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The Organization Of The Maize Photosystem Ii Light-harvesting Apparatus Under Normal And Chilling Conditions

Douglas A. Campbell

THE ORGANIZATION OF THE MAIZE PHOTOSYSTEM II LIGHT-HARVESTING APPARATUS UNDER NORMAL AND CHILLING CONDITIONS

by

Douglas A. Campbell

Department of Plant Sciences

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ABSTRACT

The maize light harvesting system is structurally and functionally perturbed by chilling stress, with alterations in fluorescence parameters and protein processing. The molecular basis for these changes was investigated through biochemical analysis of the photosystem II-associated light-harvesting complexes (LHC II) of maize mesophyll thylakoids.

LHC II complexes from thylakoids were separated into three populations by mildly-denaturing electrophoresis. LHC II-1 contains four polypeptides and is equivalent to the oligomeric LHC IIb complex of Thornber et al. (1988). A less abundant oligomeric band, LHC II-2, contains a subset of the LHC IIb polypeptides, along with the LHC IIa complex (CP 29). The LHC II-3 band contains the LHC IIa and LHC IIc complexes. The LHC IIa and LHC IIb polypeptides were identified by immunoblotting. The LHC II populations separated from PS II-enriched membranes are similar, but isolated LHC II particles generate three chlorophyll-protein bands, all derived from LHC IIb.

Interactions among LHC II complexes were investigated by protein cross-linking with 3,3'-dithio-bis(propionic acid n-hydroxysuccinimide ester) (DSP), a cleavable cross-linker of 1.1 nm length. Cross-linking occurs between appressed thylakoids, as shown by resistance to unstacking following DSP treatments. DSP treatment gave

cross-linking between the LHC IIa and LHC IIb complexes, among LHC IIb complexes, between the PS II core and LHC IIa/LHC IIb and between the PS I core and LHC I. Disruption of appression prior to DSP treatment eliminates the LHC IIa/LHC IIb and the LHC IIa/LHC IIb/PS II cross-linked products. Hence, specific linkages occur between the PS II related complexes of adjacent appressed thylakoid membranes. This organization may have functional implications.

The assembly of LHC IIb oligomers is disrupted by low light chilling stress. Three LHC IIb polypeptides are heavily labelled by light-induced phosphorylation of thylakoids. The pattern of LHC IIb phosphorylation is altered by low or high light chilling stress prior to thylakoid isolation; the effect is partially reversed by the presence of high ATP concentrations during phosphorylation. These changes provide biochemical background to disruption of LHC II function following chilling. The timing of chilling stress influences the degree of chill-induced changes.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abbreviations

ATP	adenosine triphosphate
chl	chlorophyll
DMSO	dimethyl sulfoxide
DSP	3,3'-dithio-bis(propionic acid n-hydroxysuccinimideester)
EDTA	ethylenediamine tetraacetic acid
fp	free pigment
IEF	isoelectric focusing
kDa	kilodalton
LHC II	photosystem II associated peripheral light harvesting chlorophyll-a/b-protein complex
MES	4-morpholineethanesulfonic acid
Mr	relative molecular mass
PAGE	polyacrylamide gel electrophoresis
PPF	photosynthetically active photon flux
PS I	photosystem I
PS II	photosystem II
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.	Standard error of the mean
^{32}p	phosphorus 32

Chapter One. General Introduction

1.1 Thylakoids - Energy Transducing Membranes

Photosynthesis, the transduction of light to chemical energy, can be conceptually divided into two stages. The first consists of the harvesting of photons to drive electron transport and the generation of a proton gradient, with concomitant production of ATP and reducing equivalents. In higher plants, this process is localized to the thylakoid membranes of chloroplasts (Marder & Barber, 1989; Staehelin, 1986), and is interpreted based on the chemiosmotic hypothesis of Mitchell (1966), as confirmed by the proton gradient experiments of Jagendorf (1967).

In plants, electron transport involves the oxidation of water, releasing oxygen and protons. Through the coordinated activity of two photosystems and associated electron carriers, electrons from water are transferred to NADPH. Figure 1.1 presents a diagram based on current consensus thinking on the relative organization of the various components of the light-harvesting and electron transport system of thylakoids. Subsequent sections and chapters will consider the structure and function of the PS II-associated integral membrane chlorophyll-protein complexes (LHC II) which harvest photons and pass energy to the photosystems.

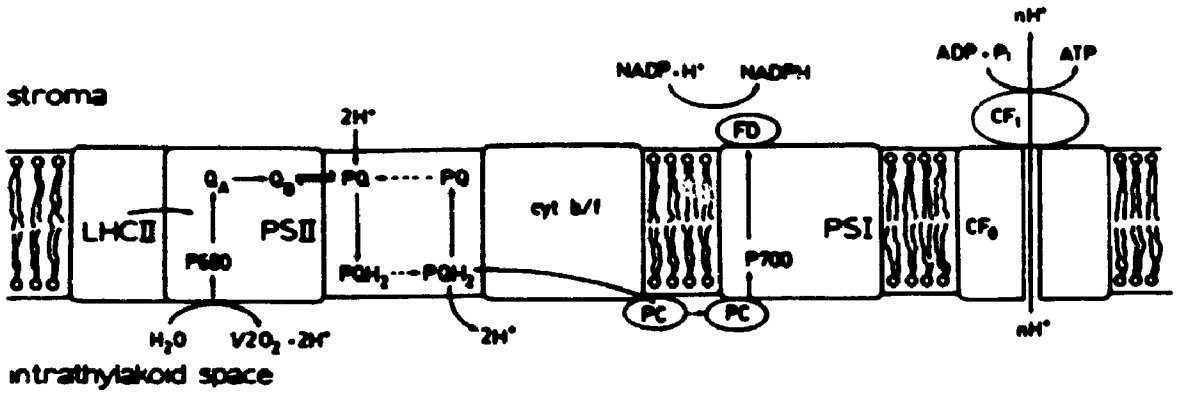


Figure 1.1 Schematic Model of Light Energy Transduction by Thylakoids.

Modified from Hayden and Baker (1990). CFo, intrinsic coupling factor complex; CF1, extrinsic coupling factor complex; cyt<u>b/f</u>, cytochrome <u>b/f</u> complex; FD, ferredoxin; LHC II, light-harvesting chlorophyll <u>a/b</u> protein complexes associated with photosystem II; P680, reaction centre chlorophyll(s) of photosystem II; P700, reaction centre chlorophyll(s) of photosystem I; PC, plastocyanin; PQ, plastoquinone; PS I, photosystem I; PS II photosystem II; Q_A, primary quinone acceptor of photosystem II; Q_B, secondary quinone acceptor of photosystem II.

The second stage of photosynthesis comprises the classic dark reactions, which can potentially proceed in the absence of light. These processes use ATP and reducing equivalents to drive carbon fixation, nitrogen fixation, metabolite synthesis, and the general metabolism of plants. The enzymology and regulation of the dark reactions are dependent upon the thylakoid based energy transduction.

1.2 The Chloroplasts of Higher Plants - An Overview

The chloroplasts of most higher plants are found primarily in the cells of leaves, although in some plants, chloroplasts in stem or other tissue are important (Esau, 1977). Leaves are constructed of defined layers of cells, with non-pigmented epidermal layers covering pigmented palisade and spongy mesophyll layers. Typically, all cells are within a few cellular diameters of vascular tissue, and most cells are in contact with air spaces (Nobel, 1991). The air spaces exchange with the atmosphere through pores termed stomates, whose closure is regulated by epidermal guard cells.

Bundle sheath cells surround leaf veins, and play an important role in CO_2 concentrating mechanisms in plants such as maize, which initially fix CO_2 into four carbon carboxylic acids. This type of fixation mechanism is termed C4 metabolism (Hatch and Slack, 1968). The bundle

sheath chloroplasts of C4 plants are photosynthetically specialized for energy production, with a lowered capacity for linear electron transport from water to NADPH. However, this thesis deals only with chloroplasts from the mesophyll cells of maize and other species.

Chloroplasts are cellular organelles bounded by double envelope membranes. Higher plant chloroplasts are typically ovoid, 3-10 μm in diameter (Staehelin, 1986). They arise from preexisting chloroplasts by fission (Staehelin, 1986) or by differentiation of other plastid forms (Esau, 1977). In addition to their central role in photosynthetic carbon fixation, they play important roles in metabolism and lipid synthesis (Stumpf & Shimakata, 1983).

Chloroplasts contain DNA and protein synthesizing machinery; the organization of the DNA and ribosomes resembles that of prokaryotic organisms (Staehelin, 1986). Chloroplast DNA codes for many of the thylakoid proteins and other proteins involved in photosynthesis. However, many chloroplast proteins are nuclear-encoded (Gray, 1988). Proteins which are nuclear-encoded must be imported from the cytosol across the envelope membranes and assembled into mature form. The process involves chloroplast envelope receptor sites (Cline et al., 1985), cleavable transit peptides (Clark et al., 1990) and molecular chaperone molecules (Lubben et al., 1989; Roy 1989).

The genetic and structural similarities between chloroplasts and cyanobacteria led to the endosymbiotic hypothesis for the origin of chloroplasts from an originally free living progenitor resembling modern cyanobacteria (Margulis, 1982). This hypothesis recently received support with the discovery of prochloron, an oxygen-evolving prokaryote which closely resembles chloroplasts in organization (Staehelin, 1986)

1.3 Thylakoid Structure and Composition

Within chloroplasts, an extensive array of membranes forms a system of anastomosing sacs termed thylakoids (Menke, 1962; Staehelin, 1986). The chloroplast matrix is thus divided into three compartments. The stroma is the non-membranous region where ribulose bisphosphate carboxygenase/oxygenase (RUBISCO) and other enzymes of CO2 fixation are localized. The thylakoid membranes are the site of light capture and vectorial electron transport. The thylakoid lumen is the space enclosed by the thylakoid membranes, which serves as a proton reservoir and contains electron carriers (Staehelin, 1986).

Thylakoid membranes are composed of 25-35% acyl lipids by mass, with the remainder proteins and pigments (Murphy, 1986). A large fraction of the total acyl lipid is uncharged glycolipids; phospholipids generally comprise less than 20% (mol/mol). Polyunsaturated acyl residues

account for 85-90% of the fatty acids. About 30% of the total thylakoid acyl lipid consists of monogalactosyldiacylglycerol (MGDG), which, when isolated, does not form lamellar bilayers in aqueous dispersions. A related neutral glycolipid, digalactosyldiacylglycerol (DGDG) accounts for a further 20%. Sulphoquinovosyldiacylglycerol, phosphatidlyglycerol (PG) and phosphatidylcholine (PC) are also present in significant amounts. (Murphy, 1986).

The acyl lipids display both lateral and transverse asymmetry in their distribution. This parallels the asymmetric distribution of thylakoid protein complexes. The acyl lipid content is low relative to the protein content, hence, the protein complexes are densely packed (Staehelin, 1986), with little or no bulk lipid phase present in thylakoids. There is evidence for specific covalent and non-covalent associations between lipid species and various protein complexes (Krupa et al., 1987; Mattoo & Edelman, 1987; Sigrist et al., 1988).

The photosynthetic chlorophyll, phaeophytin and carotenoid molecules are arranged in specific, non-covalent associations with proteins, forming pigment-proteins, which are themselves organized into macromolecular complexes (Murphy, 1986; Staehelin, 1986).

The thylakoid membranes of higher plant chloroplasts form appressed (stacked) and non-appressed regions. The

appressed regions are visible under light microscopy and are termed grana; they consist of numerous thylakoid sacs in a stack (Anderson & Andersson, 1982; Esau, 1977). Thylakoid appression is mediated by interactions between chlorophyll protein complexes in adjacent appressed membranes, particularly the light-harvesting complexes associated with PS II (LHC II) (Mullet & Arntzen, 1980).

Maintenance of appression requires divalent cations, in particular Mg^{2+}, and can be disrupted in isolated thylakoids by removal of Mg^{2+} with chelators such as ethylene diamine tetraacetic acid (EDTA), or by mild trypsin treatment to remove exposed protein fragments (Steinback et al., 1979). In systems with reduced levels of LHC II complexes, such as maize bundle sheath cells or chlorophyll-b deficient mutants, thylakoid stacking is reduced (Staehelin, 1986).

The margins of appressed regions form tight curves. There is some evidence for specific lipid arrangements to accommodate this curvature (Murphy, 1986), along with speculation about distinct functional roles for the grana margins (Webber et al., 1988; Anderson, 1989).

Certain pigment-protein complexes in the reaction centres of Photosystem I and II are photochemically active, performing charge separations. Other complexes serve as light harvesting antennae, transferring energy to the reaction centre complexes (Marder & Barber, 1989).

The membrane protein complexes of the photosynthetic apparatus display lateral heterogeneity, with the CF_o/CF_1 ATP synthase, photosystem I (PS I) and associated light-harvesting complexes (LHC I) localized primarily to the non-appressed region (Anderson & Andersson, 1982; Marder & Barber, 1989: Staehelin, 1986). The cytochrome b/f complex serves as an intermediary between the photosystems during electron transport and is apparently present in both appressed and non-appressed regions (Staehelin, 1986). It may be divided into specific subpopulations typical of each region (Barber, 1985).

Photosystem II (PS II) and its associated light-harvesting chlorophyll a/b-protein complexes (LHC II) are located primarily in appressed regions (Anderson & Andersson, 1982; Marder & Barber, 1989; Staehelin, 1986).

1.4 The Light-Harvesting System of Photosystem II of Higher Plants.

Photosynthetic photon capture results when a pigment molecule is photochemically excited from ground state to an excited state, with the energy from the photon transferred to an electron. The excited electron can decay to ground state though heat emission, fluorescence or transfer of energy to an adjacent pigment molecule (Krause & Weis, 1991; Nobel, 1991). The characteristic

absorbance spectra of the pigment molecules results from capture of photons whose energy matches the energy gaps between various excitation levels (Sauer, 1986; Nobel, 1991). Chlorophyll and carotenoid molecules contain extensive series of conjugated double bonds, which allows delocalization of excited states. This serves to broaden the absorbance peaks, since many closely spaced energy levels are available to an electron in such a system. The net absorbance spectra of the molecules forming the pigment bed result in the action spectra for photosynthesis, with peaks in the red and blue regions and a trough in the green region (Baker and Webber, 1987).

Fluorescence kinetic measurements indicate that reaction centres share an antenna pigment bed with nearby centres (Baker and Webber, 1987). Energy transfer from the pigment bed to the reaction centre occurs through delocalization of the exciton over several adjacent pigment molecules, followed by a series of Forster resonance transfers of exciton energy (Sauer, 1986; Baker and Webber, 1987; Nobel, 1991). Ultimately the exciton is passed to a reaction centre, where the energy is trapped by a charge separation, resulting in ionization of a chlorophyll-a molecule in the reaction centre (Sauer, 1986; Nobel, 1991). The ionization marks the first step in vectorial electron transport.

A system of chlorophyll-a/b protein complexes are

responsible for funnelling the energy from captured photons into the reaction centres, with specialized complexes organized around the two photosystems. The major component of the light capture system is the main photosystem II-associated light harvesting complex, termed LHC IIb. LHC IIb contains 50% of total thylakoid chlorophyll and 90% of the chlorophyll-b (Thornber et al., 1988). In cooperation with several less abundant chlorophyll-a/b proteins, LHC IIb channels exciton energy into PS II, and may be involved in regulating energy distribution between PS II and PS I (Bennett et al., 1980). There are about two hundred chlorophyll molecules associated with LHC II units for each PS II reaction centre (Hannson & Wydrzynski, 1990).

The LHC IIb chlorophyll-proteins are derived from a family of polypeptides with molecular masses ranging between 25 and 30 kDa (Thornber et al., 1988). A family of nuclear 'cab' genes codes for precursors of these and the related polypeptides of other light-harvesting chlorophyll a/b proteins such as LHC IIa (CP 29) LHC IId (CP 24) and the LHC I proteins (Gray, 1988; Green, 1988). The precursor polypeptides are synthesized on cytoplasmic ribosomes (Machold, 1983) and post-translationally imported into the chloroplasts in an ATP dependent process (Grossman et al., 1980) which may involve specific receptors on the chloroplast envelope (Cline et al.,

1985;).

Precursors are cleaved to mature size and inserted into the thylakoids through a series of steps (Apel and Kloppstech, 1978; Kohorn et al., 1986; Clark et al., 1990). Insertion is believed to occur into non-appressed (stromal) regions of thylakoids, with subsequent assembly with pigment and migration to appressed (grana) regions (Yalovsky et al., 1990). A stromal protein factor is involved in the insertion process (Chitnis et al. 1987, Thornber et al., 1988). Uncleaved precursors can accumulate in the thylakoid membranes (Chitnis et al., 1986; Covello et al., 1988; Hayden et al., 1986; 1988), and assemble into complexes (Yalovsky et al., 1990).

Further polypeptide heterogeneity may result from differential cleavage (Clark et al. 1990) during the processing steps or palmitoylation of the apoproteins (Mattoo et al., 1987). The heterogeneity has contributed to a confusing nomenclature (Green, 1988); the situation is exacerbated by significant variation between species (Campbell and Hayden, unpublished results) and shifts in electrophoretic mobility depending on the buffer system used to separate the polypeptides (Hayden, unpublished results).

The light-harvesting polypeptides form specific, non-covalent associations with pigment molecules. Reconstitution of LHC II complexes from an apoprotein from

Pisum sativum has shown that chlorophylls a and b in equimolar amounts along with lower levels of the xanthophylls lutein, neoxanthin and violaxanthin promote maximum assembly of pigment-proteins (Paulsen et al., 1990). The number of chlorophylls per pigment protein is controversial, Thornber et al. (1988) give consensus values of 5-7 chl a, and 4-6 chl b, along with 2 to 3 xanthophyll molecules for the major LHC IIb complex. The same authors note that there are sufficient conserved basic amino acid residues (histidine, arginine and glutamine) in the polypeptide sequence of LHC IIb apoproteins to ligate 13 chlorophyll molecules (Chitnis and Thornber, 1988). Estimation is imprecise because of difficulty in maintaining the non-covalent protein-pigment interactions during isolation, along with inaccuracies in estimation of protein concentration. Nevertheless, distinct pigment compositions are criteria which distinguish complexes such as LHC IIa (CP 29) and LHC IId (CP 24) from the major LHC IIb complex (Green, 1988).

If chlorophyll b levels are lowered by light treatments (Cuming and Bennett, 1981) or use of mutant plants (Greene et al., 1988; Ouijja et al., 1988), the light-harvesting apoproteins are rapidly turned over, with little net accumulation in the thylakoids. Thus, assembly with the proper complement of pigments is essential for stability of the complexes. Furthermore, various

complexes show differential sensitivity to reduced chlorophyll b, indicating a hierarchy in partitioning of chlorophyll b (Greene et al, 1988; Morrissey et al., 1989). The levels of light-harvesting complexes are reduced by growth under high light (De la Torre & Burkey, 1990).

The LHC IIb pigment-proteins are arranged in the thylakoid membrane with the amino terminal end exposed to the stromal side of the membrane (Mullet, 1983), accessible to trypsin attack (Steinback et al., 1979) and phosphorylation by an endogenous kinase (Bennett, 1983). Models based on protein sequences suggest three membrane spanning alpha helices, placing the carboxy terminus in the thylakoid lumen (Murphy, 1986; Thornber et al., 1988) (Fig. 3.8). Tyrosine labelling by iodization supports this view (Burgi et al., 1987). However antibody mediated agglutination of thylakoids indicated a stromal exposed carboxy terminus and hence four membrane spanning alpha helices (Anderson and Goodchild, 1987)

Individual LHC IIb chlorophyll-proteins assemble into complexes, which are visible in electron micrographs of thylakoid membranes (Staehelin, 1986) or of artificial crystalline arrays (Kuhlbrandt, 1984; Kuhlbrandt and Wang, 1991). The artificial arrays display a trimer arrangement of pigment proteins; even higher oligomers are believed to occur in the thylakoids (Thornber et al., 1988). The

number and organization of complexes changes during development or under different light regimes (Morrissey et al., 1989).

Recent evidence shows that the LHC IIb proteins are organized into several distinct macromolecular complexes, differing in polypeptide composition, proximity to PS II, and distribution between appressed and non-appressed thylakoid regions (Albertsson et al., 1990; Bassi and Simpson 1987; Bassi et al. 1987a; 1987b; 1988; 1990; Larsson and Andersson 1985; Morrissey et al. 1989). These complexes are believed to form at least two pools of LHC IIb (Bassi et al., 1988; Larsson et al. 1987a, 1987b; Morrissey et al., 1989), a bound population which remains closely associated with PS II centres, and a peripheral or mobile population which dissociates from PS II centres in response to phosphorylation or other cues. The pools contain complexes differing in the relative levels of the various LHC IIb polypeptides present (Albertsson et al., 1990; Bassi et al., 1988; Larsson et al., 1987a; 1987b; Spangfort et al., 1987; Spangfort and Andersson, 1989; Thornber et al. 1988;). It is possible that genetic (Jannson et al., 1990) or processing (Clark et al., 1990; Mattoo et al., 1987) heterogeneity gives rise to specialized chlorophyll-proteins with specific structural and functional roles in LHC IIb organization.

Recently LHC II complexes have been implicated in

proton release from the PS II water oxidizing centre. In vivo modification of LHC II polypeptides with dicyclohexylcarbodiimide results in a short circuit of PS II proton pumping (Jahns and Junge, 1990). Hence, the organization of the LHC II system may need to be reconsidered in terms of a function other than strict light-harvesting.

A thylakoid membrane lipid, Δ^3-trans-hexadecenoic acid-containing phosphatidylglycerol, has been shown to play a role in mediating assembly of the major LHC IIb oligomer (Garnier et al., 1990; Huner et al., 1987; Krupa et al., 1987). At least in algae, presence of this oligomeric form has been correlated with ability to regulate distribution of excitation energy through state I-state II transitions (Garnier et al., 1990)

1.5 State Transitions

State transitions are a mechanism for the reorganization of the antenna system; it appears the transitions are involved in regulating the energy distribution between the two photosystems (Baker & Webber, 1987; Bennett, 1983; Bonaventura and Myers, 1969; Myers, 1971). In the dark, higher plant chloroplasts are in State I, with the bulk of the LHC II pool functionally connected to photosystem II (Baker and Webber, 1987). PS II requires a larger antenna because it is less

photochemically efficient than PS I, which is energetically more favoured for exciton trapping (Staehelin, 1986).

Under very low light, this state is maintained, but increasing light levels or light which preferentially excites photosystem II results in overexcitation of PS II and hence reduction of the plastoquinone pool which carries electrons away from PS II. Reduction of the plastoquinone pool activates a membrane bound protein kinase which phosphorylates some of the LHC II chlorophyll-proteins (Allen & Horton, 1981; Bennett, 1983; Bennett et al., 1980; 1988; Horton, 1983). State transitions can also be driven by changing metabolic demands (Markwell et al., 1982; Turpin & Bruce, 1990).

Phosphorylation apparently results in functional uncoupling of some LHC II units from PS II, perhaps by stimulating movement away from the reaction centre (Bassi et al., 1988; Torti et al., 1984). Excitation of PS II declines; it is possible that additional excitation of PS I occurs (Baker & Webber, 1987). This condition is termed State II, while the mechanism is termed a state transition. If excitation of PS II declines, activation of the kinase is reduced and a constitutive phosphatase removes phosphates from the LHC II proteins, allowing a return to State I (Baker & Webber, 1987). However, Islam (1989a) has found that GTP-induced phosphorylation of LHC

II complexes does not generate the same changes in fluorescence as ATP treatment. Furthermore, an ATP analogue was a poor substrate for the kinase, but caused fluorescence changes similar to those generated by ATP (Islam, 1989b). Thus, the state transition mechanism is complex, and incompletely understood. Factors such as adenylate charge and redox conditions may play important roles (Islam, 1989a;b).

1.5 The Minor Photosystem II Light-Harvesting Chlorophyll-Protein Complexes.

In addition to the major LHC IIb chlorophyll-protein complexes, several minor light-harvesting chlorophyll a/b complexes are closely associated with PS II, distinguished from LHC IIb by chlorophyll a/b ratios, apparent molecular mass of the apoproteins, immunological properties and protein sequences (DiPaolo et al., 1990; Green, 1988; Morishige et al., 1990; Thornber et al., 1988; White & Green, 1987). LHC IIa (CP 29) (Camm and Green, 1980) is closely associated with photosystem II reaction centres, with a probable stoichiometry of one per centre (Camm & Green; 1989). It has an apparent chlorophyll a/b ratio of 3:1 (Henrysson et al, 1989; Camm & Green, 1989), but may not require chlorophyll b for stability in the membrane (Morrissey et al., 1989). It may serve as a linker between an LHC IIb population and the reaction centre

(Camm & Green 1989; Bassi & Dainese, 1989). The processing of the apoprotein is apparently disrupted in chill-stressed maize, resulting in accumulation of an abnormal form of higher apparent molecular mass (Covello et al., 1988; Hayden et al., 1986; 1988).

LHC IIc (CP 26) and LHC IId (CP 24) are also closely associated with PS II centres, with chlorophyll b contents lower than LHC IIb (Thornber et al., 1988; Bassi & Dainese, 1989; Bassi et al., 1990). Based on membrane fractionation and spectral properties, a PS II model places these complexes as linkers between LHC IIb complexes and the PS II centres (Bassi & Dainese, 1989); Bassi et al., 1990). Table One presents a summary of some of the terminologies used to classify the light-harvesting chlorophyll proteins, with particular reference to the nomenclature used in this thesis.

Table 1.1 Summary of terminology of PS II related light-harvesting chlorophyll-a/b protein complexes.

The presence or absence of each complex in thylakoid subfractions is indicated by + or -. Variation depending on exact preparation is indicated by +/-. Deoxycholate (DOX) gel LHC II-2 contains a subset of the LHC IIb population, indicated by (+). Terms were collated from many references, particularly reviews by Green (1988), Bassi et al. (1990) and Thornber et al. (1988). Composition data for the O_2 evolving PS II membranes (Ghanotakis et al., 1984), LHC II particles (Steinback et al., 1982), and the LHC II-1, 2 and 3 chlorophyll-protein bands separated by the deoxycholate (DOX) gel electrophoresis system are presented in Chapter 2 of this work. The composition data for PS II reaction centres is from Camm and Green (1989) and Bassi et al. (1990)

		Presence in Membrane Subfractions					
Complex	Equivalent Terms	PS II membranes	PS II centres	LHC II particles	DOX gel LHC II-1	DOX gel LHC II-2	DOX gel LHC II-3
LHC IIa	CP 29 $Chl_{a/b}$-P1 CP III	+	+/-	+/-	-	+	+
LHC IIb oligomer	LHC II LHC II* CP 64 CP II* $Chl_{a/b}$-P2* LHCP[1]	+	-	+	+	(+)	-
LHC IIb monomer	LHC II CP 27 CP II $Chl_{a/b}$-P2 LHCP[3]	+	-	+	-	-	tr.
LHC IIc	CP 26	+	+/-	-	-	-	+
LHC IId	CP 24	+	?	-	-	-	?

1.6 The Effect of Chilling on Maize Photosystem II Light Harvesting.

Chilling of maize leaves in the light results in perturbation to the thylakoid membrane. Under high photosynthetically active photon flux (PPF) of ca. 1500 μmol photons m^{-2} s^{-1} this includes a marked decrease in the ability of these thylakoids to bind atrazine, suggesting photoinhibitory damage to the D1 protein of the PS II reaction centre. In addition, a 31 kDa polypeptide, suggested to be an unprocessed precursor of the apoprotein of LHC IIa (CP 29), accumulates in the thylakoids, binds chlorophyll and is found in association with isolated LHC II particles (Hayden et al., 1986; 1988). These membranes are unable to perform state transitions.

Chilling under low light (250 μmol m^{-2} s^{-1}) results in the accumulation of the 31 kDa polypeptide seen following high light chilling treatment, but no loss of atrazine binding sites or impairment of the ability to perform state transitions is observed. (Hayden et al., 1988; Covello et al., 1988; See recent review by Hayden & Baker, 1990).

Phosphorylation of thylakoid proteins is thought to be a factor in regulating excitation energy distribution and electron transport (Bennett et al., 1980). Recently, Val and Baker (1989) have examined phosphorylation profiles and associated photochemical changes in control

maize and maize chilled under both high and low light. They found that low light chilling changed the pattern of phosphorylation of PS II polypeptides, although high light chilling did not. They speculated that the low light chill treatment modifies the physical interactions between adjacent PS II complexes.

These observations suggest that the low light chill treatment may be perturbing the assembly and organization of the LHC II population and the association of components of this population with PS II complexes in the thylakoid.

1.7 Research Objectives

The initial objectives of the project were to characterize the changes in the structure and function of the light-harvesting apparatus of maize which occur during chilling stress; thus continuing the work previously described by Hayden and Baker (1990). These workers and colleagues (Covello et al., 1988; Hayden et al., 1986· 1988; Percival et al., 1987) had noted perturbations in the fluorescence properties and processing of protein components of LHC II, and in the interaction between LHC II and PS II (Val & Baker, 1990).

I believed that a detailed examination of the polypeptide constituents of the LHC II complexes would prove useful in further characterizing the interaction between chilling stress and light-harvesting function.

The principal analytical techniques used, and their relation to some relevant results from other laboratories are described in Chapter Two.

During the course of the physiological work, I realized that the relative arrangement of the LHC II complexes in the thylakoid membrane was not fully characterized. Therefore, the analytical techniques developed were used to pursue a protein cross-linking study of interactions between LHC II chlorophyll-proteins; this material is presented in Chapter Three.

I hypothesized that the perturbations of LHC II function previously noted (Hayden & Baker, 1990) were related to disruptions of LHC II complex assembly and changes in the regulation of LHC II/PS II interactions. Hence, I examined the assembly of LHC II complexes in chill stressed maize. I then studied the effect of chilling on phosphorylation of LHC II proteins, since protein phosphorylation is known to be involved in regulation of LHC II/PS II interactions. These results, along with directions for future research, are described in Chapter Four.

Finally, a general discussion of the results is offered in Chapter Five, along with a model of LHC II organization with possible sites of chilling perturbation.

Chapter Two. Characterization of LHC II complexes separated by mildly-denaturing electrophoresis

2.1 Introduction

The LHC II chlorophyll-proteins are organized into several distinct macromolecular complexes, differing in polypeptide composition, pigment content, proximity to PS II, and distribution between appressed and non-appressed thylakoid regions. Membrane fractionation studies have shown that LHC IIa, c and d are closely associated with PS II, perhaps serving as energetic or structural linkers between LHC IIb populations and the integral PS II chlorophyll a antenna complexes (Camm and Green, 1989; Dunahay and Staehelin, 1986; Morrissey et al., 1989; Bassi et al., 1990). Each of these minor complexes is in approximately one to one stoichiometry with the PS II reaction centre.

The major LHC IIb population has been shown to be heterogeneous by a series of studies using phosphorylation of LHC II complexes (Andersson et al., 1982; Larsson and Andersson, 1985; Islam, 1987), isoelectric focusing of isolated LHC II particles (Spangfort et al., 1987; Spangfort and Andersson, 1989), membrane fractionations following various treatments (Larsson et al., 1987a,b; Bassi et al., 1988) and immunological analysis (DiPaolo et al., 1990). The apparent number and molecular mass of LHC

IIb polypeptides varies, depending on the species and experimental technique used (Bassi et al., 1990). The heterogeneous populations have been interpreted as mobile and PS II-bound subsets of LHC IIb, with the bound population depleted in some polypeptides, but enriched in others (Bassi et al., 1988; Larsson et al., 1987; Spangfort and Andersson, 1989). The 'bound' LHC IIb population should not be confused with the minor LHC IIa, c and d complexes, which are also closely associated with PS II. The bound and mobile forms of LHC IIb are apparently preserved primarily as oligomeric structures under mild solubilization conditions, but readily dissociate under harsh conditions. However, the oligomeric organization of the LHC IIb population is affected by cold-hardening processes in some species (Huner et al., 1987).

Further information on the organization of the LHC II system has been obtained by isolation of membrane subfractions containing several distinct chlorophyll-protein complexes (Ghanotakis et al., 1984; Camm and Green, 1989; Bassi and Dainese, 1989; Bassi et al., 1990). Fractions containing various combinations of the PS II core, LHC IIb subpopulations and the minor LHC IIa, c and d complexes are interpreted to reflect <u>in vivo</u> associations, and can be conceptually combined to create models of PS II/LHC II organization (see Figure 5.1).

This chapter presents an analysis of LHC II populations from maize mesophyll chloroplasts, separated by mildly-denaturing electrophoresis using deoxycholate and SDS solubilization. This electrophoretic system has been used in studies on cold acclimation (Huner et al., 1987; 1989; Krol et al., 1988; Krupa et al., 1987), toxic stress (Krupa, 1987), chilling damage to maize thylakoids (Chapter 4, this work; Campbell et al., 1989) and LHC II organization (Chapter 3, this work).

During the course of this research, extensive characterizations of LHC II chlorophyll-protein complexes separated by two other methods have been published (Thornber et al., 1988; Bassi and Dainese, 1989). Hence, I attempted to relate the LHC II populations separated by this system to those of other authors. Two of the LHC II populations resolved using this system match well with those described by others using well characterized solubilization methods (Thornber et al., 1988; Bassi et al., 1987a,b). A third population appears unique to this system. The nomenclature of Thornber et al. (1988) is followed, but where applicable the 'CP' equivalents (eg. Green, 1988; DiPaolo et al., 1990; Bassi et al., 1990) are given (see Table 1.1).

2.2 Materials and Methods

2.2.1 Plant Growth

Maize (Zea mays L. cv. LG 11) seedlings were grown at 25 ^{o}C, 250 μMol photons m^{-2} s^{-1} with a 16 h lights on period commencing at 4:00 AM, ending at 8:00 PM. Chilling treatment, when applied, was for 6 h at 5^{o}C, 250 μMol photons m^{-2} s^{-1}, commencing at 09:00 h.

2.2.2 Mildly-denaturing electrophoresis of chlorophyll-protein complexes (green gels)

Thylakoids were isolated from maize mesophyll chloroplasts according to Hayden and Hopkins (1976). A deoxycholate/SDS detergent system (Waldron and Anderson, 1979) as modified by Huner et al. (1987) was used to solubilize thylakoids for separation of chlorophyll-protein complexes. Thylakoids were unstacked by washing twice in 1 mM EDTA, and solubilized by rapid sequential addition of 1% SDS and 2% deoxycholate to a final ratio of 20:10:1 deoxycholate:SDS:chlorophyll. Solubilized samples containing 15 μg of chlorophyll were loaded on 8% (w/v) acrylamide gels containing 1% deoxycholate and electrophoresed using a tris-HCl buffer system. This technique is highly reproducible and yields stable results over a two-fold detergent concentration range (data not presented).

2.2.3 Polypeptide analysis of chlorophyll-protein complexes

Bands or lanes excised from chlorophyll-protein gels were incubated in denaturing solution: 2.3% SDS, 10 % glycerol, 5% ß-mercaptoethanol in 0.125 mM Tris pH 6.8. Separation of polypeptides was by SDS-PAGE using the Laemmli (1970) buffers and 4% (w/v) stack/12% (w/v) separating acrylamide gels containing 0.1% SDS (Sigma) and 4 M urea. Electrophoresis was carried out at 6 ^{o}C.

Densitometric scans of Coomassie R-250 stained polypeptide gels were obtained on a LKB Ultroscan laser scanning densitometer at 633 nm. Staining intensity was linear over the protein concentrations used.

2.2.4 Immunoblotting

Polypeptides were electrophoretically transferred from gels to Immobilon (Millipore) using the ABN Polyblot system and the tris/glycine buffer system recommended by Millipore. Blots were probed with the MLH 2 antibody against LHC IIa (CP 29), and the MLH 9 antibody against all LHC IIb polypeptides (Darr et al., 1986). Antibodies were detected by probing with goat anti-mouse IgG coupled to horse radish peroxidase (Sigma), and developed using 4-chloro-1-napthol as a colourgenic substrate.

2.2.5 Membrane Fractionation

LHC II particles were prepared according to Steinback et al. (1982) by Triton X-100 solubilization of thylakoids, differential centrifugation in a sucrose gradient and precipitation of LHC II particles by addition of $MgCl_2$ and KCl. PS II enriched membranes (BBY particles) were prepared according to Ghanotakis et al. (1984) by mild Triton X-100 solubilization of thylakoids followed by centrifugation.

2.3 Results

Figure 2.1 shows unstained mildly-denaturing electrophoresis gels of pigment-protein complexes. Pigment proteins were separated from maize mesophyll thylakoids (Fig.2.1A), PS II enriched membrane subfractions (Fig. 2.1B), and LHC II particles (Fig. 2.1C). The LHC II-related bands were identified by their characteristic absorption spectra. LHC II related complexes migrate in three main bands in this system, termed LHC II-1, LHC II-2 and LHC II-3. The identities of the specific *in vivo* complexes which comprise these bands are discussed below. The PS I and LHC I bands are reduced or eliminated in the PS II membrane preparation (Fig. 2.1B). The PS II chlorophyll-a-protein complex band is eliminated from the LHC II preparation (Fig. 2.1C).

Low levels of free pigment (approx. 5% of total chlorophyll) are present on the separations of thylakoid or PS II membrane derived pigment-protein complexes. Very little free pigment is present on the separation of complexes from the LHC II preparation (Fig. 2.1C). The spectra of the free pigment zones indicate they contain primarily chlorophyll a, with little or no chlorophyll b. Taken together, this suggests that little LHC II-derived chlorophyll is present in the free pigment zone.

The monomeric forms of LHC IIa (CP 29), LHC IIc (CP 26), and LHC IId (CP 24) are not resolved, as they

comigrate in the LHC II-3 region. The octyl glucopyranoside/SDS (Camm & Green, 1980), SDS/glycerol (Bassi et al., 1987a,b) and Deriphat/glucoside (Peter & Thornber, 1988) systems resolve some or all of these monomers. LHC II polypeptides also comigrate with LHC I polypeptides in a faint band immediately above LHC II-1. The LHC II polypeptides in this band are identical to those in LHC II-1 (Fig.2.2A).

The three LHC II bands from thylakoid preparations (Fig. 2.1A) were excised and re-electrophoresed on a second mildly-denaturing gel; LHC II-1 (Fig. 2.1D); LHC II-2 (Fig. 2.1E) and LHC II-3 (Fig. 2.1F). Note that the pigment-protein complexes in the LHC II-1 and LHC II-2 bands undergo significant dissociation, generating a band which comigrates with LHC II-3.

To identify the polypeptide composition of the LHC II complexes separated by mildly-denaturing electrophoresis, lanes (Fig. 2.2; Fig. 2.3) or individual bands (Fig. 2.4) were subjected to denaturing polypeptide electrophoresis.

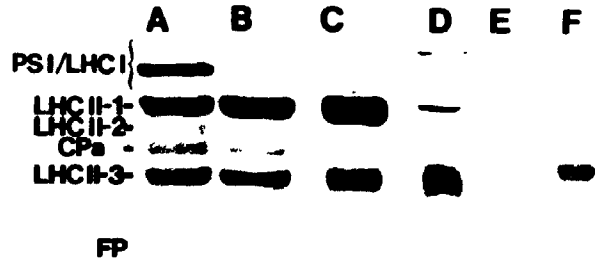


Figure 2.1. Chlorophyll-protein complexes separated by mildly-denaturing deoxycholate electrophoresis.

Gel is unstained. Lanes were loaded with; 2.1A: maize mesophyll thylakoids; 2.1B: a PS II enriched subfraction; and 2.1C: LHC II particles. Bands containing LHC II pigment proteins are numbered 1-3. Other bands are PS I/LHC I derived bands above LHC II-1 and a band (CPa) containing the chlorophyll a protein complexes of PS II, between LHC II-2 and LHC II-3. Lanes 2.1D-2.1F were loaded with excised LHC II bands from thylakoids; 2.1D: LHC II-1; 2.1E: LHC II-2; 2.1F: LHC II-3.

In Figures 2.2A, B and C the diagonal array of polypeptides results from migration approximately according to molecular mass in both the mildly-denaturing first dimension and the fully denaturing second dimension. The PS II chlorophyll a/b light-harvesting polypeptides are present between 24 and 33 kDa, in the boxed region of Figure 2.2A. LHC II-1 and LHC II-2 give rise to polypeptides off the diagonal in the denaturing dimension, indicating that they are derived from oligomeric associations of individual chlorophyll a/b proteins. The LHC I and PS I polypeptides are reduced or absent in the PS II membrane preparation (Fig. 2.2B), while the PS II apoproteins are relatively enriched. Only a subset of LHC II polypeptides is present in the LHC II particle preparation (2.2C).

The apparent molecular masses of most LHC II polypeptides separated by this system agree with consensus literature values (Thornber et al., 1988); however, the LHC IIa apoprotein migrates at an apparent molecular mass of 33 kDa (Fig. 2.4A), higher than the consensus value of 30-31 kDa (Thornber et al., 1988; Hayden et al., 1988). This shift is apparently related to the acrylamide concentration of the SDS-PAGE gels used (see Fig. 4.1).

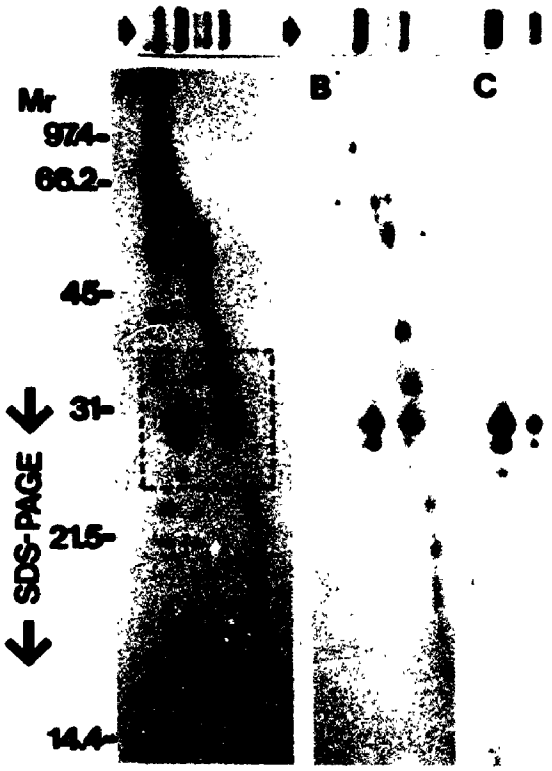


Figure 2.2. Polypeptides from lanes of mildly denaturing gels.

Lanes from mildly-denaturing gels were treated with denaturant solution and placed on slab gels; polypeptides separated by SDS-PAGE and stained with Coomassie R-250.

2.2A: Polypeptides from thylakoids. LHC II related polypeptides migrate within the enclosed region.

2.2B: Polypeptides from PS II enriched membranes; note loss of LHC I polypeptides (21-23 kDa), PS I polypeptides (66 kDa) and the coupling factor polypeptide (60 kDa).

2.2C: Polypeptides from LHC II particles; note that the polypeptide profile of the LHC II-1 and LHC II-3 bands are identical. Some LHC II related polypeptides are missing from this preparation.

Figure 2.3 represents an enlargement of the enclosed region of Figure 2.2A. The LHC IIa and LHC IIb polypeptides were identified by immunoblotting with the MLH 2 and MLH 9 antibodies (Darr et al., 1986) (Fig. 2.5), while the LHC IIc polypeptides were identified by comparison with published profiles (Thornber et al., 1988; DiPaolo et al., 1990). The polypeptides derived from LHC II-1 have apparent molecular masses of 30, 29, 28 and 26 kDa (Fig. 2.2A, 2.3, 2.4A). This population is compositionally equivalent to the LHC IIb oligomeric complex characterized by Peter and Thornber (1988); all four polypeptides are recognized by the MLH 9 monoclonal antibody against LHC IIb (Fig. 2.5B).

LHC II-2 and LHC II-3 from thylakoids and PS II membranes are each compositionally distinct from LHC II-1 (Figs. 2.2A, 2.2B, 2.3, 2.4A, 2.4B). LHC II-3 contains the LHC IIa apoprotein (33 kDa), identified by immunoblotting with the MLH 2 monoclonal antibody (Fig. 2.5A). LHC II-3 also contains two polypeptides at 29.5 and 28.5 kDa (Figs. 2.3, 2.4A, 2.4B), presumed equivalent to the LHC IIc (CP 26) polypeptides described by DiPaolo et al. (1990), also in maize. These polypeptides are of slightly different molecular mass than those derived from LHC IIb (Table 2.1). Very low levels of all four LHC IIb polypeptides are present in the LHC II-3 band (Fig. 2.5B). In thylakoids, these trace polypeptides are intensely

phosphorylated (Fig. 4.8), in agreement with Bassi et al. (1990)

The chlorophyll-proteins of LHC II-2 are associated into oligomeric structures. LHC II-2 from thylakoids and PS II membranes contains the LHC IIa apoprotein (Figs. 2.4A, B; 2.5A) and the 30 and 29 kDa LHC IIb polypeptides (Fig. 2.5B). However, LHC II-2 is distinct from LHC II-1 in that it does not contain the 28 and 26 kDa polypeptides characteristic of LHC II-1 (Figs 2.4A; 2.5B; Table 2.1). The distinct composition of LHC II-2 is partially obscured in Fig. 2.3 by the abundant polypeptides of LHC II-1, however it is evident in Figs. 2.4A and 2.5B.

Thylakoids from chill stressed maize were used to prepare Figures 2.2A and 2.3; thus the chill-stress induced polypeptide related to LHC IIa (CP 29) (Hayden et al., 1986, 1988) is present, (designated LHC IIa*). Note that this polypeptide migrates above and to the left of the normal LHC IIa apoprotein in LHC II-2 and LHC II-3. Thus, LHC IIa* forms an oligomeric structure (marked by dashed line), but does not associate into the normal oligomeric LHC II-2 structure (compare Fig. 3.5A).

In complexes derived from thylakoids, LHC I complexes migrate in oligomeric structures just below LHC II-2 (Fig. 2.2A) and above LHC II-1. LHC I polypeptides are absent from PS II membranes (Figs. 2.2B, 2.4B).

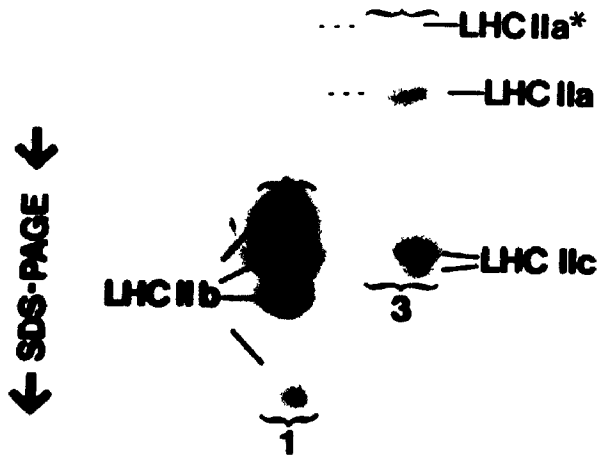


Figure 2.3. Enlargement of LHC II region of Figure 2.2A.

Polypeptides from the LHC II-1 and LHC II-3 bands are enclosed in horizontal brackets, labelled 1 and 3 respectively. Polypeptides were identified by immunoblotting (LHC IIa and b) and by comparison with published profiles (LHC IIc). LHC IIa* is the chilling stress induced polypeptide related to LHC IIa. Four polypeptides are resolved from LHC IIb and two putatively from LHC IIc. Note the presence of the LHC IIa polypeptide in the LHC II-2 band; a subset of LHC IIb polypeptides is also present in LHC II-2, but is obscured by the abundant polypeptides from LHC II-1. The LHC IIa* polypeptide is to the left (above in the first dimension) of the LHC II-2 and 3 bands.

Immunological identification of the LHC IIa and LHC IIb polypeptides is presented in Figure 2.5. The LHC IIa apoprotein is present in LHC II-2 and LHC II-3 (Fig. 2.5A). LHC II-1 contains only LHC IIb polypeptides; LHC II-2 contains a subset of two LHC IIb polypeptides and LHC II-3 contains low levels of all four LHC IIb polypeptides (Fig. 2.5B).

Table 2.1 presents a quantization of the relative polypeptide compositions of the LHC II-1 and 3 bands from thylakoids, PS II membranes, LHC II particles and excised LHC II-1 bands re-electrophoresed on a second mildly-denaturing gel. The abundance of polypeptides derived from LHC II-2 was too low to allow quantization.

Comparison of the polypeptides of LHC II-1 and 3 from LHC II particles and re-electrophoresed LHC II-1 shows that they are not distinct, and lack the polypeptides characteristic of LHC II-3 separated from thylakoids or PS II membranes (Figs. 2.2C, 2.4C, 2.4D, Table 2.1). This suggests that the LHC II particles are derived from the LHC IIb population.

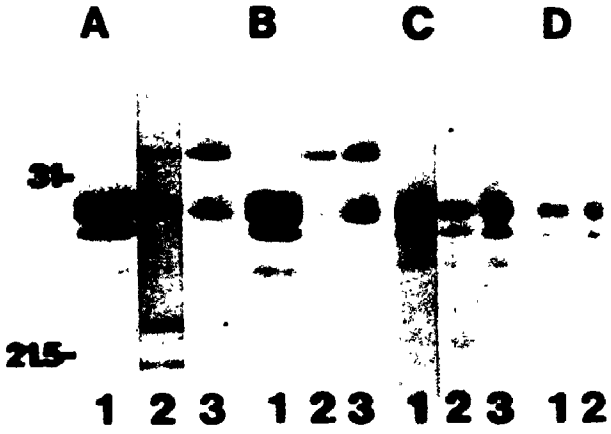
Figure 2.4. Polypeptides separated by SDS-PAGE from LHC II chlorophyll-protein bands excised from mildly denaturing gels.

2.4A. Polypeptides from complexes separated from thylakoids. Lane 1, LHC II-1; 2, LHC II-2; 3, LHC II-3. Note distinct polypeptide compositions of complexes. Lane 2 was silver stained for better visualization of faint bands; other lanes were stained with Coomassie R-250.

2.4B. Polypeptides from complexes separated from PS II membranes. Lanes 1, 2 and 3 as above.

2.4C. Polypeptides from complexes separated from LHC II particles. Lanes 1, 2 and 3 as above; note that the polypeptide compositions of lanes 1, 2 and 3 are identical.

2.4D. Polypeptides separated from bands derived from LHC II-1 band rerun on a second mildly denaturing gel. Note that the compositions are identical.


A
B
C
D
31-
215-
1 2 3 1 2 3 1 2 3 1 2

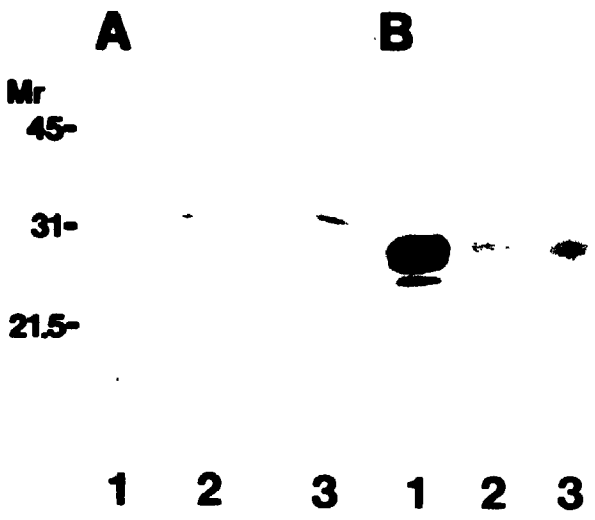


Figure 2.5. Immunoblotting of LHC II polypeptides.

Electrophoretic blots of gels similar to those represented in Fig. 2.4A were probed with the MLH 2 monoclonal antibody against the LHC IIa apoprotein (Fig. 2.5A); or the MLH 9 monoclonal antibody which recognizes all four LHC IIb polypeptides Fig. 2.5B).

Fig. 2.5A. The LHC IIa apoprotein is present in the LHC II-2 and LHC II-3 chlorophyll-protein bands.

Fig. 2.5B. All four LHC IIb polypeptides are present in the LHC II-1 band, a subset of two LHC IIb polypeptides is present in the LHC II-2 band, and low levels of all four are present the LHC II-3 band.

Table 2.1. Polypeptide compositions of LHC II chlorophyll-protein bands.

The approximate polypeptide distributions in the LHC II-1 and LHC II-3 complexes were determined by densitometric scans of stained denaturing SDS-PAGE gels loaded with chlorophyll-protein bands excised from mildly-denaturing gels (Fig. 2.4). The relative abundance of each polypeptide is expressed as the percentage of total LHC II related protein in the chlorophyll-protein band. Standard errors of measurements were less than 2% in all cases. tr - trace.

Complex	Polypeptide (kDa)	LHC II-1 from all Sources (% of total protein in band)	LHC II-3 (% of total protein in band)			
			from Thylakoids (%)	from PS II membranes (%)	from LHC II particles (%)	from re-run LHC II-1 (%)
LHC IIa	33	0	42	39	0	0
LHC IIb	30	33	tr	tr	38	35
	29	38	tr	tr	29	34
	28	20	tr	tr	26	27
	26	9	tr	tr	7	4
LHC IIc	29.5	0	34	34	0	0
	28.5	0	15	20	0	0

2.4 Discussion

Thornber et al. (1988) used a glucoside/Deriphat solubilization system to resolve LHC II complexes from PS II membranes. The major oligomeric structure, termed by them LHC IIb, is compositionally equivalent to LHC II-1 described here. Bassi et al. (1987a,b; DiPaolo et al., 1990), using a system employing a glycerol/SDS solubilization of maize mesophyll thylakoids, report a similar oligomeric form comprised of six polypeptides. The published polypeptide profile (DiPaolo et al., 1990) is very similar to that shown in Fig. 2.4A.

Both systems resolve the chlorophyll-protein monomer bands comprising LHC II-3 into three distinct complexes, termed LHC IIa, c and d (Thornber et al., 1988) or CP 29, CP 26 and CP 24 (DiPaolo et al, 1990). The polypeptide profiles of CP 29 and CP 26 match well with the results presented (Fig. 2.4A), while the minor CP 24 complex was not identified.

Nothing comparable to LHC II-2, containing LHC IIa (CP 29) and an LHC IIb subset, was present in either system. Green et al. (1982) and Metz et al. (1984) have previously noted oligomers containing LHC IIa. However, within the LHC II-2 band, the LHC IIa apoprotein exactly comigrates with two LHC IIb polypeptides, in spite of differing molecular masses. This suggests that LHC II-2 may be a single oligomeric structure, containing LHC IIa

and an LHC IIb subpopulation. As discussed in the introduction, the LHC IIb population is known to be heterogeneous, (Spangfort & Anderson, 1989; Spangfort et al., 1987; Bassi et al., 1988), with a PS II-bound population depleted in the lower molecular mass apoproteins. The LHC II-2 population may be an association between LHC IIa and an LHC IIb subset equivalent to the bound population described by these authors.

The chill-induced LHC IIa* polypeptide migrates above the LHC II-2 band, and thus is not incorporated in the normal structure(s) comprising LHC II-2. This abnormal structure may contribute to the perturbation in energy transfer observed in chill-stressed maize thylakoids (Hayden et al, 1986) (see Chapter 4).

Some confusion exists because dissociation of the LHC IIb oligomeric complex itself generates green bands which co-migrate with the LHC II-2 and LHC II-3 bands. The degree of dissociation of LHC IIb complexes varies with species, growth conditions and solubilization method. Some of the variable dissociation is related to changes in membrane composition and LHC II organization (Huner et al., 1987; 1989). A survey of LHC II chlorophyll-protein complexes separated from a range of species demonstrated that in each species, the LHC II-1, 2 and 3 bands had distinct polypeptide compositions (Campbell and Hayden,

unpub. results). However, in rye, wheat, triticale and barley high levels of LHC IIb polypeptides were present in the monomeric LHC II-3 band. This phenomenon was especially prominent in complexes separated from the thylakoids of cold-hardened C-3 cereals, and partially obscured the distinct polypeptide compositions of LHC II-1 and 3. This contrasts with the situation in maize (Fig. 2.4a) and the dicots examined (spinach, pea, faba bean, canola) (Campbell and Hayden, unpub. results) where most LHC IIb polypeptides were present in the oligomeric LHC II-1 band, with low to trace levels in the LHC II-3 band. Thus, the organization of LHC II in C-3 cereals appears distinct from that in maize and many dicots. This distinction correlates with the cold-hardening capability of the C-3 monocots.

High detergent to protein ratios or heating also result in increased dissociation, which obscures compositional distinctions. This dissociation is demonstrated when an excised LHC II-1 band or LHC II particles from detergent fractionated thylakoids are electrophoresed on mildly-denaturing gels. Both generate three pigmented bands, which co-migrate with LHC II-1, 2 and 3 from thylakoids (Fig. 2.1). However, they are compositionally identical; all resembling the original LHC II-1 band or LHC II particle. Thus, mildly-denaturing separations of chlorophyll-proteins from submembrane

fractions are not necessarily equivalent to the results obtained with thylakoids. This point is important since many studies of LHC II structure (eg. Spangfort and Andersson, 1989) use isolated LHC II particles. Furthermore, harsher solubilization systems such as the SDS/octylglucopyranoside technique (Dunahay and Staehelin, 1986) generate sufficient dissociation of the oligomeric LHC IIb complexes to partially obscure the distinct composition of the monomeric chlorophyll-protein bands.

The analysis of LHC II chlorophyll-protein complexes presented in this chapter relates the results obtained with the SDS/deoxycholate system to the current consensus concepts of LHC II organization. These concepts are used for interpretation of the results presented in subsequent chapters.

Chapter Three. Detection of Neighbouring LHC II Complexes by Protein Cross-linking

3.1 Introduction

The characterization of LHC II populations described in Chapter Two provided the technical and conceptual background for consideration of the organization and function of LHC II. Therefore, I sought to investigate how the LHC II populations of maize interact with each other, and with the integral chlorophyll a binding antennae proteins of PS II. A protein cross-linking study was pursued to detect neighbouring complexes. Previous workers have used protein cross-linkers to study thylakoid membranes (Henriques and Park, 1978) or PS II membranes (Enami et al, 1987; 1989; Adir and Ohad; 1988) and have noted linkages between LHC II polypeptides. However, no examination of interactions between specific LHC II complexes has been published.

Intact thylakoids were used for this study to minimize disruption of *in vivo* interactions. Use of intact thylakoids, as opposed to submembrane fractions, was possible since the PS II related proteins and complexes were identified based on the work presented in Chapter Two. 3,3'-dithio-bis(propionic acid n-hydroxysuccinimideester) (DSP), is a cleavable protein cross-linker of 1.1 nm length (Tae, 1983).

DSP reacts with amine groups of amino acid residue side chains, particularly lysine. It is a homobifunctional, cleavable cross-linker, with two identical reactive moieties connected by a disulphide bridge. This bridge is cleavable by treatment with reducing agents such as ß-mercaptoethanol. Cleavage facilitates analysis of cross-linked products by allowing two dimensional electrophoresis, with the products linked in the first dimension, but cleaved in the second. Migration of cross-linked products is proportional to the sum of the molecular masses of the constituents. After cleavage of the linker molecules, the constituents can be separated on the basis of individual molecular mass.

At the 1.1 nm maximum linkage distance, most PS II related interactions occurred between the chlorophyll-protein complexes of adjacent thylakoid membranes, although extensive cross-linking of PS I related complexes occurred within the plane of the membrane.

The significance of the specific interactions between LHC II and PS II complexes of adjacent appressed membranes is discussed in terms of the organization of LHC II.

3.2 Materials and Methods

Unless otherwise specified, sample preparation, separation of chlorophyll-protein complexes and separation of polypeptides was as described in Chapter Two.

3.2.1 Assessment of thylakoid stacking

Changes in 180° light scattering were monitored essentially according to Mullet and Arntzen (1980). Thylakoid samples were diluted to 10 μg chl/ml. Apparent absorbance (optical density) at 550 and 671 nm was measured using a Shimadzu UV-160 spectrophotometer. The ratio A_{550}/A_{671} is used to express light scattering to correct for small variations in chlorophyll concentration between samples.

3.2.2 Assessment of Fluorescence Induction Parameters of Thylakoids

Dark adapted control or freshly cross-linked thylakoids were diluted to 10μg chlorophyll/ml with cross-linking buffer. Fluorescence induction was assessed using a Plant Stress Meter Mk II (BioMonitor AB S.C.I., Sweden) (Oquist and Wass, 1988). Recorded parameters were the instantaneous fluorescence level (Fo), maximal fluorescence (Fm), the time at which fluorescence intensity equals half of Fm (t1/2) and the ratio of variable to maximal fluorescence (Fv/Fm).

3.2.3 Cross-linking of Thylakoids

Chloroplasts were osmotically ruptured in cold 50 mM MES pH 6.5 (HCl), 10 mM NaCl and 5 mM $MgCl_2$. Thylakoids were pelleted at 10000g for 10 min, then washed with the same buffer (for stacked thylakoids) or twice with 5 mM EDTA pH 8 (for unstacked thylakoids). When performed, trypsin digests of thylakoid membranes were essentially according to Steinback et al. (1979).

Cross-linking of membrane proteins was according to Enami et al. (1989) with modifications. The washed thylakoids were resuspended to a concentration of 1 mg chl/ml in 0.4 M sucrose, 50 mM MES pH 6.5 (HCl), 10 mM NaCl and 5 mM $MgCl_2$; NaCl and $MgCl_2$ were omitted for unstacked thylakoids. Aliquots 7 µl of 100 mg DSP/ml DMSO were added to 1 ml of thylakoid suspension at 10 min intervals for 1 h at room temperature with continuous gentle shaking. Cross-linking was terminated by addition of 200 µl of 2 M glycine. Thylakoids were then frozen at -70°C until use.

Thylakoids for fluorescence analysis were treated as above except that the cross-linking treatment was performed at 6°C in the dark; 10 µl aliquots of 100 mg DSP/ml DMSO were added every 5 minutes for 30 minutes.

3.2.4 Denaturing and non-denaturing analysis of cross-linked membrane proteins

Cross-link treated thylakoids were washed with 50 mM tricine, pH 7.8 and pelleted; the clear supernatant was decanted. The thylakoids were solubilized by addition of a solution containing 2% SDS, 1 mM EDTA, 12% sucrose and 60 mM Tris, pH 7.8, to a SDS:chl ratio of 20:1. The resuspended thylakoids were incubated at 60°C for 30 min, then briefly centrifuged at 14000 rpm in an Eppendorf microcentrifuge. 15 µl of the solubilized extract was applied to 0.2 X 10 cm acrylamide tube gels (4% stacking gel, 10% resolving gel containing 1% SDS and 4 M urea); polypeptides were resolved by SDS-PAGE using the Laemmli (1970) buffers. Electrophoresis was carried out at a constant voltage of 100 V.

The gels were then extruded and soaked in two changes of denaturing solution; 10% SDS, 10% ß-mercaptoethanol, 8 M urea and 0.125 mM Tris pH 6.8 for 70 min to cleave cross-links. The gels were rinsed and applied to 0.15 cm X 15 cm acrylamide slab gels (4% stack, 12% resolving gel containing 1% SDS and 4 M urea); polypeptides were resolved by SDS-PAGE using the Laemmli (1970) buffer system. The gels were silver stained (Merrill et al., 1981).

A variation of this procedure was employed to investigate linkages between chlorophyll-protein complexes

(Fig. 3.3). Deoxycholate/SDS mildly denaturing electrophoresis (Chapter Two) was used to separate the complexes from cross-link treated thylakoids. The links were then cleaved in conjunction with denaturation of the constituent polypeptides of the complexes. The polypeptides were then separated from lanes of the mildly-denaturing gels by SDS-PAGE.

3.3 Results

Preliminary experiments indicated that unstacking of appressed thylakoids greatly reduced the extent of PS II related cross-linking. This unexpected result seemed to promise insight into the organization of the LHC II system. Hence, I modified the original objective of using cross-linkers to characterize interactions between PS II and LHC II complexes in the plane of the membrane. To pursue a study of the relationship between thylakoid appression and LHC II organization, a convenient measure of the extent of thylakoid appression was required. In addition, two complementary techniques to achieve unstacking were used.

To assess the degree of stacking of thylakoid samples, optical density was measured at 550 nm, a wavelength where chlorophyll absorbance is low and most apparent absorbance is attributable to light-scattering (Mullet and Arntzen, 1980). The data were standardized using the ratio A550/A671. A671 is an absorbance maximum for chlorophyll in thylakoids and served to correct for changes in chlorophyll concentration. This ratio is plotted in Figure 3.1A over a four-fold range of chlorophyll concentration. Little change occurs over this range, which encompasses the concentrations of approximately 10 μg chl/ml used for subsequent measurements. Note that light scattering by thylakoids

unstacked by treatment with two EDTA washes is 20% lower than that of thylakoids maintained in stacked configuration by presence of Mg^{2+}. Low salt treatments such as EDTA washing are known to completely unstack thylakoids, as confirmed by electron microscopy, but apparently do not disrupt lipid-protein interactions or capacity for oxygen evolution (Li et al., 1990). Thus a 20% reduction in light scattering reflects complete unstacking of thylakoids.

To assess whether cross-linked thylakoids could be unstacked if cross-links were broken, ß-mercaptoethanol treatments of cross-linked thylakoids were used. A plot of A550/A671 versus increasing concentrations of ß-mercaptoethanol added to control, unstacked thylakoids, (Fig. 3.1B) shows some reduction in light scattering in response to the treatment. Thus, changes in scattering in response to ß-mercaptoethanol must be compared to this pattern.

Mild trypsin treatments also unstack thylakoids by removing exposed polypeptide fragments which apparently mediate the stacking mechanism (Steinback et al., 1979). A time course of changes in light scattering during trypsin treatment is presented in Fig. 3.1C. Note that a trypsin-induced 20% reduction in light scattering is complete after a 10 min treatment. The extent of reduction in light scattering matches that induced by EDTA

(Fig. 3.1A). Steinback et al. (1979), using electron microscopy, have shown that similar trypsin treatments completely unstack thylakoids. Note that different thylakoid preparations exhibited somewhat variable levels of scattering in the stacked condition (compare Fig. 3.1A and C); thus unstacking was assessed as the reduction in scattering relative to the control, stacked condition. Furthermore, EDTA washing subsequent to trypsin treatment induced some further decline in scattering, perhaps by disrupting residual stacked regions.

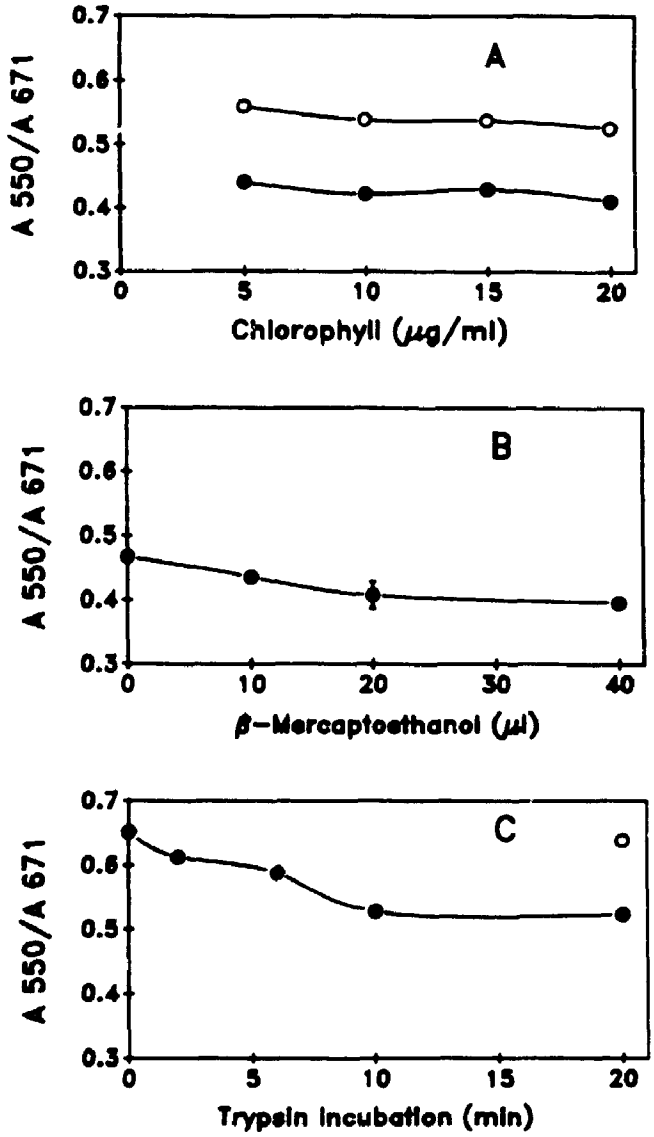
Figure 3.1 Control Curves for Measurement of Thylakoid Stacking By Light Scattering.

Standard errors (n = 3-6) are within the symbol size.

Fig. 3.1A Assessment of Thylakoid Stacking by Light Scattering over a Range of Chlorophyll Concentrations. Stacked ( ) or Unstacked ( ) thylakoid preparations were diluted to measurement concentrations, apparent absorbance at 550 and 671 nm measured and the ratio plotted.

Fig. 3.1B The Effect of ß-Mercaptoethanol Treatments on the Ratio A550/A671. Aliquots of ß-mercaptoethanol were added to 0.4 ml, 10 ug chl/ml samples and A550/A671 was assessed.

Fig. 3.1C Trypsin Treatment Reduces Light Scattering. Trypsin treatment ( ) and control incubation ( ) were followed for 20 min. Chlorophyll concentration approx. 10 ug/ml.


A
A 550/A 671
0.7
0.6
0.5
0.4
0.3
0
5
10
15
20
Chlorophyll (μg/ml)
B
A 550/A 671
0.7
0.6
0.5
0.4
0.3
0
10
20
30
40
β−Mercaptoethanol (μl)
C
A 550/A 671
0.7
0.6
0.5
0.4
0.3
0
5
10
15
20
Trypsin incubation (min)

Unstacking thylakoids by EDTA washing did not change the polypeptide composition relative to stacked thylakoids (data not presented). To assess the changes in the trypsin treated membranes, the polypeptides (Fig. 3.2A) and chlorophyll-protein complexes (Fig. 3.2B) from thylakoids treated with trypsin were examined. Over the time course of 0 - 20 min, the LHC IIa, b and c apoproteins were cleaved to generate products reduced in mass by approximately 2 kDa. Cleavage was complete after 10 min of trypsin treatment. Few other changes in the polypeptide profile were evident, indicating that other than in the light-harvesting chlorophyll-proteins, few trypsin cleavable fragments are accessible. In spite of the quantitative conversion of LHC II apoproteins to smaller forms (Fig 3.2A, compare lanes 10 and 20 with lane CON), no change is evident in the profile of chlorophyll-protein complexes from thylakoids treated by trypsin for 20 min (Fig. 3.2B). Thus the chlorophyll-binding regions of these complexes are not affected by the trypsin treatment, nor are the interactions between constituent pigment-proteins of the complexes.

Figure 3.2 Polypeptide and Chlorophyll-Protein Analysis of Trypsin Treated Thylakoids.

Samples were those used for Fig. 3.1C.

Fig. 3.2A: Polypeptide Profiles of Trypsin Treatment Time Course. Trypsin treatment from 2 to 20 min results in a reduction of apparent molecular mass of approx. 2 kDa for LHC II apoproteins, indicated by dashes connecting equivalent polypeptide bands in the 20 min trypsin treated (20) and control (CON) lanes. Four other digestion products, marked with dots beside the 20 min lane, are evident. Three are in the 20 kDa range, and one is about 50 kDa. Digestion is essentially complete after 10 min.

Fig. 3.2B Chlorophyll-proteins from Control (CON) and 20 min Trypsin Treated (20) Thylakoids; separated by mildly-denaturing electrophoresis. The three LHC II related complexes, 1, 2 and 3, are labelled; no change is evident. Some change occurs in the relative abundance of the various PS I/LHC I bands near the top of the lane following trypsin treatment

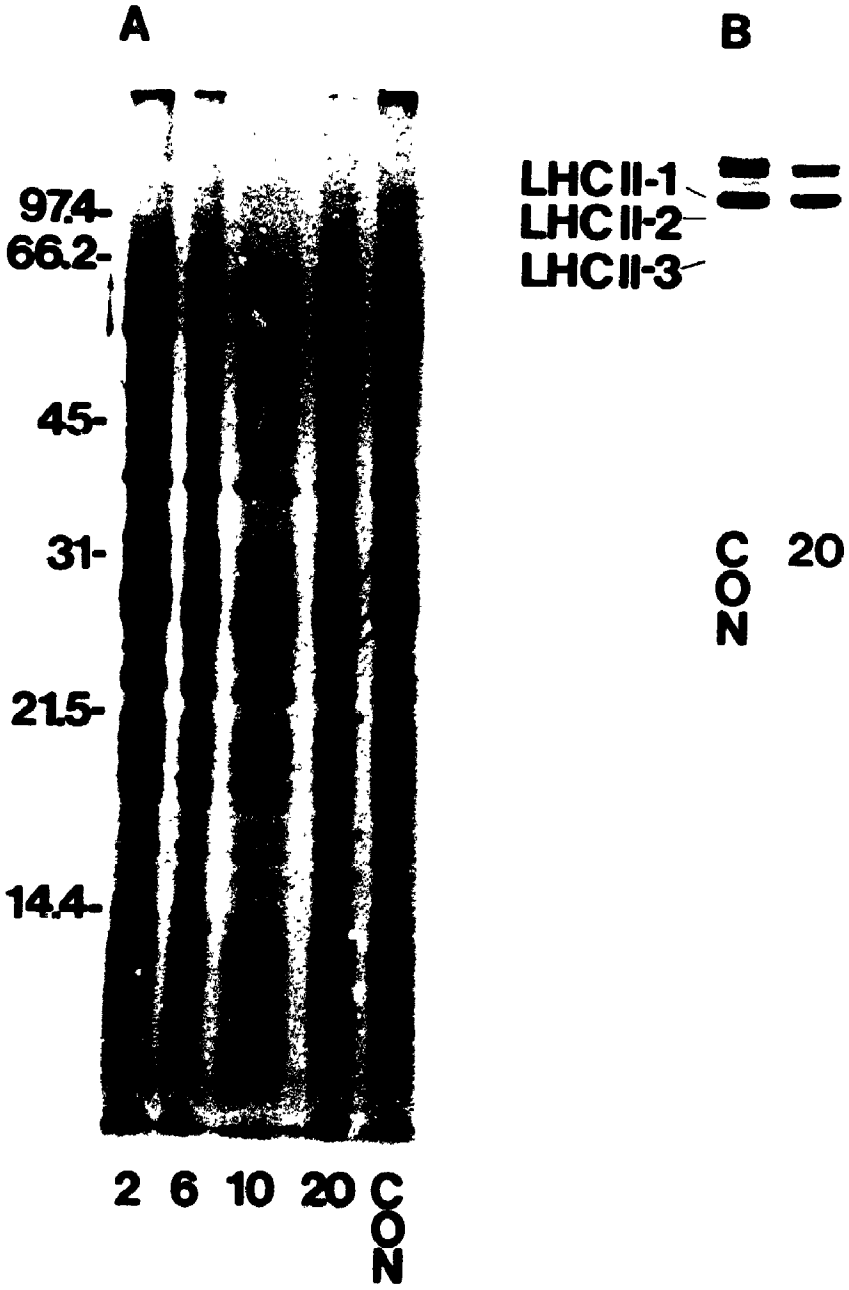

A
B
97.4-
66.2-
45-
31-
21.5-
14.4-
LHC II-1
LHC II-2
LHC II-3
2 6 10 20 CON
C 20
ON

Table 3.1 presents a summary of the changes in light scattering in response to various treatments. Treatment of stacked or unstacked thylakoids with DSP does not greatly change the degree of stacking; nor does treatment with DMSO, the solvent used to solubilize DSP. However, if membranes treated with DSP while stacked are subsequently washed with EDTA, little reduction in scattering/stacking is observed, although EDTA washes of control thylakoids result in a 20% reduction of scattering. This suggests that cross-links occur between adjacent appressed thylakoids. Treatment of DSP treated stacked membranes with ß-mercaptoethanol followed by EDTA washes reduces scattering/stacking. However, the effect of ß-mercaptoethanol alone on scattering and apparent difficulty in fully cleaving the cross-links at concentrations of ß-mercaptoethanol low enough to avoid disrupting the membranes reduce the extent of unstacking of previously cross-linked membranes.

Table 3.1 Assessment of Thylakoid Stacking by Light Scattering After Various Treatments.

Expressed as A550/A671, n = 3-8; S.E. in parentheses. Stacking was maintained by presence of 5 mM $MgCl^{2+}$ during isolation and treatments (Stacked Thylakoids) or disrupted by washing with 1 mM EDTA (Unstacked Thylakoids).
Details of Treatments: Control - thylakoids incubated at 25°C in dark for 1 h after preparation. Incubation at 5°C gave the same scattering measurement (data not presented).

DSP - 7 μl aliquots of 10 mg DSP/ml DMSO added every 10 min during 1 h at 25°C, cross-linking then terminated by addition of glycine. Addition of DMSO alone did not change the scattering measurement (data not presented)

DSP, EDTA - cross-link treated thylakoids (previous treatment) washed with 1 mM EDTA, sufficient to unstack control thylakoids.

DSP, Trypsin, EDTA - cross-link treated thylakoids incubated for 20 min with trypsin followed by 1 mM EDTA wash.

DSP, ß-mercaptoethanol, EDTA - cross-link treated thylakoids treated with 20 μL ß-mercaptoethanol for 30 min at 25 °C (to cleave cross-links) followed by 1 mM EDTA wash.

	A550/A671	
Treatment	Stacked Thylakoids	Unstacked Thylakoids
Control 25°C	0.510 (0.026)	0.394 (0.006)
DSP	0.533 (0.010)	0.401 (0.004)
DSP, EDTA	0.511 (0.009)	0.381 (0.014)
DSP, Trypsin, EDTA	0.509 (0.003)	0.309 (0.029)
DSP, ß-mercaptoethanol, EDTA	0.405 (0.003)	

Note that EDTA washes alone did not unstack thylakoids cross-linked while in the stacked configuration.

The goal of the study was to understand *in situ* organization of the LHC II/PS II system, hence a measure of the physiological function of the cross-link treated membranes was desired. Chlorophyll fluorescence measurements provide a convenient, rapid, intrinsic probe of aspects of PS II and LHC II function (Krause and Weis, 1991). Several fluorescence parameters of control, cross-linked and unstacked thylakoids were measured; a summary is presented in Table 3.2. Fo is the instantaneous fluorescence level after application of actinic light to dark adapted leaves; it is believed to reflect LHC II organization. An increase in Fo results from treatments which cause dissociation of LHC II complexes, or their functional uncoupling from PS II centres. No apparent changes in Fo occur in response to any of the treatments. The small changes observed were probably concentration dependent, since Fo is highly sensitive to chlorophyll concentration.

Table 3.2 Fluorescence Parameters of Thylakoids after Cross-linking and Unstacking Treatments.

Data obtained from dark adapted thylakoids at 10 μg chl/ml with a Plant Stress Meter (Biomonitor AB S.C.I., Sweden). Fluorescence parameters are described in text. S.E. in parenthesis; n = 4 for all treatments.

Details of Treatments:

Fresh thylakoids were rapidly isolated, washed and maintained in a stacked configuration by presence of cations.

Control Thylakoids were incubated for 30 min with shaking at 6°C in the dark. Note moderate deterioration of Fv/Fm from fresh levels upon incubation.

Cross-linked Thylakoids were treated as for control, but aliquots of cross-linking reagent were added. Note that the cross-linking partially prevents the deterioration of Fv/Fm noted in control relative to fresh thylakoids.

Unstacked Thylakoids were washed with EDTA and resuspended without cations to unstack appressed thylakoids. Note large decline in Fv/Fm relative to other samples. Restacked thylakoids were treated as for unstacked, but readdition of cations, followed by a 10 min incubation on ice resulted in partial recovery of Fv/Fm.

Thylakoid Treatment	Fo (fluorescence)	Fv/Fm (ratio)	$t_{1/2}$ (ms)
Fresh	0.28 (0.002)	0.76 (0.005)	118 (4)
Control	0.25 (0.05)	0.65 (0.006)	69 (0)
Cross-linked	0.25 (0.005)	0.70 (0.005)	83 (0)
Unstacked	0.27 (0.005)	0.50 (0.01)	138 (6)
Restacked	0.27 (0.004)	0.63 (0.002)	135 (4)

Large changes occur in Fv/Fm in response to membrane unstacking, or prolonged incubation. Fv/Fm is a ratio calculated as (Fmaximum - Fo)/(Fmaximum). It reflects the photochemical efficiency of PS II, and correlates well in most systems with capacity to evolve oxygen (Krause and Weis, 1991). Since Fv/Fm is a ratio, it is less sensitive to small changes in chlorophyll concentration than is Fo. The deterioration in Fv/Fm upon incubation is partially prevented by a cross-linking treatment, probably because the cross-linker serves to lock apoproteins and complexes in place. Unstacking induces a large drop in Fv/Fm, which is partially reversible upon restacking; thus the stacked configuration is of major importance to the function of the LHC II/PS II assemblage. $T_{1/2}$ is the time at which fluorescence is at half of the maximal level. For the present purposes it is sufficient to note that the cross-linking treatment does not greatly disrupt PS II/LHC II function as measured by these fluorescence parameters.

With the knowledge that extensive cross-linking occurred between appressed membranes, and that cross-linking was not disruptive of PS II function, identification of the cross-linked products was pursued. To identify neighbouring chlorophyll-protein complexes, cross-linked thylakoids were subjected to mildly-denaturing electrophoresis, as described in Chapter Two. The results are presented in Figures 3.3 and 3.4. Changes

in the migration of chlorophyll-protein complexes in response to cross-linking treatments results from linkages between adjacent complexes, since links within one multi-polypeptide complex would presumably not change the migration of the complex.

Cross-linking stacked membranes changes the migration of chlorophyll-protein complexes. The pattern of PS I/LHC I bands is altered to favour very large oligomeric associations, which migrate near the top of the lanes. This occurs in all cross-linking treatments, and provides an internal control that the various treatments do not interfere directly with the cross-linking chemistry. The cross-linked PS I/LHC I products migrate at the positions of oligomer structures present in variable amounts on separations of complexes from control thylakoids (compare Fig. 3.3A and Fig. 3.1A). Thus, the cross-linker appears to stabilize interactions normally present in the plane of one membrane.

Figure 3.3 Separation of Chlorophyll-protein Complexes from Cross-link Treated Thylakoids.

Photograph of unstained, mildly-denaturing electrophoresis gels shows separation of pigmented complexes.

Fig. 3.3A Control chlorophyll-protein complexes are labelled (see Fig. 2.1 for details)

Fig. 3.3B Complexes from thylakoids cross-linked while in stacked configuration. Extensive cross-linking between chlorophyll-protein complexes results in lower abundance of normal bands, with generation of dark bands near top of lane, consisting of large associations of LHC I/PS I and LHC II/PS II.

Fig. 3.3C Complexes from thylakoids cross-linked after unstacking by EDTA washing to remove cations. The pattern of LHC II and PS II related bands is similar to the control; however extensive cross-linking of LHC I and PS I results in stabilization of large oligomers of these complexes, which are present under control conditions as faint bands near top of lane (Fig. 3.3A).

Fig. 3.3D Complexes from thylakoids cross-linked after unstacking by trypsin treatment (see Figs. 3.1, 3.2 and Table 3.1 for details). The pattern of products resembles that of Fig. 3.3C.

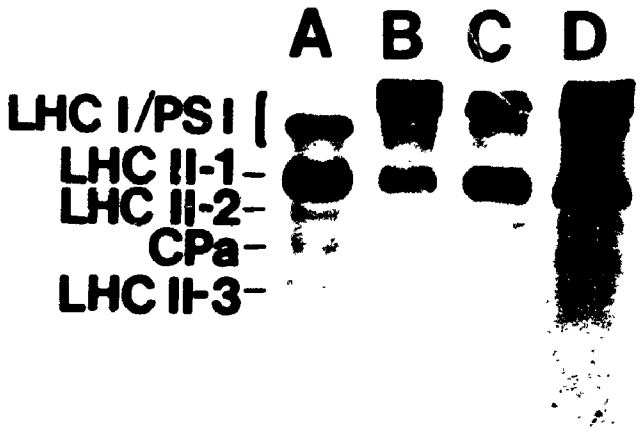

A
B
C
D
LHC I/PS I
LHC II-1
LHC II-2
CPa
LHC II-3

Figure 3.4 Densitometric Profiles of Chlorophyll-Protein Complexes separated from Cross-link Treated Thylakoids.

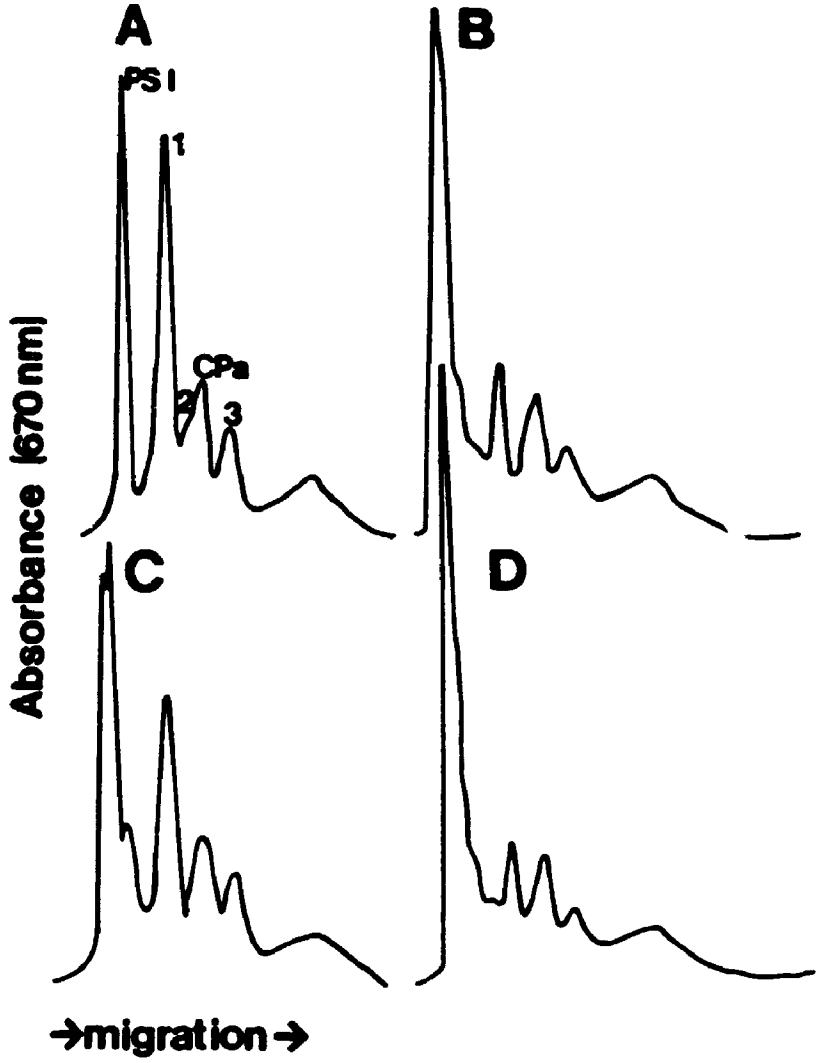
Lanes from gels similar to those presented in Figure 3.1 were scanned at 670 nm; peaks reflect chlorophyll content.

Fig. 3.4A Profile of chlorophyll-protein complexes from control thylakoids. The peaks are labelled; PS I - several closely spaced bands containing oligomeric associations between PS I and LHC I complexes; 1 - LHC II-1 band, equivalent to LHC IIb; 2 - LHC II-2 band, containing oligomers of LHC IIa and an LHC IIb subset; CPa - the PS II integral chlorophyll a protein complexes; 3 - LHC II-3 band, containing monomers of LHC IIa and LHC IIc.

Fig. 3.4B Chlorophyll-protein complexes from thylakoids cross-linked while stacked. Note major reduction of LHC II-1 peak.

Fig. 3.4C Complexes from thylakoids cross-linked while unstacked. Note that PS I/LHC I peak is shifted to larger size (to left), but LHC II-1 peak is similar to control.

Fig. 3.4D Complexes from thylakoids cross-linked after unstacking, thorough washing and restacking by addition of cations. Profile resembles that of stacked membranes.


A
B
C
D
PS I
1
CPa
2
3
Absorbance (670 nm)
→migration→

In thylakoids cross-linked while stacked (Figs. 3.3B, 3.4B), the abundance of the LHC II-1 band is greatly reduced, while the LHC II-2, 3 and CPa bands are somewhat less abundant. Concomitantly, cross-linked products appear above the LHC II-1 band, extending to the top of the lane.

These effects are largely reversed in thylakoids unstacked by EDTA washing (Figs. 3.3C, 3.4C) or trypsin treatment (Fig. 3.3D). Hence, the PS II related linkages occur largely between the complexes of adjacent appressed membranes, while the PS I related linkages occur within the plane of the membrane.

The complexes from membranes cross-linked after EDTA unstacking and subsequent restacking resemble those from stacked membranes (Fig. 3.4D). This provides evidence that the linkages observed in the stacked membranes are directly between the complexes of appressed membranes, and are not mediated by extrinsic factors.

Two-dimensional electrophoresis was employed to identify the polypeptide constituents of the cross-linked complexes. Lanes from gels similar to those presented in Fig. 3.3 were excised and treated with a denaturant solution containing ß-mercaptoethanol to cleave cross-links. The denatured polypeptides were then separated from the gel strips by SDS-PAGE, with the results presented in Figure 3.5.

Most polypeptide constituents of the cross-linked products could be identified based on the membrane fractionation and immunological experiments presented in Chapter Two, although several were identified only as PS II related. The cross-linked products are described in the legend to Fig. 3.5. Note that residual linkage of LHC IIb polypeptides occurs in unstacked thylakoids (Figs. 3.5C, D). However, the LHC IIa/LHC IIb/PS II products appear only in stacked or restacked membranes (Figs. 3.5B, E).

In stacked membranes, the staining intensity indicates an approximate 1:1 ratio between each cross-linked PS II apoprotein and the LHC IIa apoprotein, while the LHC IIb cross-linked product is more abundant. However, a cross-link between LHC IIa and an LHC IIb polypeptide would shift the migration of the entire oligomeric LHC IIb complex. This contributes to the apparent excess of LHC IIb polypeptides in the cross-linked products from stacked membranes (Figs. 3.5B, E). In addition, some of this LHC IIb product is presumably equivalent to the LHC IIb-only product present in unstacked membranes.

Figure 3.5 Separation of Polypeptides from Chlorophyll-protein Complexes of Control and Cross-linked Thylakoids.

Changes between the control (Fig. 3.5A) and the others reflect cross-links between protein complexes.

3.5A Polypeptides from chlorophyll-protein complexes of control thylakoids. Some polypeptides identified; also note the coupling factor polypeptide at 60 kDa.

3.5B Thylakoids cross-linked while stacked. Note extensive cross-linking of LHC IIa and b polypeptides into large oligomeric structures, to the left of the position of control LHC IIb polypeptides (middle bracket). PS II CPa polypeptides and other PS II polypeptides are also cross-linked into large structures (upper bracket). The PS I and LHC I complexes are cross-linked into large oligomers. CFo is also cross-linked, at 60 kDa.

3.5C Thylakoids cross-linked after unstacking by EDTA washing. The PS I/LHC I and CFo products are present, but LHC II/PS II cross-linking is eliminated; low levels of LHC IIb cross-linked product remain.

3.5D Thylakoids cross-linked after unstacking by trypsin treatment. Cross-linking pattern resembles 3.3C, note that LHC II polypeptides are at lower molecular mass because of cleavage by trypsin (see Fig. 3.1)

3.5E Thylakoids unstacked by extensive EDTA washing, restacked by resuspension with cations, then cross-linked. Cross-linking pattern resembles 3.3B.


A
B
C
D
E
97.4-
66.2-
45-
31-
21.5-
14.4-
PSI
PSII
LHCIIa
b
c
LHCI
SDS-PAGE
LINKS CLEAVED

To complement the identification of cross-linked complexes, cross-linked polypeptides were separated directly from thylakoids. Figure 3.6 presents silver stained second dimension gels of polypeptides derived from DSP treated stacked (Fig. 3.6A) and unstacked (Fig. 3.6B) maize mesophyll thylakoids. The polypeptides were first separated by denaturing SDS-PAGE under conditions which maintain cross-links if present. These gels were then treated with ß-mercaptoethanol, to cleave the disulphide bridge of the cross-linker. The polypeptides were subsequently separated in the second dimension by SDS-PAGE (Fig. 3.6). Polypeptide spots off the diagonal are derived from products cross-linked in the first dimension, since normal denatured polypeptides migrate approximately according to molecular mass in both dimensions. Control thylakoids not treated with DSP do not generate off-diagonal spots in this system (data not presented).

In membranes cross-linked while stacked (Fig. 3.6A), cross-linked products containing the apoproteins of LHC IIb, LHC IIa/LHC IIb and LHC IIa/LHC IIb/PS II are evident, marked with arrows. PS I/LHC I and LHC I products, and a coupling factor derived product are also present. In membranes cross-linked after unstacking by EDTA washing, most PS II related products are eliminated, leaving only an LHC IIb product. This supports the

results of Fig. 3.5, that some cross-linking of LHC IIb complexes occurs within the plane of the membrane, but that most PS II related linkages occur between appressed membranes. PS I and coupling factor related products remain, at the right side of the gel (Fig. 3.6B).

Cross-linking was also performed on isolated LHC IIb particles prepared by the detergent solubilization method (Steinback et al. 1982) as modified by Hayden et al. (1986). The results are presented in Figure 3.7, with the treatment as in Fig. 3.6. Use of this purified preparation allowed resolution of the 30/29 kDa doublet of LHC IIb, which is not usually resolved on separations of total thylakoid proteins. Fig. 3.7 shows that some linkage occurs within isolated LHC IIb particles. Since LHC IIb exists primarily as an oligomer (forming the LHC II-1 band on mildly-denaturing gels), intra-complex cross-linking would not be detected by the mildly-denaturing separations of Fig. 3.3.

Table 3.3 presents a collation of the cross-linked products found in stacked and unstacked thylakoid membranes.

Figure 3.6 Diagonal Gel Electrophoresis of Polypeptides from Thylakoids treated with Cross-linker (DSP).

The gels were silver stained following the two dimensional separation of polypeptides. Linkages were maintained in the first dimension, (right to left), and cleaved in the second dimension, top to bottom.

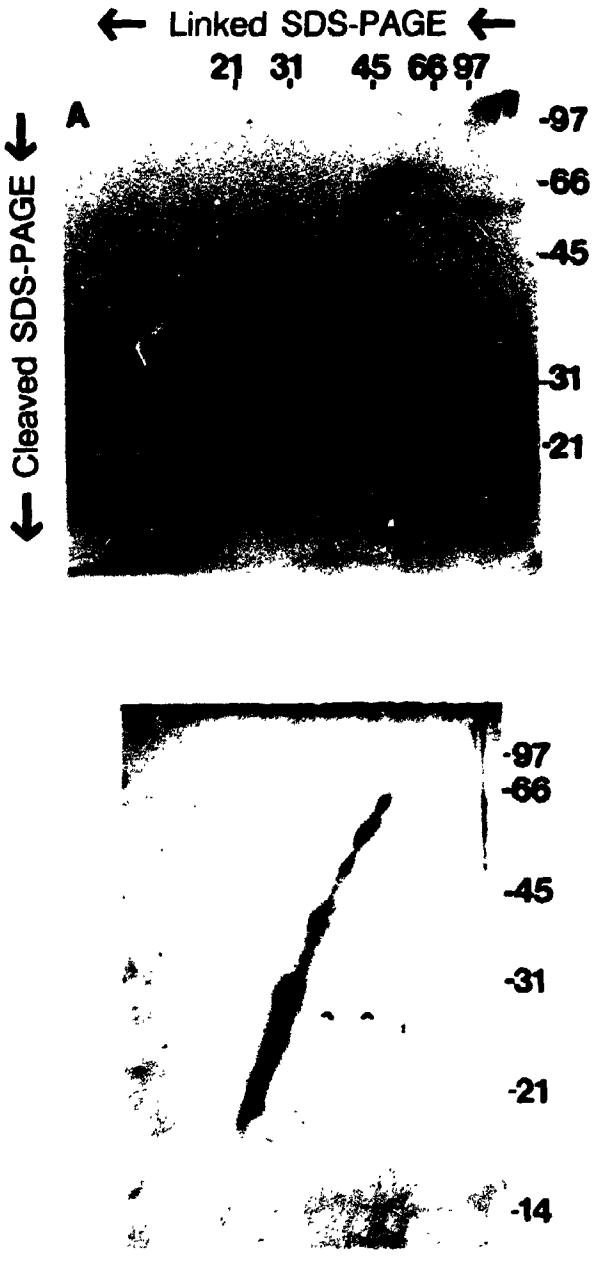
Fig. 3.6A Thylakoids were maintained in stacked configuration during the cross-linking treatment.

Fig. 3.6B Thylakoids were unstacked with two EDTA washes just before the cross-linking reaction, which was performed in the absence of Mg^{2+}.

3.4 Discussion

The light-scattering data (Table 3.1) are readily explained by cross-links between adjacent appressed thylakoids. Cross-linking of membranes while in the stacked configuration prevents subsequent EDTA induced unstacking, while cleavage of the cross-links in conjunction with EDTA washing results in unstacking. The presence of cross-links also prevents the unstacking which normally accompanies trypsin treatments of thylakoids, probably because the covalently bound linker molecule protects the exposed peptide regions from trypsin attack.

Specific inter-membrane associations are supported by the data from separations of chlorophyll-protein complexes and polypeptides from membranes treated with cross-linker (Figs. 3.3, 3.4, 3.5, 3.6). In membranes cross-linked while in a stacked configuration, LHC IIa is linked with LHC IIb (Figs. 1A, 3B). These linkages are eliminated by unstacking the membranes prior to DSP treatment, indicating that they occur between chlorophyll-protein complexes in adjacent membranes. Both of these LHC II complexes form linkage products with PS II apoproteins, in approximately 1:1 stoichiometry. LHC IIb by itself forms products in the plane of membrane, between neighbouring complexes (Fig. 3.5C) and perhaps within individual LHC IIb oligomers (Fig. 3.7). The cross-linking of trypsin treated membranes (Fig. 3.5D) indicates that some cross-


← Linked SDS-PAGE ←
21 31 45 66 97
A
← Cleaved SDS-PAGE
-97
-66
-45
-31
-21
-97
-66
-45
-31
-21
-14

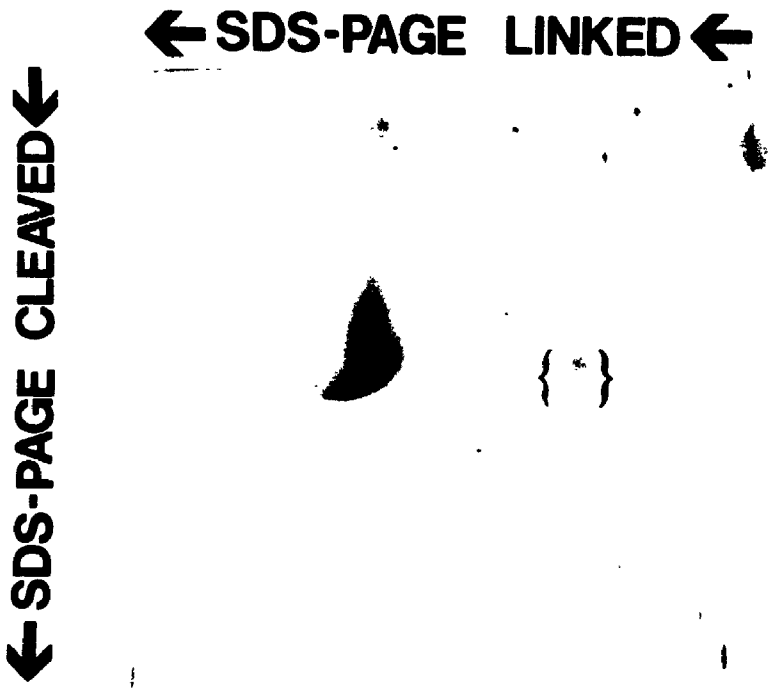


Figure 3.7 Diagonal Gel Electrophoresis of Polypeptides from LHC II Particles Treated with Cross-linker.

Preparation is as in Fig. 3.6 except that the cross-linked sample was LHC II particles prepared by detergent solubilization of thylakoid membranes. The cross-linked product is enclosed in brackets. It consists of a prominent doublet at ca. 30/29 kDa, along with lower levels of the other LHC IIb polypeptides.

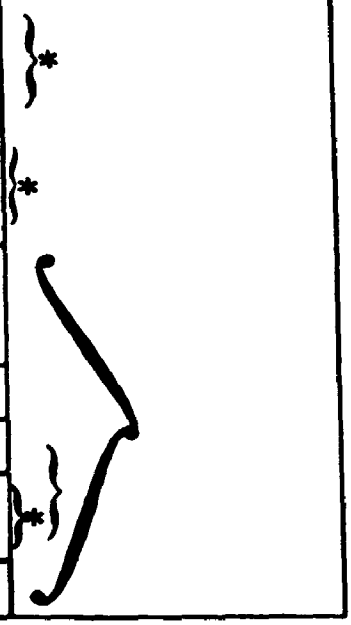
Apparent Molecular Mass (kDa)	Identity of Apoprotein	Crosslinked Products
65	PS I reaction centre	
24, 20	LHC I	
60	ATP synthase coupling factor	
51, 45	PS II integral antennae (CPa's)	
39	PS II related	
33	LHC IIa	
30.5, 30, 28.5, 26	LHC IIb	
17	PS II related	

Table 3.3 Summary of Molecular Masses and Identities of Cross-linked Polypeptides.

LHC II apoproteins were identified by immunoblotting; other apoproteins were identified based on molecular mass and membrane fractionation experiments. Constituents of each cross-linked product are encompassed by brackets in right-most column; nested brackets indicate that a given apoprotein is present in several cross-linked products. Products marked with an asterisk are present at some level in membranes cross-linked after unstacking; other products are present only in membranes cross-linked while stacked.

3.4 Discussion

The light-scattering data (Table 3.1) are readily explained by cross-links between adjacent appressed thylakoids. Cross-linking of membranes while in the stacked configuration prevents subsequent EDTA induced unstacking, while cleavage of the cross-links in conjunction with EDTA washing results in unstacking. The presence of cross-links also prevents the unstacking which normally accompanies trypsin treatments of thylakoids, probably because the covalently bound linker molecule protects the exposed peptide regions from trypsin attack.

Specific inter-membrane associations are supported by the data from separations of chlorophyll-protein complexes and polypeptides from membranes treated with cross-linker (Figs. 3.3, 3.4, 3.5, 3.6). In membranes cross-linked while in a stacked configuration, LHC IIa is linked with LHC IIb (Figs. 1A, 3B). These linkages are eliminated by unstacking the membranes prior to DSP treatment, indicating that they occur between chlorophyll-protein complexes in adjacent membranes. Both of these LHC II complexes form linkage products with PS II apoproteins, in approximately 1:1 stoichiometry. LHC IIb by itself forms products in the plane of membrane, between neighbouring complexes (Fig. 3.5C) and perhaps within individual LHC IIb oligomers (Fig. 3.7). The cross-linking of trypsin treated membranes (Fig. 3.5D) indicates that some cross-

links occur within the plane of the membrane in regions of LHC IIb complexes impervious to trypsin attack.

Taken together, the results demonstrate specific associations within 1.1 nm distances between LHC IIa, LHC IIb and PS II complexes of adjacent appressed thylakoids. However, most models of thylakoid function and structure consider associations between chlorophyll-protein complexes only within the plane of one membrane (eg. Fig. 1.1).

The fluorescence data (Table 3.2) indicate that the cross-linking treatment does not disrupt in situ organization of PS II, and therefore the cross-linked products reflect neighbouring complexes, and not artificial aggregations. Hence, PS II/LHC II organization should be considered in the context of appressed membranes and inter-membrane organization.

Several independent lines of inquiry have implicated thylakoid appression as important in energy distribution within the PS II related antenna system. PS II fluorescence induction kinetic analysis supports a lake model of PS II/LHC II functional organization (Webber and Baker, 1987). In this model, individual PS II centres are embedded in a common pigment bed, composed of LHC II units. Excitation energy captured by an LHC II unit is thus potentially available to numerous reaction centres. The reaction centres require a time interval (on average

approx. 500 μs) to trap an exciton, perform charge separation and return electron transport components to initial redox states (Hansson and Wydrzynski, 1990). During this period, the centre is closed to further excitation energy.

Fluorescence competes with charge separation as a deexcitation mechanism. After dark adaptation, all reaction centres are open for trapping; when light is applied, initial fluorescence yield is low. As photons are absorbed and their energy trapped, reaction centres are closed to further excitation, and fluorescence yield increases. If each PS II centre is assumed to possess a discrete, independent antenna, the fluorescence induction curve would be expected to be exponential, as each centre is independently closed by exciton capture. The sigmoidal nature of fluorescence induction indicates that multiple PS II centres share an antenna. This implies that at least some of the LHC II units are not specifically associated with any one PS II centre, but serve to connect the PS II centres.

In systems in which thylakoid appression is reduced, the sigmoidicity of the fluorescence induction curve is also reduced (Baker and Webber, 1987). The change is related specifically to membrane appression, and is not caused by changes in the overall ratio of LHC II units to reaction centres. Thus, appression of thylakoid membranes

is involved in formation of the common PS II antenna pigment bed, perhaps because PS II and LHC II units are more densely packed in appressed membranes relative to unappressed membranes. However, an alternative interpretation is that the common pigment bed extends over several appressed membranes, as well as within the plane of a given membrane.

Transfer of excitation energy between LHC II units is by an inductive coupling of favourably oriented pigment dipoles termed Forster transfer. This process is effective between chlorophylls over inter-molecular distances of 10 nm (Nobel, 1991). Our cross-linking studies show that LHC II complexes of adjacent membranes approach within 1.1 nm. An estimate of the size of individual LHC II chlorophyll-proteins can be made based on molecular mass and a typical value for protein partial specific volume. Such an estimation yields a diameter of about 4 nm for a single LHC II chlorophyll-protein. Hence, the chlorophylls of LHC II complexes of adjacent membranes are within the effective range of the Forster transfer mechanism. Furthermore, a speculative model of an individual LHC II chlorophyll-protein places several chlorophylls near the appressed membrane surface (Thornber et al., 1988) (Fig. 3.8). Based on this model, some chlorophylls are as close to those of the appressed membrane as to those within the plane of the membrane.

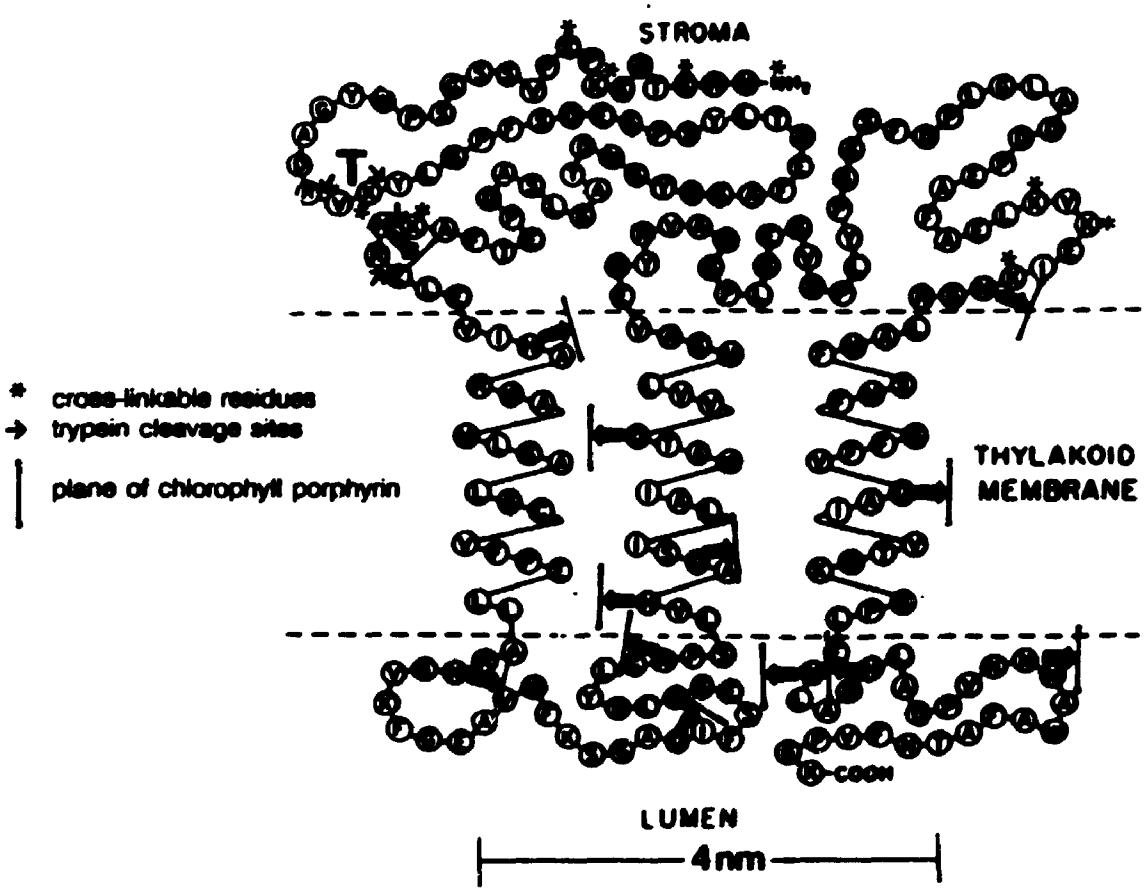


Figure 3.8 Model of Individual LHC II pigment-protein complex.

Modified from Thornber et al. (1988). Approximate diameter (4 nm) calculated based on estimated molecular mass. Polypeptide backbone of model based on computer modelling of hydropathy, alpha helical regions and probable folding. Most LHC II chlorophylls are known to be in an aprotic environment, inaccessible to acidic attack (Siefermann-Harms and Ninnemann, 1983).

A more recent model by Kuhlbrandt and Wang (1991), based on electron crystallography of crystalline LHC II is significantly different than the model depicted in Fig. 3.8, but also places several chlorophyll molecules near or at the membrane surface. The orientation of the porphyrin head groups of chlorophyll a in LHC II is believed to be predominantly perpendicular to the membrane plane (Kuhlbrandt and Wang, 1991).

A structural organization of LHC II and PS II units extending over appressed thylakoids is supported by series of circular dichroism studies (Garab et al., 1988a, b). The authors interpret their results to indicate the presence of helical arrays of LHC II units with embedded PS II centres, extending over hundreds of nanometers in three dimensions; clearly such a structure involves numerous appressed membranes.

Measurements of the fluorescence parameters of cross-linked thylakoids provide an approach to determine the extent of functional connections between LHC II complexes within the plane of individual thylakoids, and between adjacent thylakoids. Such measurements, in conjunction with treatments to remove divalent cations and treatments to induce state transitions, are currently in progress.

Chapter Four. Chilling Stress Perturbs LHC II Structure

4.1 Introduction

Previous work in this laboratory, in collaboration with the group of N.R. Baker, has shown that the 77 K fluorescence emission spectrum of isolated LHC II particles is perturbed following chilling stress (Hayden et al., 1986). Chilling also induces accumulation of a modified form of LHC IIa, hypothesized to be an incompletely processed precursor, and herein termed LHC IIa*. The accumulation of LHC IIa* is temperature and light dependent (Covello et al., 1988). LHC IIa* was found to be an integral thylakoid polypeptide which co-isolated with a membrane subfraction containing normal LHC IIa (Hayden et al., 1986). In isolated thylakoids at 20°C, degradation of LHC IIa* required Mg^{2+} and was halted by various protease inhibitors. However, the inhibition did not match the profiles of known classes of proteases (Hayden et al., 1988).

The synthesis of LHC II polypeptides is light-regulated through the phytochrome system (Apel, 1979; Simpson and Herrera-Estrella, 1990) and shows circadian rhythmicity (Kloppstech, 1985). The normal period of maximal synthesis is in the morning, with synthesis declining to near zero by late afternoon; when grown under constant light this pattern is retained for at least three

days. I reasoned that maximal accumulation of the LHC IIa* polypeptide would occur if chilling under light started just before the period of normal maximal synthesis, since the entire chilling period of 5-6 h would fall within the maximal synthesis period. Therefore, with some exceptions, I generally started chilling treatments at 3:00 AM, one hour before the normal lights on time.

Subsequent studies by others (D. Ort, pers. comm.; Anderson and Wilkins; 1989) led to a reassessment of the results. It is now apparent that chilling stress disrupts the circadian timing of protein synthesis. Hence, an early morning chilling treatment may give very different results from a mid-morning chilling treatment.

The synthesis and assembly of chlorophyll-protein complexes during chilling stress was monitored by accumulation of radiolabelled amino acid into chlorophyll-protein bands. A relative drop in synthesis or assembly of the oligomeric LHC IIb complex was detected.

The phosphorylation profile of LHC IIb polypeptides was then examined following chilling stresses. Changes in relative phosphorylation were detected, perhaps because of changes in the accessibility of the various LHC IIb polypeptides to the thylakoid protein kinase(s).

4.2 Materials and Methods

Unless otherwise specified, techniques were as described in Chapter Two.

4.2.1 Plant Growth and Temperature Treatment.

Maize seedlings were grown at 25 ^{o}C, 350 μmol photons m^{-2} s^{-1} (16 h day). For the low light chilling treatment, plants at the third or fourth leaf stage (10-12 days after planting) were shifted in the morning from control growth conditions to 5 ^{o}C, 250 μMol photons m^{-2} s^{-1} for six hours. The high light chilling treatment was the same but at 1500 μmol photons $m^{-2}s^{-1}$. Details of timing of chilling treatments are given in the results section.

4.2.2 ß-Octyl-glucopyranoside/SDS mildly-denaturing electrophoretic gels.

The solubilization procedure was according to Dunahay and Staehelin (1986) as modified by Hayden et al. (1988). Washed thylakoids were solubilized by addition of tris-maleate (2 mM) Glycerol (10 % w/v) ß-Octylglucopyranoside (Sigma) (8.8 mg/ml) and SDS (2.2 mg/ml) to a final ratio of 20:5:1 ß-octylglucopyranoside:SDS:chlorophyll, followed by incubation for 5 min on ice with stirring. Samples were briefly centrifuged, then aliquots of 30 μg chlorophyll (70 μl) were loaded on 8 X 12 X 0.3 cm 12% acrylamide gels containing tris-HCl (pH 9.2) with 5%

stacking gels containing tris-HCl (pH 6.1). Electrophoresis was performed using tris/glycine running buffer (pH 8.2) containing 0.1% SDS, at 6 °C for 3 h at a constant current of 25 mA.

4.2.3 Radiolabelling of newly synthesized polypeptides.

Attached leaves 3 or 4 were gently abraded to remove the cuticle, labelled with 0.37 MBq aliquots of ^{3}H-lysine (NEN) in 0.4 % Tween-20 (Fisher) solution, incubated for 1 h, and then harvested onto ice. Subsequent analysis of chlorophyll-protein complexes was as described in Chapter Two or in section 4.2.2.

The LHC II-1 and LHC II-3 bands were excised from deoxycholate/SDS gels, while the oligomeric LHC IIb, monomeric LHC IIa and monomeric LHC IIb/c bands were excised from ß-octylglucopyranoside/SDS gels. The acrylamide bands were bleached and dissolved using 30% hydrogen peroxide for 2 h at 60 °C. Incorporated radiolabel was measured by liquid scintillation.

4.2.4 Assessment of Fluorescence Parameters of Leaves.

Control or chill stressed attached leaves were dark adapted at the treatment temperature for 1.5 h. Fluorescence induction was assessed using a Plant Stress Meter Mk II (BioMonitor AB S.C.I., Sweden) (Oquist and Wass, 1988). Recorded parameters were the instantaneous

fluorescence level (Fo), maximal fluorescence (Fm), time at which fluorescence is at half the maximal level (t1/2) and the ratio of variable to maximal fluorescence (Fv/Fm).

4.2.5 Light-Activated Phosphorylation of LHC II Polypeptides.

Phosphorylation of thylakoid polypeptides from control, low light chilled and high light chilled maize was performed essentially according to Val and Baker (1989). Leaves were harvested from dark adapted plants. Thylakoids prepared as described in Chapter Two were suspended to a concentration of 150 μg chlorophyll/ml in 2 ml of 50 mM tricine (pH 7.8, NaOH) containing 5 mM $MgCl_2$, 10 mM NaCl and 5 mM NaF. ATP was added to the reaction mixture to a concentration of 200 or 400 μM, including 40 or 80 μCi of ^{32}P-ATP (Amersham, 5000 Ci/mmol). Phosphorylation was carried out at 25 ^{o}C and 250 μmol photons $m^{-2}s^{-1}$ for 15 minutes. The reaction was stopped by 50 fold dilution of the reaction mixture with ice cold 1 mM EDTA (pH 8.0) containing 5 mM NaF. NaF served to inhibit phosphatase activity (Bennett, 1983), while the EDTA served to unstack the thylakoids to facilitate subsequent solubilization for mildly-denaturing electrophoresis. The thylakoids were pelleted by centrifugation, resuspended in the same buffer; aliquots were then recentrifuged and frozen. Subsequent analysis

of ^{32}P labelled thylakoids was according to the mildly-denaturing and denaturing electrophoretic procedures described in Chapter Two. Phosphorylation of LHC IIb polypeptides was quantified by excision of bands from SDS-PAGE separations of polypeptides from LHC II-1 chlorophyll-protein bands. ^{32}P was measured by liquid scintillation counting of excised bands.

4.2.6 Reductant Activated Phosphorylation of Thylakoid Polypeptides.

Reductant activated phosphorylation was performed essentially according to Torti et al. (1984), based on an activation system described by Bennett (1979). The reaction mixture described in Section 4.2.5 was supplemented with 1 mM NADP, 5 mM glucose-6-phosphate, 5 μM ferredoxin and a large excess of glucose-6-phosphate dehydrogenase (2 μl/ml of Boehringer-Mannheim grade II suspension from yeast). The enzyme was added last to start the reaction, which was performed as described in Section 4.2.5, except that the reaction vial was foil wrapped to maintain darkness.

4.3 Results

In most chilling experiments, the accumulation of LHC IIa* polypeptide was much lower than the levels reported in previous work (Fig. 4.1B). On some occasions, virtually no LHC IIa* was present. Some isolations were performed using buffers which omitted $MgCl_2$, a cofactor in the degradation process (Hayden et al., 1988). Little improvement in yield of LHC IIa * resulted (data not presented). Occasional exceptions occurred, with relatively high yields (Fig. 4.1A), but a correlation between morning chilling and high yields was recognized only after experiments were complete.

Figure 4.1 also presents a comparison of polypeptide migration on a 10-18% acrylamide gradient gel (Fig. 4.1A) with migration on a 12% acrylamide gel (Fig. 4.1B). Note the shifts in the relative mobility of polypeptides (and hence their apparent molecular mass). Particularly evident is the reduced mobility of the LHC IIa and LHC IIa* polypeptides on the 12% gel (Fig. 4.1B) compared to the gradient (Fig. 4.1A). This shift largely accounts for the anomalously high apparent molecular mass of the LHC IIa polypeptide noted in Chapter Two. The reason for this shift is unknown; but the result is improved resolution of the LHC IIa polypeptide from the LHC IIb polypeptides.

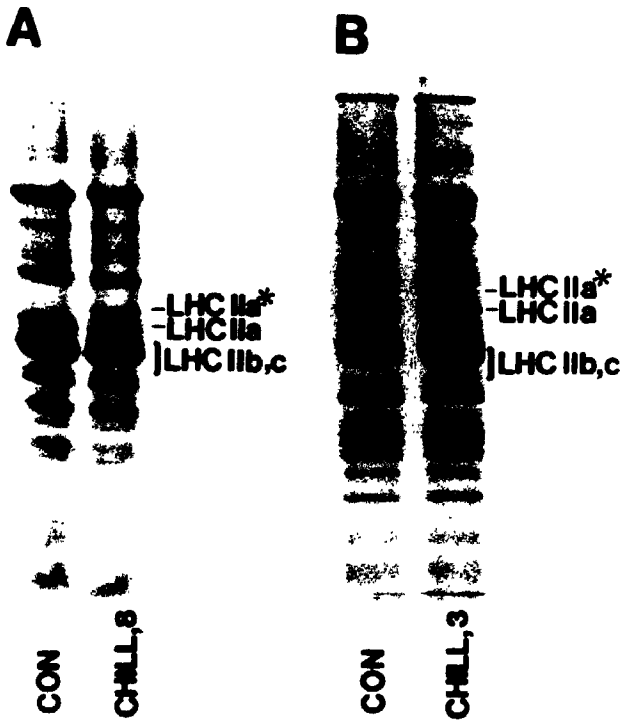


Figure 4.1 Accumulation of LHC IIa* chill-induced polypeptide varies with timing of chilling.

Polypeptides separated by SDS-PAGE from mesophyll thylakoids of control (CON) or chill-stressed (Chill,8; Chill,3) maize. Gels stained with Coomassie R-250.

Fig. 4.1A Comparison of control with low light chilling stress commencing at 8:00 AM, 4 h after lights on. Note accumulation of LHC IIa* to levels comparable to LHC IIa. 10-18% acrylamide gradient gel.

Fig. 4.1B As Fig. 4.1A, but low light chilling stress commencing at 3:00 AM, 1 h before normal lights on. A low level of LHC IIa* is present. 12% acrylamide gel.

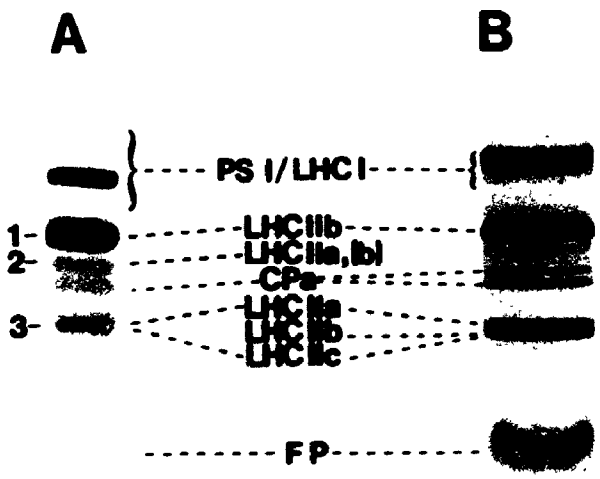
With the knowledge that aspects of LHC II polypeptide processing were disrupted by chilling stress, I sought to examine the assembly of LHC II complexes during chilling stress. For these investigations, two mildly-denaturing electrophoretic separation systems were used. The first (Fig. 4.1A) was the deoxycholate/SDS system described in Chapter Two.

The second mildly-denaturing system was based on a ß-octyl-glucopyranoside/SDS solubilization (Fig. 4.2B). This technique resolves 6 main bands: a PS I/LHC I band, oligomeric LHC IIb, the PS II-related chlorophyll a-protein complexes CP 47 and CP 43, monomeric LHC IIa, and monomeric band containing LHC IIb and c. A significant amount of free pigment is generated, on the order of 20-30% of chlorophyll. Compared to the deoxycholate method, PS II associated complexes are preferentially extracted and resolved. This improved resolution is at the expense of greatly increased dissociation of LHC IIb oligomers (data not presented) and higher levels of free pigment (Fig. 4.2B).

Figure 4.2 Comparison of methods for separation of chlorophyll-protein complexes.

Fig. 4.2A Deoxycholate/SDS mildly denaturing gel of chlorophyll-protein complexes from thylakoids. LHC II related bands are labelled at left, 1, 2 and 3. Composition of bands indicated at centre. LHC II-2 contains LHC IIa complexes and a subset of LHC IIb polypeptides, indicated by [b]. Note low level of free pigment.

Fig. 4.2B ß-Octylglucopyranoside/SDS mildly denaturing gel of chlorophyll-protein complexes from thylakoids. Composition of bands given at centre. This system resolves the two complexes comprising the CPa band of the Deoxycholate/SDS system, and two bands at the position of LHC II-3. No equivalent to the LHC II-2 band is present and high levels of free pigment are generated.


A
B
PS I/LHC I
1
2
3
LHCIIb
LHCIIa,bl
CPa
LHCIIa
LHCIIb
LHCIIc
FP

When using the ß-octylglucopyranoside/SDS system the incorporation of ^{3}H-lysine into the LHC IIa and LHC IIb/c bands was summed for more relevant comparison with the data from the deoxycholate/SDS system, which does not resolve the monomeric forms of the various LHC II complexes which migrate in the LHC II-3 region. However, the two systems are not equivalent since significantly more dissociation of LHC IIb occurs with the ß-octylglucopyranoside system. High levels of dissociated LHC IIb complexes co-migrate with LHC IIc polypeptides in the bottom band of the LHC II-3 region, obscuring the distinct LHC IIc polypeptides (data not presented).

The LHC II-2 band was omitted from analysis because it contains very low levels of LHC II polypeptides and is heavily contaminated with comigrating polypeptides (Fig. 2.4A). ^{3}H-lysine was chosen as the label molecule because the LHC II polypeptides are rich in lysine (Fig. 3.8), while the D1 protein of PS II, which is turned over very rapidly, lacks lysine (Kyle, 1986).

In Figures 4.3 and 4.4 incorporation of ^{3}H-lysine is presented as the ratio between LHC II-1 and LHC II-3. Absolute incorporation declined to approximately one-third control levels during low light chill treatments. Absolute incorporation also varied between experiments, partly because of differences in the timing of chilling, and partly because of differences in label application.

However, in every experiment, regardless of details of technique or timing of chilling, label incorporation into the LHC II-1 band declined relative to the LHC II-3 band during low light chilling, and recovered to control levels within 24 h after chilling stopped. Hence, the data from four separate experiments were pooled, to give the summary results of Figures 4.3 and 4.4. This was done with the recognition that discrepancies between experiments are reflected only in the error bars of this presentation. In reality, the timing of chilling may influence relative accumulation in LHC II complexes. Further work is required to elucidate the timing effect.

Both separation systems demonstrated similar changes in label accumulation following low light chilling. However, in neither case does the ratio of control label incorporation between LHC II-1 and LHC II-3 match the ratios of chlorophyll or polypeptide content between these bands. With the deoxycholate/SDS system, the ratio of chlorophyll content is approximately 3:1 LHC II-1:LHC II-3, while the ratio of control label incorporation is approximately 1.5:1 (Fig. 4.4). With the ß-octylglucopyranoside system, the ratio of chlorophyll content is approximately 2:1 LHC II-1:(LHC IIa+LHC IIb/c), while the ratio of control label incorporation is approximately 0.5:1.

Figure 4.3 Relative incorporation of ^{3}H-lysine into LHC II bands separated by ß-octylglucopyranoside/SDS method.

Chlorophyll-protein complexes separated from thylakoids as in Fig. 4.2B. The oligomeric LHC IIb band, the LHC IIa band and the monomeric LHC IIb/c band were excised from the gel and incorporated radioactivity measured by liquid scintillation. The graph plots radioactivity in the LHC II-1 band divided by the sum of radioactivity in the LHC IIa and LHC IIb/c bands. Four separate labelling experiments were performed, with low light chilling treatments commencing at times between 3:00 and 10:00 AM. Mean results from each experiment were summed and averaged, S.E. plotted as error bars (n=4). Relative labelling of the oligomeric LHC IIb band is reduced during the chilling treatment.

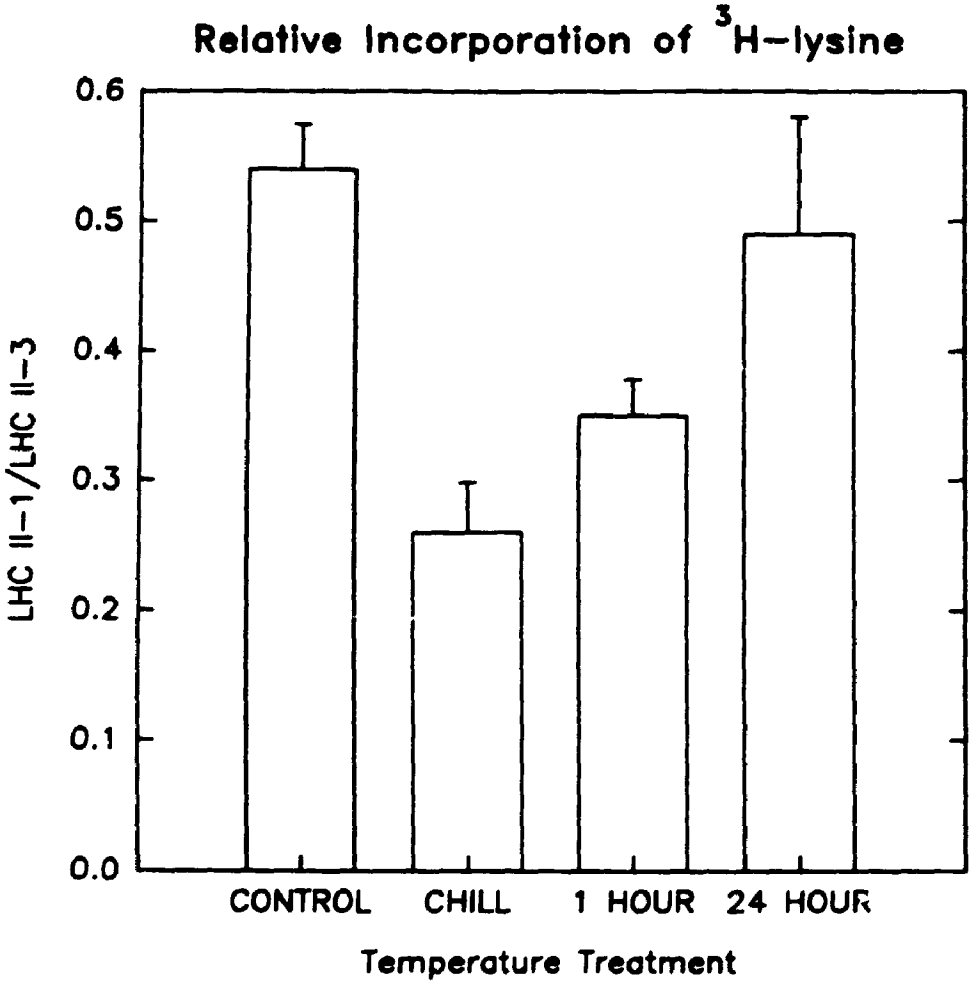
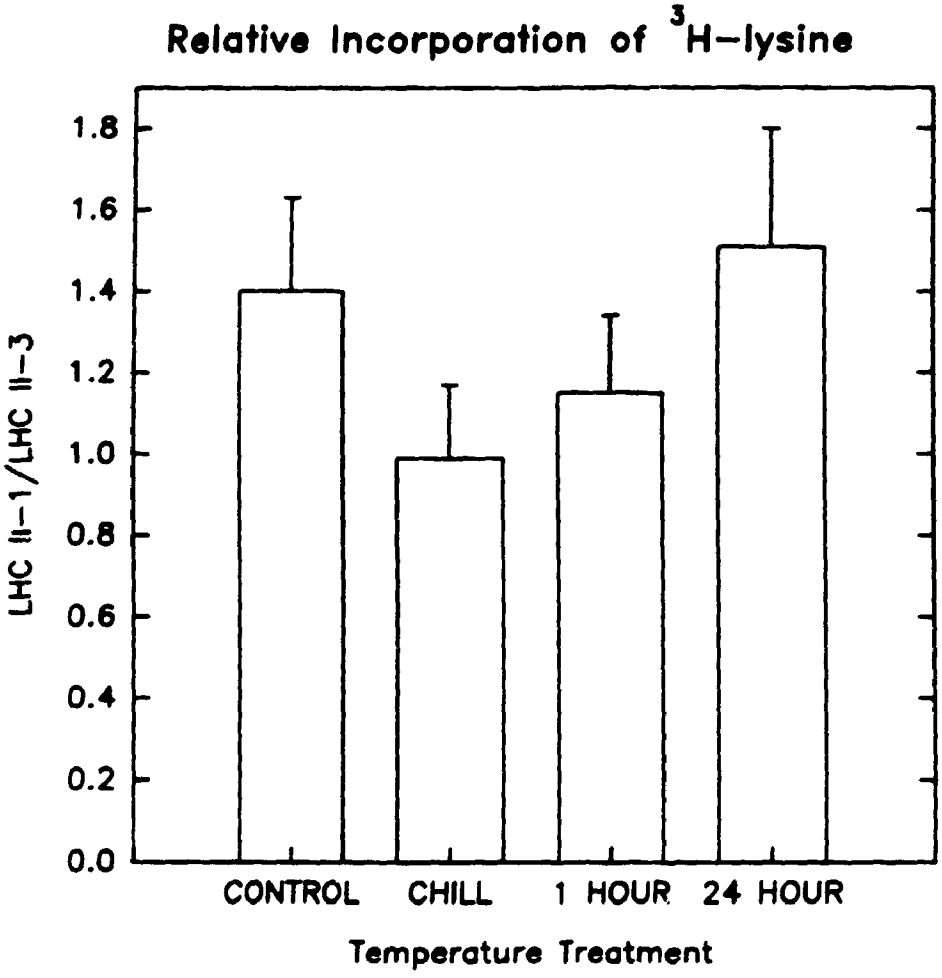

Relative Incorporation of ^{3}H-lysine
LHC II-1/LHC II-3
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CONTROL
CHILL
1 HOUR
24 HOUR
Temperature Treatment

Figure 4.4 Relative incorporation of ^{3}H-lysine into LHC II bands separated by Deoxycholate/SDS method.

Chlorophyll-protein complexes separated from thylakoids as in Fig. 4.2A. The oligomeric LHC II-1/LHC IIb band, and the monomeric LHC II-3 band were excised from the gel and incorporated radioactivity was measured by liquid scintillation. The graph plots radioactivity in the LHC II-1 band divided by the sum of radioactivity in the LHC IIa and LHC IIb/c bands. Four separate labelling low light experiments were performed, with chilling treatments commencing at times between 3:00 and 10:00 AM. Mean results from each experiment were summed and averaged, S.E. plotted as error bars (n=4). Relative labelling of the oligomeric LHC II-1 band is reduced during the chilling treatment. Note that relatively more label is incorporated into the oligomeric LHC II-1 band using this separation system, compared with ß-Octylglucopyranoside/SDS system used for Fig. 4.3.


Relative Incorporation of ^{3}H-lysine
LHC II-1/LHC II-3
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CONTROL
CHILL
1 HOUR
24 HOUR
Temperature Treatment

The fluorescence parameters of chill stressed leaves were assessed following chilling treatments commencing in the midmorning. Following chilling under low light, Fv/Fm was slightly depressed (Fig. 4.5). Fv/Fm is a measure of the photochemical efficiency of PS II (Baker, 1991; Krause and Weis, 1991;). Light dependent reductions in the photochemical efficiency of PS II are termed photoinhibition of PS II. Thus, the leaves subjected to low light chill were mildly photoinhibited. Following chilling under high light, Fv/Fm was depressed to 60% of control levels, indicating more severe photoinhibition. The instantaneous fluorescence level, Fo, is believed to reflect LHC II organization (Baker, 1991; Krause and Weis, 1991). Fo was somewhat reduced after both the low light and high light chilling treatments (Fig. 4.6), although variability among leaves was high.

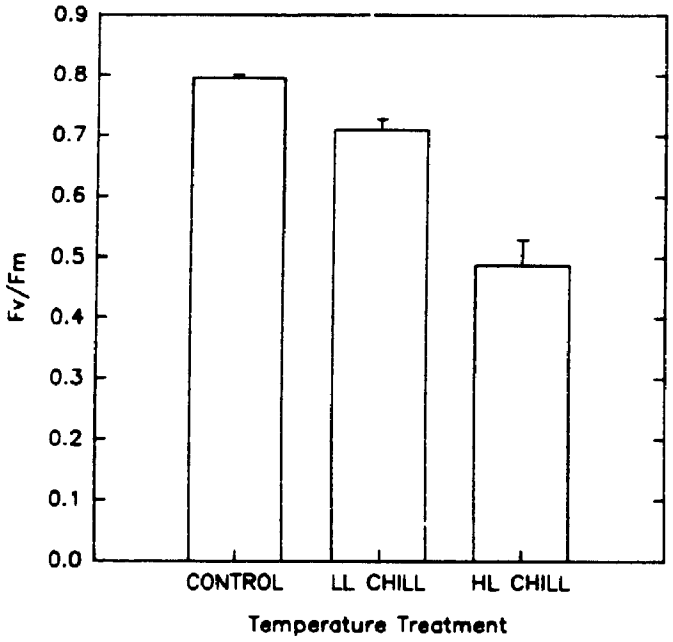


Figure 4.5 Chilling stress lowers Fv/Fm.

Chilling treatments as described in materials and methods, commencing at midmorning, 4-5 h after normal lights on. Fv/Fm recorded using Plant Stress Meter. The low light chill causes some depression of Fv/Fm, while the high light chill induces a larger drop. S.E. plotted as error bars, n=5.

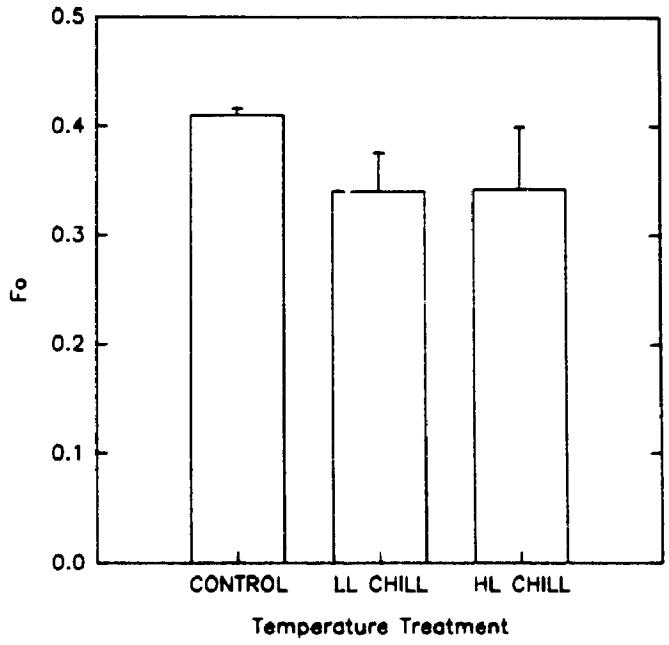


Figure 4.6 Fo is slightly affected by chilling stress.

Leaf samples the same as Fig. 4.5. Both chilling treatments induce a small drop in Fo level fluorescence, although leaf to leaf variability is high. S.E. plotted as error bars, n=5.

Phosphorylation of thylakoid membrane polypeptides was performed with gamma-^{32}P-ATP. Phosphorylation was followed by separation of the LHC II populations by mildly-denaturing electrophoresis. The LHC II bands were excised, and the constituent polypeptides separated by denaturing electrophoresis. ^{32}P labelling of the polypeptides was detected by autoradiography (Figs. 4.7, 4.8) and quantified by excision of the labelled LHC II polypeptide bands followed by liquid scintillation counting (Figs. 4.9, 4.10, 4.11).

Two methods are widely used to activate thylakoid protein kinases; reductant systems which drive the plastoquinone pool to a reduced state (Bennett, 1979; Torti et al., 1984) and light activation (Bennett, 1979; Val and Baker; 1989), which presumably mimics the in vivo activation process. Figure 4.7 presents an autoradiograph of SDS-PAGE separations of polypeptides from thylakoids phosphorylated using the two activation methods. The reductant system generates significantly more non-LHC II phosphorylation than the light-activated system (compare 4.7C with 4.7D), even if light activated phosphorylation proceeds for over 30 min. (data not presented). Furthermore, experiments using isoelectric focusing followed by SDS-PAGE to separate LHC II polypeptides indicated that light-activated phosphorylation resulted in labelling of a subset of LHC IIb polypeptides, while

reductant activated phosphorylation resulted in labelling of most LHC IIb polypeptides (data not presented). Thus, the reductant activated phosphorylation appears to result in more extensive phosphorylation than occurs *in vivo*, and hence does not accurately reflect physiological events. Therefore, light activated phosphorylation was chosen for assessing the effect of chilling on the phosphorylation of LHC II apoproteins.

Phosphorylated LHC II complexes were separated by mildly-denaturing electrophoresis using the deoxycholate/SDS system, followed by SDS-PAGE to resolve the polypeptides from each complex (as in Figure 2.2 Phosphorylated polypeptides were then detected by autoradiography (Fig. 4.8). Note the heavy phosphorylation of LHC IIb polypeptides in LHC II-3, even though staining shows that LHC IIb polypeptides are present at only very low levels (Fig. 2.2). Bassi et al. (1990) also note this intense phosphorylation of monomeric LHC IIb. The LHC IIa and LHC IIc apoproteins are not phosphorylated.

Figure 4.7 Comparison of light and reductant activation of thylakoid protein kinases.

Fig. 4.7A Stained SDS-PAGE of polypeptides separated from thylakoids phosphorylated using the reductant-activated system.

Fig. 4.7B As 4.7A except light-activated phosphorylation

Fig. 4.7C Molecular mass standards

Fig. 4.7D Autoradiograph of 4.7 A

Fig. 4.7E Autoradiograph of 4.7B

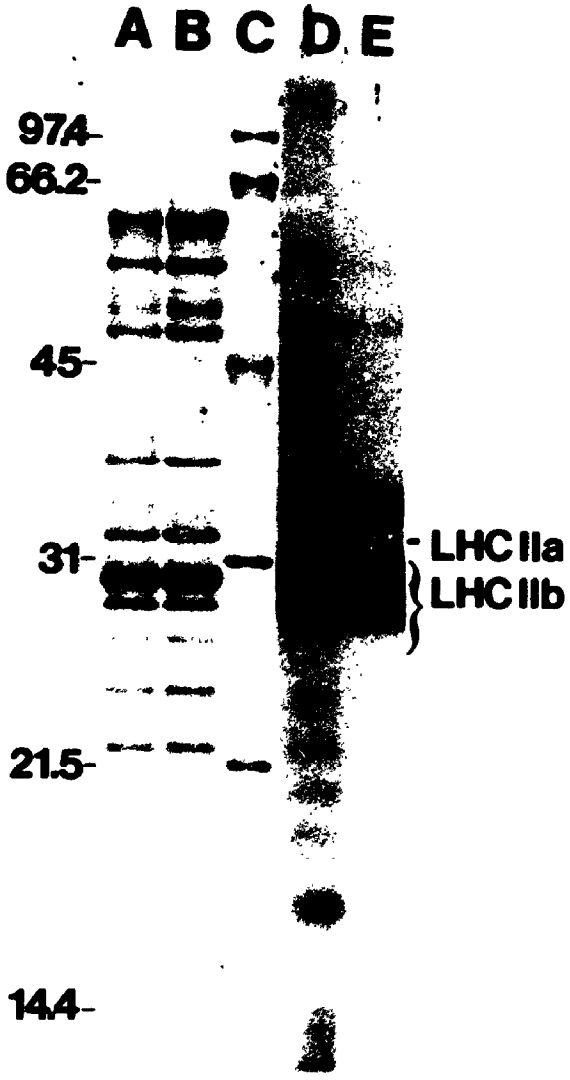

A B C D E
97.4-
66.2-
45-
31-
21.5-
14.4-
LHC IIa
LHC IIb

Figure 4.8 Autoradiographic detection of ^{32}P phosphorylation of LHC II proteins.

Fig. 4.8A presents the phosphorylation profile of the polypeptides separated from a lane of a mildly denaturing gel (see Fig. 2.2). Polypeptides from LHC II-1, 2 and 3 are noted from left to right. LHC IIb polypeptides are heavily labelled. Low levels of other polypeptides along the diagonal are labelled, particularly the D1/Q_B binding protein, but LHC IIa and the LHC IIc polypeptides are not labelled.

Fig. 4.8B presents the phosphorylation profile of the LHC IIb polypeptides separated from LHC II-1. A shorter exposure time than Fig. 4.8A shows the resolution of the 30/29 kDa doublet. Note that the 28 kDa polypeptide is most heavily phosphorylated, while the 26 kDa polypeptide is not phosphorylated.

Fig. 4.8C presents the profile of stained LHC IIb polypeptides from LHC II-1 for comparison.


^{32}P AUTORAD
A
B C
SDS-PAGE
1 2 3
30
29
28
26
kDa

For further analysis, the abundant, heavily phosphorylated LHC IIb polypeptides from the oligomeric LHC II-1 band were used. Gels similar to that presented in Fig. 4.8B were stained, and the three phosphorylated LHC IIb polypeptide bands were excised. ^{32}P was detected by liquid scintillation counting of the excised bands. The relative phosphorylation pattern of the three polypeptide bands was determined at two concentrations of ATP following chilling treatments. In Figures 4.9, 4.10 and 4.11 phosphorylation of each polypeptide band is presented relative to total phosphorylation of the three phosphorylated LHC IIb polypeptides.

When phosphorylation is performed at an ATP concentration of 200 μM, a chill induced decrease in relative phosphorylation of the 28 kDa LHC IIb polypeptide is apparent after an early morning low light chill treatment. The control pattern at this ATP concentration agrees with results of Larsson et al. (1985; 1987a; 1987b) in that the 28 kDa LHC IIb polypeptide is most heavily phosphorylated, in spite of the lower abundance of this polypeptide compared to the 30 and 29 kDa LHC II polypeptides.

When phosphorylation is performed at an ATP concentration of 400 μM (Figs. 4.10, 4.11) the control phosphorylation more closely reflects relative polypeptide abundance (Table 2.1). No change in the phosphorylation

profile is apparent following the early morning low light chilling treatment with phosphorylation at 400 μM ATP (Fig. 4.10).

Following mid morning chilling under low and high light, the relative phosphorylation of the 28 kDa LHC IIb polypeptide drops slightly (Fig. 4.11). In these experiments phosphorylation was only performed at 400 μM ATP. It is possible a more marked effect would be observed using 200 μM ATP.

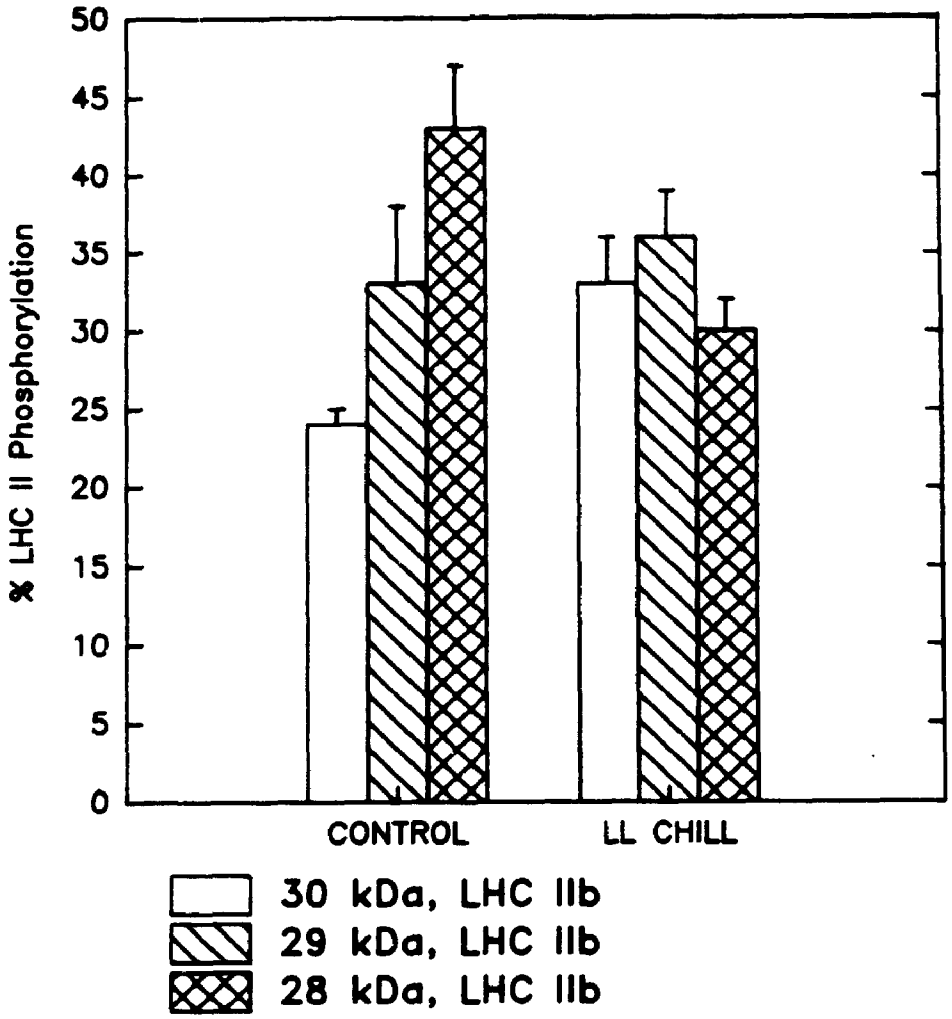


Figure 4.9 Relative phosphorylation of LHC IIb polypeptides following early morning chilling; 200 μM ATP.

Low light chill commencing at 3:00 AM, 1 h before normal lights on. Thylakoids isolated at 10 AM, after 1 h dark adaptation at treatment temperature. 200 μM ATP present in phosphorylation mixture. Note marked reduction in relative phosphorylation of the 28 kDa LHC IIb polypeptide following chilling stress. S.E. plotted as error bars, n=3.

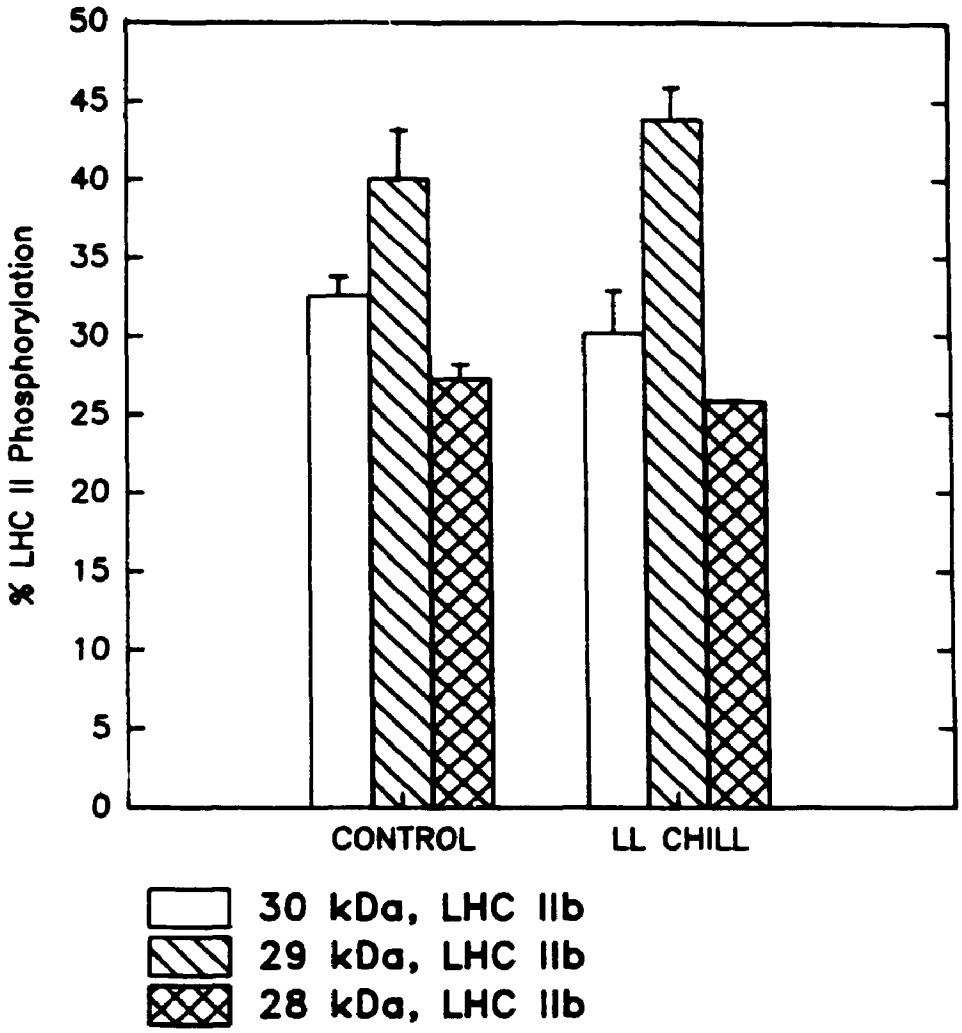


Figure 4.10 Relative phosphorylation of LHC IIb polypeptides following early morning chilling; 400 μM ATP.

Low light chill commencing at 3:00 AM, 1 h before normal lights on. Thylakoids isolated at 10 AM, after 1 h dark adaptation at treatment temperature. 400 μM ATP present in phosphorylation mixture. Control pattern is distinct from that of Fig. 4.9, and relative phosphorylation does not change following chilling. S.E. plotted as error bars, n=3.

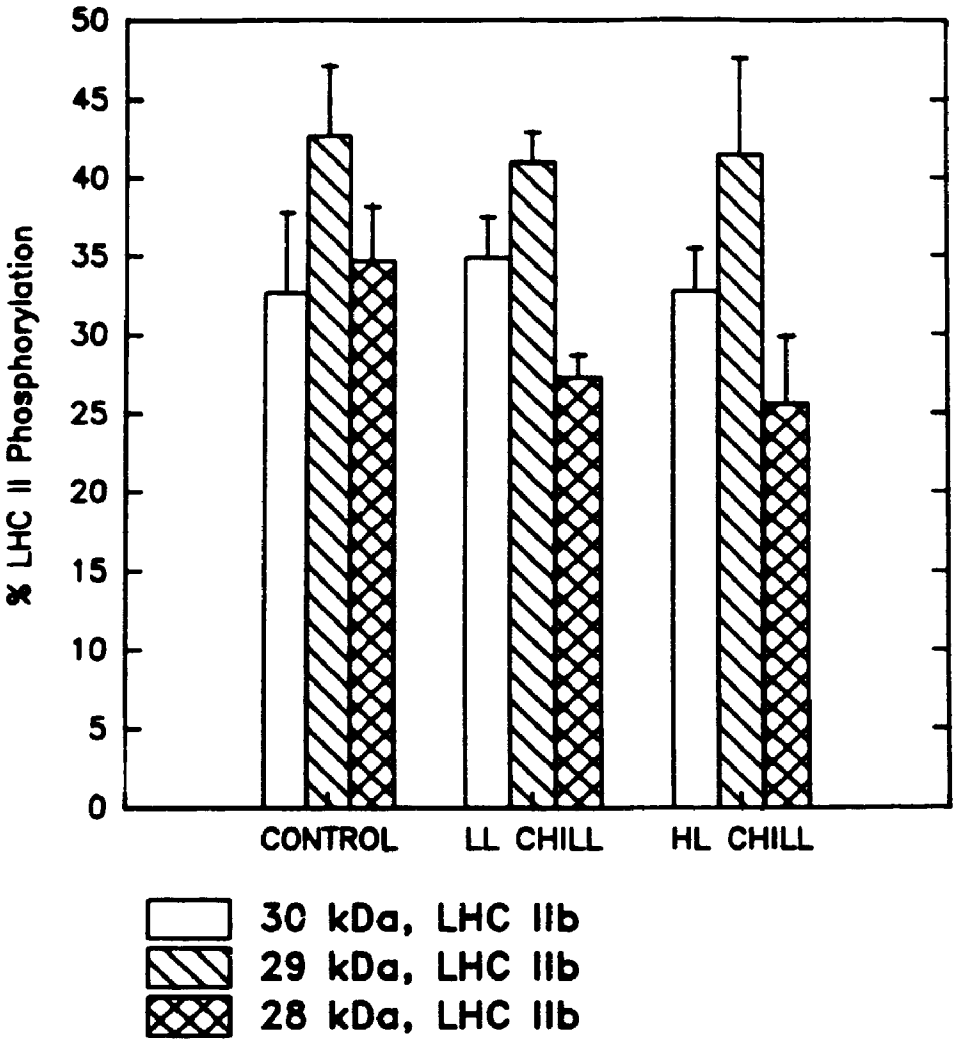


Figure 4.11 Relative phosphorylation of LHC IIb polypeptides following mid-morning chilling; 400 μM ATP.

Low or high light chill commencing at 9:00 AM, 5 h after normal lights on. Thylakoids isolated at 4 PM, after 1 h dark adaptation at treatment temperature. 400 μM ATP present in phosphorylation mixture. Control pattern is distinct from that of Fig. 4.9, with a slight drop in relative phosphorylation of the 28 kDa LHC IIb polypeptide following both chilling treatments. S.E. plotted as error bars, n=3.

4.4 Discussion

Recent work (D. Ort, pers. comm.; Anderson and Wilkins, 1989) has shown that in some chilling sensitive species such as maize, chilling stress effectively arrests progression of circadian timing of protein synthesis and carbon fixation. The normal cycles of protein synthesis are interrupted during the chilling stress.

Based on this information, the timing of chilling treatments was compared with the timing used in the earlier work on accumulation of the LHC IIa* polypeptide (Hayden et al., 1986; 1988; Covello et al., 1988). In all cases in the earlier work, chilling had commenced at 8-9:00 AM, 4-5 h after normal lights-on. In trials in which large accumulations of LHC IIa* were observed (Fig. 3.1A), chilling had commenced at mid-morning. In trials in which low levels of LHC IIa* accumulated, chilling commenced in early morning, before normal lights-on. This timing dependence of the effects of chilling stress will be pursued in future experimental work, initially using accumulation of LHC IIa* as a convenient marker.

Some of the variability in the amino acid incorporation studies probably results from differences in the timing of the chilling as well. However, the general consistency of the response in spite of different techniques and times of chilling indicates that the change is real; perhaps related to changes in lipid synthesis or

organization. No changes were detected in the stoichiometry of newly synthesized LHC IIb polypeptides (data not presented), but such experiments are technically difficult because of the low rates of protein synthesis during chilling treatments. Perhaps such analysis will be more feasible once the optimum time for chilling for maximum accumulation of LHC IIa* is established.

The discrepancy in the ratios of label accumulation between the gel systems probably relates to greatly increased dissociation of LHC IIb complexes when using the ß-octylglucopyranoside solubilization. It is possible that entire sub-populations are maintained in oligomeric form with the deoxycholate solubilization, but dissociated with the ß-octylglucopyranoside solubilization.

The discrepancy between control label net accumulation and relative chlorophyll content is less easy to understand. Label accumulation reflects newly synthesized polypeptides, which probably must assemble with chlorophyll molecules in a monomeric form before final assembly in multi-apoprotein oligomers. The nascent monomeric chlorophyll-apoproteins would co-migrate with the LHC II-3 band, before assembly into oligomers migrating at the LHC II-1 position.

Conversely, relative chlorophyll content between bands on gels reflects the total pool of complexes. Among the trials summed for Figs. 4.3 and 4.4, those with

longest labelling periods (5 h vs. 1 h) had the highest ratios of LHC II-1 : LHC II-3 label accumulation, although still not as high as the LHC II-1 : LHC II-3 chlorophyll ratios. It is possible that some fraction of the nascent LHC IIb apoproteins require considerable time for assembly into oligomeric forms. Yalovsky et al. (1990), using an *in vitro* system, found that up to 50 minutes after insertion of labelled LHC IIb precursors into thylakoids, most incorporated label was in the monomeric form of LHC IIb, although some was detected in the oligomeric form. Furthermore, reduced temperatures slowed the movement of precursor to grana regions, where assembly into pigmented complexes occurred.

Alternately, the turnover time of the LHC IIa and/or LHC IIc complexes of the LHC II-3 band may be shorter than the turnover time of the LHC IIb complexes. This would skew label accumulation towards the less abundant but shorter lived LHC IIa or LHC IIc complexes.

The scatter in the data may be reduced by further work with consistent timing of chilling to correspond to the period of maximal protein synthesis, with accumulation of LHC IIa* as a convenient marker.

A disruption of LHC II organization following chilling may cause the small drop in Fo of leaves following both chilling treatments (Fig. 4.6). This effect is apparently independent of the photoinhibitory

changes shown by Fv/Fm, since the drop in Fv/Fm was much more pronounced after the high light chill than after the low light chill. Hence, the drop in Fo is perhaps more related to a disruption of LHC II function reflected in the change in relative label incorporation during the low light chill treatments. These results agree with those of Hayden et al. (1986), who measured the fluorescence parameters of thylakoids isolated following a high light chilling treatment.

Phosphorylation of LHC IIb polypeptides is widely thought to be involved in the regulation of energy distribution between the photosystems through the mechanism of state transitions (Baker and Webber, 1987). However, evidence is accumulating that factors other than LHC IIb phosphorylation are involved in both state transitions and the regulation of energy distribution between the photosystems.

Havaux (1988) and Percival et al. (1987) showed that high light chilled maize is unable to perform state transitions, but Val and Baker (1989) and this work show that extensive LHC II phosphorylation occurs following high-light chilling. In two studies Islam (1989a; 1989b) has shown that LHC IIb phosphorylation using an ATP analogue does not induce the characteristic fluorescence changes indicative of a state transition. Conversely, use of another ATP analogue which is a poor substrate for LHC

IIb phosphorylation does result in the characteristic fluorescence changes. A proviso is that these studies used the reductant activated phosphorylation system, rather than light activated phosphorylation. Habash and Baker (1990) showed that phosphorylation treatments inhibit electron transport independent of LHC IIb phosphorylation. Baker (1991) postulates that a mechanism other than state transitions is required to explain the physiological role of LHC II phosphorylation.

Considering these studies, it is perhaps no longer valid to think of LHC II phosphorylation strictly in terms of the state transition mechanism. Furthermore, the state transition mechanism involves factors other than LHC II phosphorylation.

However, the pattern of phosphorylation at the lower ATP concentration (200 μM), and possibly the pattern of phosphorylation at the higher ATP concentration (400 μM) following the mid-morning chill, indicate that phosphorylation of LHC IIb polypeptides is somewhat disrupted by chilling stress. More work would be necessary to understand the effects of ATP concentration and timing of chilling. The chilling treatment may change the Km of the kinase for the LHC IIb polypeptides, perhaps by a conformational change rendering the 28 kDa LHC IIb polypeptide less accessible to the kinase. The effect is apparently wholly or partially overwhelmed by high

concentrations of ATP substrate, and may be more severe following mid-morning chilling than following early morning chilling.

The major impact of chilling timing on accumulation of LHC IIa* is readily demonstrated and assessed. The other chill-induced changes are more difficult to measure and interpret. Several attempts to detect changes in thylakoid membrane fluorescence emission spectra were inconclusive, perhaps partially because of timing problems. The changes in relative net accumulation of label between LHC II-1 and LHC II-3 probably reflect a disruption of assembly of LHC IIb oligomers. A chill-induced disruption of LHC IIb organization is supported by the small depression of Fo fluorescence, and possibly by the changes in relative phosphorylation of LHC IIb polypeptides following chilling stress.

Chapter Five. General Discussion and Summary

5.1 LHC II organization

The characterization of the deoxycholate/SDS gel system demonstrated that the compositions of the LHC II-1 and LHC II-3 bands are in accordance with the results obtained using two other mildly-denaturing separation systems described by Thornber et al. (1987, 1988) and Bassi et al. (1987a). The major LHC II-1 band contains LHC IIb in an oligomeric form. LHC II-3 contains the LHC IIa (CP 29) and LHC IIc (CP 26) complexes, and possibly the LHC IId (CP 24) complex, although this minor complex was not identified. Bassi et al. (1990) have proposed a model for the organization of LHC II and PS II in the plane of the thylakoid, based on the compositions of various submembrane fractions. They place LHC IIa in close proximity to the PS II core (Camm and Green, 1989; Henrysson et al., 1989). LHC IIc, along with LHC IId, are also thought to be close to the PS II core, although not as tightly associated as LHC IIa.

Many studies have shown that the LHC IIb complexes form a heterogeneous population. Bassi et al. (1990) postulate at least three functionally distinct subsets of LHC IIb. One is thought to undergo phosphorylation and be mobile, since it is enriched in stromal membranes following high light treatments to induce state

transitions. However, PS II centres damaged by high light are thought to migrate to stromal regions for reassembly (A.K. Mattoo, pers. comm.); hence the LHC IIb units may migrate along with the PS II centres.

A second LHC IIb subset may phosphorylate, but is apparently not mobile. The third is relatively closely associated with PS II centres in a monomeric form, and is depleted in the highly phosphorylated 28 kDa polypeptide.

The LHC II-2 band resolved on deoxycholate/SDS gels may be related to the third, non-mobile LHC IIb subpopulation. Furthermore, since the LHC IIb polypeptides exactly comigrate with the LHC IIa polypeptides in the LHC II-2 band, LHC IIa may be in a specific association with this LHC IIb subpopulation.

The separations of chlorophyll-protein complexes from membrane subfractions demonstrate that caution must be exercised when comparing green bands from thylakoids with those from subfractions. Although isolated LHC II particles generate three green bands migrating at the LHC II-1, 2 and 3 positions, all three are derived from LHC IIb complexes, have the same polypeptide constituents and apparently result from dissociation.

A parallel problem arises under conditions which generate dissociated LHC IIb complexes from thylakoids. This situation occurs with over-solubilization and some harsh membrane treatments. Furthermore, high levels of

dissociated LHC IIb appear to be normal in thylakoids from some species such as wheat, rye and barley, particularly when grown under cold-hardening conditions. Any of these phenomena may partially or wholly obscure the distinct compositions of the LHC II-2 and LHC II-3 bands.

The cross-linking study demonstrated specific interactions between LHC IIa and LHC IIb complexes of adjacent appressed thylakoids. LHC IIb complexes also cross-linked between thylakoids. Some PS II centres cross-linked with LHC II complexes of adjacent thylakoids, although only a fraction of the total PS II centres were so involved. Cross-linking within the plane of the membrane occurred among LHC IIb complexes, extensively between PS I centres and LHC I complexes and within the coupling factor complex. Fluorescence induction parameters indicate that photochemistry is not disrupted by cross-linking, and that cross-linking actually stabilizes thylakoids against time dependent deterioration.

The 1.1 nm length of the cross-linker, combined with models of LHC II complexes which show chlorophyll molecules near the membrane surface (Thornber et al., 1988; Kuhlbrandt and Wang, 1991), suggest that many light-harvesting chlorophylls of adjacent thylakoids are within distances allowing Forster energy transfer; this possibility is under investigation.

5.2 The effects of chilling on LHC II organization

Previous work (Hayden and Baker, 1990) had shown that chilling of maize in the light resulted in a disruption of LHC IIa processing, with generation of a higher molecular mass form, termed in this work LHC IIa*. Chilling stress also disrupts LHC II fluorescence emission, and high light chilling impairs photochemistry and the ability to perform state transitions.

The work presented in Chapter Four extends these observations. Chilling has been found to arrest progression of the circadian rhythm of carbon dioxide fixation in other species (Anderson and Wilkins, 1989). Furthermore, chilling of sensitive plants disengages the circadian timing of protein synthesis (D.R. Ort, personal communication). I noted that when chilling commenced in mid-morning, after normal lights-on, high levels of LHC IIa* accumulated. Conversely, early morning chilling, commencing before normal lights-on led to little or no accumulation. This phenomenon may explain some of the variability noted in the label incorporation and phosphorylation experiments. The effect of timing on chilling stress will be pursued in future work, as the accumulation of LHC IIa* provides a convenient marker.

Chilling under low light results in reduced relative accumulation of oligomeric LHC IIb, presumably by disrupting the assembly of oligomers. This notion is

supported by the results of Yalovsky et al. (1990) who found that *in vitro* assembly of LHC IIb precursors into complexes was slowed at low temperatures. The disruption of LHC IIb oligomer assembly may explain a small drop in the Fo fluorescence of both low light and high light chilled maize plants.

A discrepancy in the stoichiometry of label accumulation versus abundance of mature complexes may reflect long assembly times for oligmeric LHC IIb. Alternately, if the turnover rate of the monomeric LHC IIa and LHC IIc complexes is faster than that of oligomeric LHC IIb complexes, label accumulation would favour less abundant but shorter-lived complexes.

Both low light and high light chilling resulted in changes in the relative phosphorylation of LHC IIb polypeptides. Phosphorylation of the 28 kDa LHC IIb polypeptide was relatively reduced after early and mid-morning chilling treatments. This effect was apparently wholly (early morning chill) or partially (mid-morning chill) reversed by phosphorylation in the presence of higher ATP concentrations.

Debate continues over the role of phosphorylation of LHC IIb polypeptides under physiological conditions. It is now evident that the classic state transition mechanism (Bennett, 1983) is not mediated solely by LHC IIb phosphorylation, and that LHC IIb phosphorylation is not

explicable solely in terms of state transitions (Islam, 1989a, b; Baker, 1991). High light chilled maize is unable to perform state transitions, while low light chilled maize remains competent. However, the changes in LHC IIb phosphorylation appeared similar following both chilling treatments. Hence, it seems likely that the minor changes in relative phosphorylation following chilling are secondary effects of membrane disruption, rather than causative agents of the disruption of light-harvesting function.

5.3 A model of the LHC II system

Figure 5.1 presents a model of PS II and LHC II organization. The requisite data are drawn from three sources.

Kuhlbrandt and Wang (1991) describe a model of an individual LHC IIb complex, based on electron crystallography of purified complexes. They place chlorophyll molecules near the membrane surface, with a monomeric LHC IIb of dimensions 5 X 4 X 3 nm. Their model places the N-terminus at the stromal side of the thylakoid, with the carboxy terminus in the thylakoid lumen. The dimensions agree well with a theoretical calculation based on the apparent molecular mass of the monomer (Section 3.4). They organize these monomers into cylindrical trimeric structures, of diameter 7.3 nm and thickness 6 nm. Hence, the trimer protrudes approximately 1 nm from each side of the 4 nm membrane plane.

A review by Bassi et al. (1990) summarizes that group's view of LHC II/PS II organization in the plane of the membrane, based on various membrane subfractions with distinct but overlapping compositions (Bassi and Dainese, 1989). They postulate that two PS II centres with distinct antenna compositions form and organizational unit. Finally, a review by Thornber et al. (1988) summarizes the research of that group on the organization of LHC II complexes.

The interactions between complexes of adjacent membranes are postulated from this work. The composition of LHC II-2 supports the placement of LHC IIa and an LHC IIb monomer in close proximity in the model of Bassi et al. (1990).

Potential sites of chilling action include the accumulation of the abnormal LHC IIa*, with the possibility for disruption of LHC IIa function. Assembly of LHC IIb oligomers is probably disrupted, with concomitant perturbation of phosphorylation.

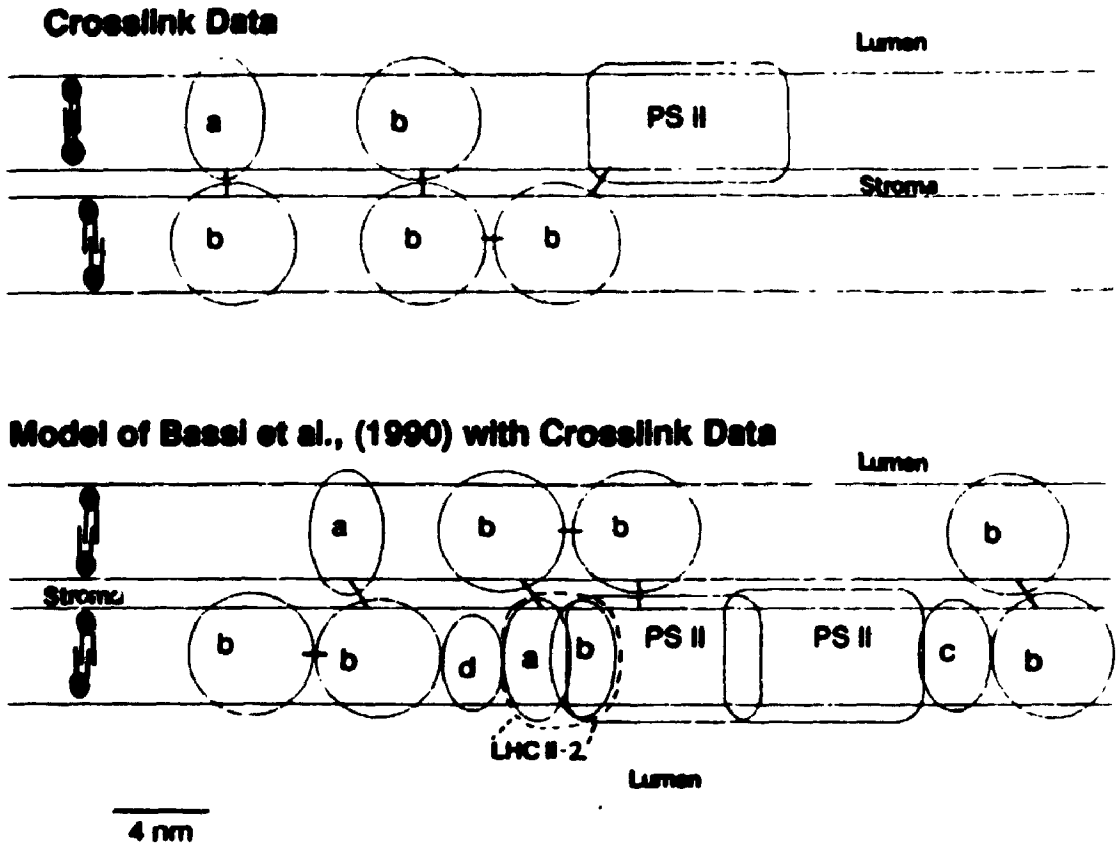


Figure 5.1 A Model of LHC II/PS II Organization

Cross-links between complexes indicated by dark dashes. Complexes are shown in side view, with the membrane bilayer indicated. LHC IIb unit superimposed on PS II centres is a monomer; others are trimers. Proposed components of LHC II-2 chlorophyll-protein band outlined. a - LHC IIa; b - LHC IIb; c - LHC IIc; d - LHC IId;

5.4 Concluding Remarks

The research described in this thesis is continuing through several directions of inquiry. The interaction between chilling stress and the circadian timing of protein synthesis is under investigation by J. Chernys and D.B. Hayden. This project should give insights into the disruption of circadian rhythms by chilling. In addition, the effects of chilling on thylakoids will be better characterized by consistent timing of chilling at periods leading to maximum disruption. The disruption in LHC IIa protein processing during chilling will be investigated through localization of the processing enzymes.

The effects of chilling on the organization of LHC IIb complexes are under investigation in a collaborative project between D. Bruce and J. Lazenby of Brock University and D. Campbell and D.B. Hayden of The University of Western Ontario. We have isolated thylakoids from control, high-light and low-light chilled maize. The Brock workers are measuring the circular dichroism spectra of these thylakoids. Comparison of the spectra reveal changes in pigment orientation, and hence changes in chlorophyll-protein complex organization. We have also separated LHC IIb complexes from thylakoids by mildly-denaturing electrophoresis; the circular dichroism spectra of these complexes will also be measured.

Fluorescence measurements of cross-linked thylakoids

are under way by D. Campbell and D.B. Hayden, to probe the functional interactions between LHC II and PS II units. Thylakoids will be treated with chelators to remove divalent cations, a treatment that normally disrupts fluorescence parameters by altering interactions between chlorophyll-protein complexes. We will then determine whether cross-linking protects against such disruption. Control and cross-linked thylakoids will be treated with ATP, which induces state transitions in the control situation and the effect of cross-linking will be assessed. PS II connectivity an functional antenna size will be measured in thylakoids following various treatments. Through these experiments we hope to gain further insight into the functional interactions between LHC II and PS II units. Preliminary results indicate that most energy transfer between LHC II and PS II occurs within the plane of the membrane. However, our preliminary data indicate that some energy transfer is dependent on thylakoid appression.

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