, 1988). This may affect the binding of PSII to the column, perhaps reducing the total amount of PSII that could be isolated using this method. It is also possible that having copper bound at the C-terminal end of PsbH affects the activity of PSII. In order to counteract this problem, H-his₆ C. reinhardtii cells were grown in media lacking either copper or copper and cobalt (another metal than can bind histidine residues). This did produce PSII lacking the copper EPR signal, which is more suitable for biophysical analysis, but the yield was not improved.

Despite recovering only a small percentage of the total PSII expected and as yet not being able to identify the precise reason, it should be noted that the column wash (or run through) does not evolve oxygen even when treated exactly as the eluted PSII. This suggests that although SDS-PAGE and Western blotting can detect PSII polypeptides in the column run-through, the PSII is not active, and therefore it could be argued that we were isolating all the oxygen-evolving PSII.

Given the problems I have experienced in working with the D2-his-tagged strains (detailed in Chapter 3), it appears that the tagging of a peripheral polypeptide has a lesser effect on the function of the PSII complex and hence, is a superior method by which to isolate active PSII from C. reinhardtii. During the optimisation procedure it was discovered that the alteration of conditions allowed complexes with differing polypeptide composition to be isolated. These various PSII preparations may prove useful in further studies of PSII, such as its assembly, and perhaps in structural studies or crystallography. In summary, the his-tagging of PsbH to isolate PSII has proved to be an efficient method. The use of the H-his₆ tagged strain to purify mutated PSII is detailed in other chapters of this thesis.
5.1 Introduction

Epitope tagging, first described by Monro & Pelham in 1984, is a recombinant DNA method for making a gene product immunoreactive to an existing antibody. Very simply, a polynucleotide encoding a short continuous epitope is inserted in the correct frame into a gene of interest and the modified protein is expressed in an appropriate host. The expressed fusion protein can then be detected using the appropriate antibody.

Epitope tagging facilitates rapid localisation and purification of the tagged polypeptide. It is also a powerful method by which to assess expression of the tagged polypeptide and to follow the movement of proteins. The technique has also been utilised to investigate the topology of integral membrane proteins and to investigate protein-protein interactions.

Epitope tagging is extremely useful for detecting proteins that are poorly immunogenic or in cases where cross-reaction of the specific antibody with related proteins complicates analysis. It also eliminates the time consuming and unpredictable task of producing an antibody specific to the protein being studied.

In this work I have attempted to tag the *C. reinhardtii* PsbH polypeptide with two epitope tags, the poly-histidine tag and the haemagglutinin (HA) tag. In addition, I have taken the rather unorthodox approach of trying to tag the small PsbH polypeptide with the mature plastocyanin protein.

As discussed in Chapter 4, histidine tagging of PsbH allows purification of active PSII from the green alga, *C. reinhardtii*. In an attempt to increase the yield of PSII, using the method described in this thesis, I aimed to increase the histidine tag from six to ten residues. We believed that such action could help to prevent the degradation of the tag by proteases and, hence increase the amount of PSII recovered from the column (Section
4.5). The extension of the his-tag at the C-terminal end of PsbH is presented in this chapter (Section 5.2.3).

Polyhistidine tags are very popular for purification purposes because the tagged protein can be isolated using IMAC (Section 4.1). However, the use of the his-tag as an epitope has still not found favour, perhaps because many of the available antibodies, so far, lack high specificity and low background (Jarvick & Telmer, 1998). Detection of the his-tagged PsbH polypeptide using an anti-his antibody was not observed in my research and the detection of PsbH using a specific antibody has also proved unsuccessful in this laboratory (Cain, 1998). In a further attempt to detect PsbH a HA epitope tag was used in tandem with a poly-histidine tag for PSII/protein purification. The influenza haemagglutinin epitope (YPYDV PDY A) is widely used as a general epitope tag and antibodies are commercially available. The standard HA tag was altered to incorporate amino acid codons favoured by C. reinhardtii and a 6x histidine tag (his6) was incorporated at the 3’ end of the HA epitope tag. (Smith, 2000). This HA-his6 cassette was added to the 3’ end of the psbH gene. The incorporation of this tag, its expression and detection are described in this chapter (Section 5.2.2 & 5.4).

As the general procedure for tagging the PsbH polypeptide proved favourable we decided to try a new approach to tagging the C. reinhardtii protein. As discussed elsewhere in this thesis, EPR spectroscopy is a technique used routinely to study photosynthetic RCs. EPR spectroscopy allows signals specific to certain transition metals to be identified and any changes due to RC manipulation to be followed. Plastocyanin is a small, (10 kDa) Type I copper protein that functions as an electron donor in PSI. As a copper containing protein, plastocyanin has specific spectral qualities, suitable for EPR analysis, and a good optical spectrum, so would be good as a general spectroscopic marker. Our reasoning for tagging a PSII polypeptide with this copper-containing protein was twofold. Firstly, and
most importantly, as PSII does not contain any copper we hoped to be able
to use the protein as a specific, spectroscopic marker allowing a new PSII
EPR signal to be seen. We predicted that this could be used to probe the
PSII structure by studying its interaction with intrinsic PSII signals.
Secondly, we hoped it would provide more information regarding the
constraints of tagging small proteins. Tagging the C-terminal end of PsbH
with plastocyanin is presented in Section 5.2.1.

The following chapter presents the construction of tagged psbH
genes and the analysis of the C. reinhardtii transformants.

5.2 Creation of Tagged psbH Genes

5.2.1 Construction of the pH-plastocyanin plasmid

The pea petE mature coding sequence was tagged onto the 3' end of
the C. reinhardtii psbH gene as described below and in Figure 5.1.

The mature protein coding sequence for pea plastocyanin was
amplified from the plasmid pUCPCR, (Gift to Saul Purton from John Gray,
Cambridge University) which contains the pea petE gene, using the
following oligonucleotides:

Forward primer
NRPC1 - 5'-gcaatcaattggctgttgagg-3'
Reverse primer
NRPC2 - 5'-gcaggtccgactctagagg-3'

The reaction conditions were as given in Section 2.6.6 with an
annealing temperature of 63°C for 1 min. A product of ~350 bp with a MfeI
site at the 5' end and an XbaI site at the 3' end was cloned into the MfeI and
NheI sites of pH-his to create pH-plastocyanin (Figure 5.1). The primer
NRPC1 altered the first alanine in the ALA cleavage signal of the transit
peptide to glutamine, thus disrupting the signal for cleavage by the
thylakoid processing peptidase.
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Figure 5.1a The thylakoid targeting sequence and mature protein regions of the pea petE gene in pUCPCR.

Figure 5.1b Schematic summary of the construction of pH-plastocyanin. This plasmid contains the psbH gene tagged at the C-terminal end with the mature plastocyanin protein from pea. pUCPCR was used as a template to create a PCR fragment containing the mature pea plastocyanin with the restriction sites MfeI and XbaI upstream and downstream of the mature protein. Digestion of the PCR product with these enzymes allowed it to be cloned into the plasmid pH-his6 cut with MfeI and Nhel. This created the plasmid pH-plastocyanin.
5.2.2 Construction of pH-HA<sub>2</sub>his<sub>6</sub> plasmid

The *psbH* gene of *C. reinhardtii* was tagged at its carboxy-terminal end with a haemagglutinin (HA) epitope (YPYDVPDYAGYPYDVPDYAPWHHHHHH stop) as detailed below and in Figure 5.2.

A haemagglutinin/6xhis epitope cassette was blunt-cloned into the *EcoRV* site of the vector pSK (Stratagene) creating pSKHATc<sup>R</sup>H<sub>6</sub> (Smith, 2000). The HATc<sup>R</sup> epitope cassette was excised from pSKHATc<sup>R</sup>H<sub>6</sub> with *EcoRI*, yielding a 1.33 kb cassette. This was cloned into the *MfeI* site of pH-his<sub>6</sub> creating the plasmid pH-hisHATc<sup>R</sup>. The cassette conferring tetracycline resistance was then dropped out of pH-hisHATc<sup>R</sup> by digestion with *NcoI* creating pH-HA<sub>2</sub>his<sub>6</sub>, a plasmid in which *psbH* is tagged with the HA epitope and six histidine residues (Figure 5.2).

5.2.3 Construction of pH-his<sub>10</sub> plasmid

An extension of 10 histidine residues was tagged onto the carboxy-terminal end of the *C. reinhardtii* psbH gene as described below and in Figure 5.3.

The 3' region of the *psbH* gene was amplified from the plasmid p72B-SH using the following oligonucleotides, the ten histidine codons shown in red:

**Forward primer**
NRHD1 -5'-aaagctggcagttctgaagg-3'

**Reverse primer**
NRH10R -5'-gggctagctta(gtg)iocaattgagaaactttagc-3'

The reaction conditions were as given in Section 4.2.1. A product of ~550 bp with a *BstXI* site at the 5' end and an *NheI* site at the 3' end was cloned into the *BstXI* and *NheI* sites of p72B-SH to create pH-his<sub>10</sub> (Figure 5.3).
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Figure 5.2a The codon optimised 6x histidine-tagged 3x haemagglutinin epitope-tagged cassette (Smith, 2000).

Figure 5.2b Schematic summary of the construction of pH-HA$_2$his$_{6}$. This plasmid contains the $psbH$ gene tagged at the C-terminal end with a HA tag followed by six histidines. pSKHATc$^{R}$H$_{6}$ was cut with EcoRI and an 1.33kb fragment was recovered containing the HA cassette. This fragment was cloned into pH-his$_{6}$ linearised with MfeI, creating the plasmid pH-hisHATc$^{R}$. The tetracycline resistance cassette was removed from this plasmid using NcoI. Religation yielded the plasmid pH-HA$_2$his$_{6}$. 
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**Eco19 Fragment - 4.4kb**

![Diagram of Eco19 Fragment - 4.4kb]

*Figure 5.3a* The *Eco19* fragment of the *C. reinhardtii* chloroplast genome.

**PCR Amplification**

incorporating ten histidines at the C-terminal end of the *psbH* gene

![Diagram of PCR Amplification incorporating ten histidines at the C-terminal end of the *psbH* gene]

*Figure 5.3b* Schematic summary of the construction of pH-his\(_{10}\). This plasmid contains the *psbH* gene tagged at the C-terminal end with ten histidines. *p72B* was cut with *SmaI* and *HpaI* to create *p72B-SH* (Table 2.7). PCR was used to create a tag of ten histidines at the 3' end of the *psbH* gene using *p72B-SH* as a template. *p72B-SH* and the PCR product were each digested with *BstXI* and *Nhel*, the endogenous fragment of *p72B-SH* was replaced with the PCR fragment creating the plasmid pH-his\(_{10}\).*
### 5.2.4 Sequencing of tagged psbH genes

All plasmids were sequenced (Section 2.7) to confirm that the tags were present and in-frame with the \( \text{psbH} \) gene. The sequencing primer H2P (Table 2.9) was used to sequence through the \( \text{psbH} \) gene and subsequent tag. The resulting sequences are given in Table 5.1.

<table>
<thead>
<tr>
<th>plasmid</th>
<th>psbH tagged sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{pH-plastocyanin} )  (*amino acid numbering following from ( \text{psbH} ))</td>
<td>\begin{align*}</td>
</tr>
<tr>
<td>5' tgg gaa act tta gct aaa gtt tct caa &amp; ttg W E T L A K V S Q L <em>90 \quad \text{gct gtt gag gtt tgg ctt ggt gcc agt gat} \quad \text{A V E V L L G A S D 100} \quad \text{ggg gtt tgg gct ttt gtt cca agc agt tgg} \quad \text{G G L A F V P S S L 110} \quad \text{gaa gtt agc gct gga gag acc att gta ttc} \quad \text{E V S A G E T I V F 120} \quad \text{aag aac aat ctt cct ctc cac aat gtt} \quad \text{K N N N A G F P H N V 130} \quad \text{gtc ttt gat gaa gac gag att cct gct ggg} \quad \text{V F D E D E I P A G 140} \quad \text{gct gat gca tgc aat tcc atg cct gct gga} \quad \text{V D A S K I S M P E 150} \quad \text{gaa gat ctt ctc aat ggc cct ggt gat gtt} \quad \text{E D L L L N A P G E T 160} \quad \text{tac agg gtc aag ttg gat gct aag gtt acc} \quad \text{Y S V K L D A K G T 170} \quad \text{tac aat ctc tac tgc tca cct cac caa gga} \quad \text{Y K F Y C S P H Q G 180} \quad \text{gct ggt atg gtt gga caa gtc act gtt aat} \quad \text{A G M V G O V T V N 190} \quad \text{taa 3'} \quad \text{•} \end{align</em>}</td>
<td></td>
</tr>
<tr>
<td>( \text{pH-HA2hiS6} )</td>
<td>\begin{align*}</td>
</tr>
<tr>
<td>5' tgg gaa act tta gct aaa gtt tct caa &amp; ttc W E T L A K V S Q F <em>90 \quad \text{tac cca tac gac gta cca gag tac gct ggt} \quad \text{Y P Y D V P D Y A G 100} \quad \text{tat cct tac gat gtt cct gat tac gct cca} \quad \text{Y P Y D V P D Y A P 110} \quad \text{ttg cac cac cac cac cac cac ctaa 3'} \quad \text{W H H H H H H H H H 117} \end{align</em>}</td>
<td></td>
</tr>
<tr>
<td>( \text{pH-his10} )</td>
<td>\begin{align*}</td>
</tr>
<tr>
<td>5' tgg gaa act tta gct aaa gtt tct caa &amp; ttg W E T L A K V S Q L <em>90 \quad \text{cac cac cac cac cac cac cac cac cac cac cac cac} \quad \text{H H H H H H H H H 100} \quad \text{taa} \quad \text{•} \end{align</em>}</td>
<td></td>
</tr>
</tbody>
</table>

*Table 5.1* Sequences of the epitope tag on the 3' end of \( \text{psbH} \) plasmids. The normal amino acid sequence of PsbH is shown in blue, the normal terminus being KVS.
5.2.5 Transformation of *C. reinhardtii* using tagged *psbH* genes

The *C. reinhardtii* mutant H-null opp. (O'Connor et al., 1998) was transformed with the plasmid pH-plastocyanin, pH-HA\textsubscript{his\textsubscript{6}} or pH-his\textsubscript{10} using the biolistic technique, as described in Sections 2.10.1 and 4.2.3. Transformants were selected for by recovery of photoautotrophic growth under moderate light (~45μE/m\textsuperscript{2}/s).

5.3 Southern Analysis of *Chlamydomonas reinhardtii* Transformants

Colonies arising from the transformation of H-null with the plasmids described above (Section 5.2) were picked and taken through three rounds of single colony isolations on the selective medium HSM. In each case a random number of colonies were picked for further analysis. Total DNA was purified from these transformants, H-null and CC-1021 by the method given in Section 2.5.1. Southern blotting analysis was used to confirm whether or not the *psbH*-disrupted strain, H-null, had been transformed with the tagged *psbH* gene. In each Southern blot, total genomic DNA from wild-type and H-null strains of *C. reinhardtii* were included as controls.

Total genomic DNA was digested with *EcoRI* (Section 2.6.1) and was then separated on a 0.8% TAE gel by electrophoresis at 40V. The DNA was transferred to a nylon membrane (Section 2.8.1) and hybridised to a probe specific for the *psbH* gene or specific to the plastocyanin tag itself. Figure 5.4 shows the autoradiogram of the DNA blot of the putative plastocyanin-tagged transformants probed with a fragment of the *psbH* gene. The presence of a single hybridising band of 4.4 kb, rather than the expected 4.8 kb, in each of the transformants shows that after transformation with pH-plastocyanin the *psbH* gene is taken up by *C. reinhardtii* but the plastocyanin tag is lost. In order to confirm that the tag is not present an identical blot was made including linearised pH-plastocyanin as a control. This blot was probed with the 300 bp *MfeI/PacI* plastocyanin
fragment from the plasmid. The autoradiogram of this blot is shown in Figure 5.5. This confirms that the plastocyanin tag is not present as the probe hybridises to the control but not to the putative H-plastocyanin transformants.

Total genomic DNA from wild-type, H-null and putative HA$_2$his$_6$ tagged transformants was digested with EcoRI and doubly digested with EcoRI and Ncol (Section 2.6.1). These digestions were separated on a 0.8% TAE gel by electrophoresis at 40V. The DNA was transferred to a nylon membrane (Section 2.8.1) and hybridised to a probe specific for the psbYl gene. An autoradiogram of this blot is shown in Figure 5.6. The presence of a single hybridising band of approximately 4.4 kb for the transformants shows that after transformation with pH-HA$_2$his$_6$ the psbYl gene has correctly inserted into the H-null genome. The blot also confirms the homoplasmicity of the transformants as there is no trace of the 6.4 kb hybridising band detected in H-null. When the DNA is digested with both EcoRI and NcoI the single hybridising band in the transformant is reduced to approximately 3.7 kb. This suggests that the HA$_2$his$_6$ tag is present, a single NcoI site is found on the tag and the hybridised band is the size expected after the double digestion described (Figure 5.6 a & d).

Unfortunately, and contrary to expectations, transformation with pH-his$_{10}$ does not yield tagged transformants. As for other blots, total genomic DNA digested with EcoRI (Section 2.6.1) was separated on a 0.8% TAE gel by electrophoresis at 40V. The DNA was transferred to a nylon membrane (Section 2.8.1) and hybridised to a probe specific for the psbH. Figure 5.7 shows the autoradiogram of the DNA blot. The presence of a single hybridising band of 6.4 kb, rather than the expected 4.4 kb, in each of the transformants shows that transformation of H-null with pH-his$_{10}$ has not produced any positive transformants.
Genomic DNA was prepared from wild-type, H-null and transformants. DNA was digested with EcoRI. The filter was probed with the 250bp BstXI/NheI fragment of *psbH*. Hybridised band sizes are shown in black, the expected band size for positive transformants is shown in red.

**Figure 5.4** Southern analysis of putative H-plastocyanin transformants. The Southern blot shows that the putative H-plastocyanin transformants contain the *psbH* gene (a&b). However, the plastocyanin tag does not appear to be present (a&d). Figures b,c and d show the expected sizes of fragments to which the probe hybridises in wild-type, H-null and the transformant strains respectively.
Genomic DNA was prepared from wild-type, H-null and transformants. DNA was digested with EcoRI. As a control, the plasmid pH-plastocyanin, which contains the pea plastocyanin mature sequence, was linearised with MfeI. The filter was probed with the 300bp MfeI/PacI plastocyanin fragment from this plasmid. The hybridised band size for the positive control is shown in black, the expected band size for positive transformants is shown in red.

**Figure 5.5** Southern analysis confirming the absence of the plastocyanin tag in transformants. When transformants were probed with the sequence of the tag itself no hybridising bands are seen on the autoradiogram (a). The plasmid containing the *psbH* gene tagged with plastocyanin has been used as a positive control and a hybridising band of the expected size is seen (a&c). Figure 5.5b shows the expected size of fragment to which the probe should hybridise in the transformant. This blot also shows that the pea plastocyanin probe does not recognise *C. reinhardtii* plastocyanin.
Genomic DNA was prepared from WT, H-null and H-HA\textsubscript{2}his\textsubscript{8} transformants. DNA was digested with EcoRI or with EcoRI and Ncol. The filter was probed with the 250bp BstXI/NheI fragment of \textit{psbH}.

**Figure 5.6** Southern analysis of putative H-HA\textsubscript{2}his\textsubscript{8} transformants. The Southern blot shows that the H-HA\textsubscript{2}his\textsubscript{8} transformants contain the \textit{psbH} gene and digestion with EcoRI and Ncol indicates that the HA tag is present (a). Figures b, c and d show the expected sizes of fragments to which the probe hybridises in wild-type, H-null and the transformant strains respectively.
Genomic DNA was prepared from WT, H-null and H-his\textsubscript{10} transformants. DNA was digested with EcoRI. The filter was probed with the 250bp BstXI/NheI fragment of \textit{psbH}.

**Figure 5.7** Southern analysis of putative H-his\textsubscript{10} transformants. The Southern blot shows that the putative H-his\textsubscript{10} transformants do not contain the tagged \textit{psbH} gene. Figures b, c and d show the expected sizes of fragments to which the probe hybridises in wild-type, H-null and the transformant strains respectively.
5.4 Characterisation of the H-HA$_2$his$_6$ Transformant

5.4.1 PCR and sequencing analysis of transformants

PCR was used to amplify the $psbH$ gene from total genomic DNA isolated from wild-type, H-null and the H-HA$_2$his$_6$ transformant cells. The PCR fragments can be used as a quick alternative to a Southern blot to ascertain whether the $psbH$ gene has been restored. More importantly the PCR product can be sequenced to confirm the restoration of the gene and the incorporation of the tag.

The PCR experiments were carried out using two primers, shown below, which flanked the $psbH$ gene.

**Forward primer**
NRH2P - 5'-tcaattatggcaacagg-3'

**Reverse primer**
NR$psb$Hi3' - 5'-cccggatccaagaaaagtgagcta-3'

The reaction conditions were as given in Section 4.3.2. This combination of primers produced an ~500 bp fragment for the transformant, ~450 bp for wild-type and ~2450 bp for H-null (Figure 5.8).

In order to verify that the HA-tag was present on the $psbH$ gene, the PCR products from amplification of transformant DNA with primers NRH2P and NR$psb$Hi3' were sequenced with primer NRH2P (Section 2.7). Figure 5.9 shows an extract from the automated DNA sequencing and the HA tag can be seen in the blue shaded boxes while the his-tag can be seen in the grey shaded boxes. This sequence shows the tag to be in-frame with the $psbH$ gene as expected.
Figure 5.8 PCR analysis of H-HA$_2$his$_6$ transformants. The gel photograph shows wild-type, H-null and H-HA$_2$his$_6$ transformant PCR products when the given primers, flanking the psbH gene, are used. The product of ~500bp for the transformant indicates that the psbH gene is present. The product of ~2450bp for the H-null strain indicates the disruption of the psbH gene with the aadA cassette. The wild-type fragment of 450bp is as expected. Lane 1 is a control where the PCR reaction took place in the absence of template DNA.
Figure 5.9 Automated DNA sequence of the sense strand of the PCR-amplified \textit{psbH-HA}_2\text{his}_6 gene in the \textit{C. reinhardtii} strain H-HA\textsubscript{2}\text{his}_6 (Figure 5.8). The 2xHA tag and the six-histidine tag are seen at the C-terminal end in blue and grey shaded boxes respectively.
5.4.2 Growth analysis of transformants

The phenotype of the H-HA_2his_6 transformant was examined by comparing growth in the light on TAP, TAP supplemented with spectinomycin, and HSM. Under these conditions the H-HA_2his_6 transformant grows as wild-type, this is shown in Figure 5.10a. The loss of the aadA cassette in the transformant is shown by its inability to grow on TAP supplemented with spectinomycin. The plates were incubated for the same period of time, showing the slower photoautotrophic growth of both wild-type and the H-his_6 transformant on HSM, compared to the faster mixotrophic growth on TAP.

Growth curves were carried out as in Section 2.3.7 in liquid TAP and HSM. These growth curves (Figure 5.10b, growth in TAP) show the growth of the transformant to be similar to that of wild-type.
Figure 5.10a Spot tests showing the growth characteristics of H-HA$_2$his$_6$. Fifteen microlitre aliquots of cells from liquid culture were spotted onto solid media and grown under moderate light. These spot tests show the growth phenotype of the H-HA$_2$his$_6$ transformant compared with the wild-type and H-null strains. The transformant behaves as wild-type under these conditions.

Figure 5.10b H-HA$_2$his$_6$ transformant growth curves. This graph shows that the growth pattern of H-HA$_2$his$_6$ is similar to that of wild-type. Cells were grown in TAP media in moderate light.
5.4.3 Oxygen evolution

In order to assess whether the addition of the HA$_2$his$_6$-tag on the C-terminal end of PsbH had any detrimental effect on the oxygen-evolving activity of the PSII complex, transformant cells were subjected to steady-state oxygen evolution analysis as described in Section 2.3.6. Measurements were taken in the presence of the electron acceptors DMBQ and K$_3$Fe(CN)$_6$. The oxygen evolution rates obtained for wild-type and transformant cells are comparable (Table 5.2), indicating that the tag does not have a significant detrimental effect on the oxygen evolving capacity of PSII.

\[
\begin{array}{|l|c|c|}
\hline
\text{Sample} & \text{O}_2 \text{ evolution rate} & \text{Chl } a / \text{Chl } b \\
 & \mu\text{molO}_2/\text{mg Chl}^*/\text{hr} & \text{ratio}^* \\
\hline
\text{Wild-type cells} & 248 \pm 16 & 2.5 \pm 0.1 \\
\text{H-null (PSII') cells} & 0 & --- \\
\text{H-HA$_2$his$_6$ transformant cells} & 231 \pm 11 & 2.4 \pm 0.1 \\
\hline
\end{array}
\]

*Chlorophyll assay by the method of Porra et al. (1989)

**Table 5.2** The oxygen evolution characteristics of wild-type, H-null and H-HA$_2$his$_6$ transformant cells. Each value is from measurements in duplicate from two different cell cultures.

5.4.4 Western analysis

A 15% protein gel was prepared and run as given in Section 2.9. Total cell extracts were prepared from wild-type, H-null and H-HA$_2$his$_6$ transformant cells as described in Section 2.3.8. Equal amounts of these cell extracts were loaded on the basis of chlorophyll concentration. In order to detect the PsbH polypeptide tagged with haemagglutinin, Western analysis was carried out as given in Section 2.9.3. The primary antibody used was High Affinity Anti-HA, rat monoclonal antibody (Boehringer Mannheim)
and the secondary antibody was Anti-rat IgG, HRP-linked (Sigma). The HA-tagged PsbH gene was not detected in this preliminary analysis.

5.5 Discussion

This chapter details the construction of the *C. reinhardtii* *psbH* gene tagged with a variety of epitopes. The success with which the tagged genes are incorporated into the chloroplast genome varies depending on the tag used. Results from Chapter 4 of this thesis show that tagging the C-terminal end of the PsbH polypeptide with six histidines is feasible and has little or no detrimental effect on the function of the PsbH polypeptide or on PSII function.

Results presented in this chapter show that the PsbH polypeptide has been successfully tagged with a HA$_2$his$_6$ tag at its C-terminal end. Preliminary analysis indicates that the tag has little or no detrimental effect on the structure of the protein and PSII appears to function normally. Unfortunately, initial attempts to detect the HA-tag on PsbH using antibody detection proved unsuccessful.

The other extensions used to tag the PsbH polypeptide were the mature pea plastocyanin protein sequence and a tag of ten histidines. Construction of plasmids to be used to transform the *C. reinhardtii* chloroplast genome was successful, however, analysis of transformants showed that the alterations to the genome were different to those expected.

Transformation of the H-null strain with pH-plastocyanin produced transformants which were shown to be homoplasmic. Interestingly, the *psbH* gene had been successfully incorporated into the chloroplast genome but the plastocyanin fragment was not present in the transformed genome. This suggests that the selective pressure to incorporate PsbH, facilitating photoautotrophic growth, is very powerful. The *psbH* gene coding regions are present in the H-null strain, but PsbH is not functional due to disruption with the *aadA* cassette. It is possible that the region flanking the cassette
recombined via with the homologous regions of the gene in the plasmid but did not take up the plastocyanin sequence. A second, and more likely event, is the incorporation of the plastocyanin sequence into the chloroplast genome with the \textit{psbH} gene, followed by subsequent removal. This removal could be for a number of reasons. It is probable that such a tag on the C-terminal end of the small PsbH protein is going to affect the structure of the protein. What is important is the extent in which such an extension will affect the function of the tagged protein. In this case it appears that the tag significantly disrupts protein function and hence the function of PSII. These transformants were selected for by their ability to grow photosynthetically and it appears that this pressure is strong enough to cause the removal of this detrimental fragment of DNA and hence only these altered transformants can grow in the selective conditions. It is also possible that the codons are significantly different to those normally used in the chloroplast genome of \textit{C. reinhardtii} and this may cause the selective cleavage of the plastocyanin DNA.

Interestingly, the same arguments cannot be used when looking at the reasons for the 10x his-tag not being incorporated into the chloroplast genome. In contrast to the case of transformation with the plastocyanin tag, the transformants analysed had not taken up the \textit{psbH} gene and Southern analysis showed the transformants to be as the H-null strain - that is the fragment of DNA to which the probe hybridised to was the same size as that in the H-null strain. H-null itself is unable to grow photoautotrophically, therefore the transformants must be altered in some way as they are able to grow on the selective media. The question raised here is why is the \textit{psbH} gene not incorporated into the genome as in the case for plastocyanin to make a wild-type genome and functional PSII? It is possible that the 10-histidines bind metals which are toxic and any positive transformants are not viable. This would mean any transformants analysed were essentially false positives, a small percentage which do occur randomly in any
transformation experiment. Due to all the positive transformants being lethal the probability of analysing false positives could be greatly increased up to 100% in this case.

Further analysis of these transformants would provide further information about what has happened to the chloroplast genome following transformation.
CHAPTER 6

Mutagenesis of the D1 Polypeptide in

*Chlamydomonas reinhardtii*
6.1 Introduction

D1 is a well-studied component of PSII being part of the heterodimer that forms the photochemical core of PSII (Section 1.4.2.1). It is also one of the PSII proteins that is light regulated (for a review see Aro et al., 1993). In this work it is the amino acid residues that are involved in the mechanism of water oxidation that are of interest. It is relatively difficult to identify residues on D1 that are important for the optimal functioning of water oxidation partly because there are no analogous residues in the bacterial reaction centre, as water oxidation does not occur in this system. Models of the tetranuclear Mn cluster require the presence of 22-24 ligand binding sites. Additionally, ligands are required for the calcium and chloride necessary for water oxidation. Hence, it is likely that a large number of amino acids are essential for water oxidation to take place, and perhaps many more are necessary for its optimum function.

An important approach in assigning ligands to the Mn cluster has been the site-directed mutagenesis of amino acids thought to be likely candidates. Such studies have been undertaken using Synechocystis 6803 (Debus, 1992; Nixon and Diner, 1992; Nixon et al., 1992; Chu et al., 1995 a&b; Debus, 2001; Diner, 2001) and C. reinhardtii (Diner, 2001). It has been postulated that the C-terminal end of the D1 protein is particularly important with respect the water oxidation. The C-terminus protrudes from the thylakoid membrane on the luminal side where the Mn cluster is located. Svensson and co-workers (1991) showed that a portion of the C-terminus is very highly conserved within the thirty-one species they compared, suggesting that it is a very important region. Despite mutagenesis studies of this region of the D1 protein, along with other luminaly exposed regions, only a few changes to residues expected to act as ligands led to phenotypes consistent with the disruption of Mn or Ca ligation. Site-directed mutagenesis has highlighted a number of residues proposed to be
important in the mechanism of water oxidation including D59, D61, E65, D170, E189, H190, H332, E333, H337, D342 and A344.

In this work I have attempted to add more information to that already collected on the roles of specific amino acid residues and their role in the mechanism of water oxidation. I have used the green alga *C. reinhardtii* as a model system in which to manipulate the D1 protein and assess the effects of such manipulation on water oxidation. In *C. reinhardtii* there are two copies of the *psbA* gene located on the inverted repeat sequence of the chloroplast genome. The gene is located on a 7 kb fragment of the genome and is disrupted by four Type I introns. Although mutagenesis of the gene in this form is feasible, it is very difficult and analysis of transformants is complicated. Two approaches to simplify the genetic manipulation of *psbA* have been attempted. Both reduce the *psbA* gene from ~7 kb down to ~1.1 kb by creating intronless *psbA* genes. Johanningmeir and co-workers (1993) used cDNA to create the intronless *psbA* plasmid while Minagawa and Crofts (1994) removed the introns and fused the exons of the *psbA* gene together. In both approaches transformed *psbA* deletion strains recovered to give wild-type phenotypes.

The analysis of PSII site-directed mutants in *C. reinhardtii* has been hindered by the lack of an efficient PSII isolation method. This has recently been overcome by using IMAC (Section 4.1) whereby PSII polypeptides tagged with histidines can be used to purify PSII complexes. In order to exploit this to analyse D1 mutants, a suitably tagged mutant strain had to be created. A PsbA-null/PsbH-null mutant lacking D1 and PsbH was constructed. This created a suitable background which could be co-transformed with a tagged gene to facilitate purification of PSII, along with a gene harbouring the mutation of interest. In this work the PsbA-null/PsbH-null strain was co-transformed with plasmids (Table 2.7) pH-his$_6$ and pBA157 (a plasmid containing the intronless *psbA* gene) and also with pH-his$_6$ and pBA153.W317F (a plasmid containing the intronless *psbA*
gene with a mutation changing the amino acid residue 317 from tryptophan to phenylalanine). Tryptophan 317 on D1 was targeted as it was postulated to be indirectly involved in the mechanism of water oxidation. Tryptophan residues are often found at the membrane interface because they have a large hydrophobic aromatic ring which is likely to be, preferentially, buried in the hydrophobic part of the membrane lipid bilayer, while the amide group can be expected to localize, preferentially, in the more polar environment at the interface (Killian & von Heijne, 2000). Tryptophan 317 is unlikely to be involved directly in water oxidation but it is the nearest amino acid co-factor which may be oxidised other than tyrosine 160 and, hence, may have some role in the fine-tuning of the water oxidising mechanism. Construction and analysis of the PsbA-null/PsbH-null background strain is presented along with construction of the transformants D1-W317F, D1-W317F/H-his6 and an intronless PsbA/H-his6 strain.

6.2 Construction of a D1-W317F Mutant

6.2.1 Construction of the pBA153.W317F plasmid

The tryptophan to phenylalanine change at residue 317 of the *C. reinhardtii* PsbA polypeptide was incorporated as described below.

The *psbA* gene has four large introns which make its genetic manipulation rather cumbersome. These difficulties were overcome by splicing together all of the exons of the *psbA* gene by PCR to give a compact intron-free gene (Minagawa & Crofts, 1994) and creating a series of plasmids, including pBA153 and pBA157 used in this work. The W317F change was incorporated into the *psbA* gene by PCR. As there were no useful restriction enzyme sites in the region of W317, straightforward mutagenic PCR could not be used and a “Mega Primer” method was utilised (Landt *et al.* 1990). Two PCR amplifications were required, the first to
incorporate the W317F change. A 650 bp region of the psbA gene was amplified from the plasmid pBA157 using the following oligonucleotides:

**Forward Primer**
P615 - 5'-CTACATGGGTCGTGAGTGGG-3'

**Reverse Primer**
D1-W317FR - 5'-GATGTCTGCGAAAGTGTTTAGTACACG-3'

The primer D1-W317FR contains the base changes to convert tryptophan (TGG) to phenylalanine (TTC), the changed bases are shown in red. The reaction conditions were as given in Section 2.6.6 with an annealing temperature of 45°C for 45sec. A product of ~590 bp was recovered. This product was then used as a forward primer in a second PCR amplification using the following oligonucleotide:

**Forward Primer**
Original PCR product

**Reverse Primer**
P2296R - 5'-CACGAGCCATGGACATTTTCAC-3'

The reaction conditions were as given in Section 2.6.6 with an annealing temperature of 42°C for 1 min and a "Hot Start" was used. A product of ~1.4 kb with a unique PstI site upstream of the W317F change and HpaI site downstream was cloned into the PstI and HpaI sites of pBA153 to create pBA153.W317Fspecl. The aadA cassette from pKS-483-aad-483 (kind gift from Jun Minagawa, RIKEN, Japan) was cloned into the KpnI site downstream of psbA to produce pBA153.W317F conferring resistance to spectinomycin (Figure 6.1).
PCR Amplification
to incorporate a W to F change at position 317 of the D1 polypeptide. The first PCR created the mutation, the second PCR allowed useful RE sites to be incorporated into the PCR product allowing the fragment of the psbA gene with the change to be incorporated into plasmid pBA153.

Figure 6.1 Schematic summary of the construction of the plasmid pBA153.W317F. This plasmid contains the intronless psbA gene with two base changes causing residue 317 to be converted from tryptophan to phenylalanine. PCR was used to incorporate the change into the gene, the PCR product was digested with Psfl and Hpal as was plasmid pBA153. The endogenous psbA gene fragment was then replaced with the mutated fragment. To allow selection for spectinomycin resistance, the aadA cassette was cloned into a KpnI site downstream of the psbA gene, creating plasmid pBA153.W317F.
6.2.2. Sequencing of pBA153.W317F

pBA153.W317F was sequenced (Section 2.7) to confirm the incorporation of the W to F change. The sequencing primer A944 (5’ cgaaggttacgttccgg 3’) was used to sequence through the W317F change in the *psbA* gene. Resulting sequence is given in Table 6.1.

<table>
<thead>
<tr>
<th>plasmid</th>
<th>Altered <em>psbA</em> sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBA153.W317F (<em>amino acid numbering of PsbA</em>)</td>
<td>5’ aac ttc aac caa tca gta gta gac tca caa</td>
</tr>
<tr>
<td></td>
<td>N F N Q S V V D S Q *310</td>
</tr>
<tr>
<td></td>
<td>ggt cgt gta cta aac act ttc gca gac atc</td>
</tr>
<tr>
<td></td>
<td>G R V L N T F A D I 320</td>
</tr>
<tr>
<td></td>
<td>atc aac cgt gct aac tta ggt atg gaa gta</td>
</tr>
<tr>
<td></td>
<td>I N R A N L G M E V 330</td>
</tr>
<tr>
<td></td>
<td>atg cac gag cgt aac gct cac aac ttc cct</td>
</tr>
<tr>
<td></td>
<td>M H E R N A H N F P 340</td>
</tr>
<tr>
<td></td>
<td>cta gac tta gct tca act aac tct aac tca</td>
</tr>
<tr>
<td></td>
<td>L D L A S T N S S S 350</td>
</tr>
<tr>
<td></td>
<td>aac aac taa 3’</td>
</tr>
<tr>
<td></td>
<td>N N * 352</td>
</tr>
</tbody>
</table>

*Table 6.1* Sequence confirming the W to F change at residue 317 of *psbA*. An extract of the sequence is presented showing the W to F change in red.

6.2.3 Transformation of C. reinhardtii with pBA153.W317F

The *C. reinhardtii* strain ac-u-ε (mt+), lacking PsbA, was transformed using the biolistic technique (Section 2.10.1) with the plasmid pBA153.W317F. Transformants were selected for spectinomycin resistance under low light (~5μEm⁻²s⁻¹) on TAP.

6.2.4 Southern analysis of C. reinhardtii transformants

Transformants were picked and taken through three rounds of single colony isolations on selective media. A number of colonies were selected for further analysis. Total DNA was purified from these transformants, ac-
u-ε (mt+), lacking PsbA, and wild-type by the method given in section 2.5.1. This DNA was digested with XhoI (Section 2.6.1) and separated on a 0.8% TAE gel by electrophoresis at 40V. The DNA was transferred to a nylon membrane (Section 2.8.1) and hybridised to a probe specific for the psbA gene. Figure 6.2 shows the autoradiogram of the Southern blot probed with a 715 bp XbaI fragment of the intronless psbA gene. This blot confirms that there are two copies of the psbA gene in wild-type, as expected (Figure 6.2 a & b). It also confirms the presence of the two intronless copies of psbA in the transformants (Figure 6.2 a & b).

Unfortunately, the blot does not confirm the homoplasmicity of the transformants. The PsbA deficient strain, ac-u-ε (mt+), has not been fully characterised or sequenced, this makes the Southern analysis of this strain difficult. This Southern blot shows that the deletion in this strain over the psbA region spans the 715 bp XbaI fragment used as a probe, hence the probe cannot hybridise to ac-u-ε (mt+) DNA because the homologous region is not present. Further analysis is described in subsequent sections of this chapter.
Figure 6.2 Southern analysis of putative D1-W317F transformants. The Southern blot shows the two copies of the intronless psbA gene present in the transformants. The blot also shows the increased size of the two copies of psbA in wild-type, in which the introns are present. This blot does not confirm the homoplasmicity of the transformants, it is not possible compare band sizes with the ac-u-ε (mt+) background strain as the probe does not bind to the DNA from this strain due to the deletion.
6.3 Construction of a PsbA Mutant in a His-tagged Background

6.3.1 Construction of a C. reinhardtii PsbA-null/PsbH-null double deletion mutant

A double deletion mutant was required in order to allow mutations in the \textit{psbA} gene to be expressed in a his-tagged background thus facilitating the isolation of PSII from such mutants for detailed analysis. The construction of this double mutation is described below.

\textit{C. reinhardtii} strains ac-u-ε (mt+) and FuD7 (mt+) are deletion strains at both copies of the \textit{psbA} gene (Palmer \textit{et al.}, 1985). Spot tests were set up to test both strains for their susceptibility or resistance to the antibiotics spectinomycin, kanamycin and amikacin. This was required in order to confirm their suitability for transformation using the \textit{aadA} cassette (Goldschmidt-Clermont, 1991) and the \textit{aphA-6} (Bateman & Purton, 2000) cassette as selectable markers. Both strains were susceptible to spectinomycin but FuD7 (mt+) was resistant to kanamycin and amikacin (Figure 6.3) making it unsuitable for transformation using the \textit{aphA-6} cassette. The strain ac-u-ε (mt+) was, therefore, used as the \textit{psbA} deletion mutant in which to make the double deletion.

\textit{C. reinhardtii} strain ac-u-ε (mt+) was transformed, using the biolistic technique, with plasmid p72BKmR as described in section 2.10.2. This plasmid harbours the \textit{psbH} gene disrupted with the \textit{aphA-6} cassette (Bateman & Purton, 2000). Transformants were selected for kanamycin resistance under low light (~5μEm^{-2}s^{-1}). Transformants were picked and taken through three rounds of single colony isolations on kanamycin-containing selective media to drive the genome to a homoplasmic state. Colonies were selected for further analysis.
Figure 6.3 Spot tests showing the growth characteristics of the FuD7 and ac-u-ε (mt+) PsbA deletion strain, compared to wild-type. Three 15μl aliquots (labelled A, B and C) of cells from liquid culture were spotted onto solid media and grown under moderate light (45μEm⁻²s⁻¹). Both strains are unable to grow photosynthetically, as expected and both are susceptible to the antibiotic spectinomycin. FuD7 is resistant to both kanamycin and amikicin, making it unsuitable as a background strain for transformation using the aphA-6 cassette as the selection tool. In contrast, ac-u-ε (mt+) is susceptible to both amikicin and kanamycin.
6.3.2 Southern analysis of C. reinhardtii PsbA-null/PsbH-null transformants

Transformants were analysed by Southern blotting to confirm that the PsbA deletion strain, ac-u-ε (mt+), had been transformed with the **aphA-6** disrupted **psbH** gene, creating a PsbA-null/PsbH-null strain. Total DNA was purified from these transformants, ac-u-ε (mt+) and CC-1021 by the method described in Section 2.5.1. This DNA was digested with **EcoRI** (Section 2.6.1) and separated on a 0.8% TAE gel by electrophoresis at 40V. The DNA was transferred to a nylon membrane (Section 2.8.1) and hybridised to a probe specific for the **psbH** gene. Figure 6.4 shows the autoradiogram of this blot. The presence of a single hybridising band of approximately 1.8 kb for the transformants shows that the **psbH** gene is disrupted with the **aphA-6** cassette as expected (Figure 6.4c). The blot also confirms the homoplasmicity of the transformants as there is no trace of the 4.4 kb hybridising band that is detected in wild-type and ac-u-ε (mt+) strains.
Genomic DNA was prepared from wild-type, ac-\(u^{-}\) and transformants. DNA was digested with EcoRI. The filter was probed with the 250bp BstXI/Nhel fragment of psbH.

**Figure 6.4** Southern analysis of putative PsbA/PsbH double deletion transformants. The Southern blot (a) shows that wild-type and the ac-\(u^{-}\) (mt+) strain contain the psbH gene. The presence of a single hybridising band of 1.8kb in the PsbA-null/PsbH-null transformants indicates that the psbH gene has been disrupted with the *aphA-6* cassette and the transformants are homoplasmic. Figures b and c show the expected sizes of hybridising bands.
6.3.3 PCR and sequencing analysis of transformants

PCR was used to amplify the psbH gene from total genomic DNA isolated from wild-type, ac-u-e (mt+) and PsbA-null/PsbH-null transformant cells. The PCR fragments confirm the disruption of the psbH gene and, more importantly, sequencing of this product confirmed the disruption (data not shown).

The PCR experiments were carried out using two primers, shown below, which flanked the psbH gene.

**Forward Primer**
NRH2P - 5' -tcaattatggcaacagg-3'

**Reverse Primer**
NRpsbHiS' - 5'-cccgggatccaagaaaagtgagctattaacg-3'

The reaction conditions were as given in Section 2.6.6, including 20 cycles with an annealing temperature of 56°C for 1 min. This combination of primers produced a fragment ~450 bp for wild-type and ac-u-e (mt+), while the PsbA-null/PsbH-null fragment was ~1.9 kb confirming the disruption of PsbH in the PsbA deletion strain (Figure 6.5).

6.3.4 Growth analysis of transformants

The phenotype of the PsbA-null/PsbH-null transformant was examined by comparing growth in moderate light (45μEm⁻²s⁻¹) on TAP, TAP supplemented with spectinomycin or kanamycin and HSM (Figure 6.6). Under these conditions the transformant is unable to grow photoautotrophically on HSM, it is resistant to kanamycin as expected but is sensitive to spectinomycin, a factor that is required for further genetic manipulation. The plates were incubated for the same period of time, showing the slower photoautotrophic growth of wild-type HSM, compared to the faster mixotrophic growth on TAP.
1 = wild-type (CC-1021)
2 = ac-u-ε
3 = PsbA-null/PsbH-null transformant No.1
4 = PsbA-null/PsbH-null transformant No.2
5 = PsbA-null/PsbH-null transformant No.3
6 = PsbA-null/PsbH-null transformant No.4
7 = PsbA-null/PsbH-null transformant No.5
8 = control (no template DNA)
M = markers (MBI 100bp plus ladder)

Figure 6.5 PCR analysis of PsbA-null/PsbH-null transformants. The gel photograph shows wild-type, ac-u-ε (mt+) and PsbA-null/PsbH-null transformant PCR products when the given primers, flanking the psbH gene, are used. The product of ~1.9kb for the transformants indicates that the psbH gene is disrupted with the aphA-6 cassette. The product of ~450bp for wild-type and the ac-u-ε (mt+) shows that the psbH gene is present. Lane 8 is a control where the PCR reaction took place in the absence of template DNA.
Figure 6.6 Spot tests showing the growth characteristics of the PsbA-null/PsbH-null transformants compared to wild-type and the ac-u-ε (mt+) PsbA deletion strain. Three 15μl aliquots (labelled A, B and C) of cells from liquid culture were spotted onto solid media and grown under moderate light (45μE m⁻² s⁻¹). The transformants could not grow photosynthetically, as expected. They are resistant to kanamycin indicating that the psbH gene has been disrupted with the aphA-6 cassette, but are susceptible to spectinomycin providing a suitable background for subsequent transformation using the aadA cassette for selection.
6.3.5 Co-transformation of C. reinhardtii with pBA153.W317F and pH-his₆

The PsbA-null/PsbH-null C. reinhardtii strain created above was co-transformed using the biolistic technique (Section 2.10.1) with the plasmids pBA153.W317F and pH-his₆ or pBA157 (containing the aadA cassette conferring spectinomycin resistance for selection) and pH-his₆. Transformants were selected for spectinomycin resistance and photosynthetic growth. Transformants were taken through at least three rounds of single colony isolations prior to analysis.

6.3.6 Southern analysis of C. reinhardtii transformants

Transformants were analysed by Southern blotting to confirm that the PsbA-null/PsbH-null deletion strain had been co-transformed with the W317F mutated-psbA gene (or the original intronless psbA gene) and the tagged psbH gene.

To confirm the presence of the psbH gene Southern analysis was carried out exactly as described in Section 4.3.1. Figure 6.7 shows the autoradiogram of this blot. The presence of a single hybridising band of approximately 4.4 kb for the transformants shows that the psbH gene has correctly inserted into the PsbH-null genome. The blot also confirms the homoplasmicity of the transformant as there is no trace of the 1.8 kb hybridising band detected in the PsbA-null/PsbH-null strain Figure 6.4. The digestion of total genomic DNA with EcoRI and MfeI confirmed the presence of the unique MfeI site in transformants 1-4, indicating that the his-tag is also present in these transformants (see Section 4.3.1 for details).

To confirm the presence of the psbA gene in the transformants a Southern Blot was carried out as in Section 6.3.4. This blot was probed with a 1.25 kb HindIII fragment in the intronless psbA plasmid. This fragment of DNA overlapped exon I of the psbA gene and the upstream flanking region. This probe was chosen in order to overcome the problem of using just a
psbA gene fragment. By using this overlapping region a direct comparison between the transformants and the PsbA-null strain should be possible. In Section 6.3.4 the XhoI probe did not bind to the DNA extracted from the ac-u-ε (mt+) strain because the region spanning the probe was deleted, in this case whether the transformant chloroplast genomes were homoplasmic or heteroplasmic could not determined. It was foreseen that by using a probe that could detect both the psbA gene and the flanking region, that had not been deleted in the ac-u-ε (mt+) strain, we would be able to confirm whether or not transformants were homoplasmic. The autoradiogram of this blot is shown in Figure 6.8. Two hybridising bands can be seen for wild-type (16 kb and 11.5 kb) and in the ac-u-ε (mt+) strain (14 kb and 8 kb) as expected from analysing sequence maps of the inverted repeat sequence in which psbA is located. From this sequence the sizes of bands expected in the transformants were estimated, the expected bands (3 kb and 8.5 kb) are shown in Figure 6.8d. The probe does bind to XhoI DNA fragments of these sizes, but also to fragments corresponding to the ac-u-ε deletion strain. On first inspection this suggests that the chloroplast genome has not been driven to homoplasmicity. However, when the hybridising band pattern on the autoradiogram from intronless psbA/H-his₆ and D1-W317F/H-his₆ transformants are compared they are seen to be identical. If the W317F change was affecting PSII activity or was causing some toxicity in the cell we would expect a heteroplasmic state to persist in the chloroplast genome which could explain the banding pattern seen. However, as the same pattern is seen in the intronless psbA/H-his₆ transformants, which are essentially wild-type with a his-tag on PsbH, it suggests that the transformants have all been driven to homoplasmicity. It appears that an XhoI site may have been incorporated into the chloroplast genome on transformation which has changed the restriction enzyme map around the psbA gene. Without the complete genome sequence in the psbA region both in wild-type and the ac-u-ε (mt+) strain it is difficult to predict the expected band sizes when using
this probe. Also seen on the Southern blot is a hybridising band of approximately 10kb (labelled ?) in the transformants which cannot be accounted for, again suggesting that the mapping may be incorrect. Complete sequencing of the pBA153 plasmid would provide a more complete knowledge of the restriction enzyme sites present in the inserting DNA and more precise mapping of expected hybridising band sizes would be possible.
Genomic DNA was prepared from wild-type, psbA-null/PsbH-null and transformants. DNA was digested with EcoRI or with EcoRI and MfeI. The filter was probed with the 250bp BstXI/Nhel fragment of psbH.

**Figure 6.7** Southern analysis of D1-W317F/H-his6 (1-3) and the intronless PsbA/H-his6 (A & B) transformants. The Southern blot shows that the transformants all contain the psbH gene. Only transformant B does not contain the unique restriction enzyme site MfeI, the presence of which suggests the histidine tag is present as in the other four transformants. See Figure 4.3 for a details of expected sizes of hybridising bands.
Genomic DNA was prepared from wild-type, ac-u-c (mt+) and D1-W317F transformants. DNA was digested with *XhoI*. The filter was probed with the 1.25kb *PstI* fragment of the intronless *psbA* gene plus upstream flanking region.

Figure 6.8 Southern analysis of putative D1-W317F/H-his₆ and intronless PsbA/H-his₆ transformants. The Southern blot shows that the transformants are positive as hybridising bands of the expected size are seen (a & d). This blot does not confirm the homoplasmicity of the transformants, as the probe also binds to DNA fragments the size of those in the ac-u-c (mt+) strain (a & c) in both the D1-W317F/H-his₆ and intronless PsbA/H-his₆ transformants. There is also an extra band (?) to which the probe binds. This is not expected from the mapping of the *psbA* gene and the probe used. See text for further details.
6.3.7 Growth analysis of transformants

The phenotypes of the D1-W317F, D1-W317F/H-his₆, intronless PsbA/H-his₆ transformants were examined by comparing growth in the light on TAP, TAP supplemented with spectinomycin and HSM (Figure 6.9). Under these conditions the transformants are all able to grow photoautotrophically on HSM and they are all resistant to spectinomycin. These growth tests suggest that the W317F change does not have a major effect on PSII activity in the mutants.
Figure 6.9 Spot tests showing the growth characteristics the D1-W317F and D1-W317F/H-his$_6$ transformants. Growth is compared to wild-type, the PsbA-null/ PsbH-null double deletion strain and a control transformant in which the intronless $psbA$ gene and the H-his$_6$ tag are incorporated (PsbA/H-his$_6$). Fifteen microlitre aliquots of cells from liquid culture were spotted onto solid media and grown under moderate light (45$\mu$Em$^{-2}$s$^{-1}$). The transformants could grow photosynthetically while the PsbA-null/PsbH-null strain could not. They are also resistant to spectinomycin indicating that the $aadA$ cassette is present.
6.4 Discussion

This chapter details the creation of a *C. reinhardtii* strain deficient in both the PsbA and PsbH polypeptides, PsbA-null/PsbH-null. A *C. reinhardtii* strain, ac-u-c (mt+), in which both copies of the *psbA* gene are absent, was used as the starting strain in which to create the double deletion mutant. The PsbA-null/PsbH-null strain is resistant to the antibiotic kanamycin as the *aphA-6* cassette has been used to disrupt the *psbH* gene. The PsbA and PsbH polypeptides are essential for photosynthetic activity, hence this background is suitable for co-transformation with plasmids harbouring changes to the *psbA* and *psbH* genes and photoautotrophic growth can be selected for. If the changes are likely to yield non-photosynthetic mutants the *aadA* cassette can be used for selection, as this has not been used when creating the background strain, PsbA-null/PsbH-null.

Successful co-transformation of this background has been achieved using the plasmid pH-his6. A mutant with a his-tag on PsbH and a site-directed change on D1 (D1-W317F/H-his6) has been produced. In depth analysis of this mutant and its effect on PSII activity is yet to be undertaken. Southern analysis suggested that the transformants had been driven to homoplasmicity but the interpretation of the blots was complicated. Sequencing analysis (not shown) confirmed that the W to F change was present in the transformant but more complete analysis is required.

Demonstrated here is an easy procedure for creating D1-mutants from which PSII can be isolated using histidine tagging technology. It is perceived that a more in depth analysis of these mutants is possible using isolated PSII, which will hopefully provide more information as to amino acid residues important in the mechanism of water oxidation and their actual role.
Chapter 7 - Discussion

CHAPTER 7

Discussion
7.1 Final Discussion

The mechanism of photosynthetic water oxidation is still very much under debate although as data gradually becomes available a more detailed view of how this important reaction takes place is being formed. The overall aim of the research undertaken whilst working towards this thesis was to use site-directed mutagenesis of the green alga *C. reinhardtii* to gain more information about the reactions that lead to water splitting. It was hoped that a spectroscopic study of site-directed mutants could be completed using EPR spectroscopy and, subsequently, using ENDOR.

Biophysical analysis of PSII, using techniques such as EPR, has proved invaluable in investigating the mechanism of water oxidation. These methods routinely use isolated PSII from higher plants. Unfortunately, higher plants are not as amenable to genetic engineering as the green alga *C. reinhardtii*. The major advantage of *C. reinhardtii* is its ability to grow on acetate, allowing a range of photosynthetic mutants to be created that would be non-viable in higher plants. Although using *C. reinhardtii* facilitates mutagenesis, it had already been demonstrated that the study of PSII from this alga was not as straightforward as studying higher plant PSII due to difficulties in isolating *C. reinhardtii* PSII (Shim *et al.*, 1990). In order to use EPR spectroscopy to study PSII the absence of PSI is required because a number of PSI EPR signals mask those of PSII. Our initial strategy to overcome this problem was to create the *C. reinhardtii* site-directed mutants in a PSI-deficient background. This strategy successfully removed the PSI signals, and some of the PSII EPR signals could be seen (Section 3.2.4), however a number of PSII signals routinely manipulated to study PSII function (e.g. Mn multiline) could not be seen. Unfortunately, it appeared that this technique was not suitable for studying PSII function in *C. reinhardtii* mutants and an alternative strategy was required. The strategy we used was to isolate PSII using IMAC and histidine tagging technology as detailed in Chapter 4.
Chapter 7 - Discussion

Having developed an efficient method of isolating PSII from \textit{C. reinhardtii} efforts could again concentrate on mutagenesis of amino acid residues thought to be involved PSII function. Site-directed mutants were created in the his-tagged background (Chapter 3 and Chapter 6), allowing the isolation of the mutated PSII for analysis.

In this work we used the PSII polypeptide PsbH as the site of the histidine tag in the hope that we could purify a more complete PSII complex than that of Sugiura and co-workers (1998), who tagged the central PSII polypeptide, D2 (PsbD). The choice of PsbH as the tagged peripheral protein allowed selection of tagged transformants without the use of a selectable marker, transformants were selected for by the recovery of photosynthetic growth (Section 4.2.3). This is advantageous as it facilitates subsequent mutations to be made in the his-tagged mutant using the limited selectable markers available (as shown in Chapters 3 & 6). This method of purifying PSII proved successful and allowed site-directed mutants to be created in a background from which PSII could be isolated.

During the optimisation procedure it was discovered that the alteration of conditions allowed complexes with differing polypeptide composition to be isolated. Further work would include an analysis of these complexes as this may provide further information about the assembly of PSII. It is also perceived that the PSII isolated my be useful in structural studies and crystallography. Tagging of polypeptides could provide a way to identify the small PSII polypeptides using the structural data available at present.

In order to allow analysis of PSII from D1 mutants a PsbA-null/PsbH-null deletion strain had to be created allowing co-transformation with the gene encoding a his-tagged PsbH polypeptide and the \textit{psbA} gene harbouring the site-directed change. This mutant was successfully constructed and its co-transformation is demonstrated in Chapter 6. This co-transformation method provides a simple system to create any D1 mutation.
in a his-tagged background, facilitating the isolation of PSII and allowing the affect of mutations on PSII function to be analysed. Although no amino acid ligand of the Mn cluster has been identified unambiguously, many candidates have been identified (Debus, 2001), of these a good number are residues of D1. The method demonstrated here should allow these candidate ligands to be targeted for site-directed mutagenesis and PSII isolated for further investigation using advanced spectroscopic methods.

The D2-Y160F mutant lacks the redox active tyrosine Y_D. By studying this mutant we hoped to gain an understanding of Y_D's role in water oxidation and also obtain further information about the redox active tyrosine Y_Z in the absence of the complicating EPR signals arising from Y_D. The creation of this mutant in a his-tagged background allowed it to be studied by EPR. Initially the mutation was created in a background in which the PSII D2 polypeptide was tagged. Initial characterisation of this mutant showed it to be unstable (Section 3.3). This finding led us to create the mutation in a his-tagged background in which PsbH was tagged (Chapter 4). This mutant has been shown to be stable and suitable for spectroscopic analysis.

P680⁺ reduction kinetics presented in Chapter 3 show that the loss of Y_D has no effect on the oscillatory nature of P680⁺ reduction, but it has affected the proton/hydrogen coupled electron transfers which occur with a microsecond rate constant. The data suggests that the presence of Y_D does not affect the redox potential of P680/P680⁺. In this work it is proposed that Y_D affects the proton/hydrogen transfers which accompany the relaxation phases of P680⁺ reduction. This does not necessarily mean that Y_D is directly involved in a proton transfer pathway, but merely that its electrostatic or structural contribution is required for normal proton/hydrogen transfer. This work also shows that Y_D is not essential for assembly of the Mn cluster.
Further information about the redox active tyrosine Yz has not been investigated in this work due to time limits, however it has been shown that the PSII isolated from the PsbH-his-tagged variant of the D2-Y160F mutant is suitable for EPR analysis and experiments can now be undertaken.

Results from Chapter 4 of this thesis show that tagging the C-terminal end of the PsbH polypeptide with six histidines is feasible and has little or no detrimental effect on the function of the PsbH polypeptide or on PSII function. Introduction of further tags at the C-terminal end of PsbH was attempted because of the ease with which this could be done given the method developed for the H-his6 strain. Three tags were made, a HA2his6 tag a H-his10 tag and the mature pea plastocyanin sequence was incorporated at the C-terminal end of PsbH.

Only the HA2his6 tag was successfully incorporated into the chloroplast genome of C. reinhardtii. This tagged strain could allow further investigation into the role of the PsbH polypeptide now the isolation and detection of the protein is possible. PsbH is essential for PSII function and has been shown to be phosphorylated (Dedner et al., 1988) although a specific role is yet to be proven. Using the epitope tagged PsbH strains such a role may become clearer, perhaps confirming the proposal that PsbH is essential in the dimerization of PSII (Hankamer et al., 1999). Further analysis of the false H-plastocyanin and H-his10 transformants, such as sequencing in the region of the psbYi gene, could provide further information about what has happened to the chloroplast genome following transformation.

It is envisioned that the method of purifying PSII from C. reinhardtii developed in this work will be useful in studying the structure of the PSII complex. Although investigation into the roles of specific amino acids in the mechanism of water oxidation was not as thorough as initially planned the PSII isolated from this alga has been shown to be suitable for EPR spectroscopy and is likely to be useful for other spectroscopic techniques.
Chapter 7 - Discussion

The methods developed in this work will facilitate such research allowing *C. reinhardtii* to remain useful as a model organism in the study of PSII and the mechanism of water oxidation.


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