The Structural Organization of Photosystem II Polypeptides.

William Robert Odom
Louisiana State University and Agricultural & Mechanical College

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The structural organisation of photosystem II polypeptides

Odom, William Robert, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1991
THE STRUCTURAL ORGANIZATION OF PHOTOSYSTEM II POLYPEPTIDES

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Botany

by

William Robert Odom
B.A., University of New Orleans
December 1991
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ABBREVIATIONS

BCIP = 5-bromo-4-chloro-3-indolyl phosphate
Chl = Chlorophyll
CNBr = Cyanogen bromide
Cyt = Cytochrome
DCBQ = 2,5-dichloro-p-benzoquinone
EDC = 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
Fd = Ferredoxin
LDS-PAGE = Lithium dodecyl sulfate polyacrylamide gel electrophoresis
NADP = Nicotinamide adenine dinucleotide phosphate
NBT = Nitro blue tetrazolium
NHS = N-hydroxysuccinimide
PS I = Photosystem I
PS II = Photosystem II
P$_{680}$ = Primary Chl $a$ dimer of PS II
P$_{680}^*$ = Excited P$_{680}$
P$_{680}^+$ = Oxidized P$_{680}$
P$_{700}$ = Primary Chl $a$ dimer of PS I
P$_{700}^*$ = Excited P$_{700}$
P$_{700}^+$ = Oxidized P$_{700}$
Q$_A$ = Plastoquinone A
Q$_B$ = Plastoquinone B
Q$_B$$H_2$ = Plastoquinol
TMBZ = 3,3',5,5'-tetramethylbenzidine
TS buffer = 10 mM Tris-HCl, pH 7.4, 150 mM NaCl
Y$_D$ = Redox reactive tyrosine 160 on D2
Y$_Z$ = Redox reactive tyrosine 161 on D1
ABSTRACT

The structural organization of Photosystem II polypeptides has been investigated using the protein crosslinking reagent 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (EDC), and a library of mono- and polyclonal antibodies. The first phase of this research was to generate the antibody probes against specific PS II polypeptides. A murine monoclonal antibody, FQC3, was characterized. It recognized the D2 protein of *Spinacia oleracea* L. The second phase of this research utilized antibodies against D1, D2, CPa-1, CPa-2, Cyt b$_{599}$, 33 kDa manganese stabilizing protein (MSP), 24 kDa extrinsic protein, and the 17 kDa extrinsic protein to identify PS II subunits crosslinked by EDC. Several intra-complex crosslinked products were identified. One crosslinked species at 110 kDa (called XL) was identified by two different antibodies and is composed of CPa-1 and the extrinsic 33 kDa MSP. The third phase of this research characterized XL. This was of particular interest considering both CPa-1 and the 33 kDa MSP are required for maximal O$_2$-evolving rates. EDC modified PS II membranes evolve normal rates of O$_2$. An increase in EDC concentration results in increased retention of the O$_2$-evolving rate by CaCl$_2$ washed EDC modified PS II membranes. XL (the crosslinked species between CPa-1 and the 33 kDa MSP) was isolated. Chemical and proteolytic cleavage techniques were used to identify peptide fragments of CPa-1 and the 33 kDa MSP involved in crosslinkage. CNBr generated fragments
from XL were identified at 50 kDa and 25 kDa. N-terminal sequence analysis of the 50 kDa species indicated that the 15.7 kDa C-terminal CNBr fragment of CPa-1 is unequivocally crosslinked to the 33 kDa MSP. Additional N-terminal sequence analysis of the 25 kDa species strongly suggests that the 15.7 kDa C-terminal CNBr fragment of CPa-1 is crosslinked to the 7 kDa N-terminal CNBr fragment of the 33 kDa MSP. The large extrinsic loop of the apoprotein of CPa-1 thus appears to anchor the extrinsic 33 kDa MSP of the O2-evolving complex to the thylakoid membrane through charge pair interactions. By helping elucidate the nature of the non-covalent interactions among PS II proteins, and these results provide a more integrated view of the structural and functional organization of PS II polypeptides.
INTRODUCTION

Photosynthesis is the metabolic process by which carbohydrates are formed from carbon dioxide and water in the chlorophyll-containing tissues of plants exposed to light. In 1862, the appearance of starch in the "chlorophyll corpuscle" led Sachs to the following conclusion: "the whole of the carbon, in whatever organic combination it may be found later, occurs originally in the form of starch" (Green 1967). Importantly, the magnitude of the chemical changes that occur during the formation of starch from such simple compounds was finally perceived. More than a century later, the dissection of oxygen-evolving reaction centers has been significantly advanced by a Nobel prize winning accomplishment. The X-ray crystallographic solution of the three-dimensional structure of bacterial reaction centers has provided a model for examining the structure and function of Photosystem II (PS II) (Deisenhofer et al. 1985).

PS II is the multimeric enzyme complex that is responsible for the photochemical splitting of H$_2$O in most photosynthetic organisms. The oxygen produced from that water splitting reaction is vital to the sustenance of life on earth. The other products of water oxidation, the hydrogen ions and electrons, are ultimately used to convert atmospheric carbon dioxide to organic compounds. PS II is also the current target for many of the commercially
available herbicides. The significance of this basic research is far reaching. Current knowledge of photosynthetic reaction centers (oxygenic and non-oxygenic) has already inspired seminars on chemical electronics and artificial photosynthesis.

While the functional characteristics of the proteins associated with PS II have been extensively analyzed in many organisms using classical biochemical, advanced spectroscopic, and molecular biology techniques, relatively little is known of the structural organization of these proteins in the photosynthetic membrane. In this study, the protein crosslinker 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) has been utilized to investigate protein-protein associations in oxygen-evolving PS II membrane preparations from Spinacia oleracea L. In order to determine PS II protein architecture, mono- and polyclonal antibodies were used to identify crosslinked proteins. Once identified and isolated, various chemical and enzymatic cleavage techniques were employed to determine specific regions of interaction of the juxtaposed polypeptides.
Photosynthesis As early as 1937, R. Hill demonstrated that photochemical oxygen-evolution could be separated from carbon fixation by using non-physiological electron acceptors. Photosynthetic electron transport from H₂O to electron acceptors such as potassium ferricyanide has become known as the "Hill Reaction". Isotopic experiments by Ruben and Kamen using H₂¹⁸O demonstrated that the O₂ evolved was from water. Isolated spinach chloroplasts were used by Arnon, Allen and Whatley in 1954 to demonstrate cell-free photosynthesis: light driven CO₂ assimilation and O₂ evolution. There are three integral membrane protein complexes forming the "Z scheme", first proposed by Hill and Bendall in 1960, of photosynthetic electron transport from water to NADPH: Photosystem II (PS II), Cytochrome (Cyt) b₆/f, and Photosystem I (PS I) (Figure 1).

Energy from the sun is absorbed by the light-harvesting chlorophyll-protein antennae complexes and ultimately funneled to the reaction center chlorophyll (Chl) a dimer of each respective photosystem. In PS II P₆₈₀⁻ becomes P₆₈₀⁺ and this initial charge separation event is the beginning of photosynthetic electron transfer. The electron is transferred to the primary acceptor, pheophytin a, within 3 picoseconds, then transferred sequentially to two quinone acceptors, Q₀ and Q₁. Q₀ is a protein-bound plastoquinone, and upon reduction by
Figure 1. The Z Scheme of Photosynthetic Electron Transport
pheophytin, forms a stable semiquinone. Electron transfer from $Q_A$ to $Q_B$ also forms a stable protein-bound semiquinone but $Q_B$ is a two electron acceptor. A second light induced charge separation event completely reduces the terminal quinone which is protonated and $Q_BH_2$ is released into the plastoquinone pool within the lipid bilayer. This quinone pool is the link between PS II and the Cyt b$_6$f complex. The electron carriers of the Cyt b$_6$f complex are two cytochrome b$_6$ moieties, one cytochrome f, and a "Rieske" iron sulfur center (Cramer et al. 1987). The Cyt b$_6$f complex is also called the plastoquinol : plastocyanin oxidoreductase complex. This multimeric electron transport pathway between the photosystems translocates protons from the stroma to the lumen, most likely by a mechanism similar to the Q cycle in the mitochondrial cytochrome b-c$_1$ complex. Cyt b$_6$f may also participate in cyclic electron transport with PS I using the stromal ferredoxin pool as the mobile electron carrier. Plastocyanin, the mobile electron carrier that accepts electrons from the Cyt b$_6$f complex, transfers electrons from the Cyt b$_6$f complex to Photosystem I. Plastocyanin is a mobile copper containing polypeptide in the lumen of the thylakoids. P$_{700}$ is the light driven reaction center Chl a dimer of PS I. Upon light excitation $P_{700}$ is excited to $P_{700}^*$ which looses an electron. The electron is then transferred along a more positive reduction potential through A$_b$, A$_i$, F$_X$, F$_b$, and F$_A$, ultimately donating an electron to the mobile stromal polypeptide ferredoxin (Golbeck 1987). Ferredoxin has a host of electron acceptors, one of which is the flavoprotein
ferredoxin nicotinamide adenine dinucleotide phosphate oxidoreductase. NADP is reduced to NADPH by this oxidoreductase.

Plastocyanin serves to reduce $P_{700}^+$ returning it to the ground state. The oxygen-evolving complex and $Y_z$ of PS II serve to reduce $P_{680}^+$. All oxygenic photosynthetic electron transport occurs through the components of these three integral membrane protein complexes. The relaxation of this trans-thylakoid proton gradient, created by the oxidation of water and quinones, through the ATP-synthase complex drives the formation of ATP (Merchant and Selman 1985). PS II, Cyt b$_6$/f, PS I, and ATP-synthase are ubiquitous in higher plant thylakoids (Murphy 1986).

There is a definite heterogeneity of these multimeric protein complexes within the architecture of the thylakoids. Thylakoid membranes can be stacked to form the grana which have appressed external surfaces or stromal lamellae whose outer surface is exposed directly to the stroma. Cytochrome b$_6$/f is found in both the grana and the stromal lamellae whereas PS II is found primarily in the grana. PS I and ATP-synthase are predominantly located in the stroma lamellae (Anderson 1981).

**PS II Membrane Isolations**  
PS II research was significantly advanced by the development of isolation procedures for thylakoid membranes enriched in PS II. The straightforward isolation of PS II oxygen-evolving membrane particles used herein is derived from several complex procedures. Four seminal isolation procedures were compared on the basis of polyacrylamide gel
electrophoresis polypeptide profiles, $O_2$ evolution, freeze-fracture electron microscopy, 77 K fluorescence, and electron paramagnetic resonance (Berthold et al. 1981; Kuwabara and Murata 1982b; Yamamoto et al. 1982; and Dunahay et al. 1984). Confirmation that the term "particle" was a misnomer and that these preparations were extremely stable inside-out appressed granal membrane leaflets (0.5 $\mu$m in diameter), enriched in PS II, and exhibiting high rates of $O_2$ evolution, was paramount in the dissection of this multisubunit membrane protein complex (Dunahay et al. 1984).

**Photosystem II Polypeptides**

PS II is composed of 15 - 20 major polypeptides. Besides the distal antenna (LHCP II) there are seven to ten major polypeptides including both nuclear and chloroplastic encoded species (Table I). D1, D2, Cyt $b_{599}$, CPa-1, and CPa-2, are integral membrane proteins and are chloroplast encoded. The three major extrinsic proteins, the manganese stabilizing protein (MSP), the 24 kDa protein, and 17 kDa protein, are associated with the oxygen evolving complex and are nuclear encoded. Several low molecular weight polypeptides (less than 5 kDa) have been identified, however their functions are not known.

**Distal Antenna (LHCP II)** Solar energy is captured by green plants and funneled to PS II in the light-harvesting Chl a/b - protein complex and transferred by delocalized exciton coupling and by Förster mechanisms (Kühlbrandt and Wang 1991). The Chl a/b binding polypeptides are encoded
Table I. Major PS II Polypeptides (excluding LHCP II)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Intrinsic/Extrinsic</th>
<th>Apparent M.W. (kDa)</th>
<th>Metallic Cofactors</th>
<th>gene</th>
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<tr>
<td>D1</td>
<td>I</td>
<td>32</td>
<td>Mg,Fe</td>
<td>psb A</td>
</tr>
<tr>
<td>CPa-1</td>
<td>I</td>
<td>49</td>
<td>Mg</td>
<td>psb B</td>
</tr>
<tr>
<td>CPa-2</td>
<td>I</td>
<td>45</td>
<td>Mg</td>
<td>psb C</td>
</tr>
<tr>
<td>D2</td>
<td>I</td>
<td>34</td>
<td>Mg,Fe</td>
<td>psb D</td>
</tr>
<tr>
<td>Cyt b\textsubscript{559}</td>
<td>I</td>
<td>9</td>
<td>Fe</td>
<td>psb E</td>
</tr>
<tr>
<td>Cyt b\textsubscript{559}</td>
<td>I</td>
<td>4</td>
<td>Fe</td>
<td>psb F</td>
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<tr>
<td>MSP</td>
<td>E</td>
<td>33</td>
<td>Ca</td>
<td>psb O</td>
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<td>24</td>
<td>E</td>
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<td>?</td>
<td>psb P</td>
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<td>17</td>
<td>?</td>
<td>psb Q</td>
</tr>
<tr>
<td>5</td>
<td>?</td>
<td>5</td>
<td>?</td>
<td>psb L</td>
</tr>
<tr>
<td>4</td>
<td>?</td>
<td>4</td>
<td>?</td>
<td>psb I</td>
</tr>
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in the nucleus (Chua 1977) by the \textit{cabII} gene family (Kindle 1987). Early designations for these genes were \textit{AB}\# (Cashmore 1984 and Coruzzi 1983). Synthesis of these polypeptides is light regulated (Apel 1978). Recent two dimensional crystallographic structure analysis at 6 Å resolution supports a trimer of nearly identical 25 kDa monomers (Kühbrandt and Wang 1991). Although LHCP II remains associated with the PS II oxygen-evolving membrane particles during isolation, it is not a requirement for oxygen-evolution. These antennae support approximately 225 - 300 Chl per reaction center (Mavankal 1989).

D1 and D2 Reaction Center, Charge Separation Presently the three dimensional structure of PS II has not been resolved. There have been many attempts to crystallize oxygenic reaction centers such that the direct structural
organization of the polypeptides and their cofactors can be determined. However, the best crystals of PS II reaction centers are only two-dimensional crystals which contain CPα-1, D1, D2, and Cyt b$_{559}$ (Dekker, et al. 1990). The photosynthetic reaction center from *Rhodopseudomonas viridis*, which is not oxygenic, was the first reaction center to be crystallized (Deisenhofer *et al.* 1985). The 3 Å resolution allowed chain tracing of the polypeptide subunits and established the specific locations of the cofactors. The structural model of the bacterial reaction center emerged before the amino acid sequences of the polypeptides were known. The extensively characterized bacterial reaction center led PS II investigators to question which polypeptides harbored the reaction center in oxygenic photosynthesis (Trebst and Depka 1985). The amino acid sequence identity between D1 and D2 in PS II is greater than the identity between L and M in the bacterial reaction center (Deisenhofer and Michel 1989). There are, however, regional sequence similarities between L and D1 and between M and D2 particularly when comparing the quinone binding regions (Trebst 1986). These sequence identities, the ability to isolate reaction center cores (Nanba and Satoh 1987), and the spectroscopic characterization of the primary radical pair P$_{680}^+$ and pheophytin* in these isolations (Danielius *et al.* 1987) has led to the universal acceptance of the D1-D2-Cyt b$_{559}$ reaction center in oxygenic organisms. Conserved amino acids between the bacterial reaction center and PS II have been examined
extensively (Michel and Deisenhofer 1988). Immunological evidence has also identified D1 and D2 as the reaction center polypeptides (Nixon et al. 1986).

These two polypeptides, D1 (Eaglesham and Ellis 1974) and D2 (Chua and Gillham 1977), are chloroplast encoded by the *psbA* and *psbD* genes, respectively. Each gene has been sequenced in many organisms (Neumann 1988). Both polypeptides migrate electrophoretically to 32-34 kDa, and the mobilities are chaotrop dependent (Marder et al. 1987). Azido-atrazine labeled with ¹⁴C was photoligated to a polypeptide in the 30 kDa region, thereby identifying the herbicide binding polypeptide (Pfister et al. 1981). The mature D1 protein of spinach does not have any lysyl residues (Zurawski et al. 1982) and normally turns over rapidly (Metz et al. 1986). Initial topography predictions based on hydropathy analysis suggested 7 transmembranous α-helices in both the D1 and D2 polypeptides (Rao 1983). However, experimental evidence suggests that each polypeptide has 5 trans-thylakoid α-helices and a stromally located N-terminus. The topology was established using antibodies generated against synthetic peptides of the proposed extrinsic portions (Sayre et al. 1986). This orientation is supported by the amino acid point mutations that are located in the stroma and confer herbicide resistance (Erickson et al. 1984). In spinach, both mature polypeptides (D1 and D2) are phosphorylated and their N-termini blocked by *N*-acetyl-*O*-phosphothreonine (Michel et al. 1988). Many studies employing mutants have attempted to characterize the transcriptional, translational, and posttranslational regulation
of the \textit{psbA} and \textit{psbD} genes as well as their products. A mutant of \textit{Scenedesmus obliquus} lacks a protease involved in the C-terminal processing of D1. In the absence of this protease, a 1.5 kDa portion of the C-terminal region remained on D1, suggesting that this region normally was removed before PS II assembly (Taylor et al. 1988). In a \textit{psbA} deleted mutant of \textit{Synechocystis} 6803, the absence of D1 resulted in minimal amounts of D2 accumulating (Nilsson et al. 1990). \textit{Chlamydomonas reinhardtii} \textit{psbA} deletion mutants and nuclear mutants are only beginning to aid in elucidation of the complex regulation of the assembly and turnover of PS II (Jensen et al. 1986, Rochaix and Erickson 1988). Coordination of both nuclear and plastid regulatory elements is indicated.

One of the most intriguing aspects of PS II electron transport from H$_{2}$O to Q$_{b}$H$_{2}$ is the coupling of the light driven single electron charge separation of P$_{680}^{+}$/Pheo', the two electron reduction of Q$_{B}$ to Q$_{b}$H$_{2}$, and the four electron concerted oxidation of H$_{2}$O to molecular oxygen (Figure 2). The redox environment of the reducing side of PS II is maintained by the integral membrane protein architecture of the D1-D2-Cyt b$_{59}$ core complex. Much of this coordination has been extrapolated from the bacterial reaction center (Rutherford 1989); however, our understanding of the mechanism for the reduction of P$_{680}^{+}$ by the Mn mediated photo-oxidation of water has not been significantly advanced since the elucidation of the "S state" model (Kok et al. 1970) (Figure 3). The protolytic and dioxygen release sites were not
established until almost two decades later (Renger et al. 1987). In the dark, 75% of the reaction centers are in $S_1$ and 25% remains in $S_0$. This fits with the pioneering work establishing a maximum $O_2$ evolution peak on the third flash and an $O_2$ evolution peak periodicity of 4 thereafter. A redox reactive tyrosine, $Y_z$, located on the D1 polypeptide, D1-Y161, has been identified using site-directed mutagenesis techniques in *Synechocystis* 6803 (Debus et al. 1988). $Y_z$ is the electron donor to $P_{680}^+$ and the distance between them is approximately 10-15 Å (Hoganson and Babcock 1989). The forward reaction time of the initial charge separation event from $P_{680}^+$ to the charge pair, $P_{680}^+/Pheo^-$, is orders of magnitude faster than the slowest, $Y_z^+/Q_b^-$ to $[Mn_4]^+/Q_b^-$. It is not known which protein or proteins serve as ligands to the Mn complex.

Crosslinkage of the 33 kDa MSP to D1, D2, and an unidentified 34 kDa polypeptide may implicate one or more of those 3 integral membrane proteins.
Figure 3. S state model (Kok et al. 1970, Renger et al. 1987)

in the Mn binding (Mei et al. 1989). Recent evidence implicates a redox reactive D1 histidine as one of the Mn ligands (Ono and Inoue 1991). Extended X-ray absorption fine structure (EXAFS) studies suggests that the manganese complex has oxygen ligands (Debus 1991). The exact location, oxidation states, and ligands of the Mn of PS II is not clear. Connections between the oxidizing side and the reducing side of PS II clearly involve these two polypeptides, as demonstrated by the identification of Yz and Yd. Yd is the homologous redox reactive tyrosine located on D2.
Cyt b_{599}  Cytochrome b_{599} (α peak at 559 nm) is an integral component of PS II in all oxygenic photosynthetic organisms. In all reaction center core complex isolations, Cyt b_{599} copurified with D1 and D2. Although known to be tightly associated with the thylakoid membrane, purification methods that maintained biological activity proved difficult since they required 2 % Triton X-100, 4 M urea, and 6 % dithiothreitol, at pH 8 and a temperature of 0-4°C (Garewal and Wasserman 1974). Cyt b_{599} was determined to be the only cytochrome present in PS II oxygen-evolving membrane particles (Bricker et al. 1983), as characterized using difference spectra, 3,3',5,5'-tetramethylbenzidine (TMBZ) staining of nondenaturing polyacrylamide gels, and lithium dodecyl-sulfate polyacrylamide gel electrophoresis (LDS-PAGE). The molecular weight of Cyt b_{599}, as determined by LDS-PAGE, is 9-10 kDa. Purification of biologically active cyt b_{599} revealed a 4 kDa subunit that was not a degradation product of the 9 kDa polypeptide. Analysis of the chloroplast genome revealed an open reading frame just downstream from the gene that encodes for the 9 kDa polypeptide. Further analysis of these two open reading frames and their corresponding proteinaceous products revealed a conserved histidine residue located within the conserved hydrophobic domain of each polypeptide (Widger et al. 1985). The two polypeptide subunits are now designated α (9 kDa) and β (4 kDa) and are encoded in the chloroplast by the psbE and psbF genes respectively. Subunit stoichiometry based on HPLC analysis of isolated Cyt b_{599} suggests α1:β1. If there is only one heme/PS II reaction center, then there
must be a heterodimeric bis-histidine coordination of the heme. If on the other hand there are two hemes/PS II reaction center then there could be two heterodimers, $(\alpha\beta)_2$, or two homodimers, $(\alpha \alpha)$ and $(\beta \beta)$ (Widger et al. 1985). This structure seems simple on the surface, however, it is complicated by an uncertainty in the number of hemes per PS II reaction center. The transmembranous topography of the $\alpha$ subunit of Cyt b$_{599}$ was established using immuno-gold techniques (Vallon et al. 1989) and antibody-determined protease-accessibility techniques (Tae et al. 1988). The carboxy terminal is on the lumenal surface of the thylakoid membrane. The heme, with both $\alpha$-histidine-22 and $\beta$-histidine-17 ligands within the membrane, is closer to the stromal surface of the thylakoid membrane. There is no direct topological evidence for the $\beta$ subunit. Additional experiments using different PS II preparations and assuming two pheophytin molecules per PS II reaction center suggest one heme/PS II reaction center (Miyazaki et al. 1989). A function for Cyt b$_{599}$ on the oxidizing side of PS II in water oxidation is possible. A possible PS II by-pass or PS II cyclic function has also been proposed (Westhoff et al. 1985). Preliminary electron paramagnetic resonance spectroscopic evidence suggests Cyt b$_{599}$ protects PS II from photoinhibition (Thompson and Brudvig 1988) however, the definitive function of this heterodimeric protein remains a mystery.

Proximal antenna (CPa-1 and CPa-2) These two polypeptides are found in all photosynthetic oxygen-evolving species and minimally serve as the proximal
MGLPWYRVHT VVLNDPGRIL SVHIMHTALV AGWAGSMALY ELAVFDPSDP 050
VLDPMWRQGM FVIPFMTRLG ITNSWGGWSI TGGTITDPSI WSYE GVAGAH 100
IMPSGLCFLA AIWHVYYWDL EIFSDERTGK PSLDLPKIFG IHLFL S GVA C 150
FGFGAFHVTG LYGPGIWVSD PYGLTGKVQP VCSAWGVEGF DP FPVRGIA S 200
HHIAAGTLGI LAGLFLHLSVR SPQRLYKGLR MGINETVLSS SIAAVFFA AF 250
VVAGTMWYGS ATTPIELFGP TRYQWDQGYF QEIYRRVS A GLAENQSFSE 300
AWSKIPEKLA FYDYIGNNPA KGGLFRAGSM DNGDGI AVGW LGHIP FRDKE 350
GRELFFRRMP TFFETFPVVL IDGDGI V RAD VPFR RAE S K Y SVEQVGVTVE 400
FYGGELNQVS YSDPATVKKY ARRAQLGEIF ELDRASSL KD CVFRSSPRGW 450
FSFGHASFAL LFFFGRHIWHG SRS RFDV VA GIDPDL DV QV EF GAP KIGD 500
PSSRRQGV. 508

Figure 4. Amino acid sequence of spinach CPa-1

chlorophyll \(\alpha\) antenna for the reaction center (Bricker 1990). CPa-1 is also
called CP47, based on electrophoretic molecular weight determinations,
similarly CPa-2 is CP43. Early reports suggested that the PS II reaction center
was located in CPa-1 (Nakatani \textit{et al.} 1984). However, the implications of the
X-ray crystallographic solution of the bacterial reaction center strongly support
a D1-D2 based PS II reaction center as described earlier. Chl \(\alpha\) and \(\beta\)-
carotene are non-covalently associated with these polypeptides. The single
copy genes encoding for CPa-1 and CPa-2 are on the chloroplast genome, \textit{psbB}
and \textit{psbC} genes respectively. The polypeptides are single-chained, intrinsic,
thylakoid-membrane proteins. The amino acid sequence for CPa-1 from
spinach has been deduced from the plastid gene sequence (Morris and
Herrmann 1984), (Figure 4). Hydropathy plot analysis suggests that each
polypeptide has six putative membrane spanning \(\alpha\)-helices, with both termini
(NH$_3^+$ & COO$^-$) on the stromal side of the thylakoid membrane (Bricker 1990). Experimental evidence using antibodies against specific hydrophilic domains of CPa-2 also suggests 6 transmembranous α-helices (Sayre and Wrobel-Boerner 1990). The hydropathy plots also predict five hydrophilic regions connecting the membrane spanning helices. There are conserved, histidyl residues that coordinate some of the 20-25 Chl α molecules within the lipid bilayer. CPa-1 is more strongly bound to the D1-D2-Cyt b$_{599}$ reaction center core than is CPa-2 as evidenced by the sequential removal of CPa-2 and CPa-1 using chaotropic agents (Ghanotakis et al. 1989) or detergents (Fotinou and Ghanotakis 1990). After the post-translational modification of the amino terminal region, the NH$_3^+$ terminal residue of CPa-2 is N-acetyl-O-phosphothreonylnine (Michel et al. 1988). The amino terminal of CPa-1 is not blocked (Bricker 1990). Little other data exists on post-translational modification of these two polypeptides. Investigations of PS II assembly using mutants of C. reinhardii (Jensen et al. 1986) and Synechocystis PCC 6803 (Vermaas et al. 1988) suggest CPa-1 and CPa-2 are required for PS II to be competent in oxygen evolution. The assembly of a functional photosystem seems to be mediated by the interaction of CPa-1 and CPa-2 with D1 and D2. Of particular interest is the large luminal extrinsic loop between helix V and helix VI that contains 191 amino acids in CPa-1 and 133 amino acids in CPa-2. Site-directed mutagenesis within these regions of the two polypeptides (Rögner et al. 1991 and Putnam-Evans, C., personal communication) are attempting to
determine additional functional roles of these polypeptides other than in the proximal antennae. There are other proposed roles for these two polypeptides. Both polypeptides are protected from tryptic attack and NHS-biotin labeling by the 33 kDa MSP (Bricker and Frankel 1987). Crosslinking experiments suggest an interaction between CPa-1 and the 33 kDa MSP, although these crosslinked species have not been well characterized (Enami et al. 1987, Bricker et al. 1988). Interestingly, CPa-1 and CPa-2 are required for O₂ evolution, and no preparation devoid of either polypeptide will evolve O₂.

**Extrinsic Polypeptides** The extrinsic polypeptides of PS II are nuclear encoded and must be transported across 3 membranes to the lumen of the thylakoid membrane where they become part of the functional oxygen evolving complex on the donor side of PS II (Westhoff et al. 1985). Various gene designations have been proposed (wax, oec, oee, psb #), but the current convention for these nuclear genes, designated psbO, psbP, and psbQ, was established at a gene nomenclature workshop held at the VIIIth International Congress on Photosynthesis (Hallick 1989). The number of amino acids in each precursor/mature protein, as deduced from the gene sequence, is: psbO, 331/247 (Tyagi et al. 1987); psbP, 267/186; and psbQ, 242/149 (Jansen et al. 1987). Calculated molecular weights for each precursor/mature protein are 35/26.5 kDa, 28.5/20.2 kDa, and 24.9/16.5 kDa, respectively. Predicted molecular weights only confuse the issue when trying to identify these mature proteins using LDS-PAGE. The mature psbO gene product is referred to as
the manganese stabilizing protein (MSP), and its LDS-PAGE determined molecular weight is 33 kDa. Similarly, LDS-PAGE determined molecular weights of the mature psbP and psbQ gene products are 24 and 17 kDa, respectively. Post-translational processing of the precursors is accomplished by two separate proteolytic cleavages of the transit sequences (Jansen et al. 1987 and Tyagi et al. 1987), directing translocation first to the stroma and ultimately to the lumen. The hydrophobic portion of the transit sequence (amino acids -1 to -24, where amino acid # +1 is the first amino acid of the mature protein) from many lumenally-destined precursors is similar in all oxygenic photosynthetic organisms (Philbrick and Zilinskas 1988). Cyanobacteria lack psbP and psbQ, however psbO is found in all oxygenic organisms.

Characterization of the three major extrinsic polypeptides of PS II was greatly advanced by use of high salt techniques to remove extrinsic proteins (Kuwabara and Murata 1982b, 1983, Åkerlund and Jansson 1981, Ono and Inoue 1983, Miyao and Murata 1984).

The 33 kDa MSP was well characterized before the current PS II isolation methods or the high salt extrinsic polypeptide removal techniques were established. Isoelectric focusing established 5.2 as the pI. The amino acid composition revealed only one methionine, one tryptophan, and two cysteine residues. Based on the intensity of light absorption at 276 nm ($A^{1%} = 6.8$) and a molecular mass of 33 kDa, the extinction coefficient was determined to be 22,000 M$^{-1}$ cm$^{-1}$. In order to explain the very hydrophilic nature of the 33 kDa
MSP and its tight association with the thylakoid membrane, it was postulated that the 33 kDa MSP must be anchored to the thylakoid membrane by a hydrophobic intrinsic membrane polypeptide (Kuwabara and Murata 1982a).

Interestingly, the amino acid sequence of the spinach 33 kDa MSP was

EGGKRLTYDE IQSKTYLEVK GTGTANQCPT VEGGVDSFAF KPGKYTAKKF 050
CLEPTKFAVK AEGISKNSGP DFQNTKLMTL LTYTLDEIEG PFEVSSDGT 100
KFEEDKDGYID AAVTVQLPGG ERVPFLFTIK QLVASGKPSF FSGDLVPSY 150
RGSSFLDPKG RGGSTGYDNA VALPAGGRGD EELQKENNKF NVASSKGIT 200
LSVTSSKPET GEVIGVFQSL QPSDTDLGAK VPKDVKIEG V 247

Figure 5. Amino acid sequence for spinach MSP

determined by solid-phase sequencing of the isolated protein (Oh-oka et al. 1986) before the amino acid sequence was deduced from the cDNA nucleotide sequence (Tyagi et al. 1987). The only difference is an additional glutamine on the C-terminal of the sequence obtained from the protein, 247 vs. 248 amino acids. The currently accepted spinach sequence is 247 amino acids, with only one glutamine on the C-terminal. The 33 kDa MSP amino acid sequence is known for several cyanobacterial and several higher plant species. There is a marked difference in the amino acid composition between the Anacystis nidulans R2 sequence and all of the other sequences, but not in the total number of amino acids (Kuwabara et al. 1987). Comparison of the sequences from A. nidulans R2 to the sequence of other species reveals 5 conserved regions, which perhaps serve as critical structural or functional domains.
two cysteine residues which form a disulfide bridge that is required for \( \text{O}_2 \) evolution (Tanaka and Wada 1988), are also conserved. In pea, the 33 kDa MSP is encoded by a multi-gene family (Wales et al. 1989). Amino acid sequence comparisons were made between the 33 kDa MSP and bacterial manganese superoxide dismutase in an attempt to assign Mn ligands to the 33 kDa MSP (Oh-oka et al. 1986). There is no direct evidence that the 33 kDa MSP binds manganese.

The extrinsic polypeptides of PS II have long been known to be necessary for optimal \( \text{O}_2 \) evolution. Their role is to maintain the ionic environment at and around the site of \( \text{O}_2 \) evolution. One of the more informative, and simple methods for determining the role of the extrinsic polypeptides is to measure \( \text{O}_2 \) evolution after salt treatments designed to selectively remove the extrinsic polypeptides and/or the inorganic cofactors, such as \( \text{Ca}^{2+} \) and \( \text{Cl}^- \). Incubation of PS II oxygen-evolving membrane particles in isolation buffer with 1 M \( \text{NaCl} \) removes the 24 and 17 kDa extrinsic proteins (Kuwabara and Murata 1983). This treatment causes the loss of \( \text{O}_2 \) evolving activity, however, a recovery of 95% of the original \( \text{O}_2 \)-evolving activity occurs when the \( \text{O}_2 \)-evolving assay is done in the presence of 15 mM \( \text{CaCl}_2 \) (Ghanotakis et al. 1984). PS II \( \text{O}_2 \)-evolving membranes, incubated in resuspension buffer containing 1 M \( \text{CaCl}_2 \), lose all three extrinsic polypeptides (Ono and Inoue 1983). PS II \( \text{O}_2 \)-evolving membranes incubated in resuspension buffer with 1 M \( \text{NaCl} \) and 2.6 M urea also lose all of the extrinsic polypeptides (Miyao and Murata 1984). The \( \text{Cl}^- \)
concentration must remain above 100 mM when the extrinsic polypeptides are removed with CaCl₂ or NaCl/urea or else the loosely bound Mn will be lost. Consequently, O₂ evolution will not be greater than 10 % of the unperturbed membranes. However, if the membranes remain in 200 mM NaCl and 5 mM CaCl₂ the tetranuclear manganese complex remains intact and O₂ evolution can be as high as 37 % of unwashed controls (Miyao and Murata 1984). PS II O₂-evolving membranes that are exposed to low pH buffers (2.0 - 6.5, 20 mM) sequentially lose the 17 kDa, 24 kDa, and 33 kDa, extrinsic polypeptides. The pH at which half of the 17 kDa polypeptide becomes dissociated from PS II is 5. Similarly, the half dissociation pH's for the 24 kDa and 33 kDa polypeptides are 4.1, and 3.6, respectively. More than 70 % of the Mn is lost after 10 minutes at pH 3 (Shen and Inuoe 1991). PS II O₂-evolving membranes, incubated in resuspension buffer made with 1 M Tris-HCl, pH 8.0-9.2, lose all three extrinsic polypeptides and all 4 of the bound Mn. O₂-evolving activity cannot be restored after this treatment (Åkerlund and Jansson 1981 and Kuwabara and Murata 1982b). Ca²⁺ was found to be optimal in the restoration of O₂-evolving activity after 1 M NaCl treatment (CaCl₂ vs. MgCl₂, SrCl₂, MnCl₂, all at 15 mM) (Ghaontakis et al. 1984). The 24 and 17 kDa polypeptides maintain the Ca²⁺ and Cl⁻ environment required for O₂ evolution (Jansen et al. 1987). The 33 kDa MSP sequence has been compared to other Ca²⁺ binding proteins and two putative Ca²⁺ binding sites have been identified (Coleman 1990). The 33 kDa MSP, even after electroblotting to nitrocellulose,
will bind $^{45}$Ca (Wales et al. 1989), however no unambiguous ligands for calcium have been identified. Two different binding sites for Ca$^{2+}$ have been identified, a high affinity site in wheat and a low affinity site in spinach (Ghanotakis et al. 1984). Complete reconstitution of O$_2$-evolving activity has never been reported. Clearly, the extrinsic polypeptides and their inorganic cofactors are required for maximal O$_2$-evolving rates.

Current Structural Models

Current structural models of PS II polypeptides place D1-D2-Cyt b$_{599}$ at the reaction center core (Brudvig et al. 1989, Coleman 1990, Hansson and Wydrzynski 1990, Rochaix and Erickson 1988, Rutherford 1989). All of these models reflect the homology to the three dimensional structure of the bacterial reaction center (Deisenhofer et al. 1985). As mentioned previously, the stoichiometry of Cyt b$_{599}$ is unclear. CPa-1 and CPa-2 are believed to be juxtaposed to the reaction center with CPa-2 bound less tightly (Ghanotakis et al. 1989). Proposed distances between the electron transfer components exist (Hoganson and Babcock 1989); even Mn-Mn and Mn-ligand distances have been estimated using EXAFS (Debus 1991 and Brudvig et al. 1989), but protein architecture is lacking. Several investigations using the extrinsic 33 kDa MSP as an affinity ligand for isolation of the reaction center complex (PS II devoid of LHCII) postulate that the 33 kDa MSP specifically binds to the reaction
center through CPa-1 or CPa-2 (Isogai et al. 1987) or through the D1-D2-Cyt b₅₅₉ core (Gounaris et al. 1988).

The interaction of the 33 kDa MSP with PS II is unclear, although the 24 kDa polypeptide is likely bound to the MSP and the 17 kDa protein is bound to the 24 kDa polypeptide (Miyao and Murata 1989). In experiments using low pH to remove the extrinsic polypeptides, negative charges on the 33 kDa MSP (aspartate and glutamate residues) have been suggested to bind the entire extrinsic polypeptide ensemble to the PS II membrane particles (Shen and Inoue 1991). It is unclear if the 17 kDa polypeptide binds only to the 24 kDa protein or to both the 24 kDa and 33 kDa polypeptides (Miyao and Murata 1989). The absence of the 24 and 17 kDa polypeptides in cyanobacteria implies the 33 kDa MSP interacts alone with the reaction center. Removing a small portion from the N-terminal of the 33 kDa MSP using limited proteolysis, indicates that this region of the 33 kDa MSP is necessary for binding to the membrane bound PS II complex (Eaton-Rye and Murata 1989). These studies are based on reconstitution techniques, which are poorly understood, and never provide maximal recovery of O₂-evolving activity. An intact manganese complex is necessary for maximal binding of the 33 kDa MSP to PS II and manganese complex and the 33 kDa MSP are required for binding the 24 kDa and 17 kDa extrinsic polypeptides to the photosystem (Kavelaki and Ghanotakis 1991).
Several studies using cleavable bifunctional crosslinking reagents and diagonal electrophoresis indicate the 33 kDa polypeptide interacts with CPa-1 (Enami et al. 1987). Similar results were obtained using various chain length disuccinimidyl esters, also revealing crosslinkage between the two peripheral extrinsic proteins (Enami et al. 1990). When PS II O₂-evolving membrane particles were labeled with [NHS]-biotin in the presence or absence of the 33 kDa MSP, labeling of CPa-1 was observed only after the removal of the 33 kDa MSP (Bricker et al. 1988). These data also support a close interaction of CPa-1 and the extrinsic 33 kDa MSP.

Several studies using electron microscopy provide estimates of molecular architecture, but only gross distances can be gleaned from these type of data. Interestingly, differences between PS II from cyanobacteria and spinach have been reported. The spinach PS II core complex (CPa-1, CPa-2, D1, D2, and Cyt b₅₉₉) has a triangular-to-circular flat cylinder, 12.5-13 nm (top view) by 6.8 nm (height) by 8 nm (diameter). PS II core complexes from *Synechococcus* sp. have similar total volumes to spinach, but the top view is more elongated and not triangular-to-circular (Irrgang et al. 1988). Analysis of two dimensional crystals from spinach, containing CPa-1, D1, D2, and Cyt b₅₉₉ are asymmetrical and 10 nm (length) x 7.5 nm (maximal width) x 6 nm (height) (Dekker et al. 1990).
Methodology to Determine Structural Relationships

Crosslinking reagents are bifunctional reagents that can react at two different sites. Crosslinking reagents were originally designed for the determination of intramolecular distances, but were later found to be useful for the examination of intermolecular interactions. Many different crosslinkers have been utilized to determine the interactions between many types of biological macromolecules (Bäumert and Fasold 1989). Crosslinking reagents can be cleavable, two-step- or photo-activated, hydrophobic, or hydrophilic. Some crosslinking reagents require the reactive groups to be within specific distances, and insert molecular bridges between the reactive groups. Specific molecular bridge lengths can be chemically engineered into the crosslinking reagent. Other crosslinking reagents act as a catalyst for the formation of a covalent bond between two adjacent reactive groups and are considered zero length crosslinkers.

Chemical crosslinking techniques encompass several phases. The first phase is to choose a crosslinking reagent that will interact with the macromolecules of interest. The primary consideration is whether or not the target is hydrophobic or hydrophilic. The second consideration is the distance between the reactive groups to be crosslinked. Once the specific crosslinker is chosen, the crosslinking conditions must be determined. The final phase is resolution and detection of the subunits that are crosslinked. The subunits and the crosslinked subunits can be separated using electrophoresis, gel filtration or
When using cleavable crosslinking reagents, two-dimensional electrophoresis is often employed. The first dimension resolves the polypeptides. The crosslinked species will migrate at a higher molecular weight. The second dimension contains a reagent which cleaves the crosslinker (for example, mercaptoethanol for disulfide containing crosslinkers). Uncrosslinked proteins will appear as a diagonal from upper left to lower right. The individual monomers previously crosslinked will migrate below the diagonal of uncrosslinked proteins and appear as individual spots in the same vertical line (Bäumert and Fasold 1989).

Carbodiimides are a class of crosslinking reagents that have the general formula $R-N=\text{C}=N-R'$ where $R$ and $R'$ are either aliphatic or aromatic. EDC is a hydrophilic carbodiimide used for the direct formation of peptide bonds in aqueous solutions. EDC catalyzes the formation of amide bonds between a free amino group and a free carboxyl group (Carraway and Koshland 1972). These groups are known to be closely associated and to form salt bridges that stabilize quaternary and tertiary protein structure. This suggests a charge-pair interaction between polypeptide subunits. EDC is considered a zero length crosslinker. The conditions for crosslinking electron transfer proteins using EDC have been extensively characterized (Mauk and Mauk 1989).

Crosslinking conditions for this study were designed to minimize intercomplex crosslinking. Parameters which preserve $O_2$-evolving activity were maintained during crosslinking.
A library of mono- and polyclonal antibodies as specific probes is advantageous from two perspectives. Detection limits are enhanced using antibodies. Antibodies also allow unambiguous identification of the subunits that are crosslinked. Monoclonal antibody technology permits the production of very specific probes. Even if it is difficult to isolate individual subunits, the entire multimeric complex can be used as an antigen for injections. Antibodies against synthetic peptides can be designed if the amino acid sequences are published. Determination of domains of neighboring subunits or membrane topography can be established (Sayre et al. 1986).

The goal of this research was to determine the structural organization of the polypeptides of PS II. Since no three dimensional structure exists for PS II, other methods must be employed to determine the structural organization of the subunits. Protein crosslinking can provide information about the tertiary and quaternary structure of multimeric proteins. Neighboring PS II subunits were covalently crosslinked using the hydrophilic crosslinking reagent EDC. This crosslinking reagent does not enter the hydrophobic membrane environment and to be crosslinked, subunits must have domains exposed to the aqueous phase. Hydrophilic segments which link the hydrophobic trans-thylakoid α-helices of intrinsic polypeptides and the three hydrophilic extrinsic polypeptides on the lumenal surface of the thylakoid membrane are candidates for EDC crosslinkage. Crosslinked species were resolved using LDS-PAGE
and the identity of the subunits crosslinked were determined using a library of mono- and polyclonal antibodies that recognize the specific subunits of PS II.

The first phase of this project was to obtain or generate a library of antibodies against PS II components. Using this library of antibodies, the second phase was to identify which PS II subunits are crosslinked by EDC. The final phase of this research is to characterize the species crosslinked. Characterization of these crosslinked species not only identified neighboring PS II subunits, but also identified domains of interaction between subunits.

There are two major analytical constraints when using crosslinking reagents to determine the structural organization multimeric proteins. The first is the ability to detect the crosslinked species. That limitation has been circumvented by the development of specific antibody probes to each subunit of PS II. The second limitation of the technique is the low amount of protein that is crosslinked. If high concentrations of EDC are used, multimeric crosslinking occurs. Concentrations of EDC low enough to form 1:1 crosslinked species can be resolved using LDS-PAGE. The high sensitivity and specificity of the antibodies detects low levels crosslinked products and also identifies the subunits involved in the crosslinkage.
MATERIALS AND METHODS

Isolation of PS II oxygen-evolving membrane particles

PS II oxygen-evolving membrane particles were isolated by the method of Berthold et al. (1981), using the modifications of Ghanotakis and Babcock (1983). Market spinach (Spinacia oleracea L.) was washed with deionized water, ground in a cold blender containing cold chloroplast isolation buffer (100 mM sucrose, 200 mM NaCl, 5 mM MgCl₂, and 50 mM Na-KPO₄ buffer, pH 7.4) and filtered through four layers of Miracloth (CalBiochem Co.). The filtrate, kept on ice, was centrifuged for 5 minutes (min) at 2,000 x g to collect the class II chloroplasts. The remainder of the PS II oxygen-evolving membrane particle isolation procedure was done at 4°C. Class II chloroplasts (intact thylakoids) were resuspended in 300 mM sucrose, 10 mM MgCl₂, 15 mM NaCl, and 50 mM Mes-NaOH, pH 6.0 (resuspension buffer) and incubated for 1.5 hr in the dark. The 1.5 hr dark incubation, believed to enhance granal stacking, was superfluous and did not significantly enhance yield or quality of PS II oxygen-evolving membrane particles. Thylakoids were then subjected to Triton-X-100 treatment for 25 min in the dark at a Chl to triton ratio of 25:1 with occasional agitation. Triton treated thylakoids were centrifuged at 2,000 x g and the supernatant was immediately recentrifuged at 40,000 x g. The high speed pellet was resuspended in resuspension buffer, and again subjected to
differential centrifugation to obtain PS II oxygen-evolving membrane particles. This second differential centrifugation served to separate any remaining stromal lamellae and remove the excess Triton-X-100. [Chl] was measured spectrophotometrically by the method of Arnon (1949). PS II oxygen-evolving membrane particles exhibited a chlorophyll a/b ratio of 1.90-2.03. PS II oxygen-evolving membrane particles are highly enriched in PS II while being depleted in other thylakoid membrane proteins (Dunahay et al. 1984).

In some experiments, PS II oxygen-evolving membrane particles were incubated 2 X for 1 hr in resuspension buffer (R membranes); resuspension buffer containing 1 M NaCl to remove the 24 and 17 kDa extrinsic proteins (N membranes); resuspension buffer containing 1.0 M CaCl₂ to remove the 17, 24, 33 kDa polypeptides and the loosely bound Mn (C membranes); or resuspension buffer containing 1.0 M Tris-HCl, pH 9.2, (instead of Mes/NaOH) to remove the all of the Mn and the 33, 24, and 17 kDa extrinsic proteins (T membranes). These modified membranes were washed twice with resuspension buffer to remove the high salt and will be called salt washed membranes, and referred to as R, N, C, or T membranes (Table II).

A non-oxygen evolving PS II core complex was isolated by the procedure of Bricker et al. (1985). PS II reaction center complex was isolated by the procedure of Ghanotakis et al. (1987).
Table II. Composition of PS II salt washed membranes

<table>
<thead>
<tr>
<th>Treatment Symbol</th>
<th>Wash Buffer</th>
<th>Proteins Lost</th>
<th>Mn Lost</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>Resuspension Buffer</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>N</td>
<td>Resuspension Buffer + 1 M NaCl</td>
<td>24 kDa</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17 kDa</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Resuspension Buffer + 1 M CaCl₂</td>
<td>33 kDa MSP</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 kDa</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>17 kDa</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>Resuspension Buffer made with 1 M Tris/HCl, pH 9.2</td>
<td>33 kDa MSP</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 kDa</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>17 kDa</td>
<td></td>
</tr>
</tbody>
</table>

Crosslinking (EDC)

In proteins, EDC induces the formation of peptide bonds between adjacent carboxyl and free amino groups (Carraway and Koshland 1972) (Figure 6). Intact PS II oxygen-evolving membrane particles, as well as those that had been selectively depleted of various extrinsic PS II polypeptides, were then washed twice with resuspension buffer to remove excess salt and a third time with resuspension buffer made with 50 mM sodium-potassium phosphate, pH 6.0. The washed membranes ([Chl] = 1 mg/ml), now in a phosphate resuspension buffer, were subjected to protein crosslinking. Initial crosslinking
Figure 6. Structure of 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide, EDC (Bauminger and Wilchek 1980)

Concentrations were 0, 10, 50, 100, 250, 500, 750, and 1,000 mM. Extensive crosslinkage occurred at and above 50 mM EDC; therefore the second range was narrowed to 0, 1, 2, 5, 10, 15, 20, and 25 mM for 20 min at 4°C, in the dark. Crosslinkage [0-25 mM] was also done on the salt washed membrane preparations. The EDC modified PS II oxygen-evolving membrane particles were washed in Mes/NaOH resuspension buffer twice, aliquoted and stored at -20°C prior to LDS-PAGE. After the optimum crosslinking concentration was determined, ([EDC] = 15 mM), and a crosslinked species of interest was identified, PS II oxygen-evolving membrane particles were crosslinked in bulk and stored at -20°C, for the subsequent isolation of crosslinked species.

Crosslinking [15 mM] was also done in the presence of saturating white light and 2,5-dichloro-p-benzoquinone (DCBQ) to determine if light and/or electron transport induced protein conformational changes might enhance or diminish crosslinking.
Identification of Intracomplex Crosslinked Polypeptides

There are several antibodies available for the detection of crosslinked PS II components (Table III). The first phase of this research was to develop and characterize these specific probes. Some of these probes were developed and characterized in other laboratories.

Table III. Antibodies used to detect PS II polypeptides

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>PS II protein</th>
<th>Poly- or monoclonal</th>
<th>producing organism</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-D1</td>
<td>D1</td>
<td>P</td>
<td>Rabbit</td>
<td>Trebst¹</td>
</tr>
<tr>
<td>FAC2</td>
<td>CPa-1</td>
<td>M</td>
<td>Mouse</td>
<td>Bricker²</td>
</tr>
<tr>
<td>anti-5</td>
<td>CPa-1</td>
<td>P</td>
<td>Rabbit</td>
<td>Delepelaire³</td>
</tr>
<tr>
<td>anti-6</td>
<td>CPa-2</td>
<td>P</td>
<td>Rabbit</td>
<td>Delepelaire³</td>
</tr>
<tr>
<td>FQC3</td>
<td>D2</td>
<td>M</td>
<td>Mouse</td>
<td>Odom⁴</td>
</tr>
<tr>
<td>anti-b₃₃₉</td>
<td>cyt b₃₃₉</td>
<td>P</td>
<td>Rabbit</td>
<td>Widger⁵</td>
</tr>
<tr>
<td>anti-MSP</td>
<td>MSP</td>
<td>P</td>
<td>Mouse</td>
<td>Bricker⁶</td>
</tr>
<tr>
<td>FBC13</td>
<td>24 ext</td>
<td>M</td>
<td>Mouse</td>
<td>Frankel⁷</td>
</tr>
<tr>
<td>FCC4</td>
<td>17 ext</td>
<td>M</td>
<td>Mouse</td>
<td>Frankel⁷</td>
</tr>
</tbody>
</table>


Mono- and polyclonal antibody production

Mice were immunized by intraperitoneal injection with spinach PS II core complex equivalent to 100 µg Chl in Freund's complete adjuvant. Ten days later mice were injected with the equivalent of 100 µg Chl of the same immunogen in Freund's incomplete adjuvant. A final immunization was performed ten days later with the equivalent of 100 µg Chl of the same
immunogen in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl (TS buffer). Injection volumes were 500 µl.

Standard hybridoma techniques were used to isolate monoclonal antibodies (Wise and Watson 1983). Three days after the final immunization, antigen-primed murine splenocytes were obtained aseptically by teasing one mouse spleen apart in incomplete (no serum) 37°C RPMI 1640 medium, with 2X penicillin/streptomycin (pen/strep). The cell suspension was transferred to a 50 ml centrifuge tube to allow the large pieces to settle after which the supernatant was removed. The supernatant was centrifuged at 400 x g and the whole cell pellet was resuspended in 20 ml of incomplete RPMI 1640 with 1X pen/strep. When the immunization schedule was started, cultures of AG8.653 myeloma cells also commenced. These variant murine myeloma cells are 8-azaguanine/6-thioguanine resistant; they do not have hypoxanthine guanine phosphoribosyl transferase (HGPRT) nor do they excrete any immunoglobulin chains. Without HGPRT, AG8.653 cannot use hypoxanthine in the medium to synthesize purines via the alternate biosynthetic pathway, however they will grow in the presence of 8-azaguanine. Cultured cells were pooled and centrifuged for 5 min at 400 x g, after counting the cells. The pellet was washed twice in incomplete RPMI 1640, with 2X pen/strep. Finally, the myeloma cells were resuspended in 20 ml of incomplete medium with 1X pen/strep. The splenocyte:myeloma ratio is critical and must be 3:1. Both cell types were placed in the same 50 ml centrifuge tube and centrifuged for 5 min
at 400 x g. After removing as much medium as possible, the following fusion protocol was adhered to strictly, starting with the addition of 1 ml of polyethylene glycol added slowly over 45 seconds. Addition of 40 ml of incomplete RPMI 1640 with 2X pen/strep over 1.5 min was done in increments of 3 ml in 30 sec, 9 ml in 30 sec, and 28 ml in 30 sec. The fusion mixture was incubated for 8 min at 25°C, and for 2 min at 37°C and immediately centrifuged for 5 min at 200 x g. The pellet was resuspended in 100 ml of complete RPMI 1640 plus hypoxanthine and thymidine (HT medium) that contained peritoneal macrophages isolated from a retired breeder mouse. The resuspended cell fusion mixture was aliquoted using an octapipettor into ten 96-well microtiter plates, each well containing 100 µl. Twenty-four hours later, an additional 100 µl of the same medium plus aminopterin (A) was added to each well. HAT medium is the basis for clonal selection. Splenocytes do not grow well in culture; myeloma cells will not grow because aminopterin blocks the endogenous synthesis of both purines and pyrimidines. After 10-14 days the only cells capable of growth are the hybridoma cells, that contain the spleen cell's HGPRT, and the rapid growth characteristics of the myeloma. These cells use the exogenous hypoxanthine and thymidine provided in the medium.

Importantly, some hybrids retain the function for antigen-specific antibody production. Screening for antibody production for PS II polypeptides was done using well supernatant from growing hybridoma colonies in a Miniblotter II
Advantages of this device are the small supernatant volume requirement (100 µl from one well of a 96 well tissue culture plate), capability to test multiple colonies at once, and the capacity to screen simultaneously for positives against individual PS II polypeptides. Once positive colonies have been identified, the antibody secreting hybrid cell line must be isolated and can then be cloned and continuously cultivated \textit{in vivo} or \textit{in vitro}.

Polyclonal antibodies were generated using selected purified polypeptides or polypeptide fragments. Mice were first immunized (day 0) by intraperitoneal injection of 100-200 µg protein/100 µl plus 100 µl of Ribi Adjuvant System, RAS, per manufacturer’s suggested immunization protocols (\textit{Ribi Immunochem Research, Inc.}). On day 21 mice were injected as before, and on day 31 serum was collected from the immunized mice and tested for antigen-specific immunopositive reaction. Positive or not, mice were again boosted on day 42, serum collected on day 52, and tested. Once serum antibody titers were high, collected antibodies were used as antigen-specific probes. This methodology was largely utilized for polyclonal antibody production of the 33 kDa MSP and fragments thereof.

\textbf{Monoclonal antibody characterization}

\textbf{FQC3 Isotype} \hspace{1cm} The isotype of FQC3 was determined by an enzyme-linked immunosorbant assay with isotyping kits (\textit{Sigma Chemical Co.} and \textit{Boehringer-...}
Mannheim Co.) and peroxidase-conjugated secondary antibodies. Assays were developed with 2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid) and H$_2$O$_2$ (Groome 1980).

**Metabolic labeling of leaf discs** For metabolic labeling, leaf discs (2.4 cm diameter) were punched from washed spinach leaves. These discs were floated on deionized water with 0.05 % Tween 20 and irradiated under a 60 W incandescent lamp for 30 min. After this preincubation, the leaf discs were placed under the same irradiation on deionized H$_2$O containing 0.05 % Tween 20 and a mixture of $^{35}$S cysteine and $^{35}$S methionine, 4.625 x10$^4$ Bq/ml, (Trans 35, ICN Radiochemicals) for various times (0, 0.5, 1, 2, and 4 h). Leaf discs were macerated with mortar and pestle and chloroplasts isolated as described above. PS II oxygen-evolving membranes were isolated from these labeled thylakoids. After electrophoresis, the polyacrylamide gel was stained with coomassie blue, destained (7 % acetic acid and 25 % MeOH, in deionized H$_2$O overnight) and impregnated with 2,5-diphenyloxazole (20 g in 100 ml of glacial acetic acid) for 1 h (Skinner and Griswold 1983). The gel was washed twice for 1 h in deionized H$_2$O, once in 25 % MeOH and dried. The dried gel was exposed to X-OMAT AR film for 10 days at -80°C and developed.

**Fusion Protein Isolation** E. coli JM101 containing either the recombinant plasmid $p$PND1 or $p$PND2 (kind gift of Drs. P. M. Nixon and J. Barber) were grown overnight in 5.0 ml of Luria broth medium with 100 µg/ml of ampicillin at 37°C and with moderate agitation. One ml of this culture was used to
inoculate 500 ml of Luria broth medium containing 100 μg/ml ampicillin and either (non-induced) 0.0 or (induced) 500 μM isopropylthiogalactoside (IPTG). The 500 ml non-induced and induced cultures were grown overnight at 21°C with no agitation, cell concentrated by centrifugation at 10,000 x g for 10 min, and resuspended in 50 mM Tris-HCl, pH 6.8, with 2 % LDS, 6 % sucrose, 5 % β-mercaptoethanol, and 200 μl of saturated bromophenol blue. This buffer initiates cell lysis. For more complete cell lysis, the cells were heated for 5 min at 100°C. The disrupted cells were cooled on ice and centrifuged at 14,000 x g for 5 min. The resulting supernatant was the working protein lysate.

**Endoproteinase Lys-C treatment** For treatment with the endoproteinase Lys-C, alkaline tris-washed PS II reaction center complex (each sample, 3 μg Chl) was resuspended in a total volume of 15 μl of 10 mM Tris-HCl, pH 8.65 with 10 % sucrose containing various concentrations of the endoproteinase Lys-C (0, 2, 10, and 20 μg) and incubated at 37°C for two hours. After solubilization, the Lys-C treated samples were heated at 70°C for 5 min prior to electrophoresis.

**Electrophoresis and Blotting**

Prior to electrophoresis, samples were solubilized in 1.4 % LDS, 3.6 % β-mercaptoethanol, 6 % sucrose, 3.6 % saturated bromophenol blue and 8.0 M urea for the urea gels. Proteins were resolved using several electrophoretic methods:
1) 12.5-20 % gradient LDS-PAGE, 22-30 W*h at 4°C (Delepelaire and Chua 1979);

2) 15 % LDS-PAGE with 8 M urea, 34 W*h at 25°C;

3) 12.5 % preparative LDS-PAGE, 25 W*h at 4°C;

4) 13-16.5 % LDS-PAGE, with a tris-tricine reservoir buffer system, 34 W*h at 4°C; or

5) a step gradient, 4-10-16.5 % LDS-PAGE, with a tris-tricine reservoir buffer system, 75 W*h at 4°C (Schagger and Von Jagow 1987). Often electrophoresis was followed by immunoblotting to pure nitrocellulose (Towbin et al. 1979) or polyvinylidene difluoride membrane, PVDF (Immobilon P, Millipore Co.). Blots were either stained with amido black, coomassie brilliant blue or blocked for 1 hour with 5 % non-fat dry milk in TS buffer. Blocked panels were incubated overnight with primary antibody at room temperature. Blots were then washed with TS buffer, and appropriate peroxidase-conjugated (4 hour incubation) or alkaline phosphatase-conjugated (1 hour incubation) secondary antibodies were applied. Secondary antibody-enzyme conjugates were diluted 1:2000 in TS buffer with 1 % BSA prior to application. After the appropriate incubation with continuous agitation, blots were washed with TS buffer and developed using H₂O₂ and 4-chloro-1-naphthol (Hawkes 1986) for the peroxidase, and NBT/BCIP for the phosphatase.
Oxygen evolution

Oxygen evolution was measured using a polarographic apparatus that quantifies the electrolytic reduction of \( O_2 \). The total reaction volume was 1 ml and contained the following constituents: assay buffer composed of 50 mM Mes/NaOH, pH 6.0, 400 mM sucrose, and 15 mM CaCl\(_2\); 10 \( \mu \)g Chl; 250 \( \mu \)M DCBQ (added as 2.5 ul of a 100 mM stock in 100 % ethanol). Reaction was allowed to proceed for 1 min. The light was provided by a slide projector with a 500 W lamp filtered by a saturated solution of copper sulfate such that the PAR upon the cuvette was 1250-1500 micromoles (m\(^2\) sec\(^{-1}\)). Slopes were calculated by hand measurement and \( O_2 \)-evolving rates were determined in \( \mu \)moles of \( O_2 \) (mg Chl\(^{-1}\)) hr\(^{-1}\).

To determine the amount of \( O_2 \)-evolving activity retained by crosslinked membranes, PS II oxygen-evolving membrane particles were isolated as above and treated with EDC concentrations of 0, 5, 15, and 50 mM, then subjected to the same treatment used to generate R membranes and C membranes. R membranes and C membranes that were never exposed to the crosslinkage procedure served as unperturbed controls. R membranes and C membranes were assayed 5 times at each EDC concentration. The entire experiment was replicated four times.
Isolation of the CPa-1/MSP Crosslinked Species (XL)

A crosslinked species, identified by both anti-MSP and FAC2, was isolated using preparative electrophoresis followed by electroelution. Entire PS II oxygen-evolving membrane particle isolations were crosslinked in bulk using 15 mM EDC and frozen in 5 ml aliquots with a [2 mg/ml] Chl. Eight preparative 1.5 mm LDS-PAGE gels were each loaded with 1.25 mg Chl from one of the frozen 5 ml aliquots. After electrophoresis, gels were incubated in acid free Coomassie Blue stain (0.1 % Coomassie Blue, w/v, in 25 % methanol) for 4 h, and destained in 25 % methanol until the crosslinked species could detected using a light box. Crosslinked bands were cut from all 8 gels, incubated for 30 min in 25 mM Tris, 190 mM glycine buffer pH 8.8, with 1 % LDS and electroeluted in the same Tris/glycine buffer except with 0.1 % LDS (Jacobs and Clad 1986). Typical final electroelution volumes were 0.4-0.5 ml. Electroeluted samples were washed using several mls of H₂O in a Centricon 30 in an attempt to reduce the detergent concentration. Samples were concentrated to 250 μl and stored at -20°C or -80°C just prior to chemical or proteolytic cleavages.

Chemical and proteolytic cleavages of XL

MSP, CPa-1, and XL were isolated by preparative LDS-PAGE followed by electroelution and subjected to cleavage with cyanogen bromide (CNBr) (Gross 1969). All cleavages were done in a chemical safety hood with proper
ventilation. CNBr cleaves on the carboxy of methionine with excellent efficiency except when threonine or serine is on the carboxyl side of methionine (Schroeder et al. 1969). Since the only methionine in the 33 kDa MSP is followed by threonine, this cleavage method was optimized for maximal cleavage of the methionine/threonine bond. Cleavage of CPa-1 under conditions optimized for MSP went to apparent completion. Cleavages were done using excess (30 fold) CNBr at a concentration of 120 mg CNBr/ml in 50 % formic acid. For smaller amounts of protein, reaction volumes were 1 ml. Pooled electroelutions were cleaved using 10 ml reaction volumes. Cleavage time was 4 h at 25°C, with occasional agitation. After cleavage, samples were aliquoted to 1 ml Eppendorf tubes and lyophilized (with the vacuum pump, under the hood) until dry. Cleaved samples were solubilized in 1.4 % LDS, 3.6 % β-mercaptoethanol, 6 % sucrose, 3.6 % saturated bromophenol blue, and fragments were resolved using LDS-PAGE in an analytical gel for direct fragment analysis or prep gels for the isolation of specific fragments.

LDS-PAGE electroeluted polypeptides were also cleaved at the most acid labile bond between aspartate and proline using formic acid (Landon 1979). Protein was dissolved at 0.5 mg/ml in 70 % formic acid and incubated at 40°C for 12 h. Cleavage was less efficient than the CNBr reaction, and only the major fragments generated were considered in the analysis. After cleavage, samples were lyophilized and solubilized in 1.4 % LDS, 3.6 % β-mercaptopoethanol, 6 % sucrose, 3.6 % saturated bromophenol blue, and fragments were resolved using LDS-PAGE in an analytical gel for direct fragment analysis or prep gels for the isolation of specific fragments.
to ethanol, 6% sucrose, 3.6% saturated bromophenol blue. Fragments were resolved using analytical LDS-PAGE.

Limited proteolytic cleavage was done on MSP and XL that were electroeluted and washed 3 X in a Centricon 30 with 5 mM Mes/NaOH, pH 6.5. Proteins were treated with α-Chymotrypsin type VII (Sigma Chemical Co.), isolated from bovine pancreas, and treated with 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK) which inactivates any contaminating trypsin. Cleavages were done in 5 mM Mes/NaOH, pH 6.5 and the approximate protein to enzyme ratio was 100:1 (Eaton-Rye and Murata 1989) Cleavage times were 0.5 and 1.0 h at 20°C. Reactions were stopped by addition of a small volume of 100 mM PMSF (phenylmethanesulfonyl fluoride) solubilized in isopropanol such that the final PMSF concentration was 5 mM. Samples were solubilized in 1.4% LDS, 3.6% β-mercaptoethanol, 6% sucrose, 3.6% saturated bromophenol blue. Fragments were resolved using analytical LDS-PAGE followed by immunological analysis of electroblots.

**Detection of polypeptides biotinylated on Immobilon**

Often after cleavage, normal detection methods after LDS-PAGE were not possible. LDS-PAGE resolved fragments were electrotransferred to PVDF and the blot was washed for 5 min in 0.1 M NaHCO₃, pH 8.3. The blot was then incubated with rocking with 100 ml of 5 μM NHS-Biotin in the same buffer for 1 h at 25°C. The excess NHS-biotin was quenched with 10 mM glycine in the
same buffer for 5 min (LaRochelle and Froehner 1986). The blot was washed in the same buffer 15 min three times and then blocked with 5 % dry milk in TS buffer. The blot was probed with a 1:5,000 dilution of Avidin-peroxidase for one hour and developed as above using 4-chloro-1-naphthol (Hawkes 1986).

Sequence analysis

N-terminal amino acid sequence analysis was done by automated phenyl isothiocyanate or Edman degradation. Samples of varying degrees of purity were electrophoresed using LDS-PAGE and electroblotted to a PVDF membrane. Blots were stained with acid free Coomassie Blue stain (to prevent N-terminal blockage), destained with 25 % methanol, and rinsed with 3 changes of ultrapure water. These washes remove the high amount of residual glycine from the transfer buffer. The membrane was allowed to dry and bands were cut with a sterile razor blade and placed in a sterile 1.5 ml Eppendorf tube for shipment to the sequencing facility. The minimum amount of protein needed for unequivocal sequence analysis was about 3-5 picomoles. If the bands were seen after drying, there was enough protein to obtain a sequence. The maximum area for one sequence analysis is about 25 mm². Sequence analysis was performed at the Sequence Facility, Baylor University.
RESULTS and DISCUSSION

Monoclonal antibody characterization: FQC3

Specific identification of crosslinked polypeptides permits the examination of the structural organization of PS II. The monoclonal antibody, FQC3, identified a polypeptide in the 30-35 kDa range, in PS II. It was necessary to determine which PS II component was recognized by FQC3 because there are several PS II polypeptides in this molecular weight range, and the antigen used to generate this antibody was a mixture of PS II polypeptides.

The isotype of the monoclonal antibody FQC3 is lambda IgM. The monoclonal antibody FQC3 recognized a protein with an apparent molecular mass of 32 kDa in thylakoid membranes, PS II oxygen-evolving membranes, and PS II reaction center complex (Fig. 7). The component recognized by FQC3 comigrated with the extrinsic 33 kDa MSP and thus appeared as a doublet. An antibody raised against the D1 protein (kindly supplied by Dr. A. Trebst) recognized a component with an apparent molecular mass of 34 kDa in these protein samples. The components recognized by the antibodies FQC3 and α-D1 are distinct. In the PS II reaction center complex an additional protein band with an apparent molecular mass of about 97 kDa was recognized by both antibodies and it represents the putative D1/D2 heterodimer (Nixon et al. 1986).
Figure 7. Immunoblot analysis of proteins present in chloroplasts, oxygen-evolving PS II membranes, and PS II core complex, probed with FQC3 and α-D1. Panel A was stained with amido black. Panel B was probed with FQC3. Panel C was probed with polyclonal α-D1 (kindly provided by A. Trebst). In each panel, lane 1 contained 20 μg Chl of class II chloroplasts, lane 2 contained 8 μg Chl of oxygen-evolving PS II membranes, and lane 3 contained 2 μg Chl of OERC complex. The protein recognized by FQC3 appears as a doublet in this figure because the extrinsic 33 kDa protein migrates at nearly the same location as the component recognized by FQC3.
The D1 protein of spinach does not contain lysyl residues (Zurawski et al. 1982) while the spinach D2 protein contains five lysyl residues (Alt et al. 1984). The closely related D1 and D2 proteins can be distinguished using the endoproteinase Lys-C (Marder et al. 1987) which cleaves to the carboxyl side of lysyl residues. In the absence of urea during electrophoresis the D2 protein exhibits a higher mobility than the D1 component while in the presence of 8.0 M urea the D1 protein migrates faster than D2 (Marder et al. 1987). When the extrinsic 33 kDa protein was removed from PS II with treatments such as alkaline Tris washing, the component recognized by FQC3 appeared as a single, well-defined band (Figure 8). The intensity of the protein recognized by FQC3 decreases as the concentration of Lys C increases (Figure 8A) while the intensity of D1 is constant (Figure 8B) when urea is absent. The intensity of the protein recognized by FQC3 decreases and there is also a noticeable shift to a lower apparent molecular weight of a proteolytic fragment which is recognized by FQC3 in the presence of urea (Figure 8C). The intensity of the bands representing D1 is similar at all Lys C concentrations when electrophoresed in the presence of 8 M urea (Figure 8D). In the presence of urea, the protein recognized by FQC3 migrates slower than the D1 protein (Figure 8 C&D). Both the pattern of proteolytic digestion by the endoproteinase Lys C and the differential migration of these proteins in the absence or presence of urea strongly suggest that the protein recognized by FQC3 is the D2 protein of PS II.
Figure 8. Immunoblot analysis of endoproteinase Lys C treatment of tris-washed OERC complex resolved by LDS-PAGE in the absence and presence of 8 M urea. Panels A and B are standard LDS-PAGE (without urea), while panels C and D are LDS-PAGE with 8 M urea. Panels A and C are probed with FQC3 and panels B and D are probed with α-D1. Lane assignments are as follows: lane 1, 0 μg Lys C; lane 2, 2 μg Lys C; lane 3, 10 μg Lys C; and lane 4, 20 μg Lys C. All lanes contained 3 μg Chl of 1 M tris-washed OERC complex.
In both electrophoretic systems, the monoclonal antibody FQC3 and the polyclonal antibody α-D1 recognized two protein components. Both antibodies recognized a component with an apparent molecular mass of 67 kDa. This is the putative D1/D2 heterodimer. In the absence of urea during electrophoresis, a second component was recognized by FQC3 which migrated with an apparent molecular mass of 32 kDa while a second component recognized by α-D1 migrated with an apparent molecular mass of 34 kDa. In the presence of 8.0 M urea during electrophoresis this order was reversed. The results are consistent with the differential migration of the D1 and D2 proteins in the presence or absence of urea (Marder et al. 1987). Additionally, in either gel system, the component recognized by FQC3 exhibited sensitivity to the endoproteinase Lys-C while the component recognized by α-D1 was not affected by this protease. This result indicates that the component recognized by FQC3 contains lysine and is consistent with this component being the D2 protein.

Metabolic labeling of leaf discs with 35S-amino acids has proven useful in identifying the PS II component D1. This protein is rapidly turned-over (Marder et al. 1986; Metz et al. 1986) and can easily be identified by autoradiofluorography after metabolic labeling with a radioactive substrate. Figure 9 illustrates the results obtained after a metabolic labeling time course of leaf disks with a mixture of 35S-methionine and 35S-cysteine (Figure 9). Oxygen-evolving PS II membranes were isolated from these leaf disks, electrophoresed in LDS-PAGE and either immunoblotted and probed with
Figure 9. Time course of metabolic labeling of PS II oxygen-evolving membrane proteins with $^{35}$S-methionine and $^{35}$S-cysteine. Panel A, immunoblot stained with coomassie blue. Panel B, immunoblot probed with FQC3. Panel C, autoradiofluorograph of coomassie blue stained polyacrylamide gel. Lane 1, time 0 in the radiolabeling solution; lane 2, 30 min; lane 3, 1 h; lane 4, 2 h; lane 5, 4 h. All lanes contained 10 $\mu$g chlorophyll of PS II oxygen-evolving membrane preparation.
FQC3 or stained with Coomassie Blue followed by autoradiofluorography. A single major band accumulated relatively large amounts of label during the time-course (Figure 9C). This protein had an apparent molecular mass of 34 kDa and corresponded to a diffusely stained band in the Coomassie Blue stained panel A; these properties suggested that this was the rapidly turned over D1 protein. The protein component recognized by FQC3 comigrated with the 33 kDa extrinsic protein and not the diffuse D1 band (Figure 9B). This is consistent with the identity of the FQC3-recognized protein being the D2 protein.

The recombinant plasmids pPND1 and pPND2 contain in-frame lac Z fusions of the psbA and psbD genes, respectively (Nixon et al. 1986). In the presence of isopropylthiogalactoside (IPTG), E. coli bearing these plasmids produce large quantities of β-galactosidase fusion proteins. Figure 10 is an immunoblot analysis of cell lysates isolated from uninduced and IPTG-induced E. coli containing these plasmids (Figure 10). Cell lysates from non-induced and IPTG-induced E. coli which do not contain these plasmids do not react with the monoclonal antibody FQC3 (data not shown). The monoclonal antibody FQC3 reacted with the fusion protein present in IPTG induced E. coli containing the pPND2 plasmid. No reaction was observed in uninduced E. coli containing either pPND1 or pPND2 or in IPTG-induced E. coli containing the pPND1 plasmid. This result indicated that FQC3 recognized the D2-β-galactosidase fusion protein produced under IPTG induction.
Figure 10. Immunoblot analysis of cell lysates from transformed with either pPN1D or pPN2D and under either inducing (+ IPTG) or non-inducing conditions. Panel A, E. coli proteins stained with amido black. Panel B, E. coli probed with α-β-galactosidase. Panel C, E. coli proteins probed with FQC3. Lane assignments are as follows: lane 1, protein extract from non-induced E. coli which contain the D1-β-galactosidase fusion protein gene (pPN1D); lane 2, protein extract from IPTG-induced E. coli which contain a D1-β-galactosidase fusion protein gene; lane 3, protein extract from non-induced E. coli which contain a D2-β-galactosidase fusion protein gene (pPN2D); lane 4, protein extract from IPTG-induced E. coli which contain a D2-β-galactosidase fusion protein gene. All lanes contain 100 μg of protein. These transformed strains of E. coli were a kind gift from J. Barber.
On the basis of the five lines of evidence presented, the protein recognized by the murine monoclonal antibody FQC3 was identified as the D2 protein of PS II and not the extrinsic 33 kDa protein or the D1 protein of this photosystem. This monoclonal antibody should prove to be an extremely useful tool in structural studies of the organization of the D2 protein within PS 2.

One limitation often encountered in the use of monoclonal reagents is excessive specificity. Often a monoclonal antibody which reacts with its target antigen from a given species will not recognize the same antigen isolated from other species. However, FQC3 reacts on immunoblots with the D2 protein from *Pisum sativum* and *Brassica napus* (McCarthy, Stemler, Bricker, and Jursinic (in press)).

**Identification and analysis of multiple crosslinks observed**

Mono- and polyclonal antibodies were used to identify PS II polypeptides crosslinked using increasing concentrations of EDC. There are 8 figures (Figures 11-18) in this section. The only difference among these figures is the primary antibody used to determine whether or not that specific PS II polypeptide was crosslinked by EDC. There are either two panels, R and N, or four panels, R, N, C, and T in each figure. If there are only two panels, the polypeptide recognized by the antibody has been removed prior to crosslinking in the C and T panels. These blots were blank, and so are not shown. On the right side of each figure, the uncrosslinked PS II polypeptide is identified. The
antibody used in each figure only recognizes that PS II protein or any crosslinked species containing that polypeptide.

PS II oxygen-evolving membrane particles were incubated 2 X for 1 hr the following buffers: resuspension buffer (R membranes); resuspension buffer containing 1 M NaCl to remove the 24 and 17 kDa extrinsic proteins (N membranes); resuspension buffer containing 1.0 M CaCl₂ to remove the 17, 24, 33 kDa polypeptides and the loosely bound Mn (C membranes); or resuspension buffer containing 1.0 M Tris-HCl, pH 9.2, (instead of Mes/NaOH) to remove the all 4 of the Mn and the 17, 24, and 33 kDa extrinsic proteins (T membranes). These modified membranes, resuspended in resuspension buffer to remove the excess salt, will be called salt-washed membranes, and referred to as R, N, C, or T membranes (Table II). Salt washed membranes (R, N, C, and T) were subjected to cross linkage using 0-25 mM EDC. EDC modified proteins were resolved using LDS-PAGE and electrotransferred to nitrocellulose. Western blots were probed with various mono- and polyclonal antibodies (Table III) to determine the polypeptides crosslinked.

In figure 11 the Western blot of the crosslinked proteins was probed with a monoclonal antibody that recognizes the 17 kDa extrinsic polypeptide. There are two crosslinked products containing the 17 kDa extrinsic protein (Figure 11) at 41 and 43 kDa. The low intensity, high molecular mass bands in lane A are probably due to aggregates of the 17 kDa protein.
Figure 11. Nitrocellulose transfer of SDS-PAGE-resolved EDC modified proteins probed with the monoclonal antibody, FCC4.

Figures 11-18. EDC modified polypeptides from R and N or R, N, C, and T, membranes resolved using 12.5% - 20% gradient SDS-PAGE were electrotransferred to nitrocellulose and probed with a primary antibody (See Table II). Appropriate (anti-mouse or anti-rabbit) goat secondary IgG + IgM peroxidase conjugates were applied. Proteins were visualized by incubation of the Western blot with 4-chloro-1-naphthol and $\text{H}_2\text{O}_2$. Molecular weight standards are on the left. All lanes were loaded with $15 \mu\text{g}$ Chl. [EDC, mM] in each lane: A, 0; B, 1; C, 2; D, 5; E, 10; F, 15; G, 20; H, 25.
Figure 12. Nitrocellulose transfer of SDS-PAGE-resolved EDC modified proteins probed with the monoclonal antibody, FBC13.
Figure 13. Nitrocellulose transfer of SDS-PAGE-resolved EDC modified proteins probed with the polyclonal antibody, anti-33.
Figure 14. Nitrocellulose transfer of SDS-PAGE-resolved EDC modified proteins probed with the monoclonal antibody, FQC3.
Figure 15. Nitrocellulose transfer of SDS-PAGE-resolved EDC modified proteins probed with the polyclonal antibody, anti-D1.
Figure 16. Nitrocellulose transfer of SDS-PAGE-resolved EDC modified proteins probed with the polyclonal antibody anti-Cyt b<sub>559</sub>. 
Figure 17. Nitrocellulose transfer of SDS-PAGE- resolved EDC modified proteins probed with the polyclonal antibody, anti-6.
Figure 18. Nitrocellulose transfer of SDS-PAGE-resolved EDC modified proteins probed with the monoclonal antibody, FAC2.
In figure 12, the Western blot of the crosslinked proteins was probed with a monoclonal antibody that recognizes the 24 kDa extrinsic polypeptide. A few crosslinked products containing the 24 kDa extrinsic protein at about 29.5, 32, 46, and 66 kDa can be observed (Figure 12). These crosslinked products appear in lane D. The band observed at about 45 kDa in both blots could possibly be a crosslink between the 24 and the 17 kDa extrinsic polypeptides. This band is difficult to observe when probed with the antibody against the 24 kDa polypeptide (Figure 12, very faint). If EDC induced a crosslinkage between the 33 kDa MSP and the 24 kDa polypeptide, there should be an EDC dependent band at about 55 kDa. Such crosslinked products are not detected by the antibody against the 24 kDa polypeptide. Although faint, the crosslinkage between the 24 kDa and 17 kDa supports previously proposed topology of the extrinsic polypeptides (Miyao and Murata 1984). In figure 11 and figure 12 both N panels are blank. Both polypeptides were removed prior to crosslinkage.

In figure 13, the Western blot of the crosslinked proteins was probed with a polyclonal antibody that recognizes the 33 kDa MSP. Crosslinked products are evident at 95 and 110 kDa in both treatments. Lane D (5.0 mM EDC) in the N membranes is more intense than lane D in the R membranes. EDC concentrations below 5.0 mM (lanes A,B,C) exhibit minimal or no crosslinkage with the 33 kDa extrinsic polypeptide (Figure 13). No staining was observed in C or T membrane.
In figure 14, the Western blots of the crosslinked proteins were probed with a monoclonal antibody that recognizes the D2 protein. This is the same antibody that was characterized earlier. A possible dimer of D1 and D2 that has a molecular weight of 60-66 kDa is prevalent when the extrinsic polypeptides have been removed, but is also evident in untreated membranes. The 33 kDa MSP can be observed to split the uncrosslinked D2 band in the R and N panels, and is absent in the C and T panels. Crosslinked products can be seen in the C and T membranes above the 116 kDa molecular weight standard (Figure 14). The decrease in intensity of the 32/34 kDa band corresponds to an increase in intensity of the upper molecular mass crosslinked products in lanes E-H. This decrease in intensity is also observed in the R and N membranes without the concomitant observation of high molecular mass crosslinked products.

Upon observation of the EDC modified polypeptides detected by the polyclonal antibody, anti-D1, the D1/D2 heterodimer can be seen more clearly in the C and T series than is evident in R and N (Figure 15). This heterodimer is present in control lanes as well (all four A lanes). In all four panels (R, N, C, and T) there is a decrease in the amount of D1 uncrosslinked as the concentration of EDC increases. In the R and N membranes, there is a dark band at about 37 kDa evident even at 1 mM EDC (B lanes) and it increases in intensity as the concentration of EDC increases. It is difficult to determine if the crosslinkage involves Cyt b599 since there are multiple
crosslinked products detected by anti-Cyt b_{59} in that region. At the higher EDC concentrations, extensive crosslinkage occurs as is evidenced by the high molecular weight smear in lanes G and H. Distinct crosslinked species are present at about 60 kDa in panels R and N, in lanes D-H, however it is difficult to discern crosslinked species in the C and T panels due to the large amount of the D1/D2 heterodimer.

Multiple crosslinked products are recognized by α-b_{59} at molecular weights of 35, 37, 57, and 60 kDa. The same 35 kDa crosslinked species is present in C and T membranes as well as another band at 32 kDa (Figure 16). Additional crosslinked products appear between 30 and 97 kDa at and above 1.0 mM EDC, particularly the doublet at 55 kDa and multiple bands in the 30 kDa range.

When probed with the polyclonal antibody against the apoprotein of CPa-2, few crosslinked products were observed (Figure 17).

In figure 18, the Western blots of the crosslinked proteins were probed with a monoclonal antibody, FAC2, which recognizes CPa-1. In the R and N membranes there are crosslinked products at 95 and 110 kDa, (lanes D-H), which appear identical to those observed with α-33 (Figure 18). This 110 kDa band will be isolated and further characterized and is called XL. The absence of these crosslinked products in the C and T membranes, which are devoid of the 33 kDa protein, strongly suggests that the crosslinked products observed in the untreated and NaCl-washed membranes result from the covalent coupling
of the extrinsic 33 kDa protein to the CPa-1 apoprotein. Two other crosslinked species appear in the R and N membranes probed with FAC2 at 60 and 63 kDa.

**O_2** evolution

All three extrinsic polypeptides of PS II are required for maximal O_2 evolution. PS II membranes that have been washed with 1 M CaCl_2 lose greater than 99% of these three extrinsic polypeptides and also lose about 90% of their O_2-evolving activity. However, when only the 17 and 24 kDa polypeptides are removed (1 M NaCl wash), leaving the 33 kDa MSP, near maximal O_2 evolution is observed if the O_2-evolving assay is done in the presence of 15 mM CaCl_2. Since a crosslinked species composed minimally of the 33 kDa MSP and CPa-1 has been identified, it is possible that not all of the extrinsic 33 kDa MSP is removable by 1 M CaCl_2 washing after EDC modification.

Firstly, the O_2-evolving rates of EDC modified membranes were measured. Treatment with EDC does not inhibit O_2 evolution. The O_2-evolving rates of unperturbed PS II O_2-evolving membrane particles were similar to the O_2-evolving rates of PS II membranes exposed to increasing concentrations of EDC (0-15 mM). These control O_2-evolving rates were still greater than 90% of the untreated O_2-evolving PS II membrane particles (600 μmoles of O_2 (mg Chl)^-1 hr^-1). This slight decrease in activity (90% and not 100%) was likely
due to the additional centrifugations and the necessary dilution to 1 mg/ml Chl for EDC treatment.

The 33 kDa MSP which is crosslinked to CPa-1 by EDC is not removable by 1 M CaCl₂. That remaining 33 kDa MSP after washing with 1 M CaCl₂ may correlate to a retention of O₂-evolving activity. After crosslinking with 0, 5, 15, and 50 mM EDC, the PS II membrane particles were either washed twice with regular resuspension buffer (as in R membranes) or washed twice with resuspension buffer containing 1 M CaCl₂ (as in C membranes). In the EDC crosslinked membranes washed with resuspension buffer containing 1 M CaCl₂, there was an increase in the O₂-evolving rates as the concentration of EDC increased. These rates are reported as a percent of the non-CaCl₂ washed rates at each EDC concentration (Figure 19). The CaCl₂ washed membranes that were not exposed to EDC, but exposed to the EDC procedure (0 mM EDC) were 12 % of the rate of non-CaCl₂ washed PS II membranes. At 5 mM EDC, the CaCl₂ washed EDC modified PS II membrane particles retained 20 % of the O₂-evolving rate of their control. When membranes are crosslinked using 15 mM EDC the CaCl₂ washed membrane particles retained 25 % of the control activity. Even though 50 mM EDC is known to cause extensive crosslinking of PS II polypeptides, the O₂-evolving rates of these EDC modified PS II membrane particles is comparable to the other EDC concentrations tested and the 0 mM EDC procedural control (all approximately 600 µmoles of O₂ (mg Chl)⁻¹ hr⁻¹). The EDC modified PS II membranes that were CaCl₂
Figure 19. The retention of O₂-evolution by EDC modified CaCl₂ washed PS II membrane particles reported as a percent of the control EDC modified non-CaCl₂ washed PS II membrane particles. Means were calculated across the 4 replicate experiments. Error bars represent ± 95% confidence intervals.

washed exhibited an O₂-evolving rate that was 40% of its control. Enami et al. (1989) used a cleavable crosslinking reagent which allowed quantitation of the amount of 33 kDa MSP crosslinked; a correlation was made between the retention of O₂ evolution and the amount of crosslinked 33 kDa MSP remaining after treatment with CaCl₂ or urea/NaCl (Enami et al. 1989).
However, at high cleavable crosslinker concentrations quantitation of the immobilized 33 kDa MSP was not possible. The maximum quantifiable amount of 33 kDa MSP retained by crosslinking, which was resistant to the urea/NaCl washing, was 15 %, and for the CaCl₂ washing was 30 %. The amount of O₂-evolving activity retained after urea/NaCl washing was 18 % and after CaCl₂ washing was only 13 %. This is a weak correlation at best.

In my studies, increasing concentrations of EDC result in increasing resistances to the loss of O₂-evolving activity induced by CaCl₂ washing of EDC modified PS II membrane particles. Steady state O₂ evolution was not significantly hindered by crosslinkage with EDC, although at 50 mM EDC O₂-evolving activity declines slightly, (5 %). Crosslinkage studies were also done in the presence of saturating light and DCBQ to determine if light induced conformational changes might show a pattern of crosslinking unique from that obtained when crosslinking was done in the dark. The LDS-PAGE profile of PS II membranes crosslinked both in the dark and in the light was identical.

Implications of interaction between CPa-1/MSP

Recognition of the same crosslinked species by FAC2 and anti-33 antibodies indicated one of the more interesting interactions (Figures 13 and 18). These two proteins are required for maximal O₂ evolution (Ghanotakis and Yocum 1990). Split lane experiments confirmed that the crosslinked species detected by both antibodies (FAC2 and anti-33) was in fact the same
band. This was the first crosslinked species identified unequivocally by two different antibodies. Perhaps others exist, however my interest in the of the O₂-evolving mechanism of PS II directed the further analysis of this specific crosslink, now called XL. Other investigators in the same lab were mapping the epitope for FAC2 (Bricker and Frankel 1987) and CPa-1 was fast becoming the focus of a proposed site directed mutagenesis study in *Synechocystis* PCC 6803. Characterization of the domains from each polypeptide would help to clarify: 1) the site of the extrinsic attachment to the PS II core complex, 2) better characterize the site of water oxidation, 3) further establish the role of CPa-1 and the 33 kDa MSP in cofactor requirements of O₂ evolution. The large luminal extrinsic loop between transmembranous α-helices 5 and 6 of CPa-1 had been proposed to bind the extrinsic polypeptides of the PS II required for maximal O₂ evolution (Bricker 1990).

**Analysis of minimal crosslinked fragments of XL**

CPa-1, 33 kDa MSP, and XL were isolated using preparative LDS-PAGE, followed by electroelution and then cleaved with CNBr. There are 12 predicted CNBr fragments of CPa-1 ranging from one amino acid to 15.7 kDa. The large extrinsic loop of CPa-1 has the methionine located closest to the C-terminal. The 15.7 kDa fragment of CPa-1 is the carboxy terminal CNBr fragment and contains about half of the large extrinsic loop. There are several other fragments with predicted molecular weights around 2 to 3.8 kDa, and
also an 8.4 kDa fragment. The molecular weights of the cleavage products of the 33 kDa MSP are predicted to be 8.5 and 18.1 kDa since there is only one methionine in the published sequence. However, since the only methionine is followed by a threonine the cleavage is incomplete (Schroeder et al. 1969) and three polypeptides are observed (Figure 20). In the first panel stained by Coomassie Blue the "33+" lane shows 3 major bands, the uppermost is the uncleaved 33 kDa MSP. The second band in that lane is the 23 kDa \( M_c \) large CNBr fragment of the 33 kDa MSP from the carboxyl region of the protein. The smallest fragment migrates at about 7 kDa and is the small CNBr amino terminal fragment (Figure 5). The only visible fragment in the Coomassie Blue panel of figure 20 in the "49+" lane is the 15.7 kDa doublet indicated by a horizontal arrow. The CNBr treated crosslinked species, in lane "XL+", shows a faint band at about 50 kDa indicated by a horizontal triangle adjacent to it. The apparent band at the bottom of the gel is not the 33 kDa N-terminal fragment, but simply the dye front, as can be seen by the slightly greater mobility. In lanes "49 -" and "XL -", the uncleaved controls are easily observable. The second panel of figure 20 is probed with the monoclonal antibody FAC2, whose epitope has been mapped to a region bound by \(^{360}\)proline and \(^{391}\)serine of CPa-1. The "49+" lane identifies the large C-terminal CNBr fragment of CPa-1 (also seen faintly in the Coomassie Blue panel). The faint band at 50 kDa in the Coomassie Blue panel when XL is cleaved with CNBr is also recognized by FAC2, (indicated with a horizontal
Figure 20. Cyanogen bromide cleavage of isolated polypeptides. Polypeptides and their cyanogen bromide generated fragments were resolved using a LDS-PAGE step gradient, 4-10-16.5%. The primary probe is at the top of each panel. Control lanes not treated with CNBr, are indicated with a "-", cleaved lanes indicated with a "+". Specific proteins are indicated at the top of each lane: 33 = 33 kDa MSP, 49 = CPa-1, XL = EDC induced covalent crosslink between 33 kDa MSP + CPa-1. Molecular weight markers are on the right. Proteins of interest are indicated by name with arrows on the left. Horizontal triangles indicate fragments of interest. Protein loads were approximately even before cleavages and were previously established by comparing coomassie blue staining intensity on LDS-PAGE mini gels.
arrow, "XL+" lane, in the FAC2 panel). Since the 15.7 kDa C-terminal CNBr fragment of CPa-1 is crosslinked to the 33 kDa MSP, and is not released upon cleavage as in the cleaved "49+" lane, as indicated by recognition of a higher molecular weight species (ie 50 kDa), and lack of recognition at 15.7 in the "XL+" lane, this fragment is involved in the crosslinkage of the 33 kDa polypeptide and CPa-1.

Milligram amounts of starting material are recommended for observation of CNBr generated fragments. It is difficult to detect all fragments generated by this type of cleavage when less than optimal levels of protein are available. Monoclonal antibody epitopes may be destroyed during cleavage. When large quantities are not available, non-specific proteins stains such as coomassie or silver are not sensitive enough. Again, isolated proteins (33 kDa MSP, CPa-1, and XL) were cleaved using CNBr and an ultrasensitive lysine-specific protein probe and 2 polyclonal antibodies were employed to detect the fragments. The same 50 kDa fragment recognized by FAC2 can be seen in all three panels indicated by a horizontal arrow adjacent to all three "XL+" lanes (Figure 21). Since this fragment is recognized by the polyclonal antibody, anti-33, this is confirmation that the 50 kDa species is composed minimally of the large CNBr fragment from the 33 kDa MSP and the C-terminal CNBr fragment of CPa-1. However, notice in the middle panel, (anti-33, lane "33+"), when the 33 kDa MSP alone is cleaved, the only fragment recognized by the antibody is the large fragment at 23 kDa (indicated by a horizontal arrow on the left of the figure).
Figure 21. Cyanogen bromide cleavage of isolated polypeptides. The only difference between figure 20 and 21 are the probes used for detection of fragments. The probes for this figure are also listed at the top of each panel.
Simply adding the molecular weights of these two fragments yields insufficient mass (23 + 15.7 = 40 kDa). The first lane in all three panels is an uncleaved control ("XL -", "33 -", and "49 -"). The interesting data is in the first panel, which is probed with a sensitive lysine-specific probe. In The first panel, labeled "biotin - avidin", reveals the same 3 bands seen in the "33 +" lane of the previous figure. The darkly stained 33 kDa MSP exhibiting incomplete cleavage is subtended by its two CNBr fragments (indicated by the horizontal triangles on the left). The "49 +" lane is a necessary control, primarily to compare with the "XL +" lane. CNBr generated fragments of XL detected by the lysine specific probe reveals the release of the large CNBr fragment from the 33 kDa MSP and retention of the 7 kDa CNBr fragment (biotin - avidin panel, lane "XL +", and compare to "33 +" same panel).

The 50 kDa fragment is recognized by all five probes (Figures 20 & 21). Interestingly, there are two faint bands at 25 kDa, one recognized by anti-5 and one recognized by the biotin - avidin detection system, that are not recognized by anti-33. In bulk CNBr cleavages of XL, the 50 kDa fragment identified by all 5 probes was isolated. This isolation procedure started with 60 - 100 mg Chl of EDC modified O2-evolving PS II membrane particles. Also generated during bulk cleavages of XL, but in much lesser amounts, is the 25 kDa fragment recognized by anti-5 and the very sensitive biotin avidin probe. Once isolated and concentrated, the antibodies were again tested to confirm the identity of each species. The 50 kDa fragment will be called XC. The 25 kDa
fragment will be called 25*. Since XC is recognized by FAC2 and anti-33 it must minimally be composed of the 15.7 kDa carboxy terminal CNBr fragment from CPa-1 and uncleaved 33 kDa MSP. XC is recognized by FAC2, anti-5, and anti-33. 25* is recognized by FAC2 and anti-5 but not anti-33. This suggests that 25* is composed of the N-terminal CNBr fragment from the 33 kDa MSP and the C-terminal fragment of CPa-1. Polyclonal anti-33 does not recognize the small N-terminal 7 kDa cyanogen bromide generated fragment of the 33 kDa MSP.

Large amounts of XC were obtained and a small portion was also sent for amino acid sequence analysis and the remainder was used as starting material for subsequent chemical cleavages. With a minimal amount of 25*, one third of the sample was sent for amino acid sequence analysis. Unfortunately, once a polypeptide chain is treated with CNBr, methionine-threonine bonds that are not cleaved are converted to the homoserine lactone and are no longer susceptible to CNBr cleavage. It was therefore not possible to generate 25* from isolated XC. The amount of protein sent for the first sequence analysis of 25* was not enough to obtain sequence information. The second analysis was performed on the remainder of the isolated 25* and was just enough to predict the sequence of amino acids. Sequence analysis of XC unequivocally confirms the hypothesis that the entire 33 kDa MSP is crosslinked to the 15.7 kDa C-terminal CNBr fragment of CPa-1 (Figure 22). The sequence report for XC stated that two peptides were present, in equimolar amounts. The amount
of material sequenced was about 3 picomoles. The sequence analysis of 25* was not unequivocal. Comparison of the amino acid sequence results reveals a mismatch in four positions. One site on the 33 kDa MSP and 3 sites on CPa-1 were predicted incorrectly (Figure 22). The report on 25* stated that there were 2 N-termini being sequenced and in equimolar amounts, however the total amount of material sequenced was approximately a picomole or less. The incorrectly identified amino acids are those that repeat in the published sequence. The second G in the 33 kDa MSP sequence, the second F in the CPa-1, and the second V in the CPa-1 sequence are difficult to detect when low levels of protein are sequenced. Glutamate # 5 in the CPa-1 sequence is ambiguously identified, perhaps involved in the crosslinkage catalyzed by EDC.
Cleavage of polypeptides with 70 % formic acid is less specific than cleavage with CNBr. The most acid labile peptide bond is that between aspartate and proline, referred to as a D/P site. The C-terminal 15.7 kDa CNBr fragment from CPa-1, the 33 kDa MSP, XC, were isolated using preparative LDS-PAGE, followed by electroelution. These three isolated species were cleaved using 70 % formic acid. There is only one D/P site in the 33 kDa MSP and cleavage at that site yields two fragments. The predicted molecular weight of the N-terminal fragment is 17.1 kDa; the C-terminal fragment is 9.6 kDa. Predicted cleavage fragments of the 15.7 are (from amino to carboxyl) 6 kDa, 8 kDa, 1.8 kDa, and 0.9 kDa. Western blot analysis of the fragments generated by formic acid cleavage can be seen in figure 23. There are two panels, the left panel is probed with anti-33; the right with FAC2. The first lane is uncleaved 33 kDa MSP. Due to such high protein loads, there is a dimer seen near the top of the gel. In the second lane, ("33+"), one can see the three main bands. Due to incomplete cleavage the 33 kDa MSP is still present. The two dark bands below, indicated by horizontal triangles are the two main cleavage products. The N-terminal fragment migrates at 17.5 kDa, and the smaller fragment migrates at 9.2 kDa. XC, identified on the left with an arrow, even when not exposed to formic acid treatment exhibits some degradation (Lane 4 = "XC -"). When XC is cleaved with formic acid, a 33 kDa small fragment which also migrates at 9.2 kDa (Compare Lane "33+" with Lane "XC+") is released. This indicates that the C-terminal region of the
Figure 23. Formic acid cleavage of isolated polypeptides previously cleaved with CNBr. Cyanogen bromide generated fragments were resolved using a preparative LDS-PAGE step gradient, 4-10-16.5 % and isolated by electroelution from gel strips. Fragments were resolved by 4-10-16.5 % LDS-PAGE followed by western blot analysis. The primary probe is at the top of each panel. Control lanes not treated with formic acid, are indicated with a "-", cleaved lanes indicated with a "+". Specific proteins are indicated at the top of each lane: 33 = 33 kDa MSP, 15.7 = the C-terminal CNBr fragment of CPa-1, XC = EDC induced covalent crosslink between 33 kDa MSP + the 15.7 C-terminal CNBr fragment of CPa-1. Molecular weight markers are on the right. Proteins of interest are indicated by name with arrows on the left. Horizontal triangles indicate fragments of interest. Protein loads were approximately even before cleavages and were previously established by comparing coomassie blue staining intensity on LDS-PAGE mini gels.
33 kDa MSP is not involved in the EDC induced crosslink. There is a very faint band at 24 kDa that is identified by both antibodies in lanes "XC+" (labeled on the blot with an * just to the left of each band). This could be the large formic acid fragment of the 33 kDa MSP and the 6 kDa fragment of the 15.7. However, it is difficult to access the lability of the EDC induced peptide bond, as evidenced by the many fragments in the "-" lanes.

The 33 kDa MSP and XL were isolated using preparative LDS-PAGE (12%) followed by electroelution. The partial chymotrypsin cleavage patterns of these two polypeptides were examined using Western blot analysis. The uncleaved controls are in lanes 1 and 6, identified by C 33 and C XL (Figure 25). Both polypeptides were cleaved for 0.5 hours (lanes 2 and 3) and also for 1 hour (lanes 4 and 5). After 0.5 h, the 33 kDa MSP has one major fragment, recognized by anti-33 (lane 2). That same 22 kDa fragment is released by XL (lane 3). By comparison, the CNBr cleavage patterns of the 33 kDa MSP give a small CNBr fragment (N-terminal region) which is not recognized by anti-33; only the large fragment is recognized. However, when the 33 kDa MSP is cleaved with formic acid, both fragments are recognized. There are at least two major epitopes recognized by this polyclonal antibody, neither of which is on the N-terminal. Since there is only one fragment recognized by anti-33 when cleaved for 0.5 h with chymotrypsin, it is likely that there is one site that is most susceptible to chymotryptic attack. Since that band is recognized by anti-33, that large 22 kDa fragment that is released from the 33 kDa MSP is
Figure 24. Chymotrypsin cleavage of isolated polypeptides. XL and 33 kDa MSP were isolated using LDS-PAGE followed by electroelution. Chymotryptic fragments were resolved using gradient 12.5% - 20% LDS-PAGE and subjected to western blot analysis. The probe is anti-33. Polypeptide in each lane is indicated at the top of each lane. Outer lanes are uncleaved controls. Lanes 2 and 3 = 0.5 h cleavage. Lanes 4 and 5 = 1.0 h cleavage. Molecular weight standards are on the right. Proteins are identified on the left with arrows. The horizontal triangles identify fragments of interest. Protein loads were approximately even before cleavages and were previously established by comparing coomassie blue staining intensity on LDS-PAGE mini gels.
likely cleaved at tyrosine-45, which also has two phenylalanines before (F 38 and F 40) and two more phenylalanines after (F 50 and F 57). A larger molecular weight fragment at 25 kDa appears in after 1.0 h cleavage of the 33 kDa MSP (lane 4). This fragment is not observed in the 1.0 h cleavage of XL. There is a 44 kDa species generated in both the 0.5 h and 1.0 h from chymotrypsin cleavage of the XL (lanes 3 and 5). This could be a dimer of the lower 22 kDa species released, or the 22 kDa fragment of the 33 kDa MSP still crosslinked to some portion of CPa-1.
SUMMARY AND CONCLUSIONS

Charged amino acid side chains which are crosslinked by EDC are also the regions which are most immunogenic. Most of the immunogenic sites of integral membrane polypeptides will be located in the hydrophilic regions between the transmembranous α-helices. This suggests that there could be crosslinked products formed which are not detectable using antibody probes. Cross-linkage could mask the epitope, particularly for monoclonal antibodies. This was not a limitation in this study. Multiple crosslinked species were observed. The higher concentrations of EDC cause extensive crosslinking, as evidenced by poor electrophoretic resolution of treated polypeptides (lanes G and H in figures 11-18). Most crosslinked products are produced in 5-15 mM EDC and immunochemically observed using 15 μg of chlorophyll in each lane. Few crosslinked products were formed with the 24 and 17 kDa extrinsic proteins or the 45 kDa apoprotein of CPa-2. Conversely, D1 or D2 may be crosslinked to Cyt b_{359}. These crosslinked products appear in the high molecular weight range and it is not known if they are composed of b_{359} cytochrome polymers, D1 homopolymers, D2 homopolymers, or D1/D2 heteropolymers. The 33 kDa extrinsic MSP is unequivocally crosslinked to the apoprotein of CPa1. Considering the chemistry of EDC crosslinkage, this strongly suggests a charge pair interaction between these two polypeptides.
One cannot speculate, however, that the retention of O$_2$-evolving activity after treatment with 50 mM EDC is due solely to the crosslinkage of the 33 kDa MSP to CPa-1, most likely multiple subunit crosslinking has occurred and attributing this result to a single peptide pair is not possible at this time.

The N-terminal 16 amino acids of the 33 kDa MSP appear to be necessary for binding to PS II (Eaton-Rye and Murata 1989). However, this evidence is based on reconstitution techniques using chymotrypsin modified 33 kDa MSP. Reconstitution experiments have never completely (100 %) restored O$_2$ evolution, and the protein - protein interaction (binding site) could be different from that of unperturbed PS II O$_2$-evolving membranes. The pKa's of the R-group carboxyl of glutamate and aspartate are 4.3 and 3.9, respectively. The half dissociation pKa of the 33 kDa MSP is near the pKa values for these R-groups. The exposed carboxyl of the 33 kDa MSP binds it to PS II through electrostatic interactions (Shen and Inoue 1991). Since it is possible to isolate a crosslinked species composed of the 33 kDa MSP and CPa-1 at 15 mM EDC, the regions of interaction have been mapped. The N-terminal (7 kDa) region of the 33 kDa MSP appears to be involved in binding that protein to PS II. Sequence analysis of 25*, electrophoretic migration, and antibody recognition patterns after chemical and proteolytic cleavages support this conclusion. Interestingly, in the small 16 amino acid region cleaved from the N-terminus of the 33 kDa MSP (Eaton-Rye and Murata 1989) there are 3 acidic amino acids, (E1, D9, and E10). The XC sequence analysis unequivocally identifies
the EDC catalyzed crosslinkage between the C-terminal CNBr fragment of CPa-1 and the 33 kDa MSP. The C-terminal CNBr fragment of CPa-1 occupies about half of the large luminal extrinsic loop between transmembranous helices 5 and 6 (Bricker 1990). XL, XC, and 25* are all recognized by the monoclonal antibody, FAC2. The epitope of FAC2 has been mapped to this fragment (Frankel and Bricker 1989). Early characterization of the 33 kDa MSP proposed that it was likely anchored to PS II by a hydrophobic membrane bound protein (Kuwabara and Murata 1982).

Speculation regarding the interaction of the extrinsic polypeptides with the large luminal extrinsic loop of CPa-1 interacts with the extrinsic hydrophilic polypeptides has been advanced (Morris and Herrmann 1984). This does not eliminate interactions between PS II core polypeptides (D1-D2-Cyt b559) and the 33 kDa MSP (Gounaris et al. 1988 and Mei et al. 1989). However, these studies are based on rebinding of modified 33 kDa MSP or using the 33 kDa MSP as a chromatographic ligand to bind the core complex. EDC modified PS II membranes exhibit normal \( \text{O}_2 \)-evolving rates. An increase in EDC concentration results in increased retention of the \( \text{O}_2 \)-evolving rate by \( \text{CaCl}_2 \) washed EDC modified PS II membranes. My results demonstrate that the 33 kDa MSP is anchored to PS II through charge pair interactions with CPa-1.

One of the most difficult aspects of the characterization of the crosslinked species was the resolution and detection of the fragments generated from proteolytic and chemical cleavages (Mauk and Mauk 1989). Here antibodies
were less useful because epitopes were lost during the extensive XL isolation procedure. The highly sensitive biotinylation on the blot technique helped to identify major fragments not recognized by antibodies. In fact the 50 kDa band, XC, was first observed using this procedure. Multiple fragments are generated and one must have the appropriate control lanes and only compare the bands unique to the cleaved, crosslinked lanes. This difficulty is enhanced by the possibility of multiple crosslink between two polypeptides. It is difficult to determine if EDC induced the crosslinkage between CPa-1 and the 33 kDa MSP at one or multiple sites, even when only considering the domains identified herein. The ability to identify these two domains suggests that one dominant formed by the two proteins when exposed to EDC. However, a series of crosslinked species composed only of CPa-1 and the 33 kDa MSP could involve different combinations of these contacts.

The ground work has been done to determine which PS II subunits are within van der Waals distances. Firstly, there were many major, well resolved crosslinked species identified by only one antibody. These bands could possibly be recognized by two antibodies, however not as obviously as XL. Split lane experiments could be done using 15 mM EDC. This technique enhances the identification of species recognized by more than one antibody. Secondly, if the major crosslinked species are easily detectable using antibodies, isolation procedures used herein can provide enough for N-terminal sequencing. Since the amino acid sequences are known for each PS II component nearest
neighbor relationships could be established, potentially providing some information on the low molecular polypeptides of PS II. Antibodies to these components were not available in this study, and they have only recently been discovered.

In conclusion one must consider how these results effect the current models of the organization of PS II polypeptides (Figure 25). The 33 kDa MSP is anchored to PS II minimally through CPa-1. The 24 kDa is not bound to any integral membrane protein, and likely binds the 33 kDa MSP. The 17 kDa extrinsic polypeptide binds the 24 kDa extrinsic polypeptide. CPa-1 is more tightly bound to the reaction center core, D1-Cyt b₃₃₉-D2, than is CPa-2. This is supported by the lack of crosslinked species formed with CPa-2. The path of energy from the distal antennae to P₆₈₀ is not clear, however, CPa-1 seems to be more tightly coupled to the photochemistry than CPa-2, although CPa-2 is still required for O₂ evolution. Cyt b₃₃₉ is tightly associated with D1, or D2 or both. A stronger association with D1 was evidenced by crosslinked species identified in the 35-45 kDa range. This suggests one Cyt b₃₃₉ per P₆₈₀. The stoichiometry of the 33 kDa MSP is one per reaction center. The 33 kDa MSP is required for maximal O₂-evolving rates and the interaction between this extrinsic polypeptide and CPa-1 is sufficient to protect the O₂-evolving site. The location of that site is unknown as are the Mn ligands. The interaction of the 33 kDa MSP with CPa-1 preserves the Mn associated with O₂ evolution. No current models emphasize the interaction of CPa-1 and the 33 kDa MSP.
Figure 25. A model of the structural organization of PS II polypeptides
Most models do emphasize the interaction between the 33 kDa MSP and the reaction center core polypeptides, D1-Cyt b$_{559}$-D2, but this association is only known from a functional perspective. However the direct evidence presented herein demonstrates the van der Waals interaction occurring between the 33 kDa MSP and CPa-1. This interaction could be enhanced by a stearic fit between these two subunits (1.8 to 2 Å), which might account for the tenacious binding of the 33 kDa MSP to PS II. Future models for the organization of PS II polypeptides must accommodate these protein/protein interactions.

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VITA

William R. Odom was educated in Louisiana. He attended several public schools in grades one through twelve. In 1974, he graduated from East Jefferson High School in the top ten percent of his class. He received the Academic Freshman Honor Award from The University of New Orleans. This meager financial award was not enough to defray the rising costs of education and he was forced to work full time while attending school. A full time job opportunity at Louisiana State University Medical Center was enticing enough to delay completion of his degree. After several years as a research associate in the Department of Neurology, he returned to The University of New Orleans to complete his degree. In 1983 he received his Bachelors of Arts degree in Biology. Degree in hand and no job, he and his new bride moved to Hattiesburg, Mississippi to escape the city. After searching for a job for several months, he was employed by Innovative Technology, Inc. For two years, he was a research associate at the forefront of basic and applied biochemical research. He entered graduate school at the University of Southern Mississippi in June 1986. In the summer of 1987, he transferred, with his major advisor, to the Department of Botany at Louisiana State University to continue his postgraduate education. He received the William J. Luke Teaching Award in May 1991. He is currently a candidate for the degree of Doctor of Philosophy.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: William Robert Odom

Major Field: Botany

Title of Dissertation: The Structural Organization of Photosystem II Polypeptides

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

October 25, 1991