Assemblage of Spinach Photosystem II Proteins: CPa-1 and MSP Interactions.

Carlos Benito Queirolo
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Assemblage of spinach photosystem II proteins: CPa-1 and MSP interactions

Queirolo, Carlos Benito, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1992
ASSEMBLAGE OF SPINACH PHOTOSYSTEM II PROTEINS: CPa-1 AND MSP INTERACTIONS.

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requirements for the degree of
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in
The Department of Botany

by
Carlos B. Queirolo
M. S., The University of Chile, 1983
December 1992
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LIST OF ABBREVIATIONS

100 XL: 100 kDa crosslinked product containing at least MSP and CPa-1
150 XL: 150 kDa crosslinked product containing at least MSP, CPa-2 and CPa-1
2-D: two-dimensional
ADP: adenosine-5'-diphosphate
AN: acetonitrile
ATP: adenosine-5'-triphosphate
BCIP: 5-bromo-4-chloro-3-indolyl phosphate
β-ME: β-mercaptoethanol
BPB: bromophenol blue
BSA: bovine serum albumin
CB: Coomassie brilliant blue R-250
CF : intrinsic coupling factor of the chloroplast ATP synthase
CF : extrinsic coupling factor of the chloroplast ATP synthase
Chl: chlorophyll
CN: 4-chloro-1-naphtol
CP: Chl protein
Cyt: cytochrome
DCBQ: 2,6-dichloro-p-benzoquinone
DM: dodecyl maltoside
DMSO: dimethyl sulfoxide
DTSP: dithio-bis-(succinimidyl propionate)
EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA: ethylenediaminetetraacetic acid
EG: ethylene glycol
EPR: electron paramagnetic resonance (electron spin resonance)
Fig: Figure
FA: formic acid
Fd: ferredoxin
FeS: Rieske iron-sulphur center
FPLC: high performance liquid chromatography
Ig: immunoglobulin
IT: 2-iminothiolane
LHC: light harvesting chlorophyll a/b protein
LDS: lithium dodecyl sulphate
LMW: low molecular mass (low molecular weight)
MES: 2-(N-morpholino)ethanesulfonic acid
MSP: the 33 kDa extrinsic manganese-stabilizing protein (component of OEC)
MW : observed (or apparent) molecular mass of proteins (during electrophoresis)
NADH: reduced form of nicotinamide adenine dinucleotide
NADP: oxidized form of nicotinamide adenine dinucleotide phosphate
NADPH: reduced form of NADP
NBT: nitroblue tetrazolium
NC: nitrocellulose
NHS: N-hydroxysuccinimide
OEC: oxygen evolving complex (water oxidizing complex; donor side of PS II)
OERc: oxygen evolving reaction center
OGP: 1-O-n-octyl-β-D-glucopyranoside
P680: chlorophyll acting as primary donor of photosystem II reaction center
P700: chlorophyll acting as primary donor of photosystem I reaction center
PAGE: polyacrylamide gel electrophoresis
PC: plastocyanine
Pheo: pheophytin
PQ: oxidized plastoquinone
PQH2: reduced plastoquinone
PS: photosystem
PVDF: polyvinylidene fluoride
QA: primary plastoquinone electron acceptor of photosystem II (permanently bound PQ acting as secondary electron acceptor in PS II)
QB: secondary plastoquinone electron acceptor of photosystem II
SDS: sodium dodecyl sulphate
Solution E: 50 mM MES-NaOH pH 6.0, 400 mM sucrose, 10 mM NaCl, and 5 mM CaCl2
Solution F: 50 mM MES-NaOH pH 6.0, 20 mM NaCl, 5 mM CaCl2, and 0.05 % DM
TEMED: N,N,N',N'-tetramethylethylenediamine
TGA: thioglycolic acid
tRNA: transfer ribonucleic acid
Tricine: N-tris(hydroxymethyl)methylglycine
Tris: tris(hydroxymethyl)aminomethane
TS: Tris saline solution (10 mM Tris-HCl pH 7.4 and 150 mM NaCl)
YD (or D): side-path (auxiliary) electron donor to P680
YZ (or Z): secondary electron donor of photosystem II (electron donor to P680)
ABSTRACT

This work focuses on the production and characterization of crosslinked products obtained from PS II preparations, with the aim of better understanding the structural relationships between the manganese stabilizing protein (MSP) and the interior antenna protein CPa-1. Two oxygen evolving reaction center (OERC) preparations, OERC complex and OERC core, produced various crosslinked products with the hydrophobic crosslinker dithio-bis-(succinimidyl propionate) (DTSP).

Crosslinking experiments with OERC complex demonstrated that a close association of MSP to CPa-1 occurs, as both proteins were found to be present simultaneously in various crosslinked products revealed by one- and two-dimensional electrophoretic and immunoblotting techniques. These crosslinked products did not appear in preparations from which MSP had been removed.

These results confirm published work indicating an association between MSP and CPa-1. To better understand this association, a 100 kDa crosslinked product (100 XL) was characterized by cleavage with CNBr or formic acid (FA), followed by immunoblotting. Two-dimensional diagonal LMW-LDS-PAGE of CNBr-cleaved 100 XL demonstrated that several crosslinks occurred among fragments of MSP and CPa-1. Of these, the clearest example was a crosslink between the large CNBr
fragment of MSP and the large CNBr fragment of CPa-1. Consistent with this, diagonal electrophoresis of FA-cleaved 100 XL established that a crosslink existed between the small FA fragment of MSP and an ≈ 9 kDa fragment of CPa-1. Sequencing of a band containing these two crosslinked FA fragments demonstrated that they corresponded to the 9.6 kDa carboxyl FA fragment of MSP, and to the 8.0 kDa FA fragment of CPa-1. Within these fragments, the lysine-containing segments only extend from K$^{159}$ to K$^{236}$ for MSP, and from K$^{418}$ to K$^{438}$ for CPa-1.

The procedures established here should permit the characterization of other crosslinks shown to exist in the OERC complex. Thus, this work lays the groundwork for further studies of the physical interactions among the OERC complex components.
INTRODUCTION

1. Photosynthesis.

A. Background.

The chloroplast thylakoid membrane of photosynthetic organisms is the site for a series of reactions that involve the absorption of photons and the conversion of light energy into stable chemical energy [274, 290]. This chemical energy is stored in the products NADPH and ATP, whose formation is carried out by several integral protein complexes. Active components of some of these complexes are diagrammed in Fig 1. This figure illustrates the so called "Z-scheme", whereby the absorbed light energy drives electron transport along three of these protein complexes, Photosystem II (PS II), the Cytochrome $b_{6}/f$ complex (Cyt $b_{6}/f$), and Photosystem I (PS I). This Z scheme shows components located according to both position in the electron transport pathways, and midpoint electric potentials. The electron transport chain comprises a series of proteins and their cofactors. The ultimate electron donor is water, which is oxidized to molecular oxygen (see also section 4, donor side of PS II). The electron flow may end with the reduction of NADP (ultimate electron acceptor) during non-cyclic electron transport, or it may continue with the
Figure 1. Cyclic and non-cyclic electron transport pathways, as coupled to photophosphorylation.
rereduction of the Cyt b₆/f complex by ferredoxin during cyclic electron transport. Coupled to this electron transport, a H⁺ gradient is formed across the thylakoid membrane [82, 138, 155, 214, 226, 227, 265a]. Upon dissipation of the H⁺ gradient through the ATP synthase (CF₉₀-CF₁; a fourth thylakoid protein complex), ADP is phosphorylated to ATP [110]. The products ATP and NADPH are ultimately consumed in the chloroplast stroma during the fixation of CO₂ via the Calvin cycle [52, 356; also see 141]. This reductive photosynthetic carbon cycle (C₃ cycle) is in close association/competition with photorespiration (C₂ cycle) [160, 289]. Fixation of NH₃ via glutamate synthesis also takes place in the stroma [160] by utilizing byproducts of photorespiration (α-ketoglutarate and glutamate), as well as ferredoxin and ATP (which are produced by the light reactions in the thylakoids).

The four integral protein complexes mentioned above (PS II, Cyt b₆/f, PS I and ATP synthase) are embedded in the thylakoid membranes, whose main lipid components are glycolipids [49, 236, 261, 325, 358]. Although the function of these four membrane protein complexes has been studied in great detail, the organization of their individual components and the relationship of the complexes to the overall structure of the chloroplast membrane is still under investigation. Three areas that in the last few years have contributed tremendously to the advancement in the understanding of PS II are: the sequencing of the genes that code for PS II proteins [e.g., see 135, 281, 305], the determination of two- and three-dimensional crystal structures of reaction centers [8, 26, 54, 71 - 73, 221, 222], and the development of techniques to isolate purified integral membrane protein complexes [22, 32, 55, 56,
Of these three areas, the latter may be considered a productive approach to study the structural organization of PS II, especially if it is complemented with the highly resolved structural information provided by X-ray diffraction techniques, and by the amino acid sequences of the PS II proteins deduced from their established gene sequences. Procedures to isolate some integral membrane protein complexes are examined below, followed by a brief description of various PS II preparations.

B. Isolation of four thylakoid protein complexes in a functional form.

It has been possible to isolate, in highly resolved functional forms, all four integral thylakoid complexes: an Oxygen Evolving Reaction Center (OERC) core (an active form of PS II) [106, 119], Cyt b6/f [142, 159], PS I [238], and ATP synthase [19, 216]. Subsequently it was demonstrated that the essential components of the ATP synthase can be reconstituted into active complexes [19], and the three complexes participating in the light reactions (PS II, Cyt b6/f and PS I) can also be reconstituted to an active chain capable of catalyzing noncyclic electron transport from water to NADP⁺ when supplemented with soluble protein cofactors [195]. The availability of these resolved complexes has permitted a detailed examination of their structure-function relationships, both at the intra- and at the inter-complex levels. One of these complexes, PS II, is examined below.
2. Photosystem II (PS II).

A. Background.

Fig 1 (page 2) shows PS II cofactors in relation to the cofactors of other integral membrane protein complexes involved in the light reactions of photosynthesis [139]. The PS II complex absorbs light energy with ensuing physical separations of charges [65, 270] and concomitant loss of electrons from PS II. These electrons are replaced with electrons from water, with the consequent evolution of $O_2$, release of $H^+$, and the transfer of the electrons to the plastoquinone pool, which then delivers them to the Cyt b$_{5/6}$ complex [12, 20, 323]. A variety of functional and structural studies of PS II has been possible due, in great measure, to the development of various techniques conducive to the purification of various PS II protein complexes. A summary of these purification techniques is shown in Table 1.

B. Protein components of PS II.

More than 20 proteins are thought to be associated with PS II [see 211]. The main protein constituents of PS II are listed in Table 2. The intrinsic protein elements in PS II membranes are: a) The light harvesting-chlorophyll a/b complex
Table 1. Various PS II reaction center preparations, in increasing enrichment order.

<table>
<thead>
<tr>
<th>PREPARATION</th>
<th># OF POLYPEPTIDES FOUND BY STAINING WITH Coomassie blue</th>
<th># OF POLYPEPTIDES FOUND BY STAINING WITH Silver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thylakoids (Class II chloroplasts)</td>
<td>50</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>PS II membranes</td>
<td>20</td>
<td>35 - 40</td>
</tr>
<tr>
<td>PS II core complex (evolves oxygen)</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>PS II core (evolves oxygen)</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>D1-D2-cyt b&lt;sub&gt;559&lt;/sub&gt; core (does not evolve oxygen)</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>


(LHC II, proteins with MW<sub>obs</sub> = 24 - 30 kDa and with a Chl a / Chl b ratio of about 1.2) [146, 357]. This complex is involved in both the stacking of membranes [10, 290] and the partitioning of energy between PS II and PS I [39, 41]. The latter is mostly carried out by the so called "mobile" LHC II, phosphorylated LHC II proteins which migrate toward PS I and increase its light-harvesting capability [27, 30, 198, 358]. b) Additional Chl a/b binding proteins associated with the distal or proximal antennae (having a general Chl a / Chl b ratio of 2 - 3), such as CP 29 [53, 128], CP 26 and CP 24 [25, 86], and the 28 kDa Chl a-binding protein [119]. c) The two
Table 2. Major protein components of PS II.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW&lt;sub&gt;app&lt;/sub&gt;&lt;sup&gt;&lt;a&gt;1&lt;/a&gt;&lt;/sup&gt; (kDa)</th>
<th>Organic&lt;sup&gt;&lt;a&gt;2&lt;/a&gt;&lt;/sup&gt;</th>
<th>COFACTORS</th>
<th>Preparation&lt;sup&gt;&lt;a&gt;3&lt;/a&gt;&lt;/sup&gt; containing these proteins</th>
<th>Intrinsic or Extrinsic</th>
<th>Nuclear- or Chloroplast-encoded</th>
<th>Gene&lt;sup&gt;&lt;a&gt;4&lt;/a&gt;&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>32 - 34</td>
<td>Chl a, Pheo a, Plastoquinone</td>
<td>Mg, Fe, Mn?, Ca? phosphate&lt;sup&gt;&lt;a&gt;5&lt;/a&gt;&lt;/sup&gt;</td>
<td>PS II core</td>
<td>I</td>
<td>C</td>
<td>psbA</td>
</tr>
<tr>
<td>CPa-1</td>
<td>47 - 51</td>
<td>Chl a, β-carotene</td>
<td>Mg, Mn?, Ca?</td>
<td>OERC complex</td>
<td>I</td>
<td>C</td>
<td>psbB</td>
</tr>
<tr>
<td>CPa-2</td>
<td>43 - 45</td>
<td>Chl a, β-carotene</td>
<td>Mg, Mn?, Ca? phosphate&lt;sup&gt;&lt;a&gt;5&lt;/a&gt;&lt;/sup&gt;</td>
<td>OERC complex</td>
<td>I</td>
<td>C</td>
<td>psbC</td>
</tr>
<tr>
<td>D2</td>
<td>32 - 34</td>
<td>Chl a, Pheo a Plastoquinone?</td>
<td>Mg, Fe, Mn?, Ca? phosphate&lt;sup&gt;&lt;a&gt;5&lt;/a&gt;&lt;/sup&gt;</td>
<td>PS II core</td>
<td>I</td>
<td>C</td>
<td>psbD</td>
</tr>
<tr>
<td>Cyt b&lt;sub&gt;559,α&lt;/sub&gt;</td>
<td>9 - 10</td>
<td>Haem</td>
<td>Fe</td>
<td>PS II core</td>
<td>I</td>
<td>C</td>
<td>psbE</td>
</tr>
<tr>
<td>Cyt b&lt;sub&gt;559,β&lt;/sub&gt;</td>
<td>4 - 5</td>
<td>Haem</td>
<td>Fe</td>
<td>PS II core</td>
<td>I</td>
<td>C</td>
<td>psbF</td>
</tr>
<tr>
<td>10 kDa of OEC</td>
<td>10</td>
<td>?</td>
<td>?</td>
<td>OERC complex</td>
<td>E</td>
<td>C</td>
<td>psbG</td>
</tr>
<tr>
<td>10 kDa phosphoprotein</td>
<td>7.7 - 10</td>
<td>?</td>
<td>phosphate&lt;sup&gt;&lt;a&gt;5&lt;/a&gt;&lt;/sup&gt;</td>
<td>OERC complex</td>
<td>I</td>
<td>C</td>
<td>psbH</td>
</tr>
<tr>
<td>4.8 kDa I-polypeptide</td>
<td>4.8</td>
<td>?</td>
<td>?</td>
<td>PS II core</td>
<td>?</td>
<td>C</td>
<td>psbI</td>
</tr>
<tr>
<td>3.9 kDa K-polypeptide</td>
<td>3.9</td>
<td>?</td>
<td>?</td>
<td>PS II membranes</td>
<td>?</td>
<td>C</td>
<td>psbK</td>
</tr>
<tr>
<td>PS II L-polypeptide</td>
<td>5.0</td>
<td>?</td>
<td>?</td>
<td>OERC complex</td>
<td>?</td>
<td>C</td>
<td>psbL</td>
</tr>
</tbody>
</table>
Continuation of Table 2.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Mass</th>
<th>Subcellular Location</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP</td>
<td>30 - 33</td>
<td>?</td>
<td>OERC complex E N psbO</td>
</tr>
<tr>
<td>23 kDa polypeptide of OEC</td>
<td>23 - 24</td>
<td>?</td>
<td>PS II membranes E N psbP</td>
</tr>
<tr>
<td>17 kDa polypeptide of OEC</td>
<td>16 - 17</td>
<td>?</td>
<td>PS II membranes E N psbQ</td>
</tr>
<tr>
<td>10.2 kDa PS II polypeptide</td>
<td>10 - 11</td>
<td>?</td>
<td>PS II membranes E N psbR</td>
</tr>
<tr>
<td>LHC II 24 - 30</td>
<td>Chl a/b</td>
<td>Mg, phosphate</td>
<td>OERC complex I N cab II</td>
</tr>
<tr>
<td>CP 29</td>
<td>Chl a/b</td>
<td>Mg</td>
<td>OERC complex I ? ?</td>
</tr>
<tr>
<td>CP 26</td>
<td>Chl a/b</td>
<td>Mg</td>
<td>OERC complex I ? ?</td>
</tr>
<tr>
<td>CP 24</td>
<td>Chl a/b</td>
<td>Mg</td>
<td>OERC complex I ? ?</td>
</tr>
<tr>
<td>Chl a-binding protein</td>
<td>28</td>
<td>Chl a</td>
<td>OERC complex I N? ?</td>
</tr>
</tbody>
</table>

**NOTES:**

- Molecular masses as generally determined in SDS-PAGE systems.
- The chlorophyll molecules contain the metallic cofactor Mg shown in the inorganic column.
- All the proteins found in the Namba and Satoh core preparations [245] are also found in OERC complex preparations (page 10), and thus they are not artifacts. By the same token, proteins found in OERC complex can also be found in PS II membranes.
- The "psb__" gene denominations were adopted from a published proposal [135].
- The phosphates are the result of phosphorylation of one (or more?) protein residue(s) [30].

**REFERENCES:**

- D1 and D2: [22, 210, 223, 245, 247, 277].
- Cyt b₅₅₉: [22, 149, 245, 277, 334].
- CPa-1 and CPa-2: [42, 139].
- 10 kDa of OEC: [241]; in [293], PS II-G is described as a 24 kDa gene product of a psbG gene in maize.
- 10 kDa phosphoprotein: [164].
- 4.8 kDa l-poly peptide: [163, 335].
- K-polypeptide: [240].
- L-polypeptide: [165].
- MSP: [109, 182, 187, 189, 228, 262, 279, 310, 326].
- 23 and 17 kDa proteins of OEC: [172, 188, 267, 279].
- 10.2 kDa PS II polypeptide: [151, 201, 295, 296].
- LHC II: [10, 78, 178, 237, 286, 290, 337].
- CP 29: [53].
- CP 26 and CP 24: [25, 86].
- Chl a-binding protein: [119].

Some general references include [30, 124, 135, 139, 154, 305].
proximal antenna proteins CPa-1 and CPa-2, each binding between 20 and 25 Chl a molecules and about 5 β-carotenes [40, 42, 139]. CPa-2 becomes phosphorylated, while CPa-1 does not [220]. d) The three reaction center core proteins, namely D1 or herbicide binding protein, D2, and Cyt b$_{59}$ [22, 64, 120, 232, 245, 277]. Of the last three proteins, at least D1 and D2 become phosphorylated in thylakoids [220].

In plants, extrinsic components include three main hydrophilic proteins with apparent molecular masses of 17, 24 and 33 kDa [4, 203, 239], of which the latter is the so called Manganese-Stabilizing Protein (MSP). Smaller polypeptides appear to be extrinsic as well (see Table 2). In cyanobacteria, neither the 17 or 24 kDa or equivalent proteins are present [172, 188, 192, 262, 267, 279].

3. Oxygen evolving reaction centers (OERC).

A. OERC complex.

An OERC complex is usually obtained by treating PS II membranes [32, 188] with a non-ionic detergent such as octyl-β-D-glucopyranoside (OGP), followed by differential centrifugation [46, 118, 119, 123, 166, 346]. Although yields tend to be low (≈ 10% of the Chl in PS II membranes), the resulting particles contain 7 - 10 major polypeptides (Tables 1 and 2) and are capable of high oxygen evolution rates.
B. OERC core.

An Oxygen Evolving Reaction Center core is normally attained through chromatographic procedures involving further treatment of an OERC complex with another non-ionic detergent such as dodecyl-maltoside (DM) [119], or digitonin [106]. These core preparations contain 2 - 3 fewer polypeptides than OERC complex preparations (Table 1), but are more labile with respect to their oxygen-evolving activity. These are the smallest protein assemblages that evolve oxygen. However, the yield of their isolation procedure is relatively low (= 15 % of the chlorophyll in OERC complex). The major proteins associated with OERC core particles are CPa-1, CPa-2, D1, D2, MSP and Cyt b_{59}, plus some low molecular mass proteins (Table 2).

Further elimination of proteins render a minimal core constituted by D1, D2, Cyt b_{59}, and the 4.8 kDa product of psbl [22, 64, 120, 245, 277]. Even though these core preparations cannot evolve oxygen, reduce plastoquinones or exhibit D or Z radical formation (page 11), they are capable of producing the primary charge separations between P_{680} and Pheo, thus showing a very limited (though interesting) portion of the total PS II activity.
4. Donor side of PS II.

A. Background.

The donor side of PS II is made up mainly by the so-called Oxygen Evolving Complex (OEC). The OEC is a water-oxidizing complex involved in the transfer of electrons from water to $P_{680}^+$ (formed during the primary charge separation). Upon absorption of a photon by the antenna chlorophylls, the absorbed energy is transferred to $P_{680}$, giving rise to $P_{680}^+$ [29]. The excited electron of $P_{680}^+$ is then transferred to pheophytin, and the primary charge separation occurs: $P_{680}^+$ Pheo$^-$ [65, 270]. Pheo$^-$ transfers its electron to $Q_A$, while $P_{680}^+$ is reduced by Z (or Y$_Z$, the side chain of Tyr-161 on the D1 polypeptide) [70, 218], such that the charges are further separated. Thus, this secondary separation of charges (now located on $Z^+$ and $Q_A^-$) prevents a charge recombination between Pheo$^-$ and $P_{680}^+$. The electron in $Q_A^-$ is eventually transferred to a second plastoquinone, $Q_B$, to form $Q_B^-$. Both $Q_A^-$ and $Q_B^-$ interact magnetically with a nearby non-heme Fe$^{+2}$. Along with the reduction of $Q_B$, $Z^+$ receives an electron from the Mn cluster of the OEC complex, possibly through a histidine residue [35, 256]. Now the charges are further separated on $Q_B^-$ and [Mn]$^{3+}_{2+2}$. The Mn cluster seems to be a dimer of dimers [68]. After a second photoact, the same sequence of charge separations takes place, except that $Q_B^-$ becomes $Q_B^{-2}$ and the Mn cluster becomes [Mn]$^{+2}_{2+2}$. $Q_B^{-2}$ is then protonated, probably via bicarbonate [175, 176, 294, 315, 345], forming PQH$_{2}$. PQH$_2$ leaves PS.
II and joins the reduced plastoquinone pool (Fig 1). After 2 additional photoacts, a second PQH$_2$ is released to the plastoquinone pool, while the Mn array becomes [Mn]$^{+4+2+2}$. All four oxidizing equivalents appear to be accumulated in the Mn cluster [reviewed in 68] via the so called S states [76, 105, 184], which seem to involve transitions of Mn(II) to Mn(III) and from Mn(III) to Mn(IV). These oxidation transitions are accompanied by structural rearrangements in the OEC [133]. The four oxidizing equivalents are filled by four electrons taken from two molecules of water, which are oxidized by the OEC to produce a total of four H$^+$ [311], four electrons, and O$_2$ [150], that returns the [Mn]$^{+4+2+2}$ to an electroneutral state. The formation of O$_2$ by the OEC might be accomplished by an electron/proton pump working through helix movements [343].

The OEC is associated with the luminal side of PS II [12, 20], and it is composed of at least four extrinsic proteins: MSP, the 24-, the 17- and the 10 kDa proteins (Table 2). D1 also appears to play a role in the OEC structure/function [162, 217, 219]. In addition, CPa-1 [42, 353] and some smaller polypeptides (Table 2) may be involved.

**B. The extrinsic proteins and atomic "cofactors".**

If PS II preparations are treated with 1 M Tris buffer at pH 9.3, MSP and the 17 and 24 kDa extrinsic proteins are released from these membranes concomitantly.
with losses of both Mn (the 4 Mn atoms involved in oxygen evolution by PS II) and the oxygen-evolving activity [6, 7, 188, 348, 349], thus indicating that these three extrinsic proteins are components of the \( \text{O}_2 \)-evolving complex. These three proteins have a polarity of 47 - 49 %, demonstrating their hydrophilic nature [349]. It has been suggested that both the 17 and the 24 kDa proteins are distal to MSP, since: a) Incubation of these PS II membranes with 1 M NaCl causes dissociation of the 17 and the 24 kDa proteins (but not MSP) with partial loss of oxygen-evolving activity [190, 191]. b) Treatment of PS II membranes with OGP yields the OERC complex (page 9), which includes MSP and lacks the 17 and the 24 kDa proteins, yet exhibits high \( \text{O}_2 \)-evolution rates [118]. c) Cyanobacteria contain MSP in their PS II, but lack the 17 and 24 kDa proteins (or any equivalent) [192, 262]. d) Isolation of a PS II reaction center complex has been achieved by affinity chromatography with MSP as ligand [168]. e) Crosslinking of PS II complexes produces crosslinks between the 17 kDa and the 24 kDa proteins, and between the 24 kDa protein and MSP [92]. MSP, in turn, appears to associate with CPa-1, CPa-2 and the D1-D2-Cyt b\(_{559}\) core (see page 17).

It has been concluded that the 17- and the 24 kDa [151, 154, 213], and the 10 kDa [151, 154, 295] extrinsic proteins play structural and regulatory roles rather than being directly involved in the catalysis of water oxidation. The 17- and the 24 kDa proteins may be required for binding or sequestering calcium and/or chloride necessary for optimal oxygen evolution [4, 11, 13, 79, 121, 230, 243], and may also play a role in the preservation of the Mn cluster [122]. The three main extrinsic
proteins (17- and 24 kDa and MSP) are rich in "free" lysine residues [349] and show very large dark-light acetic anhydride labeling changes [199], so such lysines have been implicated as being part of the array of buffering groups involved in the H⁺ accumulation that drives photophosphorylation [200]. See page 17 for more information about MSP.

With respect to atomic cofactors of the OEC, Cl⁻ plays a key role in the OEC activity [60, 212]. It was observed that Cl⁻ partially restores the oxygen-evolving activity of PS II membranes that have been washed with CaCl₂ [43, 191, 253, 255] or urea plus NaCl [43, 229]. These treatments remove MSP, but preserve the Mn atoms in the membrane. This partially recovered oxygen-evolving activity can be further enhanced by the addition of the 24 kDa protein, or Ca⁺² [116, 191, 230]. Thus, besides Cl⁻, Ca⁺² also plays an important role in oxygen evolution [12, 38, 79, 254]. Furthermore, a putative binding site for Ca⁺² on the secondary structure of MSP has been suggested [326].

5. Two particular protein components of PS II.

A. The internal Chl a antenna protein CPα-1.

Until a few years ago, this Chl α-binding protein was considered the location of the primary reactants of PS II, P₆₈₀ and pheophytin [128, 129, 233, 244, 281].
Recently these primary reactants have been assigned to a D1-D2 heterodimer, based on: a) The homologies between D1 and D2 [9, 268], between the L and M subunits of the reaction center complex in purple bacteria [341, 352], and among all four proteins (D1, D2, L and M) [71, 144, 145, 221, 222, 308, 341, 342, 352]. b) The isolation of a D1-D2-Cyt b_{559} core [22, 245, 232, 277], capable of undergoing photochemical oxidation [55, 56, 64, 120, 270, 301, 303, 331]. c) The chemical identity of Z (first electron donor to P_{680}^+ and that gives rise to the EPR Signal II_{very fast}) as Tyr-161 of D1 [69, 70, 218], and the chemical identity of D^+ (dark-stable radical giving rise to EPR Signal II_{slow}) as Tyr 160 on D2 [69, 319]. d) The localization of the reducing and oxidizing sides of PS II on D1 [162, 217, 219]. e) The failure of isolated CPa-1 to undergo photochemistry [120]. f) Other reports [5, 277] also point to D1-D2 as the site of reaction center function in PS II.

CPa-1 (as well as CPa-2) is an internal, light-harvesting chlorophyll-a antenna for PS II [42, 139]. It seems to serve as an intermediary transducer of excitation energy from the external (distal) light-harvesting chlorophyll a/b antenna to P_{680} [34]. Coded for by the chloroplast psbB gene [42, 233], CPa-1 is an intrinsic protein [45, 263] consisting of seven α-helices presumed to cross the membrane [233]. Because the second helix may be too short and too hydrophilic to span the thylakoid membrane, a current hypothesis is that CPa-1 (and CPa-2) contains six transmembrane α-helices [42, 320], all of which are conserved in various species examined [42]. CPa-1 (and CPa-2) also possesses, between the transmembrane helices V and VI, a large hydrophilic extrinsic loop exposed to the thylakoid lumen [9, 233]. CPa-1
(and CPa-2) binds 20 - 25 chlorophyll \( a \) molecules [40, 80] which are organized to efficiently participate in the transference of excitation energy toward the reaction center. Some of these chlorophyll molecules are supposedly ligated to the 12 conserved histidyl residues that are present in the predicted membrane-spanning \( a \)-helices [42]. CPa-1 (and CPa-2) additionally binds about 5 \( \beta \)-carotene molecules [40]. These carotenoid molecules may play a role in light-harvesting [283], in protection against photobleaching [284], and perhaps in the assembly of an active photosystem II, a phenomenon possibly regulated by lutein [158].

CPa-1 appears to be tightly coupled to the reaction center of PS II, since CPa-1 (and CPa-2) is always present in oxygen-evolving preparations of PS II from higher plants. For example, electron transfer from exogenous donors to \( Q_A \) can take place in the absence of CPa-2 [347], but not when both CPa-1 and CPa-2 are removed [5]. Elimination of the gene for CPa-1 by site-directed mutagenesis prevents the assembly of a cyanobacterial PS II core, which implies that CPa-1 (and CPa-2 to a lesser extent) is required to assemble a functional PS II complex [318, 321]. In addition, CPa-2 seems to be less tightly bound to the PS II core than CPa-1. CPa-2 can be completely removed by treating Tris-washed OERC complex with 7.5 % dodecyl maltoside, 0.75 % taurine and 2 M LiClO\(_4\), followed by chromatography [74, 120], whereas CPa-1 is removed only after a second similar treatment [120].

CPa-1 also seems to be closely associated with MSP, since: a) A "purified" subthylakoid fraction containing mostly MSP and CPa-1 (and Mn and some other polypeptides) was isolated in early experiments [353]. b) MSP shields CPa-1 (and
CPa-2) from tryptic hydrolysis [44]. e) MSP prevents the labeling of amino groups on CPa-1 by NHS-biotin, which primarily labels lysyl residues located on the large extrinsic loop of CPa-1 [46]. d) MSP is easily crosslinked to CPa-1 in both PS II membranes [46, 90, 91, 248, 250] and in OERC complex [46, 93]. Three different crosslinkers have been utilized: DTSP [dithiobis(succinimidyI propionate)] [46, 91, 93], EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide] [46, 90, 248, 250], and IT (2-iminothiolane) [52a]; all three yield crosslinks between CPa-1 and MSP. Furthermore, oxygen evolution is stabilized after EDC [90] or DTSP [91] crosslinking of CPa-1 and MSP in PS II membranes. Thus, CPa-1 appears to provide a binding site for MSP. Additional binding sites for MSP may be present on the D1-D2-Cyt b559 core [127, 299] and on CPa-2 [168].

B. The manganese-stabilizing extrinsic protein, MSP.

This 33 kDa protein was isolated first from spinach PS II membranes by isoelectric focusing [187], and then from acetone powder of spinach thylakoids by column chromatography [189]. This protein has been well characterized chemically and physicochemically [11, 187, 189, 239].

The essential role of MSP is thought to be the preservation of the tetranuclear Mn center in the photosynthetic oxygen-evolving complex [117, 254], although there is still controversy as to whether MSP is a Mn-binding protein [2, 36, 43, 156, 253, 254, 350]. In fact, when PS II membranes are extracted under oxidizing
conditions [2], or by phase partitioning [350], MSP retains some Mn atoms. On the other hand, washing of PS II membranes with CaCl₂ [43, 253, 255] or urea plus NaCl [43, 229] removes MSP, but preserves the Mn atoms in the membrane. In this case though, only about 10 - 24 % of the oxygen-evolving activity remains. This activity can be substantially restored upon rebinding of MSP to the membrane [36, 191, 228, 255]. Studies with corn mutants indicate that other Mn-binding site(s) seem to be provided by D2. Absence of D2 correlates with the absence of Mn and the lack of oxygen evolving activity [45]. In addition, it has been suggested that Mn is bound to either or both CPa-1 and CPa-2 and that this binding is stabilized by MSP [254, 353]. Cl⁻ can partially substitute for MSP in retaining Mn and associated oxygen evolution [43, 191, 229]. Since Ca⁺² seems to play an important role in oxygen evolution [12, 38, 43, 79, 230, 254], it is interesting to note the suggestion of a putative binding site for Ca⁺² on the secondary structure of MSP [326].

The amino acid sequence of MSP was determined from the purified spinach protein [252] and from nuclear gene sequences [192, 262, 310, 333, 326]. The cyanobacterial MSP is synthesized initially with an amino-terminal extension similar to a signal sequence, which is presumably responsible for directing the polypeptide to the lumen of the photosynthetic membrane system. The higher plant 33 kDa proteins are synthesized with much longer amino-terminal extensions [310, 326, 333], required for transfer of the polypeptides across the chloroplast envelopes and into the lumen of the thylakoid membranes [67, 269, 324, 339]. It has been suggested that the amino-terminal extension contains two domains, a chloroplast import
presequence and a thylakoid transfer domain [310, 326]. This has also been shown for other imported proteins located in the thylakoid lumen, including plastocyanin [139, 287] and the 17- and 24 kDa extrinsic proteins from the spinach OEC [67, 172, 324]. The similarity between the putative thylakoid transfer domain of MSP and the amino-terminal extensions of the cyanobacterial MSP lends to the speculation that both proteins are homologous [192, 262, 326].

6. Studying CPa-1 and MSP.

A. Two-dimensional diagonal polyacrylamide gel electrophoresis.

Two-dimensional diagonal polyacrylamide gel electrophoresis (2-D diagonal PAGE) is a technique that permits to visualize proteins appearing on vertical lines below a diagonal. These proteins are released during the second dimension electrophoresis from crosslinked products that form in a protein complex. Thus, this electrophoretic technique allows identification of proteins which are close to others in a protein complex. Two-dimensional diagonal PAGE has been successfully employed in determining nearest neighbor relationships for the proteins within ribosomes [179, 307], for cytochrome c oxidase [173], NADH-ubiquinone reductase [125], ATP synthase [16, 37, 88, 174, 297], proteins in erythrocyte membranes [330], bacterial light-harvesting polypeptides [206, 224], Cyt b_{6}/f complex [278], a bacterial reaction center [153], and PS II complex [36, 46, 90 - 93, 128, 225].

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MSP seems to associate to CPa-1 in PS II [36, 44, 46, 90, 91, 93, 128, 248, 250, 353]. Two-dimensional diagonal PAGE may be utilized to better understand the relationships between MSP and CPa-1. This electrophoretic technique may be redesigned to observe low molecular mass protein fragments forming vertical lines below a diagonal, allowing identification of segments (domains) of MSP which are crosslinked to particular segments (domains) of CPa-1.

B. Crosslinking studies.

I. Background.

Proteins can be "naturally" crosslinked via their own amino acid side chains (e.g., through their cysteine moieties) or artificially by utilizing foreign chemicals. Exogenous chemicals can be used to induce direct bonds between protein side chains (e.g., via dye-photosensitized reactions) [336]. In most cases, however, the exogenous compounds become a part of the crosslinking bridges.

Some disadvantages in the utilization of artificial crosslinkers may include: a) Lack of specificity (the crosslinkers could react weakly with aminoacid types other than the targeted one). b) Artifacts due to too much crosslinking (producing extracomplex crosslinks or aggregates difficult to resolve by PAGE).

On the other hand, crosslinking proteins by artificial means has many advantages: a) It permits the specific isolation of a protein that comigrates with others
during PAGE [3]. b) It allows nearest neighbor inferences, being especially useful if two or more proteins form a structural and functional unit [16, 46, 88, 90 - 93, 125, 173, 174, 179, 206, 248, 250, 278, 307, 330, 336; reviewed in 28]. c) By using crosslinkers of different lengths, one can probe spatial relationships between one protein and its neighbors in a complex. For example, a series of bis-succinimide crosslinkers, with increasing numbers of methylene links (see DTSP; page 22), could allow crosslinking between progressively more distant proteins [204, 206, 291]. d) By utilizing crosslinkers of different hydrophilicities or hydrophobicities, one can probe protein complexes soluble in aqueous media or integrated into membranes. For example, the same series of bis-succinimide crosslinkers, which are rather hydrophobic, could be sulfonated on their succinimidyl rings to make them impermeant to membranes [206, 291, 292]. Alternatively, other types of water soluble crosslinkers could be used, such as IT [179]. e) By employing cleavable crosslinkers, it may be feasible to determine which neighboring proteins were crosslinked (e.g., by detecting them on a vertical line below the diagonal of a 2-D PAGE). The bis-succinimide series and IT are good examples of easily cleavable crosslinkers [37, 46, 91, 93, 125, 173, 179, 204, 206, 278, 307, 330]. f) Heterofunctional crosslinkers may help to establish which types of amino acids are interacting between immediate protein neighbors (by using EDC, one may determine if salt bridges are binding two proteins together [248, 250]), or between a protein and other (macro)molecules, e.g., in ribosomes [179, 307]. Alternatively, macromolecules to be crosslinked could be derivatized such that homofunctional crosslinkers (such as DTSP) could be used.
This technique was utilized to identify peptide sequences at the tRNA binding site of a methionyl-tRNA synthetase [313]. Thus, artificial crosslinkers permit not only elucidation of which protein resides close to another in a complex, but it also enables us to identify which portion of the protein sequence is being crosslinked.

Additionally, since artificial crosslinkers allow investigation of native complexes, in many instances the crosslinking procedure would not perturb the functionality of the complex, e.g., see [91]. On the contrary, it may even protect such functionality [90], in which case it could be feasible to elucidate some functional roles of portions of the proteins being crosslinked.

A crosslinker which has been successfully utilized in studies of PS II is DTSP [46, 91 - 93, 128, 225]. This crosslinker is described below.

II. Chemical description of DTSP and its mode of action.

The structure of DTSP is shown in Fig 2. By means of its active carboxylates, DTSP reacts mainly with lysine side chains and R-NH₂, having maximal reactivity toward unprotonated amine groups [23, 204]. DTSP reacts by acetylating free amino groups with great rapidity at 0 °C, which is advantageous when crosslinking labile complexes. The crosslinking span of DTSP is estimated to be 1.2 nm [23]. Thus, if two appropriate amino groups are at 1.2 nm or closer, they could be acetylated to produce a disulfide bridge between them. Such disulfides can then be cleaved during two-dimensional PAGE.
C. Chemical cleavages of polypeptides and their crosslinked products.

The use of cleavage agents allows the production of protein fragments that can be separated by PAGE designed to resolve low molecular mass polypeptides [58]. Known specific cleavage agents are hydrolytic enzymes [215, 288, 327], an oxidant electrode [59], and various chemical cleavage agents; the last two have the advantage of not adding extra polypeptides to the sample. One of the most common chemicals utilized for cleavage is cyanogen bromide (CNBr). Under acidic conditions such as 70% formic acid (FA) [112, 131, 132, 208, 282], CNBr reacts very slowly with the peptide bond adjacent to a cysteine, but it reacts readily with the peptide bond on the carboxyl side of methionine, resulting in the generation of a few discrete fragments. CNBr has been employed for a myriad of studies on protein
fragments [48, 107, 126, 131, 132, 208, 234, 246, 265, 322, 327]. CNBr has also been used to cleave at the carboxyl side of tryptophanyl residues after these have been transformed into oxindolylalanine by dimethyl sulfoxide (DMSO) in HCl [157]. CNBr cleavage can be improved under certain conditions by substituting 0.3 M HCl [265], 6 M guanidine hydrochloride [322], or 70% trifluoroacetic acid [126, 234] for the 70% FA. On the other hand, FA alone has been successfully employed to cleave polypeptides. Like other acids (see paragraph below), it cleaves at aspartyl residues, but FA cleaves aspartyl-prolyl bonds preferentially because of the greater basicity of the proline nitrogen [264]. Various polypeptides have been cleaved either at 40°C with 70% FA [196], or at 37°C with 75% FA [208].

Other chemicals have been utilized as cleavage agents as well. For example: N-bromosuccinimide cleaves at aromatic residues such as tyrosine, tryptophan and histidine [112, 266, 327]. N-chlorosuccinimide [282], o-iodosobenzoic acid [209], and 2-(2'-nitrophenylsulfenyl)-3-methyl-3'-bromoindolenine [62] cleave at tryptophan. Cysteinyl residues are cleaved by 2-nitro-5-thiocyanatobenzoic acid [171, 208, 327]. In the presence of N → O acyl rearrangements, serine and threonine are preferentially cleaved by some acids [170]. HCl or acetic acid cleave preferentially at aspartyl residues [276]. Hydroxylamine hydrochloride (2 M) cleaves at asparagyl-glycyl bonds at pH 9.3 in the presence of 6 M guanidinohydrochloride [208].

The utilization of chemical cleavage agents is particularly useful because it is possible to crosslink the protein components of a complex (such as the OERC complex), isolate a particular crosslinked product, cleave it (say with CNBr or FA), and
resolve the fragments by two-dimensional diagonal PAGE. The fragments that are not crosslinked would appear on a diagonal, whereas the fragments that are crosslinked together would migrate under the diagonal on a same vertical line. These crosslinked fragments could then be isolated for their unequivocal identification through sequencing. Currently there are many quick and relatively easy and inexpensive procedures for sequencing polypeptides, e.g., [185, 186, 304]. On the other hand, despite the extensive use of both 2-D PAGE for whole proteins and chemical cleavage agents, 2-D diagonal electrophoresis with cleavage fragments from crosslinked proteins is practically non-existent in the literature (for a variant, see [33]).

Cleavage of proteins also poses some obstacles. Possibly the worst problem, especially with integral membrane proteins, is the release of highly hydrophobic peptides, which generally tend to form large insoluble aggregates, preventing detection of individual fragments. Even though several methods have been developed to minimize aggregation (see Appendix, pages 149 - 160), there are few techniques that deal directly with aggregation of hydrophobic fragments upon cleavage. However, a wide variety of cryoprotectants [e.g., glycerol, ethylene glycol (EG), DMSO, alcohols, sorbitol, sucrose, etc.] are available (Table 3, page 158). Cryoprotectants have been utilized to reduce or avoid protein aggregation (Appendix), and to keep native conformations of proteins and peptides, even at subzero temperatures. Thus, cryoprotectants may be particularly useful since almost invariably one of the steps of a cleavage procedure is lyophilization of the cleaved sample, which may induce aggregation of hydrophobic peptides upon their concentration in the vanishing
solvent. Therefore, if published recommendations fail to keep hydrophobic fragments from aggregation, a methodic search among available cryoprotectants may follow to ensure an appropriate electrophoretic analysis of the fragments that are released by cleavage of PS II proteins and their crosslinked products.

D. Use of antibodies.

Antibodies have been used primarily for immunohistochemistry [51, 57, 94, 197, 314, 354; also see Vol. 121, 1986, of Methods in Enzymology]. Antibodies have also been successfully utilized in many types of electrophoretic studies [44, 46, 50, 66, 87, 108, 181, 183, 203, 207, 210, 247, 248, 250, 271, 300, 306]. Antibodies raised against the proteins of interest are used as primary antibodies on blots of electrophoretically separated proteins, followed by incubations with enzyme-conjugated secondary antibodies and then with chromogenic substrates. This procedure is a common technique which allows protein visualization. Antibodies are of special importance to detect proteins that have been crosslinked, since antibodies are specific and permit the identification of low concentrations of crosslinked products. Additionally, in combination with specific cleavage of crosslinked products, mono- or polyclonals can be used to identify the particular fragments that have been crosslinked. The single epitope of monoclonals may provide further information on crosslink location [107, 280].
7. Proposition.

A great amount of information has been obtained about the structure and function of PS II, yet the spatial organization of the proteins in PS II is not completely understood. This study was aimed at better understanding the structural relationships among the protein components of PS II, by characterizing crosslinked products obtained from suspensions of OERC's derived from spinach PS II membranes. Specific fragmentation of a selected crosslinked product revealed a more resolved picture of the interactions occurring between two individual proteins, MSP and CPa-1.

Suspensions of OERC complex and OERC core were examined with respect to their protein composition, yield, durability and ease of handling. OERC complex was selected for further studies because it contains a minimum of the proteins necessary for oxygen evolution, it is obtained in relatively few steps, and the yields (= 1 - 3 % of the starting Chl in chloroplasts) are high enough to permit the purification of crosslinked products. An alternative preparation could have been OERC core, which contains fewer proteins and still evolves oxygen. However, the low yields (< 20 % of OERC complex), the time consuming preparation, and the lability of OERC core particles prevented the production of suitable crosslinks.

A lipophilic crosslinker was examined with respect to its ability to crosslink proteins within both OERC complex and OERC core. DTSP reached many sites susceptible to crosslinking, given the rather hydrophobic nature of most PS II
components. This allowed identification and selection of specific crosslinked products.

To identify expected crosslinked products, mono- and polyclonal antibodies raised against particular PS II proteins were utilized, on blots of both one-dimensional and two-dimensional dual and diagonal electrophoresis gels. Preparative electrophoresis was employed to isolate sufficient quantities of at least one crosslinked product (chosen mainly on the basis of its yield and purity in its electrophoretic zone) for further characterization.

One problem that arose when cleaving the crosslinked product was the release of highly hydrophobic fragments, which formed insoluble aggregates. To circumvent this problem, the cryosolvent ethylene glycol was ultimately used, such that most of the fragments predicted to be released by cleavage were accounted for with PAGE. Two-dimensional diagonal PAGE was then employed to establish which fragments of MSP were crosslinked to which fragments of CPa-1. Selected crosslinked fragments were identified by their electrophoretic mobility and antibody recognition, as well as by their sequences (since the sequences of practically all the spinach PS II proteins were known).
MATERIALS AND METHODS

1. Preparation of chloroplasts.

Class II chloroplasts were isolated from spinach leaves, essentially as described [47]. A typical isolation involved the homogenization of market spinach leaves with chloroplast isolation buffer (50 mM Na-K-HPO₄ pH 7.4, 100 mM sucrose, 200 mM NaCl and 5 mM MgCl₂), followed by filtration through one layer of nylon fabric of very fine mesh plus one layer of Miracloth®, and then by centrifugation at 2,000 g for 5 min at 0 - 1 °C. The pellet (class II chloroplasts) was resuspended in Resuspension Buffer (50 mM MES-NaOH pH 6.0, 300 mM sucrose, 15 mM NaCl and 10 mM MgCl₂), and then used for further fractionation. The inclusion of protease inhibitors in the chloroplast isolation buffer was not necessary. The Chl concentration was determined by a standard method [18].

2. Preparation of oxygen evolving Photosystem II membranes.

Subchloroplast PS II membranes having high rates of oxygen evolution were prepared basically as originally described [115], with some modifications [104].
Briefly, freshly prepared class II chloroplasts (2.5 mg Chl/mL) were either left on ice in darkness for 1.5 h (to allow for thylakoid stacking) [121], or immediately mixed with detergent [188]; no difference was found between these two treatments. To the chloroplast suspension, 20% w/v Triton X-100 was added such that the final ratio of detergent to Chl was 25 mg : 1 mg. This detergent to Chl ratio resulted in PS II membranes with Chl a to Chl b ratios between 1.87 and 2.04.

The mixture of chloroplasts and Triton was incubated for 25 min, after which centrifugations at 0 - 2 °C were performed as follows: One centrifugation at 3,000 g for 5 min; the supernatant (containing stacked double thylakoid leaflets) was centrifuged at 40,000 g for 20 min. The pellet (PS II membranes) was resuspended in the resuspension buffer, centrifuged again at 3,000 g for 5 min, and the supernatant was recentrifuged at 40,000 g for 20 min. This last pellet (purified PS II membranes) was resuspended to 2.5 mg Chl/mL in Solution A (50 mM MES-NaOH pH 6.0, 400 mM sucrose and 10 mM NaCl) to be used for OERC preparation (see below). The Chl content of the PS II membranes was determined as for chloroplasts, and their yield ranged between 30 and 40% of the Chl contained in the chloroplasts.

3. **Preparation of OERC complex.**

   Oxygen Evolving Reaction Center complex was obtained basically as described [119]. Briefly, a freshly prepared suspension of PS II membranes (2.5 mg
Chl/mL) was mixed with 1 volume of Solution B [50 mM MES-NaOH pH 6.0, 1 M sucrose, 800 mM NaCl, 10 mM CaCl$_2$ and 70 mM OGP (octyl-β-D-glucopyranoside)] and incubated for 10 min (with occasional swirling). Then 2 volumes of Solution C (50 mM MES-NaOH pH 6.0, 1 M sucrose, 400 mM NaCl and 10 mM CaCl$_2$) were added. The suspension was incubated for 5 more min (with occasional swirling), and then subjected to centrifugation at 40,000 g for 90 min. The pellet (mostly LHC proteins with a Chl a to Chl b ratio < 1.8) [123] was discarded.

The supernatant was diluted with 3 volumes of Solution D (50 mM MES-NaOH pH 6.0, 10 mM NaCl and 5 mM CaCl$_2$) and centrifuged at 40,000 g for 10 min. The pellet was resuspended in Solution E (50 mM MES-NaOH pH 6.0, 400 mM sucrose, 10 mM NaCl and 5 mM CaCl$_2$), and either used immediately or stored at -20 °C. The samples were kept in darkness at temperatures between 0 and 2 °C throughout the preparation of OERC complex. Its Chl content was determined as for chloroplasts. The yield of OERC complex was 10 - 15 % of the Chl content of PS II membranes.

4. Preparation of OERC core.

Oxygen Evolving Reaction Center core was obtained essentially as described [119]. Briefly, a freshly made suspension of OERC complex was mixed with a 10 % solution of dodecyl maltoside (DM) to yield 0.5 % final DM. Immediately
after dissolution of the OERC complex with this detergent, the mixture was loaded on a Sephacryl* S-300 column (50 x 2.5 cm ID) which was equilibrated with Solution F (50 mM MES-NaOH pH 6.0, 20 mM NaCl, 5 mM CaCl₂ and 0.05 % DM). Elution proceeded at 1 mL cm⁻² h⁻¹ with Solution F, and 1-mL samples were collected. These samples were kept on ice and used during the same day.

5. Salt washes.

Salt washes were performed on ice as described [44]. Briefly, aliquots of OERC complex were centrifuged (in a microfuge 3 x 1 min at 4 °C, or at 40,000 g at 1 °C for 20 min, depending on the volume), and the pellets were resuspended in Solution E + 1 M salt (either NaCl, CaCl₂ or Tris-HCl pH 9.4), incubated for 1 h, centrifuged (as above), and resuspended again in the same media. After incubation for 1 more hour, they were centrifuged again (as above). The pellets were washed twice with Solution E, and finally resuspended in Solution E to 1 mg Chl/mL.

6. Crosslinking of OERC's with DTSP.

Crosslinking with DTSP was performed following published recommendations [23, 204]. Briefly, OERC complex or core aliquots in Solution E were centrifuged
(in a microfuge 3 x 1 min at 4 °C, or at 40,000 g at 1 °C for 20 min). The pellets were resuspended in a phosphate buffer (50 mM NaKHPO₄ pH 7.8, 50 mM NaCl and 5 mM CaCl₂) to 1 mg Chl/mL. At time zero, varying amounts of DTSP (50 mM in DMSO) were added up to 2 mM final (or 1 mM for isolation of the 100 kDa crosslinked product, 100 XL). These crosslinking mixtures were then incubated on ice in the dark (with occasional stirring). At the indicated times (1 h for isolation of 100 XL), glycineamide was added (100 mM final, to eliminate the excess DTSP). The incubation proceeded for 30 min more, followed by addition of iodoacetamide (40 mM final, to alkylate any sulfhydryl groups remaining, thus precluding disulfide exchange), incubation for 45 min more, and then either used immediately, or stored at - 20 °C.

7. Polyacrylamide gel electrophoresis (PAGE).

A. Preparation of gels.

I. General procedures.

Gels were prepared using standard procedures [136, 137, 263]. Gels were prepared from the following stock solutions: 30 % acrylamide + 0.8 % bis-acrylamide, 60 % sucrose (with 0.1 % NaN₃ as preservative), 10 % ammonium persulfate
(kept frozen), pure TEMED (N,N,N',N'-tetramethylethylenediamine), 10 % DM, and gel buffers. The final concentrations of these gel buffers were: a) For low molecular weight (LMW) gels: 1 M Tris-HCl pH 8.5 plus 0.1 % LDS in the resolving and spacing portions, and 0.75 M Tris-HCl pH 8.5 + 0.75 % LDS in the stacking portion. b) For all the other gels: 0.5 M Tris-HCl pH 8.8 for resolving gel portions, and 60 mM Tris-HCl pH 6.8 for stacking portions. The final concentrations of the reservoir buffers were: a) For LMW gels: 100 mM Tris, 100 mM Tricine, 0.1 % LDS for the upper reservoir, and 200 mM Tris-HCl pH 8.9 for the lower reservoir [140, 147]. b) For other gels with LDS: 25 mM Tris plus 192 mM glycine for the lower reservoir and, for the upper reservoir, the same buffers plus 1 mM EDTA and 0.1 % LDS [75]. c) For native gels: as in b), but no LDS in the upper reservoir buffer. Even though SDS was shown to provide high resolution of proteins [205], LDS was used in this work because it allows less distortion of the protein bands during electrophoresis [41a]. Electrophoresis was performed at 4 °C. Particular systems are delineated below.

II. Native gels.

i) DM gels.

DM gels were prepared as recommended [24]. Briefly, slab gels (1.5 mm thick) were prepared with a resolving linear gradient of 4 - 7 % acrylamide (= 11 cm high), topped by a 2.5 % acrylamide stacking gel (= 2 cm high). Both gel portions
contained 0.05 % DM. These gels were run at 0.6 W for = 16 h. For preparative and two-dimensional designs, see pages 38 & 39.

ii) Transverse gradient gel.

A slab gel (1.5 mm thick) was made with a uniform 4 % acrylamide resolving gel, topped by a 0.5 % agarose stacking portion. The resolving region also contained a transverse gradient of 0 - 0.25 % DM, while the stacking portion had 0.3 % DM (Fig 3). This gel was run at 0.5 W for = 16 h. This transverse gradient gel was devised to optimize a protocol to separate the protein components of OERC cross-linked samples. By using a sample of OERC complex, the best separation of native complexes and individual proteins was achieved with 0.05 % DM.

III. Regular gradient (12.5 - 20 %) gels with LDS.

Gels with a linear gradient of acrylamide were prepared using standard procedures and recommendations [75, 136, 137, 193, 263]. Briefly, gels (1.5 mm thick) were prepared with a resolving linear gradient of 12.5 - 20 % acrylamide (11 cm high), topped by a 3.75 % acrylamide stacking gel (= 2 cm high). This type of gel was used to determine the protein composition of OERC samples treated in various ways. Samples were electrophoresed at 1.2 W for = 16 h.
Figure 3. Preparation of a gel with a 0 - 0.25 % w/v transverse gradient of DM. The resolving gel was 10 cm high and 14 cm wide.
IV. Low molecular weight (LMW) gels with LDS.

Gels designed to resolve low molecular mass protein fragments were prepared essentially as described before [275], considering general recommendations given by various workers [81, 97, 137, 140, 147, 208, 246]. Briefly, slab gels (1.5 mm thick) were prepared with a resolving gel of 16 % acrylamide (= 10 cm high), topped by a spacing layer of 10 % acrylamide (= 1.5 cm), and then by a 4 % acrylamide stacking gel (= 2 cm high). LDS was used for these LMW gels instead of SDS [275]. These LMW systems were electrophoresed at 2.5 W for = 20 h.

These LMW gels were used to analyze low molecular mass fragments derived from cleavages of either purified proteins from PS II or from their crosslinked products. Sometimes these gels were used to purify small amounts of fragments to be sequenced. For this, sample lanes containing these fragments were blotted onto Immobilon*, a PVDF (polyvinylidiene fluoride) membrane [329]. The blotted lanes were stained with acid-free Coomassie blue (CB; see page 42), and the bands of interest were excised and used for sequencing.

V. Preparative gels.

Particular preparative gels (2 - 3 mm thick) were prepared and electrophoresed in accordance with sections I to IV above. These preparative gels were utilized as described in sections i) to iii) below.
i) Isolation of crosslinked products.

The 100 XL crosslinked product and others were best purified by employing a dual preparative DM/LDS gel system. The first gel was 1.5 mm thick, with a resolving linear gradient of 4 - 7 % acrylamide and a 2.5 % stacking gel, both parts containing 0.05 % DM. This "first dimension" gel was loaded with crosslinked OERC complex (1 mg Chl + 1 % DM in 1.25 mL), and electrophoresed at 0.6 W for \( \approx 16 \) h. The crosslinked complex migrated in a diffuse green band with \( R_f\)'s = 0.25 - 0.40. This band was excised, equilibrated for 30 min in Sample Buffer (50 mM Tris-HCl pH 6.8, 6 % sucrose, 3 % LDS and BPB crystals), and loaded on a second slab gel (2 mm thick, 12.5 - 20 % acrylamide resolving linear gradient gel, 3.75 % stacking gel). This "second dimension" gel was electrophoresed at 2 W for \( \approx 16 \) h, and stained with acid-free CB. The band at 100 kDa was excised for further isolation of 100 XL via electroelution and concentration (with Centricon 30\* devices) [161]. The concentrated 100 XL solution was stored at -20 °C.

ii) Purification of protein components of PS II.

For this, either regular gradient gels (2 mm thick), or gels with a uniform resolving acrylamide concentration of 15 % (2 mm thick), were utilized. For example, CPa-1 and CPa-2 were purified from gels which were loaded with OERC complex at 1 mg Chl/gel and electrophoresed at 2 W for \( \approx 16 \) h. The gels were stained with acid-free CB and the polypeptides of interest were excised for further isolation via electroelution and concentration.
iii) Purification of low molecular mass peptidic fragments.

Purified polypeptides were chemically cleaved, loaded on LMW gels (2 mm thick) at ≈ 2 mg of polypeptides per gel, and electrophoresed at 4 W for ≈ 24 h. The gels were then stained with acid-free CB (see below), and the fragments of interest were excised. These fragments were identified by immunopробing on Immobilon® membranes, to which the contents of a vertical gel strip had been electrotransferred by a described method [306] (page 44).

VI. Two-dimensional gel systems.

i) Dual system: native gel followed by a denaturing regular gradient LDS gel.

These gel systems were designed essentially as described [24]. These gel systems were employed, in conjunction with immunopробing (see page 46), mainly to identify and isolate some of the crosslinked products. For this, gels 1.5 mm thick were prepared containing a resolving linear gradient of 4 - 7 % acrylamide, topped by a 2.5 % stacking gel; both gel parts contained 0.05 % DM. These gels were prepared for a first dimension PAGE either as tube gels [24] or as slab gels (1.5 mm thick), where either the tube gels, or vertical strips of the slab gels, were used as samples for the second dimension. The first dimension tube gels were run at 40 mW per tube gel for 12 h, and the slab gels were run at 0.6 W for ≈ 16 h. Then these samples (tube gels or vertical strips) were preequilibrated in Sample Buffer for 20 min, and laid on top of slab gels to be run as a second dimension PAGE. The
second dimension slab gels (2 mm thick) were prepared as a regular gradient gel (page 35). Electrophoresis was at 2 W for ≈ 20 h. The proteins were then recognized by staining with regular CB followed by silver (see page 43), or after electro-transfer to nitrocellulose (NC) or Immobilon* [306, 329] followed by immunodetection (page 46).

ii) Regular diagonal system: regular gradient LDS gel without reductants followed by a similar gel with reductants.

Two dimensional diagonal gels were utilized, in conjunction with immunoprobing (page 46), chiefly to identify the protein components of some of the cross-linked products. This diagonal system was devised following recommendations given by several workers [16, 37, 88, 91, 93, 125, 128, 173, 174, 179, 206, 225, 278, 307]. The gels were prepared essentially as described before [46]. Briefly, a regular gradient gel (page 35) was loaded with crosslinked samples in 2 to 6 central wells = 8 mm wide, flanked by PS II samples and by protein markers. The crosslinked samples (135 μl) contained = 4 μg Chl, 1.5 % LDS, 4.5 % sucrose and a bromophenol blue (BPB) crystal. Electrophoresis was at 1.2 W for ≈ 16 h. The crosslinked lanes were excised and, as pairs, incubated for 20 min in Sample Buffer with 3 % β-mercaptoethanol (β-ME) for one strip of the pair, and without β-ME for the other. The strips were then laid on top of regular gradient gels (2 mm thick; page 35) to be run as a second dimension PAGE, along with protein markers. Electrophoresis was at 2 W for ≈ 20 h, with the upper buffer containing 0.02 % thioglycolic
acid (TGA). The proteins of these second dimension gels were then detected either by staining with regular CB followed by silver (page 43), or by electrotransfer to NC or Immobilon* membranes [306, 329] followed by immunodetection (page 46).

iii) LMW diagonal system: LMW gel without reductants followed by a similar gel with reductants.

LMW diagonal gels were utilized, in conjunction with immunoprobining (page 46), to identify crosslinked fragments of the proteins involved in a crosslinked product such as 100 XL. This gel system was designed by combining an LMW system [140, 147, 275] (page 37) with the regular diagonal system [46] (page 40). For this, an LMW gel (1.5 mm thick) was loaded with chemically cleaved samples of 100 XL in 2 to 6 central wells = 8 mm wide, flanked by PS II samples and by protein markers. The cleaved samples (135 µl) contained = 50 µg protein + 1.5 % LDS + 4.5 % sucrose and a BPB crystal. Electrophoresis was at 2.5 W for = 20 h. The lanes with cleaved samples were excised and, as pairs, incubated for 20 min in Sample Buffer with 3 % β-ME for one strip of the pair, and without β-ME for the other. The strips were then laid on top of slab gels to be run as a second dimension PAGE. The second dimension slab gels (2 mm thick) were also prepared as a LMW gel. Electrophoresis was at 4 W for = 24 h, with the upper buffer containing 0.02 % TGA. The fragments in these second dimension gels were visualized by staining with regular CB followed by silver (page 43), or by electrotransfer to Immobilon* membranes and immunodetection (page 46).
B. Staining of gels.

I. Staining with CB.

i) Regular CB.

After electrophoresis, the gels (or pieces) were stained with CB R-250 by using standard procedures [31, 355]. The electrophoresed gels were incubated in a glass tray with 400 mL of a solution containing 0.1 % CB R-250 in 25 % methanol and 7 % acetic acid. Staining was overnight, followed by a one-day background destaining in a 500-mL solution of 25 % methanol + 7 % acetic acid containing several Kimwipes* towels. The Kimwipes* were replaced several times to capture the CB dye released from the gel. Upon destaining, these gels were photographed with red filters, and then stored [272] or processed for silver staining (page 43).

ii) Acid-free CB.

For this variation of the procedure in i) above, the gels to be CB-stained were incubated in a glass tray with 400 mL of a solution containing 1 g of CB R-250 in 1 L of 25 % methanol. Staining was overnight, followed by one day of destaining of the background (3 to 4 changes of 500 mL of 25 % methanol). Upon destaining, these gels were utilized for the isolation of fragments, proteins, crosslinked fragments or crosslinked products.
II. Silver staining.

This is a sensitive method to stain proteins in general [298]. The protocol used here was an adaptation [344] for Laemmli [193] type gels run with SDS or LDS and previously stained with CB. For this, the CB stained gels (with the background cleared from stain and the surfaces cleaned from deposits) were first treated to eliminate the acetic acid: they were incubated in 60 % methanol overnight, then in deionized water overnight, and then again overnight in 60 % methanol + 2 drops of 1 N NaOH/100 mL + 2 drops of 38 % formaldehyde/100 mL. Each gel was then stained for 20 min with gentle rocking in 100 mL of a solution prepared by: first, dissolving 0.8 g silver nitrate in 6 mL of deionized water; second, mixing 21 mL of 0.36 % NaOH and 1.4 mL of NH₄OH; third, gradually mixing both solutions; and fourth, bringing this solution to 100 mL with deionized water. After the 20 min incubation in this alkaline silver nitrate solution, each gel was washed 3 times (15 min each) with deionized water, and then developed with 500 mL of a freshly prepared solution containing 25 mg of citric acid and 250 μl of 38 % formaldehyde. Development time varied between 15 and 40 min, depending on the sample loads and the thickness of the gel. When sufficient staining intensity had developed, each gel was rinsed in deionized water and then incubated in 25 % methanol + 7 % acetic acid to stop the color development. These gels were then ready to be photographed (without filters).
III. CuCl$_2$ staining.

This procedure was adopted from [202], considering other recommendations [89, 328]. Briefly, after electrophoresis, the gels were rinsed with deionized water and gently rocked in 100 mL of 0.3 M CuCl$_2$ for up to 40 min. When the negatively stained polypeptide bands (transparent) were clear enough against the whitish background (caused by the precipitate of copper-dodecyl sulfate), the gels were rinsed with and stored in deionized water. The metal staining pattern was stable for months in water. This system was as sensitive as CB staining, but it was intended to be used in the isolation of proteins without the interference of the CB dye (e.g., for isolation of 100 XL). Once a polypeptide was identified, it was excised and incubated with 15 mL of 250 mM EDTA + 250 mM Tris-HCl pH 9.0 (3 changes X 10 min) to chelate the copper. This incubation caused the precipitate to disappear.

8. Blotting of gels onto NC or Immobilon$^*$.  

A. General procedure.

The proteins electrophoresed in gels were electrotransferred to sheets of NC (earlier experiments) or Immobilon$^*$ [50, 152, 177, 306, 312, 329]. Almost complete transfer of polypeptides was accomplished after $\approx$ 90 V·h/mm of gel thickness.
Electrotransfer was done at 4 °C and the blots (or selected lanes) were either immediately stained (see section B. below), or blocked with 50 mL of 5 % non-fat dry milk (Carnation®) [152] in Tris saline solution (TS: 10 mM Tris-HCl pH 7.4 and 150 mM NaCl). Blocking was for 1 h (4 h in earlier experiments). After blocking, the blots were immunoprobed as described below. Between changes of solutions, the immunoblots were rinsed three times with TS.

**B. General staining.**

I. **Amido black.**

Nitrocellulose blots (or selected portions) were rinsed with water and incubated for ≈ 20 min with 50 mL of amido black stain (1 g of amido black 10B in 1 L of 25 % methanol and 7 % acetic acid) [113]. The background was then largely removed with 25 % methanol + 7 % acetic acid (3 - 4 changes of 50 mL for 10 min each). The blots were then rinsed with water and dried. This procedure was employed to stain protein marker lanes on NC immunoblots (see section C. below).

II. **Coomassie blue.**

Immobilon® blots (or selected portions) were rinsed with water and incubated with 50 mL of regular CB stain for ≈ 20 min [329]; the background was then largely
removed with 60 % methanol (3 - 4 50 mL-changes, 10 min each). The blots were then rinsed with water and dried. This procedure was employed to stain the protein marker lanes accompanying immunoblots on Immobilon* (see section C. below).

A few times acid-free CB (see page 42) was utilized to stain blots that were to be used for sequencing purposes. After staining (for = 30 min), the blots were destained with 60 % methanol (3 changes of 50 mL for 20 min each), thoroughly rinsed with deionized water (6 changes of 50 mL for 10 min each) and dried.

C. Immunoblottings.

I. Probing.

i) Primary antibodies (mono- and polyclonals).

The blocked blots were probed overnight at room temperature with primary monoclonal or polyclonal antibodies, specific for PS II polypeptides. Solutions of the monoclonals FAC2 [107], FAAC4 [108] and FQC3 [249] were composed of the supernatants of hybridoma cell cultures, or dilutions of ascities fluids in TS + 1 % BSA + 10 crystals of NaN₃. Polyclonals were dilutions of serum in TS + 1 % BSA + 10 crystals of NaN₃. Antibody solutions were reused several times.
ii) Secondary antibodies.

Secondary antibodies were diluted in TS + 1 % BSA to 1 : 1,000 (anti-rabbit IgG) or 1 : 2,000 (anti-mouse IgG + IgM). These antibodies were conjugated to either peroxidase (for use with NC blots) or alkaline phosphatase (for use with Immobilon® blots). Incubations were for 3.5 h for NC blots, or for 1 h for Immobilon® blots.

II. Developing.

i) Use of 4-chloro-1-naphtol to detect peroxidase activity.

To visualize the polypeptides recognized by antibodies, NC blots were incubated with a 30 mM solution of the chromogenic substrate 4-chloro-1-naphtol [83, 130, 143, 183], which was dissolved in 20 mL of methanol and mixed with 80 mL of TS + 3.5 mM H₂O₂. When sufficient color was developed (after 20 - 40 min), the blots were rinsed with water, photographed and dried.

ii) Utilization of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to detect alkaline phosphatase activity.

To visualize the polypeptides recognized by antibodies, Immobilon® blots were incubated with 50 mL of a buffered solution (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 2 mM MgCl₂ and 1 μM ZnCl₂) containing 6.8 mg of BCIP and 16.5 mg of NBT [95, 271]. When sufficient color was developed (after 15 - 20 min) by the
alkaline phosphatase activity [248, 257, 309], the blot was rinsed with water and dried. The background was clearer when the blot was dry.


A. Cyanogen bromide (CNBr).

I. Gel strips.

This procedure was adapted from [246] (also see [132], and page 23), and involved the cleavage of polypeptides (or a crosslinked product such as 100 XL) fixed in the matrix of polyacrylamide gel strips. For this, the polypeptides to be cleaved were electrophoresed (e.g., in a regular gradient gel), and the gel was stained with acid-free CB (page 42). The polypeptides of interest were excised and treated with a CNBr mixture for up to 24 h. The whole procedure was performed under a hood.

The CNBr mixture was prepared by mixing 5 mL of buffer (125 mM Tris-HCl pH 6.8), 5 mL of 0.6 N HCl, and 125 μl of acetonitrile containing CNBr. This last solution was prepared by dissolving 500 mg of CNBr in 200 μl of acetonitrile (CNBr greatly increased the volume of the solution). The control tube(s) contained only acetonitrile. The gel slices were then added to the tubes and incubated at room temperature with gentle rocking for the indicated times.
After the incubation, the CNBr medium was discarded and the gel slices were washed with buffer (125 mM Tris-HCl pH 6.8 + 0.1 % LDS) for a total of 2 h (3 changes of 10 mL each). The gel slices were then equilibrated in a buffer (50 mM Tris-HCl pH 6.8 + 3 % LDS + 6 % sucrose + BPB ± β-ME) that rendered the slices as appropriate samples to be loaded in gels.

This procedure regularly cleaved CPa-1 and CPa-2 quantitatively. The only Met-Thr bond of MSP yielded = 25 % of cleavage product. The remaining 75 % of MSP was irreversibly modified such that it could not be cleaved further by retreating with CNBr.

II. Liquid phase. Inclusion of ethylene glycol.

In early experiments of CNBr cleavage in liquid media, the polypeptides were cleaved in a mixture of 2 mL of 125 mM Tris-HCl pH 6.8, 2 mL of 0.6 N HCl, and 50 μL of CNBr-containing acetonitrile, but instead of gel strips (see section I. above), a relatively small volume of electroeluted sample was added to the CNBr mixture, and incubation proceeded for only 6 h with gentle rocking. In addition, the cleavage reaction was finished by diluting the samples with = 10 volumes of water, freezing them and then lyophilizing them (for about 5 - 7 h) under a hood. The freeze-dried samples could then be taken up in a sample buffer and loaded in gels.

Unfortunately, some hydrophobic fragments derived from CPa-1, CPa-2 and 100 XL could not be resolubilized, even after the addition of trifluoroacetic acid
[147], addition of FA [112], or the use of other published treatments (see Appendix). DMSO and EG were found to keep these hydrophobic fragments from precipitating irreversibly when included instead of acetonitrile as the CNBr solvent (see page 83, and Appendix). The optimal concentrations of EG and DMSO were between 5 and 10 % v/v in the CNBr mixture. This allowed the resolubilization of practically all the fragments released by CNBr from CPa-1, CPa-2, and 100 XL. MSP did not show problems with acetonitrile as the CNBr solvent. EG proved to be the best additive (see page 83).

B. Formic acid (FA).

The FA cleavage procedure was adapted from [196], considering recommendations from [208, 264]. It was performed as follows: 100-μL polypeptide samples (e.g., 100 XL electroeluted from gel strips) were added to tubes containing 1.9 mL of a mixture of FA (70 % final), EG (5 % final) and water. Samples were incubated at 40 °C for 6 - 20 h with continuous shaking, and then lyophilized for 2 h. The residues in the tubes (a viscous paste) were used immediately or stored frozen. When used, they were dissolved with a LMW Sample Buffer (from 1 mL LMW upper reservoir buffer + 500 μl LDS + 100 μl sucrose + 50 μl saturated BPB) and loaded in LMW gels (1.5 mm thick if for analytical purposes, or 2 mm thick if for preparative isolation of free or crosslinked fragments).
10. Oxygen evolution assays.

These assays were performed according to standard procedures [e.g., 106, 119, 188] with a Clark-type electrode, which was attached to a voltage recorder via a current-voltage converter and amplifier. The electrode's chamber (1.5 mL capacity) was closed at its bottom by the platinum electrode's fitting. This chamber was outfitted with a small magnetic bar revolving right above the nylon membrane (permeable to O₂) covering the electrodes. Prior to the measurements, the system was calibrated between the saturating O₂ line and the zero oxygen line. The saturating O₂ line was obtained with the reaction medium (50 mM MES-NaOH pH 6.4, 400 mM sucrose, 10 mM NaCl, and 10 mM CaCl₂) completely aerated and at the temperature of the assay, while the zero oxygen line was attained by adding sodium dithionite crystals to the reaction medium. From the recorded curves of oxygen evolved over time, the activities were determined from initial rates at 4 or at 22 °C. For measurements of OERC complex activity, 5 - 20 µg Chl and up to 2 mM 2,6-dichloro-p-benzoquinone (DCBQ made 100 mM in pure ethanol) were used per assay. For OERC core fractions (eluted from Sephacryl® S-300 columns), activities were measured by diluting 50 µL of each fraction with 1 mL of reaction medium and 700 µM final DCBQ.
1. Preparation of OERC complex and core.

Suspensions of spinach OERC complex and OERC core were obtained sequentially as shown in Table 1 (i.e., in the order class II chloroplasts, PS II membranes, OERC complex and OERC core; see page 6). A typical preparation of Class II chloroplasts rendered between 130 and 220 mg Chl/Kg of leaves, yields similar to those attained before [47]. The yields of PS II membranes ranged between 30 and 40 % of the Chl contained in the Class II chloroplast preparations. The protein composition of PS II membranes is illustrated in Fig 4. Similar yields and protein composition of PS II membranes were obtained before [104, 115]. A typical suspension of OERC complex produced 10 - 15 % of the Chl content of PS II membranes, so the OERC complex yield was 2 - 6 % of the Chl content of chloroplasts. The protein constitution of OERC complex is also illustrated in Fig 4. This figure shows a Coomassie Blue stained blot after an electrophoretic separation of PS II membranes and OERC complex. In accordance with the expected protein composition (Table 2, page 7), it can be seen that intensely stained components of
Figure 4. Blot of an SDS-PAGE gel of PS II membranes (10 μg Chl; lane 1) and OER C complex (5 μg Chl; lane 2). The samples were electrophoresed in a low molecular mass gel type, blotted to Immobilon® (a PVDF membrane), and stained with Coomassie Blue. The major PS II proteins are indicated, along with MW markers.
OER complex include CPa-1 (a doublet at ≈ 50 kDa), CPa-2 (at 45 kDa), MSP (at 33 kDa), various LHC proteins (Chl-binding proteins between 24 and 30 kDa), CP-29 (at ≈ 29 kDa), and Cyt b_{559} (at ≈ 9 and 4 kDa for the α and β subunits, respectively). The diffuse-band proteins, namely D1 and D2, are noticeable at ≈ 34 and 32 kDa, respectively. Other bands are also visible. In addition to all these proteins, the lane with PS II membranes includes the intensely stained LHC polypeptides (between 23 and 30 kDa), and the extrinsic 24- and 17 kDa proteins, which are removed upon treatment of PS II membranes with the detergent OGP (e.g., during preparation of OER complex) [119], or with various salt washes (pages 12 - 14).

OER complex suspensions prepared from PS II membranes had Chl a to Chl b ratios between 11 and 20 (average ≈ 14), indicating a relatively high purity (most Chl a/b LHC antenna proteins had been eliminated). The oxygen evolving activity from H_{2}O to DCBQ (700 µM) of these OER complex preparations was around 1,400 µ moles O_{2} (mg Chl)^{-1} h^{-1}. Both the Chl a/Chl b ratios and the activities of the complex were comparable to published results [119].

This type of OER complex preparation was then used to chromatographically isolate OER core. Typically, OER core particles contained fewer proteins than an OER complex preparation, had Chl a to Chl b ratios around 20, and had oxygen evolving activities of about 400 µ moles O_{2} (mg Chl)^{-1} h^{-1}. The most intensely stained proteins were CPa-1, CPa-2, D1, D2, MSP, and Cyt b_{559}. Of the eluted fractions, the only fractions containing Cyt b_{559} were those containing OER core particles (results not shown). These results agree with previous findings [119].
During the chromatographic preparation of OERC core, addition of 5 mM CaCl$_2$ to Solution F proved essential for maintaining O$_2$-evolving activity, which otherwise was lost due to the long elution times (= 30 h total). The lack of CaCl$_2$ in the media could have been the reason why an early attempt at preparing an active OERC core failed [353]. The absence of CaCl$_2$ in a Solution F used previously [119] probably did not affect the OERC core activity due to the relatively short elution times (about 1/2 h) in the utilized FPLC apparatus.

2. Crosslinking of OERC's with DTSP.

To further establish neighborhood relationships between particular PS II proteins, the hydrophobic crosslinker DTSP (1.2 nm range; Fig 2, page 22) was utilized to crosslink both OERC complex and OERC core particles. DTSP was used mainly because of the rather hydrophobic nature (excluding MSP) of the polypeptides comprising the OERC's.

A. Recognition of crosslinked products in one-dimensional LDS-PAGE.

1. Preliminary experiments.

OERC core was treated with various DTSP concentrations and then subjected to PAGE. A few high molecular mass crosslinked products appeared with
increasing concentrations of DTSP. OERC complex was also treated with various DTSP concentrations, and then electrophoresed under diverse conditions. Several high molecular mass products appeared as the concentration of DTSP increased. A particularly intense product (100 XL) was observed at 100 kDa. Also visible were a crosslinked product at ≈ 20 kDa, and two intramolecular MSP products. All the observed crosslinked products disappeared upon reduction with β-ME.

A time course of OERC complex crosslinked with 0.5 mM DTSP (Fig 5) showed that several new bands appear, especially at rather high molecular masses (> 50 kDa), as the incubation time with DTSP increases. The formation of these crosslinked products is concomitant with the disappearance of PS II polypeptides. Some positively identified crosslinked products are shown by arrowheads at ≈ 150, 110, 70, 60 and 53 kDa. In addition, the arrow points to 100 XL.

II. Salt washes.

More specific results were obtained by utilizing antibodies raised against particular PS II proteins. For example, Fig 6.A pictures a blot immunoprobed with α-33, a mouse polyclonal that specifically recognizes MSP and its crosslinked products. This figure illustrates an SDS-PAGE of time courses of salt-washed OERC complex crosslinked with DTSP (0.5 mM). The control treatment (lanes 1 -4) shows that many crosslinked products appear, at increasingly higher molecular masses, as time passes in the presence of DTSP. Crosslinked products involving MSP can be
Figure 5. Time course of OERC complex crosslinked with 0.5 mM DTSP. This figure depicts an SDS-PAGE gel stained with silver. The treatments were performed as follows: to aliquots of OERC complex (214 μg Chl) suspended in a buffer (300 μL of 50 mM phosphate, pH 7.4), 3 μL of 50 mM DTSP in DMSO were added and, after the indicated incubation times, glycinamide to 100 mM was added to stop the crosslinking reaction, followed by inclusion of iodoacetamide to 40 mM to avoid disulfide exchanges. The samples were then frozen until used. Some of the controls performed included: incubation for 120 min with DMSO without DTSP (C¹); incubation for 120 min with phosphate buffer only (C²); and non-treated OERC complex (C³). The molecular masses for protein markers are indicated, as well as for the identified PS II proteins (for the polypeptides of LHC's and the two subunits of cytochrome b559, only the general location is indicated).
Figure 6A. Nitrocellulose blotting of an SDS-PAGE of a time course of OERC complex crosslinked with DTSP (0.5 mM), in the absence of reductants. Prior to crosslinking, aliquots of OERC complex were washed (see Materials and Methods) either with resuspension buffer (lanes 1-4), 1 M NaCl (lanes 5-8), 1 M CaCl2 (lanes 9-12), or 1 M Tris-HCl pH 9.4 (lanes 13-16). The washed samples were treated with DTSP for 0 (lanes 1, 5, 9 & 13), 10 (lanes 2, 6, 10 & 14), 50 (lanes 3, 7, 11 & 15), and 100 min (lanes 4, 8, 12 & 16). These samples were then electrophoresed in a 12.5-20% acrylamide gel (along with the indicated MW markers), electrotransferred to nitrocellulose, and probed with α-33 (against MSP). The 100 kDa crosslinked product (100 XL) is marked by an arrow.
Figure 6.B. Nitrocellulose blotting similar to that of Fig 6.A, but the probe was FAC2 (a mouse monoclonal raised against CPa-1). The lanes and labels are the same as for Panel A. Particularly intense crosslinked products appear at \( \approx 100 \text{kDa} \) (arrow; 100 XL) as well as at \( \approx 120 \) and 150 kDa (arrowheads), in the resuspension and NaCl washes. A crosslinked product appearing at \( \approx 60 \text{kDa} \) (marked with an *) remains being formed with all washes. A faint crosslinked product seems to be formed at \( \approx 80 \text{kDa} \) (**) only when MSP is not present (CaCl\(_2\) and Tris washes).

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seen at ≈ 150, 110, 100, 90, 70, 56, 52, 50, 39, 37, 35 and 34 kDa. Since the particularly interesting 100 kDa product (arrow) is revealed by α-33, it indicates the involvement of MSP (also see Fig 5). Intramolecular products of MSP at 27 and 30 kDa are also apparent at the latest incubation times.

Removal of MSP by CaCl₂ and alkaline Tris washes prior to crosslinking should preclude the formation of any crosslinked product involving this protein. No crosslinked products or intact MSP could be detected by the immunoprobe (α-33) upon removal of MSP (lanes 9 - 16), as opposed to washing with resuspension buffer only (a control) (Fig 6.A). As a second control, washing of OERC complex with 1 M NaCl was performed, treatment that removes extrinsic proteins but it does not remove MSP. This treatment (lanes 5 - 8) produced a pattern similar to that of the resuspension buffer washing (lanes 1 - 4), thus confirming the involvement of MSP in the crosslinked products observed.

OERC complex was washed, crosslinked for various times, electrophoresed, transferred to NC, and immunoprobed with FAC2 (a mouse monoclonal against CPa-1) [107]. A crosslinked product appeared at ≈ 100 kDa (arrow) and two less intense products at ≈ 120 and ≈ 150 kDa (Fig 6.B). Upon removal of MSP (CaCl₂ and Tris-HCl washes), none of these three crosslinked products was apparent. This indicates that at least these three products, involving the intrinsic CPa-1, also include the extrinsic MSP, or that the presence of MSP might have allowed the formation of crosslinked products between CPa-1 and other PS II proteins. In addition, the absence of MSP may permit the formation of crosslinks between CPa-1 and other
PS II proteins. For example, in Fig 6.B another crosslinked product (at ≈ 60 kDa; marked with **) appeared only when MSP was not present (CaCl₂ and Tris-HCl washes). Thus, this product was formed between CPa-1 and another polypeptide only when MSP was not shielding the crosslinking site on CPa-1, adding to the generalization that MSP is associated with CPa-1. Salt-washing experiments performed with EDC-crosslinked PS II membranes also allowed to conclude that MSP is closely associated with CPa-1 [46, 248].

Other antibodies were also utilized to probe parallel blots of time courses of OERC complex crosslinked with 0.5 mM DTSP. These blots showed that other PS II proteins, such as CPa-2, D2 and Cyt b₅₅₉, also gave rise to crosslinked products as incubation time increased. In addition, from these blots it was deduced that 100 XL involved MSP and CPa-1, but not CPa-2, D2 or Cyt b₅₅₉ (results not shown).

III. Split-lane experiments.

Crosslinked OERC complex was electrophoresed and the gel was blotted. A blot lane was split vertically, and each half-lane was probed with α-33 (for MSP) or FAC2 (for CPa-1). At least two crosslinked products reacted with both FAC2 and α-33 (Fig 7). These crosslinked products had MW_{obs} of 100 kDa (100 XL) and ≈ 120 kDa.

The α-5 (polyclonal against CPa-1) and α-33 antibodies were used to probe blotted half-lanes from a dual PAGE (a DM gel followed by an LDS gel; Fig 7).
Figure 7. Split-lane immunoblots. Strips $C_1$, $C_2$, $I$ & $2$ were prepared with OERC complex aliquots (10 $\mu$g Chl), either crosslinked with DTSP (1 mM, 1 h; strips $I$ & $2$), or treated without it (control strips $C_1$ & $C_2$). The samples were electrophoresed in a 12.5 - 20 % gradient gel and blotted (100 V-h). The blot was blocked (5 % dry non-fat milk), the lanes were split in half and the resulting vertical strips were probed with FAC2 (strips $C_1$ & $I$), or with $\alpha$-33 (strips $C_2$ & $2$). The two uppermost arrows indicate putative crosslinked products that react with both FAC2 and $\alpha$-33, thus involving CPa-1 and MSP.

Strips $3$ & $4$ correspond to a split-lane immunoblot after dual electrophoresis. DTSP-crosslinked OERC complex (1 mg Chl) was electrophoresed in a 4 - 7 % gel containing 0.05 % DM. A 2 cm high horizontal band, containing 100 XL, was excised and electrophoresed in a 12.5 - 20 % gradient LDS-gel. The gel was blotted (120 V-h). The blot was split into vertical strips, which were probed with various antibodies. Shown here are a strip probed with $\alpha$-5 (polyclonal against CPa-1; strip 3) and an adjacent strip probed with $\alpha$-33 (strip 4). Again the two uppermost arrows indicate crosslinked products at 100 and $\approx$ 120 kDa, both of which involve CPa-1 and MSP.

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Crosslinked products, at = 100 and 120 kDa, reacted simultaneously with \( \alpha \)-5 and \( \alpha \)-33, thus involving CPa-1 and MSP. These results also suggest that the dual electrophoresis system (DM followed by LDS) should be an appropriate procedure to isolate preparative quantities of relatively pure 100 XL.

In addition, duplicate split lanes probed with other antibodies (data not shown) indicated that MSP and CPa-2 formed discrete crosslinked products and either CPa-2, Cyt b_{559} nor LHC polypeptides formed part of 100 XL.

**B. Recognition of crosslinked products in two-dimensional diagonal LDS-PAGE.**

As a fourth way to verify whether MSP and CPa-1 are crosslinked to each other by DTSP, two-dimensional diagonal electrophoresis was utilized. OERC core and OERC complex particles were crosslinked with DTSP and subjected to two-dimensional diagonal LDS-PAGE. Cyt b_{559}, LHC's, MSP, D2, D1, CPa-2 and CPa-1 are OERC complex proteins that were crosslinked to various extents (Fig 8). Each protein participated in several crosslinked products, as evidenced by the horizontal "lines" of proteins formed below the diagonal. Examples of crosslinks are represented by the three vertical lines containing CPa-2 and MSP polypeptides (arrowheads), which correspond to \( \approx 150, 120 \) and \( 95 \) kDa crosslinked products. The arrows indicate two vertical lines containing CPa-1 and MSP polypeptides, corresponding to the \( \approx 118 \) and \( 100 \) kDa crosslinked products. DTSP-crosslinked OERC core also

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A.

Figure 8.A & B. Silver staining of gels corresponding to a two-dimensional diagonal LDS-PAGE of OERC complex (6 µg Chl) treated with 500 µM DTSP (panel A) or without it (panel B; next page). Both electrophoreses were in 12.5% acrylamide uniform gels without reductants (first dimensions), or with reductants (incubation of first dimensional strips in a solution containing 50 mM Tris-HCl pH 6.8, 1.3% SDS, 6% sucrose, 2% β-ME and BPB, and 0.02% TGA in the upper reservoir buffer for the second dimension electrophoresis). Arrows indicate the CPa-1 and MSP polypeptides that participated in 100 and 115 kDa products. Arrowheads indicate the CPa-2 and MSP polypeptides which participated in the 95, 120 and 150 kDa products.

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Figure 8. Panel B.
produced vertical lines of MSP with CPa-1 and MSP with CPa-2 (not shown). These examples point, respectively, to the close association between MSP and CPa-2, and between MSP and CPa-1.

Antibodies recognizing MSP or CPa-1 were used in immunoblots of two-dimensional diagonal LDS-PAGE. An immunoblot of DTSP-crosslinked OERC complex probed simultaneously with α-33 and FAC2 showed the existence of polypeptides that fall on vertical lines below the diagonal, indicating that both MSP and CPa-1 are proteins that were crosslinked together (Fig 9). Two major vertical lines of MSP and CPa-1 polypeptides descend from crosslinked products at 100 and ≈ 118 kDa, implying that MSP and CPa-1 are in close proximity in the OERC complex. A control blot (no DTSP) exhibited no crosslinks. A similar immunoblot probed with α-33 and α-6 (against CPa-2) also showed that MSP and CPa-2 are in close vicinity in the OERC complex.

This association between MSP and either of the interior antenna proteins is consistent with published results indicating that CPa-1, and CPa-2, are in close proximity with MSP [36, 44, 46, 52a, 90, 91, 93, 128, 168, 248, 250, 353].

C. Recognition of crosslinked products by the dual electrophoresis (DM/LDS) system.

As a fifth way to ascertain whether MSP and CPa-1 are crosslinked to each other by DTSP, two-dimensional dual (native followed by denaturing) PAGE was
Figure 9. Immunoblot from two-dimensional diagonal LDS-PAGE of crosslinked OER complex (see Fig 13.A), probed simultaneously with α-33 (against MSP) and FAC2 (against CPa-1). Arrows indicate CPa-1 and MSP polypeptides appearing on a vertical line below the diagonal.
used. Preliminary electrophoresis of untreated OERC complex was performed in a gel containing a transverse concentration gradient of DM (0 - 0.25 % w/v). The best resolution of green native complexes was achieved at about 0.05 % DM (data not shown). The first dimension electrophoresis (4 - 7 % acrylamide with 0.05 % DM) should resolve non-affected complexes of PS II from complexes containing crosslinked products involving MSP (e.g., 100 XL), since MSP migrates well ahead from the rest of a "PS II core" in a DM gel [24]. The second dimension electrophoresis (12.5 - 20 % acrylamide in the presence of LDS) released, in a vertical line, the individual proteins forming native complexes (Fig 10) [24]. Furthermore, polypeptides appearing in a DTSP-treated sample (Fig 10.A, arrowheads), but not in a non-treated sample (Fig 10.B), indicate crosslinked products. Most of these crosslinked products were identified on immunoblots of gels identical to those in Figs 10.A & B.

The blots in Fig 11 were probed with α-33. In panel A, the open arrowhead shows various isomorphs of non-crosslinked MSP, whereas the arrows show groups of intramolecularly crosslinked MSP species. Arrowheads indicate intermolecular crosslinked products that include MSP. None of these crosslinked products appeared in the control immunoblot (panel B). The arrowhead marked by the * points to 100 XL (see Figs 10 & 12).

The blots in Fig 12 were probed with FAC2. In panel A the arrowheads indicate crosslinked products, since these polypeptides were not present in the control blot (panel B). The open arrowhead marks a sharp band corresponding to the ≈ 60 kDa crosslinked product mentioned in Figs 5 & 6.B, and assumed to...
Figure 10.A & B. Silver staining of a two-dimensional DM/LDS-PAGE of OERC complex treated for 1 h with 1 mM DTSP (panel A), or without DTSP (panel B). Samples (12 μg Chl in 80 μL) were diluted with 1% DM + 6% sucrose + BPB, centrifuged, and the supernatants electrophoresed in a 4 - 7% gel containing 0.05% DM. From this first dimension, lanes were excised, preincubated in a medium (50 mM Tris-HCl pH 6.8, 1.3% LDS, 6% sucrose, and BPB) for 30 min and loaded sideways on top of slab gels (12.5 - 20% acrylamide) for the second dimension electrophoresis (with 0.1% LDS in the upper reservoir buffer). In panel A, the arrowheads indicate discernible crosslinked products. The arrowhead with the * marks 100 kDa.
Figure 10. Panel B.
Figure 11.A & B. Immunoblots of two-dimensional DM/LDS-PAGE of OERC complex treated for 1 h with 1 mM DTSP (panel A), or without DTSP (panel B), like the gels pictured in Fig 10. The gels were blotted onto nitrocellulose (140 V-h). The blots were blocked (5 % non-fat dry milk, Carnation *), probed overnight with α-33, challenged for 3.5 h with a 1 : 1,000 dilution of an anti-mouse Ig conjugated to horseradish peroxidase, and color developed with 4-chloro-1-naphtol. In panel A, the open arrowhead shows various isomorphs of non-crosslinked MSP, while the arrows show groups of intramolecular crosslinks of various MSP species. Solid arrowheads indicate other crosslinked products. The arrowhead with the • points to 100 XL. * For electrophoretic conditions, see gels pictured in Fig 10.
Figure 11. Panel B.

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Figure 12.A & B. Immunoblots of two-dimensional DM/LDS-PAGE of OERC complex treated for 1 h with 1 mM DTSP (panel A), or without DTSP (panel B). These blots were obtained as these pictured in Fig 11, but they were immunoprobed with FAC2. The open arrowhead marks a 56 kDa crosslinked product of CPa-1 and a small intrinsic PS II polypeptide. Solid arrowheads indicate other crosslinked products. The arrowhead with the * points to 100 XL.

*a For electrophoretic conditions, see gels pictured in Fig 10.
Figure 12. Panel B.
involve CPa-1 and a small intrinsic PS II polypeptide. But the most interesting feature is represented by the arrowhead with the *, which points to 100 XL (Figs 10 & 11). Therefore, it is again demonstrated that 100 XL includes CPa-1 and MSP.

Thus, this new way of identifying 100 XL by two-dimensional DM/LDS-PAGE (Figs 10 - 12) illustrates once more the close association of MSP and CPa-1. This is further supported by the presence of two other crosslinked products at about 118 and 150 kDa, both of which include MSP and CPa-1 (see the two uppermost arrowheads in Figs 11.A & 12.A). The crosslinked product at 118 kDa can also be observed in Figs 5, 6.A & B, 7, 8.A & 9, and the product at 150 kDa can also be visualized in Figs 5, 6.B and 7. In addition to MSP and CPa-1, this 150 kDa product appeared to contain CPa-2 as well (results not shown).

Until now it has been observed that a variety of intermolecular crosslinked products between PS II proteins can be detected by various means. A particularly intense product that has been recognized in virtually all occasions is 100 XL, so attempts were made to isolate and characterize it.

D. Isolation of 100 XL.

Normally 100 XL was isolated by the dual (DM/LDS) PAGE system (page 39). However, in early experiments, 100 XL was isolated by multiple electrophoresis. In one of these early experiments, gel strips containing isolated 100 XL were blotted, and the blots were either stained with amido black, or immunoprobed with
α-33 or with FAC2 (Fig 13). Strip 3, containing crosslinked OERC complex (included as reference) shows 100 XL (arrow) and the = 118 kDa (arrowhead) crosslinked products, which were recognized by antibodies against MSP and CPa-1 (see Figs 6 - 12). Strip 6, containing a reduced sample of DTSP-treated OERC complex, depicts the disappearance of both crosslinked products. The isolated 100 XL appears relatively pure on the amido black-stained lanes (1 & 2). Both α-33 (strip 4) and FAC2 (strip 5) recognized 100 XL. On the other hand, half lanes with reduced samples depict the disappearance of 100 XL in conjunction with the release of CPa-1 (strip 7) and MSP (strip 8), again indicating that 100 XL represents a close association occurring between these two PS II proteins.

In addition, an approximately 150 kDa crosslinked product (150 XL) was isolated and treated as 100 XL (previous paragraph), demonstrating that MSP, CPa-1 and CPa-2 were included in this product (data not shown), which implies that MSP and both interior antenna proteins are closely associated in PS II.

Despite the usefulness of the multiple electrophoresis method (Fig 13), problems of low loads (strips 7 & 8) or streaking (lane 2) were observed. To circumvent these problems, 100 XL was then isolated by the dual (DM/LDS) PAGE system (page 39), followed by electroelution and concentration. This procedure yielded highly concentrated solutions of 100 XL, adequate to perform CNBr and FA cleavage studies.
Figure 13. Split lane immunoblots of 100 XL isolated by multiple electrophoresis. DTSP-crosslinked OERC complex was electrophoresed in a preparative 4 - 7 % gel with 0.05 % DM. A horizontal band containing 100 XL was excised and electrophoresed in a preparative 12.5 - 20 % LDS gel, which was stained with CuCl2 [202] to visualize the crosslinked polypeptides at around 100 kDa. A light diffuse band was visible as a negative staining against the white-bluish background. A narrow strip containing 100 XL was excised and incubated in a chelating solution (250 mM EDTA + 250 mM Tris-HCl pH 9.0) to remove the copper. This strip was diced and split into two samples: one half of the pieces was incubated for 30 min in a non-reducing medium (50 mM Tris-HCl pH 6.8, 1.5 % LDS, 6 % sucrose and a few BPB crystals), and the other half was incubated (30 min) in the same medium plus 2 % β-ME. The non-reduced sample (lanes 1 & 2 and strips 4 & 5) was electrophoresed in an LDS gel (2 mm, 12.5 - 20 % acrylamide), along with a lane of DTSP-crosslinked OERC complex (lane 3) and protein markers. The gel was then blotted (120 V·h), and the blot was stained with amido black (lanes 1 & 2), or probed with FAC2 (lane 3 and strip 5) or with α-33 (strip 4). The reduced sample (strips 7 & 8) was electrophoresed in an LDS gel (with 0.02 % TGA in the upper reservoir buffer), along with a lane of reduced DTSP-crosslinked OERC complex (lane 6). The gel was blotted (90 V·h) and the blot was probed with α-33 (strips 6 & 8) or with FAC2 (strip 7). Lane 1 shows isolated 100 XL, while lane 2 shows a higher load of 100 XL. Lane 3 illustrates the presence of 100 XL (arrow) and the ≈ 120 kDa (arrowhead) crosslinked product. Both α-33 (strip 4) and FAC2 (strip 5) recognized the isolated 100 XL (small arrows). Lane 6 is a reduced lane of DTSP-crosslinked OERC complex (100 XL disappears). In addition, reduction of the isolated 100 XL releases both CPa-1 (strip 7) and MSP (strip 8).
3. Cleavages of 100 XL.

A. Background.

The relative position of the CNBr cleavage site of MSP (at the M-T bond), the relative location of the FA cleavage site (at the D-P bond), and the calculated molecular masses of the fragments are shown in Fig 14. This sketch was based on the amino acid sequence of MSP, which was established by direct sequencing [252], and by deduction from its gene sequence(s) [182, 310, 326]. The deduced molecular masses were calculated as shown in the legend. The observed electrophoretic molecular mass of these fragments (= 11.5 and = 21 kDa for the small and large CNBr fragments, and = 18 and = 11 kDa for the large and small FA fragments) differs from the calculated values due to anomalous migration(s). For this same reason, the total $M_W_{obs}$ for whole MSP in a LMW-LDS-PAGE system is about 28 - 30 kDa, while the $M_W_{obs}$ of MSP with a Laemmli system is about 33 kDa (hence, MSP has been called the 33 kDa extrinsic protein). These mobilities should be compared with the MSP's molecular mass of $= 26.7$ kDa, deduced from its amino acid sequence [252, 310]. Almost any part of the MSP primary structure could be crosslinked to CPA-1 by DTSP. However, the lysyl residues tend to be located toward the ends of this protein: there are 11 lysines in the small CNBr fragment (amino terminus; 14 % of the amino acids are lysines), 8 lysines in the small FA fragment (carboxyl terminus; 9 % of the amino acids are lysines), and 4 lysines in
Figure 14. Sketch of the spinach MSP, illustrating the relative position of the CNBr cleavage site (at the M-T bond), as well as the relative location of the FA cleavage site (at the D-P bond). Also indicated are the molecular masses of the fragments, as calculated from the amino acid sequence of the spinach MSP already described [252]. The deduced molecular masses were calculated by adding the molecular masses of the fragment's amino acids \( n \), and subtracting the molecular mass of \( n-1 \) water molecules, i.e.:

\[
MW_{\text{frag}} = (\Sigma_i^n MW_{aa}) - 18(n-1)
\]
the overlapping region of the large CNBr and large FA fragments (protein middle section; 5% of the amino acids are lysines).

A line sketch of the spinach CPa-1 primary structure, with the relative positions of the CNBr and FA cleavage sites, the corresponding fragment numbers and their deduced molecular masses, are displayed in Fig 15. The molecular masses of the resulting cleavage fragments of CPa-1 were calculated as for MSP (Fig 14), based on the published spinach CPa-1 sequence [42, 233]. Only a few of the CPa-1 cleavage fragments have been thoroughly identified: for example, the CNBr-fragment # 12, which carries the epitope for the monoclonal FAC2 and comprises a large portion of the large extrinsic loop, has been observed with a mobility equivalent to 15.7 kDa [107], or ≈ 17.0 kDa in a LMW gel system (e.g., see Figs 18.B & C, page 88). By examining the sequences of the CPa-1 fragments, it can be observed that: a) CNBr fragments # 8, 10, 11 and 12 contain lysines, to which DTSP could be attached. Each of these fragments possesses lysyls which would be lumenally exposed and, therefore, may potentially be in or near (a) binding site(s) for MSP (e.g., lysine residues could form salt bridges) [248, 250]. Thus, such lysyls may participate in crosslinking CPa-1 to MSP. The free amino terminus (methionine in fragment # 1) could conceivably bind DTSP; even so, this amino group might not participate in crosslinking to MSP as it might be located on the stromal side of the membrane [42]. b) Of the FA fragments, only fragments # 6 - 10 contain lysines, to which DTSP could be bound. Of these, only fragments # 7 - 9 may participate in direct crosslinking with MSP, as they are the only segments with lysyls potentially exposed.
Cleavage sites on CPa-1

Figure 15. Sketch of the spinach CPa-1. The numbers above and below the thick line show the positions for the FA and CNBr cleavage sites. The corresponding fragment numbers and their deduced molecular masses (in kDa) are indicated by numbers in oblique writing. From the sequence of the spinach CPa-1 [233], the molecular masses of these fragments were calculated as for MSP (see legend to Fig 14). The approximate positions of the six putative transmembrane α-helices are also illustrated, along with the expected sidedness of the loops and ends [42, 320]. $S$ stands for stromal side, and $L$ for luminal side. Between helices V and VI resides the large extrinsic loop of CPa-1, which is lumenerally exposed and seems to serve as binding site for MSP [233]. In addition, the *'s under the thick line indicate lysyl residues which might become directly crosslinked to MSP, since they are thought to be exposed to the lumen of the thylakoids. Other lysyls (not shown) are expected to be located on the stromal side of the membrane.
toward the lumen. This analysis is valid only if the proteins are maintained in a sided-membrane context, so two main assumptions are made here: a) In OERC complex the protein orientation through the membrane is maintained in a relatively native state; this is possible, since OERC complex evolves oxygen with relatively high rates [1,400 μ moles O₂ (mg Chl)⁻¹ h⁻¹]. b) The transmembrane orientation of the six putative α-helices of CPα-1 exists as predicted [42, 320].

Fig 15 also depicts the approximate position of the six putative transmembrane α-helices, the expected sidedness of the loops and ends, and the lysyl residues (*'s) which may become directly crosslinked to MSP. These lysyls may be exposed to the lumen of the thylakoids, where MSP is associated [6, 12, 20, 188].

The above analysis furnishes a foundation of what should be encountered when cleaving MSP and CPα-1 with CNBr or FA. It should also help in understanding the resulting patterns of cleavage of 100 XL. For example, unique fragments containing the crosslinked fragments of MSP and CPα-1 should appear in the 100 XL cleavage patterns. These crosslinked fragments should then be detected forming a vertical line below the diagonal in a two-dimensional diagonal PAGE system. Sequencing of the released fragments should yield their unequivocal identity. The general development of this procedure is described below.
B. CNBr cleavages.

I. Preliminary experiments. One dimensional explorations.

A procedure for cleaving polypeptides electrophoresed into gel pieces [246] produced low concentration of the resulting fragments (particularly for 100 XL), streaking, and formation of multiple bands (due to poor stacking of the molecules residing in the gel pieces). It was found in preliminary experiments that cleaving polypeptides in an aqueous medium containing EG corrected most of these problems (see Appendix). The separation of CNBr cleavage fragments using 10 % EG showed (Fig 16.A) two CNBr fragments of MSP (lane 1), several individual fragments of 100 XL (lane 2), and many fragments of CPa-1 (lane 3). Some of the fragments of 100 XL seemed to coincide with some of the CPa-1 and MSP fragments, while others were unique to 100 XL, indicating that there was more than one crosslink occurring in 100 XL.

Treatment of MSP with CNBr (lane 4) or FA (lane 6), and using the EG method, produced two CNBr fragments of MSP (lanes 1 & 4) at $MW_{obs} = 21$ and $11.5$ kDa (corresponding to the carboxyl and amino ends, respectively), two FA fragments (lane 6) at $MW_{obs} = 18$ and $11$ kDa (corresponding to the amino and carboxyl ends, respectively), and showed that both CNBr and FA cleavages are incomplete. The intact MSP is seen in lanes 1, 4 & 6. These incomplete cleavages seem to be a result of the chemical nature of the cleavage sites (page 23), and they cannot be split, regardless of the incubation times or cleaving conditions.
Figure 16. Panel A: Silver stained gel from a LMW-LDS-PAGE system, illustrating the separation of fragments from CNBr cleavage in an aqueous phase. For this, 100 XL was obtained by the dual DM/LDS system (see text), where the 100 XL-containing bands in the LDS gels were excised, electroeluted and concentrated in Centricon 30 devices. MSP and CPA-1 were separated electrophoretically from CaCl₂-washed OER complex, in 12.5 - 20 % gradient gels, and the gel strips containing these polypeptides were excised, electroeluted, concentrated and cleaved for 6 h. The cleaved polypeptides were then lyophilized to a paste consistency, and electrophoresed in a LMW-LDS gel containing 5 % EG. This gel was silver stained, and lanes 1, 2 & 3 shown here are the CNBr-cleaved samples of MSP, 100 XL and CPA-1, respectively.

Panel B: Summary of the fragments of MSP resulting from cleavages with CNBr (lane 4; CB stained LMW gel) or with FA (lanes 5 - 8; blot of a LMW gel). Lane 4 is MSP cleaved with CNBr for 24 h in the presence of 10 % EG, and electrophoresed in a LMW gel containing 5 % DMSO. Lane 6 contains an MSP sample which was cleaved with 70 % FA for 6 h at 40 °C in the presence of 10 % EG, electrophoresed in a LMW gel, blotted onto Immobilon, and probed with the polyclonal α-33. Lane 5 is an MSP sample treated the same as that of lane 6, but without the FA. Lane 7 is actually a half lane containing PS II membranes (8 μg Chl), and lane 8 has OER complex (4 μg Chl). Lanes 5, 7 & 8 were electrophoresed and immunoprobred as lane 6.

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II. Recognition of crosslinked fragments with 2-D diagonal LMW-LDS-PAGE.

Even though diagonal electrophoresis has been widely utilized to establish neighboring relationships between components of protein complexes (see pages 20 & 63), very little has been reported about diagonal electrophoresis designed to study fragments of proteins [33]. In a few studies, interactions between CPa-1 and MSP have been dealt at the level of cleavage fragments [44, 107, 248, 250], but diagonal electrophoresis has not been utilized, even though this technique should show the protein domains which are participating in the associations between MSP and CPa-1. With this in mind, experiments of two-dimensional diagonal LMW-LDS-PAGE of CNBr and FA-cleaved 100 XL were performed.

i) Recognition of crosslinks in silver stained gels.

Fig 17 represents silver stained gels of a diagonal LMW-LDS-PAGE of 100 XL cleaved with CNBr. Crosslinked polypeptides are indicated by various "horizontal strings" of polypeptides below the diagonal, the major ones corresponding to the whole MSP (since not all of it is cleaved by CNBr; marked at ≈ 28 kDa), to the large CNBr fragment of MSP (at ≈ 21 kDa), and to the 16.7 kDa CNBr-fragment of CPa-1 (as marked). Some other fragments are also visible. Panel B illustrates the virtual lack of peptides below the diagonal, indicating that the crosslinked fragments are still bound to each other due to the virtual absence of reducing agents.
Figure 17.A & B. Two-dimensional diagonal LMW-LDS-PAGE of 100 XL cleaved with CNBr. The 100 XL isolated from four pairs of DM/LDS gels was cleaved with CNBr in an aqueous phase in the presence of 10 % EG, lyophilized, and electrophoresed, as a first dimension, in two lanes of a LMW-LDS gel in the absence of reductants. For the second dimension, one of the two vertical lanes was incubated for 30 min in a stacking medium (50 mM Tris-HCl pH 6.8, 1.33 % LDS, 6 % sucrose, 2 % β-ME, and BPB), laid horizontally on top of a LMW slab gel, and electrophoresed along with MW markers, with 0.02 % v/v TGA in the upper reservoir buffer (panel A). The second vertical strip was treated the same, except that the stacking medium did not have β-ME, and the upper reservoir buffer did not have TGA (panel B). Both 2-D gels were silver stained. Panel B illustrates the lack of peptides below the diagonal, while panel A shows various "horizontal lines" of peptides below the diagonal, corresponding to the whole MSP (arrowhead, arrow), to the large CNBr fragment of MSP (at ≈ 21 kDa; arrowhead), to the 16.7 kDa CNBr-fragment of CPA-1 (arrowhead, arrow), and some other fragments at lower molecular masses. The three arrowheads point to peptides lying on a same vertical line corresponding to the 16.7 kDa, the large CNBr fragment of MSP, ant the whole MSP (which is not completely cleaved by CNBr). The * shows that 16.7 kDa could have been crosslinked to the 3.3 kDa CPA-1 fragment (# 11 in Fig 15). The ** point to the 16.7 kDa fragment of CPA-1, joined by a vertical line to an 11.5 kDa fragment (the parent 100 XL fragment was ≈ 27 kDa). The first vertical line of peptides (big arrow pointing up) shows many peptidic fragments being released from either the whole 100 XL or from aggregated crosslinked fragments; this makes it impossible to assign any crosslinks to particula fragments based on the peptides found on this vertical line.
Figure 17. Panel B.
There was a crosslink between the large CNBr fragment of MSP and the 16.7 kDa of CPa-1 (lower arrowheads, Fig 17.A). Peptides occupying another vertical line (arrows, Fig 17.A) indicate that there was a crosslink between the 16.7 kDa CNBr fragment of CPa-1 and (an)other peptide(s). The presence of whole MSP on these same vertical lines (arrowheads, arrows) is due to the incomplete cleavage of the M-T bond of MSP by CNBr. The 16.7 kDa CPa-1 fragment could also be crosslinked to other CPa-1 fragments (see *, **, Figure 17.A).

ii) Recognition of crosslinks in immunoblots.

Immunoblots probed with α-33 (Fig 18.A) or with α-33 and FAC2 (Fig 18.B) show that β-ME and TGA caused the release under the diagonal of the whole MSP and of its large fragment (verifying that they are crosslinked). Since only the 16.7 kDa fragment of CPa-1 bears epitopes for FAC2, and the small fragment of MSP cannot be detected with α-33, intramolecular crosslinks of MSP and of CPa-1, and crosslinks of MSP to small fragments of CPa-1, would not be detected on these blots. However, these types of crosslinks may be inferred from peptides below the diagonal derived from crosslinked fragments with relatively low molecular masses. For example, the * shows that the 16.7 kDa fragment was crosslinked to a fragment of ≈ 4 kDa (perhaps fragment # 11 in Fig 15), since the corresponding crosslinked fragment from 100 XL had a $MW_{obs} = 21$ kDa (see Fig 17.A). Similarly, the 16.7 kDa fragment might have been crosslinked to fragments # 8 or # 10 from CPa-1,
Figure 18.A. Immunoblot of a gel from a two-dimensional diagonal LMW-LDS-PAGE of 100 XL cleaved with CNBr. Cleavage and electrophoretic conditions were as those for Fig 17.A. This blot was probed with α-33. The whole MSP and its large CNBr fragment are indicated. The small CNBr fragment of MSP is not detected by α-33. A control immunoblot (of a gel like the one in Fig 17.B) showed no peptides below the diagonal.
Figure 18.B. Immunoblot of a gel from a two-dimensional diagonal LMW-LDS-PAGE of 100 XL cleaved with CNBr. Cleavage and electrophoretic conditions were as those for Fig 17.A. The blot was probed with α-33 and FAC2. The whole MSP and its large CNBr fragment are indicated. The small CNBr fragment of MSP is not detected by α-33. The arrowheads point to a vertical line of peptides at ≈ 28, 20 and 16.7 kDa, corresponding to the whole MSP, to its large fragment, and to the largest CPa-1 fragment, respectively. The * indicates 16.7 kDa fragment molecules that were crosslinked to fragments of ≈ 4 kDa, since the parent crosslinked fragment of 100 XL had a MW$_{obs}$ ≈ 21 kDa. Similarly, the ** point to 16.7 kDa fragment molecules which might have been crosslinked to the small CNBr fragment of MSP (≈ 11.5 kDa), or to fragments # 8 or # 10 from CPa-1 (14.1 and 8.5 kDa, respectively), as the *** peptide is derived from an ≈ 27 kDa crosslinked fragment of 100 XL.
Figure 18.C. Picture of the immunoblot from panel B, after being reprobed with α-5 (a polyclonal recognizing CPa-1). For this, the blot pictured in panel B was wetted with methanol, rinsed and incubated overnight with α-5, then incubated with the appropriate secondary antibodies, and color developed. Labels are the same as those for Fig 18.B.
as the "**" peptide is derived from an = 28 kDa crosslinked fragment of 100 XL (see Figs 14, 15 & 17.A).

A vertical line of peptides at ≈ 28, 21 and 16.7 kDa (arrowheads, Fig 18.B) correspond to the whole MSP, to the MSP large fragment, and to the largest CPa-1 fragment, indicating that the large CNBr fragment of MSP (21 kDa) is crosslinked to the largest CNBr fragment of CPa-1 (16.7 kDa).

The blot in Fig 18.B was reprobed with α-5 (polyclonal against CPa-1) (Fig 18.C). The whole MSP and its large fragment are again seen on a same vertical line with the 16.7 kDa fragment of CPa-1 (arrowheads). The 16.7 kDa CNBr fragment of CPa-1 also appears being involved in crosslinks to fragments other than the large MSP fragment (∗ and ∗∗, Fig 18.C). Peptides on the diagonal are apparent here and absent in Fig 18.B, demonstrating CNBr fragments of CPa-1 that are not crosslinked to MSP (none of these fragments are recognized by α-5 under the diagonal). Other low molecular mass CPa-1 fragments (not bearing epitopes for α-5 or not properly electroblotted) might still be crosslinked; some as yet unidentified low molecular mass CNBr fragments do appear on the silver stained gel (Fig 17.A).

Similar immunoblots were performed with polyclonals raised against CPa-2, D1, D2 and Cyt b₅₅₉, and no additional peptides could be observed on or below the diagonal, thus confirming that 100 XL does not contain detectable fragments of either of these PS II proteins.

The possible crosslinks between MSP and CPa-1 that occur in 100 XL are shown in Fig 19. MSP and part of CPa-1, the putative α-helices V and VI, and the
Figure 19. Some putative crosslinking sites among components of 100 XL, as deduced from CNBr cleavages. In this figure, the whole MSP and about half (carboxyl end) of CPα-1 are represented. Also indicated are the putative α-helices # V and # VI, as well as the sidedness of the CPα-1 portion shown here. No particular crosslinking assignment to any of the lysine residues has been made. The best supported crosslink is the one indicated by the thick line, between the large MSP fragment and the large CPα-1 fragment. Another likely crosslink is indicated by the solid line, happening between fragments # 11 and # 12 of CPα-1. Other possible crosslinks (dotted lines) might include fragment # 8 of CPα-1 (located outside this figure).
sidedness of CPa-1 are shown in this diagram. Any one or a combination of the crosslinks in Fig 19 may have been formed in 100 XL. The crosslink between the large MSP fragment and the large CPa-1 fragment (thick line, Fig 19) is best supported by the two-dimensional diagonal immunoblots probed with α-33 and with α-5 and FAC2 (Figs 18.A - C). Another possible crosslink occurs between fragments #11 and #12 of CPa-1 (solid line, Fig 19). Other possible crosslinks (broken lines) involve other CPa-1 fragments (inferred from CNBr cleavages; Figs 17.A & 18.B, C).

In summary, the intimate association between MSP and CPa-1 in OERC complex is due, at least in part, to an interaction between the 21 kDa fragment of MSP and the 16.7 kDa fragment of CPa-1. Experiments similar to those with CNBr were performed with FA to restrict the size of the crosslinked fragments, since FA cleaves at three sites on the 16.7 kDa CPa-1 fragment (Figs 15 & 23), and at one site near the middle of the large CNBr fragment of MSP (Figs 14 & 23).

C. Formic acid cleavages.

I. Two-dimensional diagonal LMW-LDS-PAGE.

Preliminary experiments with FA and MSP, CPa-1 and 100 XL demonstrated that optimal conditions were achieved after = 6 h at 40 °C with 70 % FA in the presence of 10 % EG, followed by lyophilization and electrophoretic analysis in LMW-LDS-PAGE (data not shown).
i) Recognition of crosslinks in silver stained gels.

MSP and its small FA fragment occur on a same vertical line, along with other peptides (arrowheads, Fig 20.A). Several other peptides corresponding to the small FA fragment of MSP also appear (arrows); thus, the small FA fragment of MSP is involved in at least one crosslink to CPa-1. Two other "horizontal strings" of peptides below the diagonal are visible at 24.5 and 20 kDa, seemingly corresponding to the largest FA fragment of CPa-1 (see Figs 15 & 23) and to the large FA fragment of MSP (see Figs 14 & 23), respectively. However, on immunoblots of 2-D gels, \( \alpha \)-33 failed to recognize the large FA fragment of MSP under the diagonal, while it did detect the small FA fragment (see Fig 21).

ii) Specific recognition of crosslinks with immunoblots.

An immunoblot of a 2-D diagonal LMW-LDS-PAGE of FA-cleaved 100 XL, probed with \( \alpha \)-33 (Fig 21), shows that only the whole MSP (at \( \approx \) 29 kDa) and its small FA fragment (\( MW_{obs} \approx 8 \) kDa) appear below the diagonal, even though the large and small FA fragments of MSP are recognized with about equal intensity (lane 2). The large FA fragment of MSP remains relegated to the diagonal. The whole MSP under the diagonal is due to the incomplete cleavage of MSP by FA (see lane 2). The small FA fragment of MSP is derived from several fragments of FA-cleaved 100 XL, as demonstrated by the strings of peptides obtained from \( \approx 70 - 40 \) kDa (solid arrowhead; see page 101), from \( \approx 22 - 20 \) kDa (open arrowheads; see
Figure 20.A & B. Silver stained gels of a two-dimensional LMW-LDS-PAGE of FA-cleaved 100 XL. Two 100 XL samples were cleaved with 70% FA + 10% EG for 6 h at 40°C, and electrophoresed in a two-dimensional diagonal gel system (see legend to Fig 17). Both FA-cleaved 100 XL samples were electrophoresed under non-reducing conditions for the first dimension. One of the samples was then incubated with β-ME and electrophoresed with TGA (panel A), while the other (control sample) was incubated and electrophoresed without these reducing agents (panel B). The largest FA fragment of CPa-1, the whole MSP and its small FA fragment are labeled. The arrowheads indicate a vertical line containing the whole MSP (because it is not cleaved to completion by FA), the 24.6 kDa CPa-1 fragment, and the small MSP fragment, along with other peptides. The small FA fragment of MSP also appears at other places (arrows).
Figure 20. Panel B.
Figure 21. Immunoblots probed with α-33 of a two-dimensional diagonal LMW-LDS-PAGE of FA-cleaved 100 XL (panel A), and of a one-dimensional LMW-LDS-PAGE (panel B) of PS II membranes (lane 1), and of FA-cleaved MSP (lane 2). For panel A, a gel like the one in Fig 20.A was blotted (90 V • h) onto Immobilon® and the blot was probed with α-33. The whole MSP and its large and small FA fragments are labeled. The small MSP segment is derived from 100 XL parent fragments with MW_{obs} ≈ 70 - 40 kDa (solid arrowhead), ≈ 22 - 20 kDa (open arrowheads), and ≈ 11 kDa (arrow).
page 102), and from ≈ 11 kDa (arrow). The results in Fig 21 clearly indicate that only the small FA fragment of MSP was crosslinked to CPa-1.

A crosslink between the small FA fragment of MSP (9.6 kDa) and fragment # 7 of CPa-1 (2.2 kDa) is also likely to occur (solid line, Fig 24), since an ≈ 11 kDa 100 XL fragment gave rise to the small FA fragment of MSP (arrow, Fig 21.A) and an = 3 kDa fragment of CPa-1 (not recognized by α-5; Fig 22); of the CPa-1 fragments in this size category (see Figs 15 & 23), only fragment # 7 (2.2 kDa) contains a lysyl, which is putatively exposed toward the lumenal side of the membrane [42].

The small MSP fragment also appears on the diagonal (Fig 21), probably because there was reduction of some DTSP crosslinks due to trace reductants present during the first dimension electrophoresis. The large MSP fragment is not crosslinked to CPa-1, since this blot fails to demonstrate the release of the large MSP fragment below the diagonal. An immunoblot probed with a polyclonal specific for the large FA fragment of MSP also failed to reveal this fragment below the diagonal (data not shown).

Various crosslinks between CPa-1 and MSP are also depicted by immunoblots probed with α-5 (Fig 22). Several fragments with molecular masses of = 25 kDa (arrow) and above appear under the diagonal. The lowest of these corresponds to the 24.6 kDa fragment of CPa-1 (# 8 in Figures 15 & 23), since this was the smallest fragment detected by FAC2 in a similar blot (not shown) [107]. The higher molecular mass fragments could be the result of incomplete cleavage of 100 XL molecules by FA. The 24.6 kDa fragment appears as a string of peptides derived
Figure 22. Immunoblot of a two-dimensional diagonal LMW-LDS-PAGE of FA-cleaved 100 XL. A gel like the one in Fig 20.A was blotted (90 V•h) onto Immobilon®, and the blot was probed with α-5. The 24.6 kDa CPα-1 fragment (# 8 in Figs 15 & 23) and peptides containing fragment # 9 of CPα-1 (Figs 15 & 23) are labeled. The arrow indicates a horizontal string of the 24.6 kDa fragment of CPα-1. Solid arrowheads indicate peptides between 8.5 and 7 kDa being derived from 100 XL fragments between ≈ 70 and 40 kDa. Open arrowheads indicate peptides between 8.5 and 7 kDa being derived from 100 XL fragments between ≈ 22 - 20 kDa.
from \( \approx 70 - 40 \) kDa (arrow), suggesting that it was released from a series of several incompletely cleaved fragments of 100 XL. The 24.6 kDa fragment contains five lysines exposed to the lumen (Fig 15), and it could be crosslinked directly to the small MSP fragment (Fig 21), or indirectly through intramolecular crosslinks to the other three CPa-1 fragments (solid arrowheads) seen below the 24.6 kDa "string" of peptides (Fig 22).

The 3 peptides between 8.5 and 7 kDa (solid arrowheads), and derived from 100 XL-fragments between \( \approx 70 \) and 40 kDa, correspond to CPa-1 fragments that may have been crosslinked to the 24.6 kDa fragment of CPa-1 (see above), to the small FA fragment of MSP (Fig 21) directly, or through intramolecular crosslinks to the 24.6 kDa fragment, which in turn would be crosslinked to the small MSP fragment (Fig 21). There are no crosslinks between the three fragments marked by solid arrowheads, since they do not fall on a same vertical line. Rather they are released sequentially by the FA cleavage of 100 XL. This would suggest that these three fragments might be related to each other. There are only two expected fragments around this range of molecular sizes: fragment # 6 at 9.2 kDa, and fragment # 9 at 8.0 kDa (Fig 15). Of these, fragment # 6 consists of the putative \( \alpha \)-helices II and III and three loops. Only the loop between those two \( \alpha \)-helices contains lysines, but this loop is thought to be on the stromal side of the membrane [42]. So it seems unlikely that this fragment participates in any direct crosslink to MSP. And the possibility of an indirect crosslink to MSP, through an intramolecular crosslink to the 24.6 kDa fragment (which contains a lysyl in the stromally exposed

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IV - V loop), is virtually ruled out by the appearance of these same three peptides (solid arrowheads) being released from 100 XL-fragments at ≈ 22 - 20 kDa (open arrowheads). Other intramolecular crosslinks in which fragment # 6 could perhaps participate would not allow indirect crosslinks to MSP either. Therefore, it is highly unlikely that the CPa-1 fragment # 6 participates, directly or indirectly, in crosslinks to MSP. On the other hand, CPa-1 fragment # 9 is a more reasonable candidate (see below).

The 3 peptides between 8.5 and 7 kDa and obtained from 100 XL-fragments at ≈ 22 - 20 kDa (open arrowheads, Fig 22) appear to have been crosslinked to the small FA fragment of MSP, which shows corresponding peptides marked with open arrowheads in Fig 21. The uppermost fragment detected with α-5 was identified by sequencing (see Fig 23) as containing a 8 kDa fragment of CPa-1 (fragment # 9, Figs 15 & 23). Thus, it is tempting to speculate that these three peptides correspond to the incomplete cleavage of fragment # 9 plus fragments # 10 and # 11 (10.8 kDa total, uppermost peptide), to fragment # 9 plus fragment # 10 (9.9 kDa total, middle peptide), and to just fragment # 9 (8.0 kDa, lowermost peptide). Sequencing of the lower two peptides should verify this hypothesis. Consistent with this hypothesis, neither of these three fragments appears crosslinked to each other, since they do not lie on a vertical line below the diagonal. In addition, the fact that these three fragments appear on the diagonal means that subpopulations of 100 XL molecules are crosslinked through segments other than these three, between the 24.6 kDa CPa-1 fragment and the small MSP fragment, for example. These results are
SEQUENCE OF FA FRAGMENTS OF SPINACH CPa-1

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SEQUENCE OF FA FRAGMENTS OF SPINACH MSP

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The first 10 amino acid cycles.

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Figure 23. Sequencing of crosslinked fragments from MSP and CPa-1. This figure is based on the amino acid sequences for spinach MSP [252] and spinach CPa-1 [233]. From these sequences, the likely cleavage sites for FA (aspartyl-prolyl bonds) [264] were determined, and the corresponding FA fragment sequences of spinach CPa-1 and MSP are shown with their corresponding end amino acid positions (AA #'s) and fragment numbers (Frag #; assigned as in Fig 15 for CPa-1), and
Figure 23. (Continuation).

Fig 14 for MSP 2. The CPA-1 large extrinsic loop is bounded by the \( \text{t} \) (at position 271) and the \( \text{r} \) (at position 448). Two crosslinked peptides excised from Immobilon\textsuperscript{®} blots were sequenced. This sequencing was performed in an Applied Biosystems 477A Protein Sequencer at Baylor College of Medicine, Houston, Texas. The chromatograms clearly showed the presence of 2 major peptides. The obtained 10 first cycles of amino acids released during this sequencing are shown at the bottom of this figure. The chromatogram for amino acid \# 1 showed a peak for proline with almost twice the molarity of the other amino acids. The sequences of the two FA fragments that most closely resembled the experimental sequences were the FA fragment \# 9 of CPA-1 and the small FA fragment of MSP. No other sequences from PS II proteins were found to match the empirical fragment sequences.

reinforced by immunoblots of 100 XL cleaved with FA and electrophoresed under various conditions (data not shown), all of which show the same pattern of fragments below the diagonal, with vertical lines joining the three CPA-1 8.5 - 7 kDa fragments and the small FA fragment of MSP, as well as the 24.6 kDa CPA-1 fragment and the small FA fragment of MSP.

Therefore, it can be concluded that both CPA-1 fragments \# 9 (8.0 kDa) and \# 8 (24.6 kDa), individually and/or in combination, are crosslinked to the small FA fragment of MSP.
II. Sequencing crosslinked fragments of MSP and CPa-1.

i) Isolation of the crosslinked fragments.

Since there was crosslinking between the small FA fragment of MSP and the 3 fragments between 8.5 and 7 kDa (Figs 21 & 22), an attempt was made to isolate and identify these fragments by sequencing them. These fragments were electrophoresed, under reducing conditions, in a one-dimensional lane to minimize loss of material, the gel was blotted and the three peptides were localized. The three peptide bands were recognized by α-5. Simultaneously, the uppermost peptide band was also recognized by α-33, indicating that it contained two comigrating peptides, the small MSP fragment and the 8 kDa CPa-1 fragment. The lower two peptides were not sequenced initially as they had also appeared (though faintly) in non-reduced lanes of FA-cleaved 100 XL, casting some doubts about their participation in crosslinks. And since the uppermost peptide band appeared being released only under reducing conditions, it was excised from acid-free CB stained blots, and the Immobilon® pieces were sent to Baylor College of Medicine (Houston, Texas) for sequencing.

ii) Sequencing results.

Sequencing of crosslinked fragments from MSP and CPa-1 clearly showed the presence of two major peptides (Fig 23). The first amino acid in the empirical sequence was proline, with almost twice the molarity of the other amino acids. The
excised band contained the FA fragment # 9 of CPa-1 and the small FA fragment of MSP. No other sequences from PS II proteins were found to match the attained sequences for the excised band.

iii) Possible crosslinks between MSP and CPa-1.

There are a number of possible crosslinks among components of 100 XL that can be deduced from its FA and CNBr cleavages (Fig 24). Any of the possible crosslinks, or a combination of these crosslinks, may have been formed in 100 XL. As a result, 100 XL could actually consist of a population of molecules with crosslinks at different places. The crosslink that is best supported by our data is between the small FA fragment of MSP and the 8.0 kDa FA fragment of CPa-1 (fragment # 9) (thick solid line, Fig 24). This conclusion is based on the 2-D diagonal immunoblots of FA-cleaved 100 XL, probed with α-33 (Fig 21) and with α-5 (Fig 22). These blots show both fragments lying on a vertical line below the diagonal, practically comigrating to the same zone (Figs 21 & 22). Immunoblots probed with FAC2 did not show the 8 kDa CPa-1 fragment, consistent with the FAC2 epitope location outside of fragment # 9 [107]. This crosslinkage is in agreement with the inferences drawn from CNBr cleavages (Fig 19), where the large CNBr fragment (carboxyl terminus) of MSP appeared crosslinked to the 16.7 kDa CNBr fragment of CPa-1 (Figs 17 & 18); the 16.7 kDa CNBr fragment includes the 8.0 kDa FA fragment (Fig 15). Further support for this crosslinkage comes from the sequencing of bands corresponding to these FA peptides (Fig 23). The FA fragment # 9 of CPa-1
Some FA cleavage fragments of 100 XL

**Figure 24.** Possible crosslinks between MSP and CPa-1 in 100 XL. No particular assignment to the lysyls in the crosslinked fragments was made; 100 XL could actually consist of a population of molecules with crosslinks at different places. These crosslinks were deduced mainly from 2-D diagonal immunoblots of CNBr and FA-cleaved 100 XL. The best supported crosslinks (see text) are crosslinks of the small FA fragment of MSP to the 8.0 kDa FA fragment of CPa-1 (# 9) (thick solid line), and to the 24.6 kDa fragment of CPa-1 (# 8) (thick broken line). An immunoblot with FAC2 did not show fragment # 9 below the diagonal, but it did recognize fragment # 8, which contains the epitope for FAC2 [107]. Also a large amount of fragment # 8 appeared on a vertical line with the small MSP fragment (Figs 21 & 22). On the other hand, sequencing of two crosslinked peptides revealed the small MSP fragment and CPa-1 fragment # 9 (Fig 23).

A crosslink between the small FA fragment of MSP and fragment # 7 of CPa-1 (solid line) is also possible, since an ≈ 11 kDa FA fragment of 100 XL released the small MSP fragment below the diagonal (arrow, Fig 21.A), along with an ≈ 2 kDa undetected fragment. Fragment # 7 has a MW ≈ 2.2 kDa and it is the only small fragment containing lysyls probably exposed to the lumen.

Crosslinks involving CPa-1 fragments located outside the scope of this figure could also occur in 100 XL. In addition, the putative α-helices # V and VI are indicated, as well as the probable sidedness [42, 320] of the CPa-1 portion shown here. This sidedness helps to preclude the formation of crosslinks between MSP and the lysines presumably located on the stromal side, e.g., lysine # 447 occurring in fragment # 10 of CPa-1 is not expected to be crosslinked.
encompasses about one quarter (carboxyl end) of the large extrinsic loop of CPa-1, plus the putative transmembrane α-helix # VI. On its extrinsic loop region, fragment # 9 contains three lysine residues, at positions 417, 418 and 438 (Fig 23). Any of these lysyls could be crosslinked to the small FA fragment of MSP (thick solid line). This 9.6 kDa MSP segment contains 8 lysine residues (Fig 23), of which any one could be crosslinked (alone or in combination) to fragment # 9 of CPa-1. Interestingly, lysines # 230, 233 and 236 seem to be located to the same side of a putative α-helix of MSP, according to a preliminary analysis of secondary structure carried out on spinach MSP with a set of protein sequence analysis programs [63]. The unique localization of these three lysines might play a role in one of the various binding sites of MSP.

Crosslinking of the small FA fragment of MSP to the large FA fragment of CPa-1 (# 8; 24.6 kDa) is very likely to occur as well (thick broken line, Fig 24), since immunoblots with α-33 do show the small FA-fragment under the diagonal and derived from rather high molecular mass crosslinked fragments of 100 XL (Fig 21); simultaneously, fragment # 8 of CPa-1 resides on a vertical line above the small MSP fragment (Fig 22). In addition, the amount of 24.6 kDa peptides recognized by α-5 under the diagonal is seemingly higher than the amount of 8.5 - 7 kDa peptides (containing fragment # 9), suggesting that more of the small FA fragment of MSP is bound to the 24.6 kDa fragment than to the 8.0 kDa fragment of CPa-1. However, the relative staining intensities of both CPa-1 fragments is not necessarily quantitative. The number and affinity of epitopes for α-5 has not been determined.
A crosslink between the small FA fragment of MSP and fragment # 7 of CPa-1 is also likely to occur (solid line; Fig 24), since the small MSP fragment also derives from a crosslinked fragment at about 11 kDa (arrow, Fig 21.A). Crosslinks involving CPa-1 fragments located outside the scope of Fig 24 may also occur in 100 XL. On the other hand, the potential sidedness of the CPa-1 loops helps to rule out crosslinks between MSP and the lysyls on the stromal side of CPa-1 (e.g., lysine # 447 occurring in FA fragment # 10 of CPa-1 is not expected to be crosslinked to MSP).

D. Possible sites of interaction between MSP and CPa-1.

The amino end of MSP is drawn in close contact with a portion of the extrinsic loop of CPa-1 (Fig 25), based on the conclusion that the amino terminus CNBr fragment of MSP is crosslinked to the 16.7 kDa CNBr fragment of CPa-1 [248, 250]. Interacting with the carboxyl half of the CPa-1 large extrinsic loop, there is a portion of the carboxyl end of MSP (Fig 25), as it is inferred from the data garnered in this work, pointing to the notion that the carboxyl terminus FA fragment of MSP is crosslinked to the FA fragments # 9 and # 8 of CPa-1. Even though more data are required to unequivocally confirm them, other crosslinks appear to occur as well (e.g., the crosslinks of the small FA fragment of MSP to FA fragments # 7 of CPa-1, not depicted in this illustration). Thus, it seems that the main binding between CPa-1 and MSP is at least through the large extrinsic loop of CPa-1 and both end segments of MSP. By analogy, and since CPa-2 was found to become crosslinked to
Figure 25. Sketch depicting interactions between MSP (thick line) and CPa-1. The 6 putative α-helices of CPa-1 traversing the membrane environment [42, 320] are shown. This figure illustrates that the binding between CPa-1 and MSP is at least between the lumenally exposed large extrinsic loop of CPa-1 and both end segments of MSP. The amino end of MSP is drawn in close contact with a portion of the CPa-1 large extrinsic loop, according to previous results [248, 250]. Interacting with the carboxyl end of the CPa-1 large extrinsic loop (part of fragment # 9; also thick line), there is a portion of the carboxyl end of MSP corresponding to the small FA fragment. Also a part of the small MSP fragment interacts with the large FA fragment of CPa-1 (fragment # 8).

An interaction between the small FA fragment of MSP and fragment # 7 of CPa-1 (Fig 23) is not shown, as it requires further proof.
MSP (Fig 13.A), it is tempting to speculate that MSP is also bound to CPa-2 through its putative large extrinsic loop [42].

Therefore, this work confirms reports pointing to MSP as being closely associated with CPa-1 [44, 46, 90, 91, 93, 128, 353] and with CPa-2 [168]. In addition, this work extends this general notion by showing that, besides the binding of the amino end CNBr fragment of MSP to the CPa-1 large extrinsic loop [248, 250], the carboxyl end FA fragment of MSP attaches to a small segment on the carboxyl end of the large CPa-1 extrinsic loop. Furthermore, since DTSP crosslinked these proteins through their lysyl residues, the bound segments of MSP and CPa-1 can be confined to about 78 amino acids on MSP (from K159 to K236), and to around 21 amino acids on CPa-1 (from K418 to K438) (see Fig 23 and below).

Crosslinked segment of MSP:

\[
\begin{align*}
159 & \quad KG \ RGGSTGYDNA \ VALPAGGRGD \ EEELQKENN \ NVASSKGTIT \\
& \quad LSVTSSKPET \ GEIVGFQSL \ QPSDTDLGAK \ VPKDVK \ 236
\end{align*}
\]

Crosslinked segment of CPa-1:

\[
\begin{align*}
418 & \quad KK \ ARRAQLGEIF \ ELDRLSLK \ 438
\end{align*}
\]
CONCLUDING REMARKS

This work focused on the production and characterization of crosslinked products arising from PS II preparations, with the aim of better understanding the structural relationships among protein components of PS II. Crosslinking of OERC complex with DTSP rendered preparative amounts of crosslinked products. OERC core, being simpler than OERC complex, was eventually disregarded because it was a much more labile preparation, it took longer to produce and it generated much lower yields (< 20 % of OERC complex). DTSP was utilized because it proved easy to find crosslinked products in usable amounts.

The crosslinking experiments demonstrated that practically all major PS II proteins (CPa-1, CPa-2, D1, D2, MSP, LHC's and Cyt b559) formed various crosslinked products in DTSP-treated OERC complex, and that a close association between MSP and CPa-1 exists in OERC complex. Similarly, a close association was found between MSP and CPa-2, between CPa-1 and a small intrinsic protein, and between other PS II components.

The experiments also provided a framework for the preparative isolation of two crosslinked products, 100 XL and 150 XL, shown to contain MSP and CPa-1. Characterization of 100 XL was achieved by cleaving this product with CNBr or FA, and by analyzing the fragments produced by using immunoblots of two-dimensional...
diagonal LMW-LDS-PAGE. The 100 XL cleavage released highly hydrophobic fragments, which combined into insoluble aggregates. This problem was eliminated by using 10 % EG during the cleavage and electrophoretic procedures.

Two-dimensional diagonal LMW-LDS-PAGE of FA-cleaved 100 XL preparations demonstrated that several crosslinks occurred among fragments of MSP and CPa-1. The clearest examples were crosslinks of the small FA fragment of MSP (carboxyl end, 9.6 kDa) to fragments # 8 (24.6 kDa) and # 9 (8.0 kDa) of CPa-1. In agreement with this, diagonal electrophoresis of CNBr-cleaved 100 XL preparations established that a crosslink existed between the carboxyl end CNBr fragment of MSP (= 21 kDa) and the 16.7 kDa CNBr fragment of CPa-1, which encompasses part of fragment # 8 and all of fragment # 9.

Sequencing of a peptide band containing two crosslinked FA fragments verified that they corresponded to the 9.6 kDa carboxyl end FA fragment of MSP, and to the 8.0 kDa FA fragment (# 9) of CPa-1. Within these fragments, the lysine-containing segments extend from K\textsuperscript{159} to K\textsuperscript{236} for MSP, and from K\textsuperscript{418} to K\textsuperscript{438} for CPa-1.

Information collected to date indicates that the amino end of MSP appears to be in close contact with a portion of the CPa-1 large extrinsic loop [248, 250], and that the carboxyl half of the CPa-1 large extrinsic loop is closely associated with the carboxyl end of MSP (Fig 25).

Procedures established here should also allow characterizations between fragments of MSP and CPa-1 and among other OERC complex components. In addition, intramolecular crosslinks occurred at least with CPa-1, D1, MSP and LHC.
polypeptides. By isolating these intramolecularly crosslinked products, subjecting them to various cleavages followed by 2-D diagonal LMW-LDS-PAGE and fragment sequencing, it should be feasible to establish which fragments were crosslinked and, therefore, to determine the folding patterns of these proteins, including the transmembrane orientation of the integral proteins.

Finally, it is concluded that this work has laid a foundation for further study of the physical interactions between the OERC complex components. This should lead to a better understanding of not only the structural (and functional) organization of the photosystem II proteins, but also other protein assemblages in general, and membrane complexes in particular.


70. Debus, R. J.; Barry, B. A.; Sithole, I.; Babcock, G. T. and McIntosh, L. 1988b. Directed mutagenesis indicates that the donor to P680+ in photosystem II is tyrosine-161 of the D1 polypeptide. Biochemistry 27: 9071 - 9074.


85. Dunahay, T. G.; Staehelin, L. A.; Seibert, M.; Ogilvie, P. D. and Berg, S. P. 1984. Structural, biochemical and biophysical characterization of four oxygen-


297. Süss, K.-H. 1986. Neighbouring subunits of CF\textsubscript{o} and between CF\textsubscript{1} and CF\textsubscript{o} of the soluble chloroplast ATP synthase (CF\textsubscript{1}-CF\textsubscript{o}) as revealed by chemical protein cross-linking. FEBS Lett. 201: 63 - 68.


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centre, B870 antenna, and flanking polypeptides from *r. capsulata*. Cell **37**: 949 - 957.


APPENDIX

OBSTACLES FOUND DURING CLEAVAGE
OF MEMBRANE PROTEINS

1. Background.

The use of cleavage agents allows the production of discrete protein fragments which can be separated by PAGE designed to resolve low molecular mass polypeptides. For example, CNBr has been employed in a variety of studies on peptidic fragments [48, 107, 126, 131, 132, 208, 234, 246, 265, 322, 327]. Similarly, FA alone has been successfully utilized to cleave polypeptides [196, 208, 264]. Many other chemicals, as well as proteolytic enzymes, have also been utilized as cleavage agents (see page 23).

The utilization of chemical cleavage agents for studying PS II is particularly useful, since it is possible to crosslink the protein components of a PS II preparation (such as the OERC complex), isolate a particular crosslinked product, cleave it, resolve the fragments by two-dimensional diagonal PAGE (page 41), and detect them (e.g., by immunoprobing; pages 44 & 46). The non-crosslinked fragments
should appear on the diagonal, whereas the fragments that are crosslinked together should appear under the diagonal lying on the same vertical line. Crosslinked fragments could be unequivocally identified through sequencing, especially that presently the sequences of practically all the PS II proteins are known.

Unfortunately, cleavage of PS II proteins (and, by extension, other membrane proteins) poses some obstacles: Cleaving polypeptides inside the gel strips into which they were electrophoresed may result in smearing/streaking, band multiplicity, and low fragment load when performing the next electrophoresis to separate the cleaved fragments. If the cleavage is done in an aqueous medium, possibly the worst problem, especially with integral proteins, is the release of highly hydrophobic fragments, which generally tend to form large insoluble aggregates, preventing (electrophoretic) separation and detection of the individual fragments.

What follows describes these problems, which were encountered when trying to electrophoretically separate the CNBr and FA fragments of MSP, CPa-1 and the 100 kDa crosslinked product (100 XL). In addition, the search for an adequate solution is presented.

2. **Dealing with artificial band multiplicity, streaking and low loads.**

Seeing that relatively high concentration and purity could be achieved for PS II proteins separated electrophoretically, a CNBr cleavage procedure already described
[246] was modified to cleave the polypeptides inside gel pieces. These gel pieces were obtained by electrophoresing 100 XL, CPa-1, CPa-2 and MSP, staining the gel with acid-free CB (page 42), and excising the polypeptide bands. These gel bands were then treated with CNBr, loaded whole or diced into the wells of a LMW gel, and electrophoresed to separate the released fragments. The gel was then silver stained (Fig 26). Despite the ease of this procedure, three problems appeared: Low concentration of fragments, particularly for 100 XL (lane 3, which shows practically no fragments), streaking/smearing (lanes 3 - 8), and formation of multiple bands. Examples of multiple bands include the "multiplex" of the whole MSP (at ≈ 29 kDa) [both the MSP large CNBr-fragment (at ≈ 20 kDa), and its small CNBr-fragment (at ≈ 9 kDa) were somewhat negatively stained against the light-brownish background of this silver staining, so they were not reprinted here], "groups" of bands at ≈ 16.7, 10 and 6 kDa (CNBr-treated CPa-1; lane 5), and tight "doublets" at around 18, 12, 10, 7.5 and 7 kDa (CNBr-treated CPa-2; lane 7). It was later verified that the components of these multiple bands did correspond with the number, shape and position of the gel pieces (containing CNBr-cleaved polypeptides) that were placed in the wells for electrophoresis. These stacks of gel pieces were intended for increasing the concentration of the fragments to be electrophoresed. The artificial multiplicity in the banding pattern of fragments would of course pose problems for assigning bands to the appropriate expected cleavage fragments.

To obtain more specific data, antibodies against MSP, CPa-2 and CPA-1 were used in immunoblots of gel panels similar to those in lanes 1 - 8 of Fig 26. These

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Figure 26. Silver stained LMW-LDS-PAGE gel of CNBr fragments produced when the polypeptides are cleaved inside polyacrylamide gel strips. The proteins were electrophoresed in a 12.5 - 20 % gradient gel. The gel strips containing these polypeptides were excised, treated with CNBr for 24 h, washed and electrophoresed in an LMW-LDS gel. Lanes 1, 3, 5 & 7 contain CNBr-cleaved MSP, 100 XL, CPA-1 and CPA-2, respectively. Lanes 2, 4, 6 and 8 contain these same polypeptides, but treated without CNBr.
immunoblotted panels are pictured in Fig 27. These polypeptides were treated in
gel strips with CNBr or without it, electrophoresed, blotted and probed with α-5
(polyclonal against CPa-1), FAC2 (monoclonal against CPa-1); FAAC4 (monoclonal
against MSP), α-33 (polyclonal against MSP), and α-6 (polyclonal against CPa-2).
Again this blot illustrates the problems of low loads, streaking and multiple bands
observed in Fig 26. Lane 3 (α-5 probe, Fig 27) also illustrates these same phenom-
ena for 100 XL fragments more clearly than the corresponding silver stained sample
(lane 3 of Fig 26). Additionally, lane 14 shows the band multiplicity for the large
CNBr fragment of MSP (which was not visible in lane 1 of Fig 26). The small CNBr
fragment of MSP (lane 14) is not recognized by α-33. FAAC4 does not seem to
recognize either CNBr fragment of MSP (lane 7); perhaps the epitope for this
monoclonal antibody resides around the CNBr cleavage site.

Despite the mentioned problems, this blot demonstrates the participation of
MSP and CPa-1 in 100 XL, since it is recognized by α-5 (lanes 3 & 4), and by
FAAC4 and α-33 (lanes 9 - 12). Furthermore, several fragments released from the
CNBr cleavage of 100 XL are noticeable with α-5 (lane 3), thus supporting fully the
participation of CPa-1 in 100 XL. Similarly, fragments released from 100 XL are
recognized by α-33 (lane 9) and by FAAC4 (lane 12), also certifying that MSP takes
part in 100 XL. On the other hand, CPa-2 does not appear to be involved in 100
XL, since α-6 does not seem to recognize the whole 100 XL (lane 15), let alone any
of its fragments (lane 16).
Figure 27. Immunoblots of panels containing samples similar to those depicted in Fig 26 above. MSP, CPa-2, CPa-1 and 100 XL were treated in gel strips either with CNBr (lanes 1, 3, 5, 7, 9, 12, 14, 16 and 18), or without it (lanes 2, 4, 6, 8, 10, 11, 13, 15 and 17), electrophoresed, blotted and probed with α-5 (lanes 1 - 4), FAC2 (lanes 5 & 6), FAAC4 (lanes 7 - 10), α-33 (lanes 11 - 14), or α-6 (lanes 15 -18). Lanes 1, 2, 5 & 6 contained CPa-1, lanes 3, 4, 9 - 12, 15 & 16 had 100 XL, lanes 7, 8, 13 & 14 had MSP, and lanes 17 & 18 had CPa-2.
With respect to the obstacles encountered in the usage of the CNBr-cleavage procedure, it seemed that at least three approaches to avoid these problems (band multiplicity, streaking and low loads) could be followed. One procedure would be to modify the electrophoretic conditions for the separation of the fragments. A second procedure would be to change the number of electrophoretic steps for isolation of 100 XL (which would allow more purity and more concentrating steps, since the isolates of several gels could be placed into one for further gel electrophoresis). The third procedure would be to cleave the polypeptides in an aqueous phase, which would allow for a better stacking of the sample, and it would also permit to obtain a higher concentration of polypeptides/fragments. The first two approaches were pursued extensively, rendering no major relief to the problems at hand (results not shown). The third approach, despite some glitches, resulted more profitable, as described below.

3. CNBr cleavages in an aqueous medium.

A. Preliminary experiments.

At this point it was certain that the poor stacking of the samples contained in different gel pieces was due to poor solubilization of the peptides released during cleavage, since it was expected that several fragments would be rendered very
hydrophobic and, therefore, susceptible to aggregation. In fact, when cleaving the polypeptides in an aqueous medium, formation of some aggregates was observed during the incubation with CNBr. Even more, after lyophilization of the samples (to eliminate the remaining CNBr), the hard solid remnants could not be resolubilized in the Sample Buffer (50 mM Tris-HCl pH 6.8, 2 % LDS, 4.4 % sucrose and BPB), even after increasing the concentration of LDS, changing the pH of the buffer, and utilizing recommendations of published procedures [97, 126, 137, 140, 147, 208, 234, 265, 322]. The electrophoretic separation of what could be resolubilized was negligible for CPa-2, CPa-1 and 100 XL. MSP seemed somewhat unaffected by the cleavage in an aqueous phase, rendering the expected two fragments, but without band multiplicity (not shown). With the exception of MSP, practically no fragments could be observed for any proteins, and large amounts of aggregate peptides accumulated at the bottom of the loading wells, and at the stacking/spacer and spacer/resolving gel interfaces (results not shown). The search for a solution to this aggregation problem is described below.

B. Utilization of cryoprotectants.

Several procedures have been developed to minimize aggregation of hydrophobic (poly)peptides. For example, to resolubilize the residues after lyophilization of the cleaved samples, trifluoroacetic acid [147, 169] or FA [112] have been used. Trifluoroacetic acid has also been included all along the cleavage procedure to keep
the fragments in solution, as well as to eliminate problems with formylation of peptides [126, 234]. In related studies, solvents which are somewhat hydrophobic [e.g., glycerol, ethylene glycol (EG), acetonitrile, alcohols, or dimethyl sulfoxide (DMSO)], alone or in combination (e.g., see [282]), have been included to avoid or eliminate aggregation of hydrophobic polypeptides. For example, glycerol has been used during native [1, 231], "semi-native" [158], and denaturing [147, 148, 208] protein electrophoresis, in stabilization of protein conformation [111], in renaturation of proteins after denaturation by guanidine hydrochloride [317], or after SDS-PAGE [77, 271], in recovery of fragments after protein cleavage [62, 147, 208], in phase separation of membrane proteins [338], in cryoprotection of membrane phospholipids [9a] and of polynucleotide/protein complexes [180], in prevention of aggregation of membrane protein complexes [64], in studies on efficiency of picosecond electron transfer in bacterial photosynthetic reaction centers [260], etc. Various simple alcohols have been employed in stimulating the activity of ATPases [216]. Sugars [14] and some salts [15] appear to stabilize protein structure. Methanol (20%) is regularly used to keep the proteins in solution during electroblotting [114, 316]. Various organic solvents have been employed to resolubilize proteins from nitrocellulose blots [259]. Glycerol and EG have been used to avoid aggregation of PS II preparations [210, 300]. Sucrose (usually 400 mM) is regularly utilized for work and storage of PS II preparations. In addition, there is an extensive body of work with cryoprotectants being used to keep native conformations of proteins and peptides, even at subzero temperatures (Table 3). Therefore, a wide variety of
Table 3. A short summary of cryoprotectants that generally help in maintaining the native conformation of proteins and peptides at low temperatures. Cryoprotectants may prove valuable in keeping in solution the hydrophobic peptides normally released during the chemical cleavage of PS II proteins.

<table>
<thead>
<tr>
<th>CRYOSOLVENTS CURRENTLY AVAILABLE</th>
<th>USES</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supercooled water droplets</td>
<td>Enzyme systems</td>
<td>[100]</td>
</tr>
<tr>
<td>suspended in nonmiscible solvents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inverted micelles with aqueous cores</td>
<td>Enzyme systems</td>
<td>[100]</td>
</tr>
<tr>
<td>Aqueous solutions with high salt concentrations</td>
<td>Protein stability</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>Enzyme systems</td>
<td>[102, 251]</td>
</tr>
<tr>
<td>Mixed aqueous-organic solvents *</td>
<td>Enzyme systems</td>
<td>[21, 61, 100, 101, 103, 216, 251]</td>
</tr>
<tr>
<td></td>
<td>Protein folding</td>
<td>[100, 102, 251, 302, 317]</td>
</tr>
<tr>
<td></td>
<td>Folding of linear peptides</td>
<td>[235, 285]</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>[9a, 17, 99, 111, 260]</td>
</tr>
<tr>
<td>Organic solvent mixtures *</td>
<td>Enzyme systems</td>
<td>[100, 251]</td>
</tr>
<tr>
<td>Organics */detergent mixtures</td>
<td>Protein isolation</td>
<td>[338]</td>
</tr>
<tr>
<td></td>
<td>Enzyme systems</td>
<td>[216]</td>
</tr>
<tr>
<td>Vitrification solutions **</td>
<td>Enzyme systems</td>
<td>[84, 96]</td>
</tr>
<tr>
<td>Liquified rare or inert gases</td>
<td>H-bonded complexes</td>
<td>[167]</td>
</tr>
</tbody>
</table>

* Organic solvents include methanol, ethanol, glycerol, DMSO, EG, etc.
** Vitrification solutions were included because, even though solid at subzero temperatures, they may be utilized to study protein folding intermediates (e.g., by spectroscopy) thanks to their glassy transparency.

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cryoprotectants (e.g., glycerol, 7G, DMSO, other simple alcohols, sorbitol, sucrose, etc.) are available. Cryoprotectants may be particularly useful since almost invariably one of the steps of a cleavage procedure is lyophilization of the cleaved protein, which may induce aggregation of hydrophobic peptides upon their concentration in the vanishing solvent.

It was thought that the observed aggregation of hydrophobic peptides released during CPA-2, CPA-1 and 100 XL cleavages could be circumvented by employing some mild organic compounds, as well as other cryoprotectants, during the whole cleavage and electrophoretic procedures. It was expected that cryoprotectants would help not only in keeping the hydrophobic peptides in solution during the cleavage stage, but they would also aid in resolubilizing the peptides after lyophilization (a low temperature step), especially if remnants of the cryosolvent stayed in the lyophilized sample. So some mild chemicals (e.g., alcohols, DMSO, EG, sugars, detergents and other cryoprotectants) were included during the CNBr cleavage of proteins solubilized in an aqueous medium. A methodic search ensued by utilizing many cryoprotectants at various concentrations during the whole cleavage procedure, followed by analytical electrophoretic separations in minigels. Unreliable yet encouraging results were obtained with methanol, ethanol and glycerol. Better results were attained with 5 - 10 % DMSO, which increased the solubility of fragments released from cleavages in aqueous media and inside gel pieces (results not shown). Adequate results were achieved with 5 - 10 % EG during cleavage. Optimal conditions for the subsequent electrophoretic separation of the fragments
were obtained when the lyophilized samples were reduced to a paste consistency, containing about one tenth of the EG originally present in the volume sample. In this case, the hydrophobic fragments were still soluble after lyophilization and, after the sample was mixed with the Sample Buffer, the concentration of EG did not surpass 10% of the sample volume loaded (otherwise, large distortions of the polypeptide bands occurred).

This appeared to be a proper system to separate the highly hydrophobic fragments released upon treatment of CPa-1 and 100 XL, which tended to aggregate irreversibly during the cleavage and lyophilization procedure. Optimal conditions are illustrated in Fig 16.A (page 84), which pictures lanes of a silver stained gel from an LMW-LDS-PAGE system, used to separate the fragments of MSP, 100 XL and CPa-1 cleaved with CNBr in an aqueous medium containing 10% EG.

Thus, the formulation of this procedure led to the successful electrophoretic separation of fragments from cleavages of 100 XL with CNBr (Figs 17 & 18; page 85) and with FA (Figs 20 - 22; page 95), followed by the sequencing of crosslinked segments from MSP and CPa-1 (Fig 23; page 103), which permitted to establish the close association between a 78-amino acid stretch of MSP (from K159 to K236) and a 21-amino acid segment of CPa-1 (from K418 to K438) (see Figs 23 - 25).
VITA

Carlos B. Queirolo was born in December 19, 1954, in San Carlos, a small city in central Chile. He studied in the local school (Experimental Consolidated School) for 13 years, completing elementary education and five years of commercial training, obtaining certification as an accountant. Moving to Santiago, the Chilean capital, he studied Biology in the University of Chile (Faculty of Sciences), while receiving various teaching assistantships as means of support. He worked on the biological activity of hydroxamic acids from Gramineae, both under the advice of Dr. Luis Corcuera in Chile (in a laboratory of plant physiology and organic chemistry), and Drs. Rubén Vallejos and Carlos Andreo in Rosario, Argentina (in a laboratory of photosynthesis), completing a Thesis that earned him an M. S. degree in 1983. This same year, he and his newly created family (consisting of: i. wife Patricia, and ii. son Ariel), moved to the U. S. A., where he studied photophosphorylation in spinach chloroplasts under the direction of Dr. Norman Good (at Michigan State University, East Lansing). After Dr. Good's departure from academics in 1986, Carlos moved to Hattiesburg, Mississippi, to study the assembly of PS II proteins in spinach, under the fine direction of Dr. Terry M. Bricker. From the University of Southern Mississippi, Carlos moved in 1987 to Baton Rouge to continue under the direction of Dr. Bricker (who had accepted a new position at Louisiana State University). Being financially supported throughout his stay in the U. S. by various research and teaching assistantships, he finally obtained the Ph. D. degree in the Botany department at LSU in 1992.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Carlos B. Queirolo

Major Field: Botany

Title of Dissertation: Assemblage of Spinach Photosystem II Proteins: CPa-1 and MSP Interactions

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

David Long
Thomas S. Moniz
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Date of Examination:

July 10, 1992