Chapter 8

Phosphorylation of Thylakoid Proteins

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Summary

Application of novel techniques for the characterization of in vivo protein phosphorylation has revealed sixteen distinct phosphorylation sites in ten integral and two peripheral proteins in photosynthetic thylakoid membranes. In addition to phosphorylation of the photosystem II (PS II) proteins D1, D2, CP43, and PsbH, and the light-harvesting antenna polypeptides LHCII and CP29, phosphorylation has been found in photosystem I (PS I) protein PsaD and in two recently identified proteins TSP9 and TMP14. The accumulated knowledge favors an involvement of reversible phosphorylation in adaptive stress responses and cellular signaling, but not in direct regulation of photosynthetic activities like electron transfer or oxygen evolution. Enhancement of PS II protein phosphorylation by abiotic stress maintains the integrity of PS II before it migrates to the stroma regions of the thylakoids where dephosphorylation and subsequent protein turnover take place. Specific dephosphorylation of the D1, D2, and CP43 polypeptides is performed by a heat shock-inducible protein phosphatase intrinsic to the thylakoid membrane. The phosphatase activity is regulated by the lumenal peptidyl-prolyl isomerase TLP40. This regulation may coordinate the protein folding activity of TLP40 in the lumen with the protein dephosphorylation at the opposite side of the thylakoid membrane. Reversible phosphorylation of LHCII in vivo is under complex redox and metabolic control and is probably involved in regulation of the size of the PS II antennae. Cold- and high light-induced phosphorylation

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of CP29 may facilitate photoprotective energy dissipation by changing PS II-LHCII interactions under stress conditions. Phosphorylation of PsaD protein could be involved in regulation of PS I stability and ferredoxin reduction by PS I. The light-induced phosphorylation of TSP9, followed by its release from thylakoids, is implicated in plant cell signaling. The exact physiological roles of the protein phosphorylation events in thylakoids should be revealed by studies with appropriate mutants of plants and algae.

I. Introduction

Light- and redox-induced protein phosphorylation in chloroplast membranes was discovered by Bennett in 1977 (Bennett, 1977). The prevailing hypothesis during much of the last two decades has been that reversible phosphorylation of LHCII is involved in state transitions, i.e. in balancing the distribution of absorbed light energy between the two photosystems, PS II and PS I (Bennett et al., ; 1980; Allen et al., 1981; Allen, 1992, 2002, 2003; Allen and Forsberg, 2001). This hypothesis has further evolved through studies of the redox sensing that connects electron transfer and protein kinase activity in photosynthetic membranes (Allen, 1992; Vener et al., 1998; Aro and Ohad, 2003). Studies of the molecular aspects of redox-dependent thylakoid protein phosphorylation have revealed it to be an extremely complex process. A multiple factor-dependent regulation of LHCII phosphorylation has been demonstrated. In addition to the requirement of plastoquinone reduction for activation of LHCII kinase (Allen et al., 1981), the Qo site of the cytochrome bf complex operates as the redox sensor for induction of the kinase activity (Vener et al., 1995; Vener et al., 1997; Zito et al., 1999). Light-induced changes in LHCII also affect its phosphorylation (Zer et al., 1999; Zer et al., 2003) as does the thiol redox state and the ferredoxin-thioredoxin system of chloroplasts (Carlberg et al., 1999; Rintamäki et al., 2000). The latter mechanism for control of LHCII phosphorylation was uncovered largely due to the finding of an initially surprising irradiance-dependence for the amount of phospho-LHCII in vivo (Rintamäki et al., 1997). In plant leaves, LHCII was found phosphorylated only at light intensities lower than those during normal plant growth (Rintamäki et al., 1997; Rintamäki and Aro, 2001). These findings and the measurements of the excitation energy transfer between the two photosystems in plant leaves have seriously questioned the role of LHCII phosphorylation in state transitions (Elich et al., 1997; Haldrup et al., 2001; Rintamäki and Aro, 2001). Accordingly, the physiological function of LHCII phosphorylation remains an open question.

More than 1100 genes encode for protein kinases in the genome of *Arabidopsis thaliana* (The *Arabidopsis* Genome Initiative, 2000). At present there are five candidate *Arabidopsis* genes for membrane protein kinases that could phosphorylate thylakoid proteins: a family of three TAK kinases (Snyders and Kohorn, 1999, 2001) and two kinases homologous to Stt7 kinase from *Chlamydomonas reinhardtii* (Depege et al., 2003). The mechanism for the redox regulation of these kinases is elusive as is the identity and number of other possible thylakoid protein kinases. With respect to the genes for protein phosphatases operating in thylakoid membranes, the situation is even less clear. No gene or protein sequence information has yet been published concerning the enzymes involved in dephosphorylation of thylakoid phosphoproteins. The additional challenge in elucidating the redox-dependent system for thylakoid protein phosphorylation lies in the fact that it requires the integrity of the membrane and the electron transfer chain for operation. Nevertheless, there has been steady progress in the decoding of the molecular mechanisms for redox regulation of thylakoid protein phosphorylation, which has been periodically reviewed (Vener et al., 1998; Ohad et al., 2001; Rintamäki and Aro, 2001; Aro and Ohad, 2003; Zer and Ohad, 2003).

Three recent groundbreaking developments provided unprecedented possibilities for revealing the functions of protein phosphorylation in the regulation of photosynthesis. Firstly, the sequencing of plant genomes allowed the full-power application of proteomic approaches to study protein modifications in these species. Secondly, plant lines with knockouts of individual genes became commercially available. Thirdly, new analytical techniques permitted the detection of protein modifications in vivo in variable

Abbreviations: D1 – photosystem II reaction center protein; D2 – photosystem II reaction center sister protein; of 29 kDa – minor chlorophyll *a*/*b*-binding protein of photosystem II; of 43 kDa – chlorophyll *a* binding protein of photosystem II; LHCII – light harvesting chlorophyll *a*/*b*-binding proteins of photosystem II; PsbH – 9 kDa *psbH* gene product; PS I – photosystem I; PS II – photosystem II; PPIase – peptidyl-prolyl cis-trans isomerase; PP2A – protein phosphatase 2A; TLP20 – thylakoid lumen PPIase of 20 kDa; TLP40 – thylakoid lumen PPIase of 40 kDa; TMP14 – thylakoid membrane phosphoprotein of 14 kDa; TSP9 – thylakoid soluble phosphoprotein of 9 kDa

environmental conditions. The latter development has already brought new insights in the field of thylakoid protein phosphorylation, showing that this process could be crucially involved in the response of the photosynthetic machinery to stress. In this chapter, I review phosphorylation of thylakoid proteins in relation to different physiological conditions in plants. Special attention is paid to the in vivo phosphorylation sites found in the individual thylakoid proteins, which form the basis for the studies of environmentally dependent changes in these distinct modifications.

II. Thylakoid Phosphoproteins

A. Detection Techniques

Five different approaches have been used for the detection of phosphorylation of thylakoid membrane proteins: 1) radioactive labeling; 2) detection of the shift in the electrophoretic mobility of individual proteins; 3) immunological analysis with phosphoamino acid antibodies; 4) measurement of the phosphorylation-induced increase in the mass of intact proteins by mass spectrometry; 5) identification and sequencing of phosphorylated peptides obtained after proteolytic degradation of proteins. The experimental protocols for determination of phosphoproteins in higher plant thylakoids by some of these methods have recently been published (Aro et al., 2004). It is important to keep in mind that the ultimate evidence for phosphorylation requires identification of the phosphorylated amino acid in the sequence of the corresponding protein. In this respect, the first four techniques listed above are limited by their inability to determine the residue(s) phosphorylated and should therefore be complemented by protein sequencing revealing the phosphorylation sites. All five approaches provided valuable information about the status of protein phosphorylation in thylakoids from different species. However, each of these methods has its own disadvantages that should be taken into account when evaluating the accumulated literature.

Detection of phosphoproteins labeled with radioactive isotopes ^{32}P or ^{33}P is the most sensitive and common technique for studies on protein phosphorylation. Radioactive labeling of proteins also provides a dependable avenue to localize the labeled phosphoamino acids (Michel and Bennett, 1987). Radio-labeled phosphate has been used in studies of thylakoid protein phosphorylation in organello (Bennett, 1977) as well as in vivo (Elich et al., 1992; Elich et al., 1993; Elich et al., 1997; Fleischmann et al., 1999; Fleischmann and Rochaix, 1999; Depege et al., 2003). Radio-labeled ATP has been widely used for phosphorylation of proteins in isolated thylakoids (Bhalla and Bennett, 1987; Cheng et al., 1994; Vener et al., 1995; Snyders and Kohorn, 1999). The limitations of radioactive labeling consist of uneven uptake of the label in different plant tissues, the large pools of endogenous phosphate, and the presence of pre-existing phosphorylation in the proteins.

The phosphorylated D1, D2, and CP43 proteins of PS II have been found to have a slightly slower electrophoretic mobility than the corresponding nonphosphorylated proteins (Callahan et al., 1990; de Vitry et al., 1991; Elich et al., 1992; Rintamäki et al., 1997). Use of this electrophoretic property in combination with specific antibodies against each individual protein has allowed studies of changes in protein phosphorylation status under different conditions (Elich et al., 1992; Rintamäki et al., 1997). This approach has mostly been used for studies of D1 protein phosphorylation. Successful application of this technique is limited to well-characterized proteins and requires the use of specific antibodies that cross-react exclusively with either phospho- or dephospho-forms of the protein.

Phosphothreonine antibodies have also been used in studies of thylakoid protein phosphorylation (Rintamäki et al., 1997; Rintamäki and Aro, 2001). This method has an advantage over labeling experiments in allowing the detection of endogenous levels of thylakoid protein phosphorylation in vivo under particular environmental conditions. It is also suitable for studies on the regulation of thylakoid protein phosphorylation using intact chloroplasts, isolated thylakoid membranes, or membrane subfractions. However, serious attention should be paid to the fact that the immunoreactivity with different commercial antibodies differs between various phosphoproteins, and the linearity of the immunoreactivity should be monitored in each case (Rintamäki et al., 1997). Moreover, the use of phosphothreonine antibodies is rather limited to the detection of only four or five major thylakoid phosphoproteins (Aro et al., 2004).

Recent developments in proteomics and mass spectrometry have allowed the detection of phosphoproteins by measuring the phosphorylation-induced change in the mass of intact proteins. Analysis of the chloroplast grana proteome by liquid chromatography mass spectrometry (LCMS) has confirmed the phosphorylation of full-length D1, D2, CP43, PsbH, and two LHCII polypeptides (Gomez et al., 2002). Three different phosphorylated forms of the recently characterized thylakoid-associated chloroplast phosphoprotein

TSP9 have also been detected by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Carlberg et al., 2003). Measurement of the intact protein mass provides strong indication for protein modification, but requires complementary mapping of the phosphorylation site(s) in the protein.

Sequencing of phosphopeptides obtained by proteolysis of phosphorylated thylakoid proteins has provided most of the presently available information on the exact phosphorylation sites (Michel and Bennett, 1987; Michel et al., 1988, 1991; Vener et al., 2001; Carlberg et al., 2003; Hansson and Vener, 2003). Conventional chemical sequencing of phosphopeptides corresponding to the N-termini of thylakoid proteins has been rather limited (Michel and Bennett, 1987) because many of these peptides are N-terminally blocked (Michel et al., 1988, 1991; Vener et al., 2001; Hansson and Vener, 2003; Turkina et al., 2004). Application of mass spectrometry has been most efficient for mapping of the phosphorylation sites in thylakoid proteins. Mass spectrometry analysis can now be combined with plant genomic sequence information. The sequencing of phosphopeptides selected from a complex peptide mixture identifies both the site of the phosphorylation and the parent phosphoprotein. This has led to direct analyses of the phosphopeptides from thylakoid membranes without prior isolation of individual proteins or protein complexes (Vener et al., 2001; Hansson and Vener, 2003; Turkina et al., 2004). Identification of nine in vivo protein phosphorylation sites in the thylakoids of *Arabidopsis thaliana* has been achieved by "shaving" of the surface-exposed domains of thylakoid proteins with trypsin, following enrichment of the phosphopeptides by immobilized metal affinity chromatography and their subsequent sequencing using mass spectrometry (Vener et al., 2001; Hansson and Vener, 2003).

B. Identified Thylakoid Phosphoproteins

A different number of phosphoproteins has been detected in thylakoids from different plants (Bennett, 1977; Cheng et al., 1994; Vener et al., 1995; Rintamäki et al., 1997; Gomez et al., 2002; Hansson and Vener, 2003) and algal species (de Vitry et al., 1991; Fleischmann et al., 1999; Depege et al., 2003). Decisive proof for phosphorylation of any protein should include the determination of the phosphoamino acid in the protein sequence. This task has been accomplished for the major thylakoid phosphoproteins. A summary of the known phosphorylation sites in thylakoids is presented in Table 1. These principal biochemical data form the basis for addressing the questions on the physiological significance of the distinct phosphorylation events by means of molecular biology and reversed genetics. Accordingly, I make a classification of these phosphorylated proteins and then analyze the published information on each of these phosphoproteins in the following parts of this chapter.

In addition to the proteins with the mapped phosphorylation sites (Table 1), another set of proteins has been advocated to undergo phosphorylation in the photosynthetic membranes. The subunit V of cytochrome bf complex in *Chlamydomonas reinhardtii* has been reported to be reversibly phosphorylated upon state transitions (Hamel et al., 2000). It was radio-labeled in the alga incubated with radioactive phosphate and identified as a 15.2-kDa polypeptide encoded by the nuclear gene *PETO* (Hamel et al., 2000; Finazzi et al., 2001). Phosphorylation of subunit V was proposed to be involved in signal transduction during redox-controlled short and long term adaptation of the photosynthetic apparatus in eukaryotes (Hamel et al., 2000; Finazzi et al., 2001).

The thylakoid protein kinases (TAKs), represented by three family members in *A. thaliana,* have been shown phosphorylated by immunoblotting with anti-phosphothreonine and anti-phosphoserine antisera (Snyders and Kohorn, 1999). The thylakoid kinase Stt7 in *C. reinhardtii* has also been suggested to undergo in vivo phosphorylation (Depege et al., 2003). This proposal was based on indirect evidence showing a shift in the electrophoretic mobility of the protein after treatment of thylakoids with a phosphatase (Depege et al., 2003). TAKs and Stt7 have been implied in phosphorylation of LHCII and state transitions (Snyders and Kohorn, 1999, 2001; Depege et al., 2003). Phosphorylation of other thylakoid proteins, including LHCII, was reduced in antisense *TAK1 Arabidopsis* mutants (Snyders and Kohorn, 2001). The activity of all of these protein kinases and their regulation was suggested to be part of a possible cascade of redox-controlled thylakoid protein phosphorylation (Snyders and Kohorn, 1999, 2001; Depege et al., 2003). Thus, elucidation of the phosphorylation sites in these enzymes and of the distinct protein kinases involved in modification of each of them may provide a key to understanding the complex regulatory network in thylakoid and chloroplast signal transduction. The low abundance of these enzymes is a major challenge in characterization of their posttranslational modifications.

Application of LCMS for measurement of masses of intact integral membrane proteins from pea thylakoids gave a strong indication for phosphorylation of the

Table 1. **Phosphorylation sites in thylakoid proteins**. A single letter amino acid code is used in the sequences with the low case t and s designating phosphorylated threonine and serine, correspondingly. *Ac*- designates the N-terminal acetylation of the peptides.

Protein	Species	Phosphopeptide sequence	Reference
D1	Spinach	*Ac*-tAILGRR	(Michel et al., 1988)
	Arabidopsis	*Ac*-tAILER	(Vener et al., 2001)
D2	Spinach	*Ac*-tIAVGK	(Michel et al., 1988)
	Arabidopsis	*Ac*-tIALGK	(Vener et al., 2001)
CP43	Spinach	*Ac*-tLFNGTLTLAGR	(Michel et al., 1988)
	Arabidopsis	*Ac*-tLFNGTLALAGR	(Vener et al., 2001)
PsbH	Spinach	AtGTVESSSR	(Michel and Bennett, 1987)
	Arabidopsis	AtQTVEDSSR	(Vener et al., 2001)
	ArabidopsisArabidopsis	AtQtVEDSSR	(Vener et al., 2001)
LHCII	SpinachSpinach	*Ac*-RKtAGKPKT	(Michel et al., 1991)
	Spinach	*Ac*-RKtAGKPKN	(Michel et al., 1991)
	Spinach	*Ac*-RKsAGKPKN	(Michel et al., 1991)
	Spinach	*Ac*-RRtVKSAPQ	(Michel et al., 1991)
	Arabidopsis	*Ac*-RKtVAKPK	(Vener et al., 2001)
CP29	Maize	AGGIItRFESSE	(Testi et al., 1996)
	Arabidopsis	*Ac*-RFGFGtK	(Hansson and Vener, 2003)
	C. reinhardtii	*Ac*-VFKFPtPPGTQK	(Turkina et al., 2004)
PsaD	*Arabidopsis*	EKtDSSAAAAAAPATK	(Hansson and Vener, 2003)
TMP14	*Arabidopsis*	ATtEVGEAPATTTEAETTE	(Hansson and Vener, 2003)
TSP9	Spinach	GGtTSGK	(Carlberg et al., 2003)
	Spinach	KGtVSIPSK	(Carlberg et al., 2003)
	Spinach	SSGStSGK	(Carlberg et al., 2003)

PsbT protein (Gomez et al., 2002). In this study, the mature PsbT protein of PS II was assigned a mass of 4,032 Da. The putative phosphorylated form of the protein was found to have a mass increased by 80 Da, corresponding to incorporation of a phosphoryl group in the protein (Gomez et al., 2002). Detection of this previously unidentified thylakoid phosphoprotein shows the power of intact protein analysis by LCMS. The site of phosphorylation in PsbT, as well as the role of its phosphorylation in the function of PS II remains to be determined.

Tyrosine phosphorylation of a set of thylakoid proteins including LHCII has been reported (Tullberg et al., 1998). This suggestion was based mainly on the results of immunoblotting analysis with an anti-phosphotyrosine antibody. However, the recent use of antibodies against phosphotyrosine did not reveal any change in the immunoreactivity of thylakoid proteins under changing environmental conditions or in response to redox state of chloroplasts (Rintamäki and Aro, 2001). Tyrosine phosphorylation of thylakoid proteins has not been confirmed by phosphoamino acid analysis or phosphopeptide sequencing. Thus, the specificity of cross reaction of anti-phosphotyrosine antibodies with thylakoid proteins has been questioned (Rintamäki and Aro, 2001). At present, there are no convincing data on tyrosine phosphorylation of thylakoid proteins.

C. Characteristics and Classification of Thylakoid Phosphoproteins

All of the known thylakoid proteins shown to be phosphorylated undergo this modification at threonine residues (Table 1). Only one LHCII polypeptide from spinach (Table 1) has been found phosphorylated at a serine residue (Michel et al., 1991). Selective threonine phosphorylation of proteins is rather unusual for the majority of eukaryotic serine/threonine kinases and may be considered a unique feature of the thylakoid protein phosphorylation system.

Most of the thylakoid membrane proteins phosphorylated at the N-terminal threonine residues are also amino-acetylated (Table 1). Formation of N-acetyl-O-phosphothreonine is a specific trait of D1, D2, and CP43 that classifies them as a distinct group of thylakoid phosphoproteins. Importantly, these features have been suggested as determinants of the substrate specificity of the PP2A-like thylakoid membrane protein phosphatase (Vener et al., 1999, 2001) and the rapid dephosphorylation of these PS II core proteins at elevated temperatures (Rokka et al., 2000). In contrast, the PsbH protein of PS II can be phosphorylated at threonine 2 and threonine 4 (Table 1) and also differs from D1, D2, and CP43 in the environmentally dependent changes in phosphorylation, as discussed below.

The light-harvesting proteins of PS II are the most abundant phosphoproteins in thylakoid membranes and include different subunits of LHCII and the minor chlorophyll-binding protein CP29. Most of the characterized LHCII polypeptides are phosphorylated at the N-terminal threonine 3 (Table 1), and the presence of basic amino acid residues on both sides of the phosphorylation sites seems important (Table 1). This requirement was shown by experiments with synthetic peptides used as the substrates for redox-dependent thylakoid kinases (Michel et al., 1991). In the same study, it was concluded that acetylation of the amino termini of LHCII-like peptides was not required for their phosphorylation. CP29 is phosphorylated at position 6 both in *Arabidopsis* (Hansson and Vener, 2003) and the green alga *C. reinhardtii* (Turkina et al., 2004). The amino acid sequences around these phosphorylation sites are similar to those for phosphorylated LHCII polypeptides (Table 1). A second phosphorylation site, corresponding to position 83, was reported in CP29 from maize (Table 1). The phosphorylated sequence has been considered unique among thylakoid proteins since it met the phosphorylation site requirements for casein kinases (Testi et al., 1996).

Three recently identified phosphoproteins, TSP9, TMP14, and PsaD (the first phosphoprotein found in PSI), do not have sequences around their phosphorylation sites that are similar to those of either PS II-core proteins or LHCII polypeptides (Table 1). TMP14 is an intrinsic membrane protein (Hansson and Vener, 2003). In contrast, TSP9 and PsaD are peripheral proteins associated with the stromal side of thylakoid membranes. TSP9 differs from the other thylakoid proteins in that it can be phosphorylated at three different threonine residues that are situated in the middle portion, and not at the N-terminus, of the protein (Carlberg et al., 2003).

D. Assessing Changes in the Stoichiometry of Protein Phosphorylation

The first determination of the extent of phosphorylation was done for Lhcb1 and Lhcb2 polypeptides of LHCII by quantitative SDS-PAGE and scintillation counting after phosphorylation of isolated spinach thylakoids with radioactive ATP (Islam, 1987). Successful measurements of the phosphorylation level for the PS II protein D1 has been also achieved by densitometric quantification of the immunoblots and autoradiograms after separation of phosphorylated and non-phosphorylated forms of the protein by SDS-PAGE (Callahan et al., 1990; Elich et al., 1992; Rintamäki et al., 1996a). The stoichiometry of in vivo D1 phosphorylation under different conditions was assessed by immunoblotting with anti-phosphothreonine antibody as well (Rintamäki et al., 1997). All of these experiments were based on SDS-PAGE separation of thylakoid proteins prior to the analyses. An alternative approach is based on LCMS analyses of the peptides released by trypsin from the surface of thylakoid membranes (Vener et al., 2001). In this case, the stoichiometry of in vivo phosphorylation for individual proteins is determined by measuring the ratio of the phosphorylated to non-phosphorylated peptide originating from the same protein present in thylakoid membranes isolated in the presence of phosphatase inhibitors. This technique allowed monitoring of phosphorylation changes in D1, D2, CP43, and PsbH proteins, but was not quantitative for LHCII phosphorylation because of alternative cleavage of these polypeptides by trypsin (Vener et al., 2001). Assessing the extent of phosphorylation by all of the methods listed above confirmed the dynamic nature of thylakoid protein phosphorylation and demonstrated that, normally, none of the thylakoid proteins is completely phosphorylated (Elich et al., 1992; Rintamäki et al., 1996a, 1997; Vener et al., 2001; Booij-James et al., 2002).

The maximal phosphorylation level of Lhcb1 and Lhcb2 polypeptides of LHCII in vitro corresponds to 22–25% of the total amount of these proteins (Islam, 1987). Importantly, in vivo phosphorylation of LHCII has been found to occur only at light intensities lower than those used for normal plant growth, which has led to the question of the physiological role of LHCII phosphorylation (Rintamäki et al., 1997; Haldrup et al., 2001; Rintamäki and Aro, 2001). The highest extent of D1 protein phosphorylation, corresponding to 80–90% of protein content, has been detected only under reducing conditions in vitro (Elich et al., 1992) or at high light intensities in vivo (Rintamäki et al., 1997). The extent of in vivo phosphorylation for D1, D2, CP43, and PsbH proteins in *A. thaliana* under standard growth conditions corresponded to about 30–50%, being higher in the leaves harvested during the daytime (Vener et al., 2001). The only rapid and significant changes during light-dark transition were found for the phosphorylation of threonine 4 in PsbH (Table 1). The level of in vivo phosphorylation of PS II core proteins was found to be highly susceptible to elevated temperatures, indicating an involvement of this reversible protein modification in response of plants to heat stress (Rokka et al., 2000; Vener et al., 2001). In this respect, the measurements of in vivo protein phosphorylation levels has started to shift the concept for thylakoid protein phosphorylation in photosynthesis from

the direct regulation of the electron flow to the involvement in adaptive responses under stress conditions (Giardi et al., 1996, 1997; Rokka et al., 2000; Vener et al., 2001).

III. Reversible Phosphorylation of Photosystem II (PS II) Proteins

A. What is the Role of PS II Phosphorylation?

Phosphorylation of PS II core proteins was first suggested to regulate the electron transfer activity in this photosystem. Decrease of the maximum capacity of PS II electron transfer upon phosphorylation of chloroplast thylakoids has been reported (Horton and Lee, 1984). In contrast, a study on thylakoid membranes with differentially phosphorylated PS II or LHCII proteins led other authors to conclude that phosphorylated PS II polypeptides continued to support high rates of electron transport (Harrison and Allen, 1991). The binding properties of the secondary quinone acceptor site of PS II, Q(B), were found to be unaffected by phosphorylation (Harrison and Allen, 1991). However, reduced binding of photosynthetic herbicides at this site due to the phosphorylation of PS II core proteins was reported in another study (Giardi et al., 1992). It was proposed that phosphorylation of PS II polypeptides modifies the Q(B) pocket and regulates electron transfer by changing the quinone binding affinity of PS II (Giardi et al., 1992, 1995). In a more recent study focused on the relationship between PS II phosphorylation and electron transport activity, individual electron transport reactions in PS II were measured in PS II membranes with different levels of protein phosphorylation (Mamedov et al., 2002). The extent of D1 protein phosphorylation in the analyzed PS II membranes varied from 10% to 58%. Despite some minor changes in the properties of the differentially phosphorylated PS II, the major conclusion of this study was that there was no direct link between the phosphorylation of PS II core polypeptides and electron transfer activity or oxygen evolution by PS II.

PS II undergoes photoinhibition at light intensities beyond those saturating for photosynthesis (Greer et al., 1986; Cleland et al., 1990; Aro et al., 1993). It was reported that phosphorylation of thylakoid membrane proteins partially protects PS II against photoinhibition (Horton and Lee, 1985). The study of differentially phosphorylated thylakoids also suggested that phosphorylation of PS II polypeptides is required for PS II function at high light intensities (Harrison and Allen, 1991). On the other hand, the phosphorylation of D1, D2, CP43, and PsbH proteins was correlated with the decline of PS II activity during high irradiance treatments (Giardi et al., 1994). Phosphorylation of PS II proteins was also proposed as an early stage of photoinhibition involved in the disassembly of the photosystem prior to degradation of D1 protein (Giardi, 1993). However, in later studies the rate of photoinactivation of PS II electron transport and oxygen evolution was not found to be affected by PS II protein phosphorylation in thylakoids from different plants (Koivuniemi et al., 1995; Rintamaki et al., 1996b). A consensus in the interpretation of the contradictory data in this field could probably be found if the dynamics of photoinhibition and its relation to the repair of the damaged PS II are taken into account. At present, it looks plausible that PS II core protein phosphorylation does not directly influence PS II susceptibility to photoinactivation and damage but is crucial for the repair cycle of the damaged PS II.

Continuous functioning of PS II requires coordinated processes of lateral migration of the inactivated photosynthetic units from grana to stroma regions of the thylakoid membrane and their replacement (Ghirardi et al., 1990, 1993; Andersson and Aro, 1997; van Wijk et al., 1997; Baena-Gonzalez et al., 1999). The inactivation of PS II is directly proportional to light intensity (Tyystjarvi and Aro, 1996), but the photoinhibition of PS II appears only at high light intensities when the rate of inactivation exceeds that of PS II replacement (Andersson and Aro, 1997, 2001; Baena-Gonzalez et al., 1999). The light-induced inactivation of PS II is a consequence of the inactivation of the D1 reaction center protein, which has to be degraded and substituted with a newly synthesized copy (Ohad et al., 1984; Mattoo and Edelman, 1987; Andersson and Aro, 2001). This turnover of D1 is a key event in the turnover of PS II. Importantly, dephosphorylation of the photoinactivated D1 has been revealed as a prerequisite for its degradation (Koivuniemi et al., 1995; Rintamäki et al., 1996a; Andersson and Aro, 1997). The dephosphorylation of D1 as a control step for proteolysis of the damaged protein is so far the most feasible regulatory role suggested for the reversible phosphorylation of the PS II core proteins. The dephosphorylation of D2, CP43, and PsbH proteins has also been found to be involved in the partial disassembly of PS II monomers that migrate to the stroma regions of thylakoids (Baena-Gonzalez et al., 1999). Accordingly, the present paradigm considers the physiological role for reversible phosphorylation of PS II polypeptides in

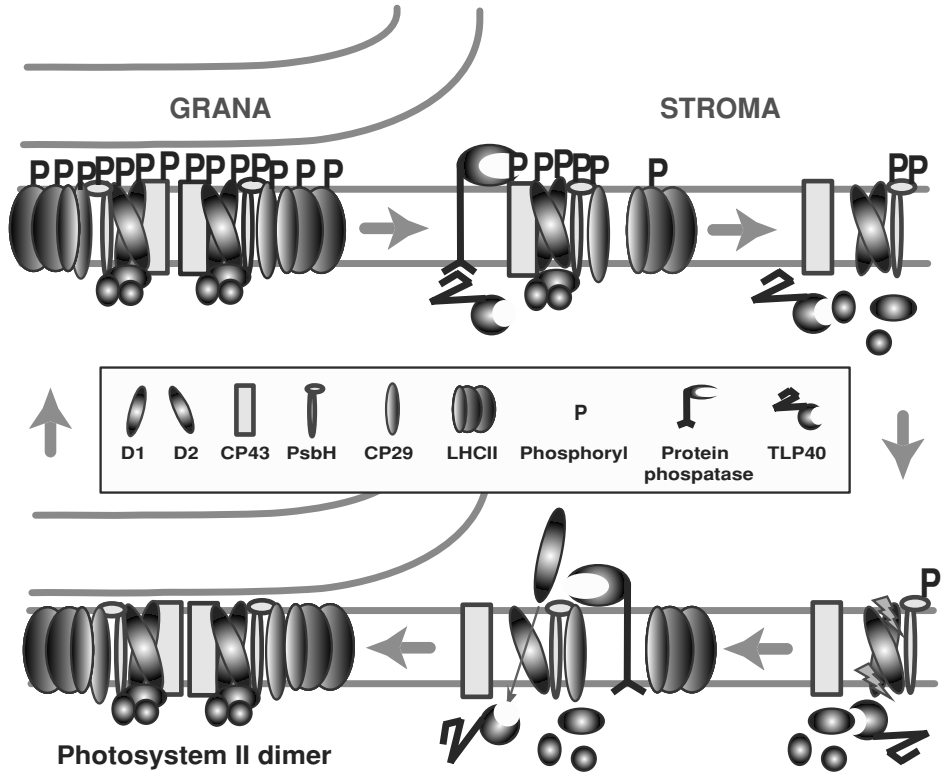

Fig. 1. Reversible protein phosphorylation during repair cycle of PS II in thylakoids of higher plants. The cycle includes light-induced phosphorylation of PS II proteins in the grana regions of thylakoids, migration of phosphorylated PS II monomers with the damaged D1 protein to the stroma membrane domains, sequential dephosphorylation of PS II polypeptides that leads to partial disassembly of PS II core complexes, proteolysis of the photodamaged D1, co-translational insertion of new D1 polypeptide in the remaining PS II complex, final assembly of the repaired PS II and its migration in the grana. The scheme shows only the PS II proteins that undergo reversible phosphorylation during the cycle. The membrane protein phosphatase responsible for dephosphorylation of PS II core polypeptides and luminal PPIase TLP40 regulating the phosphatase and probably assisting in folding of PS II polypeptides are also shown in the scheme.

the context of the PS II turnover cycle (Andersson and Aro, 2001; Rintamäki and Aro, 2001; Aro and Ohad, 2003).

B. Reversible Phosphorylation and Turnover of D1 Protein

The D1 protein is a central functional subunit of PS II, with a light-induced turnover rate higher than that of any other thylakoid polypeptide (Mattoo et al., 1981). D1 and PS II assembly as a whole undergoes a cyclic process, which is schematically outlined in Fig. 1. The "repair" cycle includes migration of PS II units containing the photoinactivated D1 from grana to stroma regions of thylakoids, partial disassembly of PS II complexes, degradation of D1 by specific proteases, co-translational insertion of the newly synthesized D1 copy in the remaining PS II complex (Andersson and Aro, 1997, 2001; Zhang et al., 1999; Rintamäki and Aro, 2001), and final assembly into functional PS II that migrates back to the stacked grana regions of thylakoids (Mattoo and Edelman, 1987). It has been demonstrated that degradation of the D1 protein necessitates this repair cycle (Aro et al., 1992; Ebbert and Godde, 1994, 1996; Rintamäki et al., 1995). The study of D1 turnover in isolated chloroplasts revealed that only the dephosphorylated protein was degraded, while D1 phosphorylated by light-activated kinase was not a subject for proteolysis (Ebbert and Godde, 1996). The proteolytic stability of phosphorylated D1 was also increased under conditions of reduced phosphatase activity in vitro (Koivuniemi et al., 1995). In leaves, the degradation of damaged D1 is prevented by sodium fluoride (Rintamäki et al., 1996a), an inhibitor of thylakoid protein phosphatases (Bennett, 1980). Accordingly, it has been suggested that dephosphorylation is a prerequisite for degradation of the damaged D1 (Rintamäki et al., 1996a). After

dephosphorylation, D1 is degraded (Fig. 1). Two different proteases, DegP2 (Haussuhl et al., 2001) and FtsH (Lindahl et al., 2000), have been implicated in the D1 degradation process. Thus, dephosphorylation of D1 allows for its degradation, necessitating the insertion of a new D1 copy into the temporarily dysfunctional PS II unit, thereby completing the 'repair' cycle (Andersson and Aro, 1997).

So far, only a single PS II-specific thylakoid membrane protein phosphatase has been identified (Vener et al., 1999). This PP2A-like protein phosphatase catalyzes the rapid and complete dephosphorylation of D1 (Vener et al., 1999; Rokka et al., 2000) and is presumably localized in the non-appressed membranes (Fig. 1) where it is anticipated to interact with a cyclophilin, TLP40 (Fulgosi et al., 1998; Vener et al., 1999; Rokka et al., 2000), as described below.

Reversible phosphorylation of the D1 protein has been found only in seed plants but not in mosses, liverworths, ferns, algae, or cyanobacteria (Rintamaki et al., 1996b; Pursiheimo et al., 1998; Rintamäki and Aro, 2001). Comparison of D1 turnover rates in higher plant and moss thylakoids under conditions promoting protein phosphorylation and PS II photoinhibition revealed a faster degradation of the moss D1 protein (Rintamaki et al., 1996). The specific phosphorylation of the D1 protein, causing retardation of its proteolysis in higher plants, has been suggested to have evolved in order to adapt the PS II repair cycle to the highly organized structure of the higher plant thylakoids with stacked grana and unstacked stroma membrane domains (Rintamäki and Aro, 2001; Aro and Ohad, 2003). It is worth noting that other hypothesis (Booij-James et al., 2002) proposed that the reversible phosphorylation of D1 in higher plants evolutionary replaced multiple D1 DNA copies in cyanobacteria for regulation of PS II core metabolism. Phosphorylation of D1 in the grana regions (Callahan et al., 1990) may serve to maintain the integrity of inactivated PS II during migration to the stroma membrane regions for dephosphorylation, degradation, and substitution of the inactivated protein with the newly synthesized polypeptide. Reversible phosphorylation of the other PS II core proteins proceeds along with that for D1 in the same cycle (Fig. 1) and likely serves a complementary role in the PS II repair process.

C. Reversible Phosphorylation of D2, CP43, and PsbH Proteins During PS II Turnover

As is the case for the D1 protein, phosphorylation of the other polypeptides of the PS II core, namely D2, CP43, and PsbH, does not appear to be involved in a direct regulation of electron transport and oxygen evolution by this photosystem. Particularly, this was demonstrated by modification of the phosphorylation sites in D2 (Andronis et al., 1998; Fleischmann and Rochaix, 1999) and PsbH (O'Connor et al., 1998) of *C. reinhardtii*. On the other hand, reversible protein phosphorylation of these proteins in thylakoids of higher plants was found to be closely related to the lateral migration of PS II between the different structural domains of the membrane system during the repair cycle. The phosphorylation of all PS II core polypeptides occurs in the grana regions of thylakoids (Ebbert and Godde, 1996; Baena-Gonzalez et al., 1999; Rintamäki and Aro, 2001). The conversion of isolated dimers of PS II to monomers was found to occur in their non-phosphorylated but not the phosphorylated forms, when studied in vitro (Kruse et al., 1997). However, equivalent phosphorylation was found in both dimers and monomers of PS II in vivo (Baena-Gonzalez et al., 1999), as illustrated in Fig. 1. The phosphorylated monomers migrated to the stroma regions of thylakoids where a stepwise dephosphorylation of CP43, D2, and D1 proteins has been demonstrated (Baena-Gonzalez et al., 1999). First, CP43 was dephosphorylated and then detached from the PS II core (Fig. 1). Second, D2 and D1 were dephosphorylated. Thus, it was suggested that phosphorylation of the PS II core proteins ensures the integrity of the monomers until repair can proceed, while dephosphorylation of CP43 and D2 proteins opens the complex for dephosphorylation of D1, its proteolysis, and the attachment of ribosomes inserting a new polypeptide (Baena-Gonzalez et al., 1999).

The reversible phosphorylation of PsbH has not been followed in the study of oligomeric PS II structures isolated from grana and stroma regions of thylakoids (Baena-Gonzalez et al., 1999). Nevertheless, an earlier study reported that dephosphorylation of both PsbH and CP43 proteins in thylakoids by exogenous alkaline phosphatase resulted in an extreme sensitivity of PS II to strong illumination (Giardi et al., 1994). It is reasonable to assume that the PsbH protein plays a role in the process of PS II disassembly and assembly (Fig. 1). The requirement of PsbH for assembly and stability of PS II was demonstrated in *C. reinhardtii* (Summer et al., 1997). A functional PsbH was also found to be necessary for rapid degradation of photoinactivated D1 and insertion of newly synthesized D1 molecules into thylakoid membrane of cyanobacteria (Bergantino et al., 2003). While the N-terminal extension containing the phosphorylation sites is typical only for eukaryotic PsbH proteins (Vener et al., 2001;

Gomez et al., 2002; Hansson and Vener, 2003), the characteristics of this phosphoprotein suggest its involvement in the assembly and disassembly of PS II (Giardi, 1993). Phosphorylation of PsbH differs from that of other PS II core proteins in two aspects. Firstly, PsbH has two phosphorylation sites (Table 1) and threonine 4 of the protein undergoes rapid reversible phosphorylation in response to light/dark transitions. This process is considerably faster than reversible phosphorylation of D1, D2, CP43 as well as of the threonine 2 in PsbH (Vener et al., 2001). Secondly, the phosphorylation sites in PsbH show principal sequence differences from those in D1, D2, and CP43 (Table 1). This difference may explain the slower dephosphorylation rates for phosphorylated PsbH compared to other PS II core phosphoproteins by the specific heat-shock-induced protein phosphatase, as discussed below.

D. Phosphorylation of PS II During Adaptive Responses

Phosphorylation of PS II polypeptides inhibits D1 protein turnover and increases PS II stability upon high light stress (Giardi, 1993; Ebbert and Godde, 1996; Baena-Gonzalez et al., 1999). Additionally this phosphorylation is likely involved in the maintenance and regulation of PS II turnover under different stress conditions. Thus, subjecting spinach to combined magnesium and sulfur deficiency was found to be accompanied by changes in D1 protein phosphorylation and turnover (Dannehl et al., 1995). In the first stages of the deficiency, the turnover of D1 was increased and D1 phosphorylation maintained in the dark. This led to a higher stability of active PS II supported by the efficient turnover of D1 protein (Dannehl et al., 1995). Prolonged stress for a few weeks, however, led to the degradation of the photosynthetic apparatus and chlorosis. Increase in the protein phosphorylation of PS II has been also found in plants in response to water deficient conditions (Giardi et al., 1996).

The phosphorylation of D1 and D2 proteins has been related to the process of photoprotective energy dissipation in plants under different environmental conditions. Energy dissipation is important for plant survival when leaves absorb more light energy than can be utilized for photosynthesis. The dissipation of the excess energy involves the xanthophyll cycle and the accumulation of deepoxidized pigments antheraxanthin and zeaxanthin (Niyogi et al., 1998; Muller et al., 2001). Chilling treatment of rice was found to decrease the photochemical efficiency of PS II in parallel with an increase in the level of zeaxanthin. Phosphatase inhibitors increased the rate of zeaxanthin accumulation under these conditions, as well as during dark-incubation of leaves at the normal temperature after chilling (Xu et al., 1999). A correlation was also found between dark-sustained phosphorylation of D1 and D2 proteins and dark-sustained zeaxanthin retention and maintenance of PS II in a state primed for energy dissipation in plants subjected to high light stress (Ebbert et al., 2001). Nocturnal retention of zeaxanthin and antheraxanthin, and their sustained engagement in a state primed for energy dissipation, have also been observed in the leaves/needles of sun-exposed evergreen species during winter (Adams et al., 2001, 2002). Phosphorylation of D1 and D2 polypeptides has been found retained along with retention of zeaxanthin and antheraxanthin and PS II remained primed for energy dissipation during nights with subfreezing temperatures, while this was rapidly reversed upon exposure to increased, non-freezing temperatures (Adams et al., 2001). In contrast to the wintertime, no nocturnal retention of zeaxanthin and antheraxanthin was found prior to sunrise on warm summer mornings (Barker et al., 2002). It is plausible that sustained phosphorylation of PS II polypeptides favors a structure of PS II and its interaction with light harvesting antennae that facilitate better nonphotochemical energy dissipation under stressful environmental conditions, particularly high light and low temperature.

The CP29 antenna protein of PS II has been found phosphorylated after exposure of *Zea mays* plants to high light in the cold (Bergantino et al., 1995). Phosphorylation of this minor light-harvesting polypeptide following chilling treatment in the light has been associated with the resistance of maize plants to cold stress (Bergantino et al., 1995). Induction of CP29 phosphorylation by cold has also been found in barley (Bergantino et al., 1998), as well as in winter rye upon high light and cold treatment (Pursiheimo et al., 2001). Recent work on the structure of the PS II supercomplexes has localized CP29 between PS II dimers and LHCII trimers associated with the photosystem (Yakushevska et al., 2003). This localization also supports an important role of CP29 in the stabilization of oligomeric PS II structure (Yakushevska et al., 2003). Thus, cold- and high light-induced phosphorylation of this protein may stabilize PS II in a way similar to that of PS II core protein phosphorylation following high light treatment. Moreover, localization of CP29 between the PS II core and the major LHCII antennae may also contribute to more efficient photoprotective energy dissipation upon phosphorylation of CP29 under stressful conditions.

Contrary to the sustained phosphorylation of PS II proteins in chilling and cold stress conditions,

extremely fast dephosphorylation of these polypeptides has been observed during short heat shock treatments of both plant leaves and isolated thylakoids. Studies on protein dephosphorylation in isolated thylakoids from spinach and *A. thaliana* revealed specific acceleration of dephosphorylation for PS II core proteins at elevated temperatures (Rokka et al., 2000; Vener et al., 2001). Raising the temperature from 22°C to 42°C resulted in a more than ten-fold increase in the dephosphorylation rates of D1 and D2 and CP43 proteins in spinach thylakoids. In contrast, the dephosphorylation rates for PsbH and LHCII polypeptides were accelerated only 2- to 3-fold (Rokka et al., 2000). The use of a phosphothreonine antibody to measure in vivo phosphorylation levels in spinach leaves revealed a more than 20-fold acceleration in D1, D2, and CP43 dephosphorylation induced by abrupt elevation of temperature, but no increase in LHCII dephosphorylation (Rokka et al., 2000). A specific dephosphorylation of D1, D2, and CP43 has also been observed by mass spectrometric techniques after a short heat shock treatment of *A. thaliana* leaves (Vener et al., 2001), although this dephosphorylation was less pronounced than in spinach. It is important to emphasize that heat-induced dephosphorylation of PS II core proteins occurs on a time scale of minutes (Rokka et al., 2000; Vener et al., 2001), while continuously high steady-state phosphorylation of these polypeptides under chilling lasts over days (Adams et al., 2001). Thus, dynamics of PS II dephosphorylation could be very flexible depending on the environmental conditions. The molecular mechanism for the adaptive response of PS II to an abrupt elevation of temperature consists of the rapid dephosphorylation of CP43, D1, and D2, which primes the photosystem to a fast turnover in response to heat shock. The fast decrease in PS II core protein phosphorylation at high temperatures is accomplished by the heat-shock-induced PS II-specific protein phosphatase regulated by the cyclophilin TLP40.

E. PS II-Specific Protein Phosphatase and TLP40

A number of different protein phosphatases have been implied in dephosphorylation of phosphoproteins in thylakoid membranes (Bennett, 1980; Sun et al., 1989; Carlberg and Andersson, 1996; Hast and Follmann, 1996; Elich et al., 1997; Hammer et al., 1997; Vener et al., 1999), although none of them were characterized at the molecular/gene level. Most soluble protein phosphatases isolated from chloroplasts were efficient in dephosphorylation of LHCII polypeptides (Sun et al., 1989; Hast and Follmann, 1996; Hammer et al., 1997). A PS II-specific membrane protein phosphatase from spinach thylakoids was purified over a thousand-fold, using detergent-engaged FPLC and thylakoid phosphopeptides for the enzyme assay (Vener et al., 1999). The purified enzyme exhibited characteristics typical of eukaryotic Ser/Thr phosphatase of the PP2A family in that it was inhibited by okadaic acid and tautomycin, irreversibly bound to microcystin-agarose, and recognized by a polyclonal antibody raised against a recombinant catalytic subunit of human PP2A. Interestingly, okadaic acid has not inhibited protein phosphatase activity in the intact thylakoid membranes, while the anti-PP2A antibody inhibited protein dephosphorylation (Vener et al., 1999). When the isolated enzyme was added to the phosphorylated thylakoid membranes, increased rates of dephosphorylation were observed for D1, D2, and CP43 proteins (Vener et al., 1999). A common trait for these three phosphoproteins is the presence of N-terminal acetylated and phosphorylated threonine residues (Table 1), which was proposed as the reason for the substrate specificity of the protein phosphatase towards these PS II core proteins (Rokka et al., 2000; Vener et al., 2001). The other distinct characteristic of this phosphatase was its association with and regulation by TLP40, a cyclophilin-like peptidyl-prolyl isomerase (PPIase) located in the thylakoid lumen (Fulgosi et al., 1998; Vener et al., 1999).

TLP40 was discovered due to its copurification with the protein phosphatase from the thylakoid membrane (Fulgosi et al., 1998). The presence of PPIases in plant chloroplasts was established more then a decade ago (Breiman et al., 1992; Mattoo, 1998). However, TLP40 was the first complex, multi-domain cyclophilin-like PPIase found in chloroplasts as well as in plant species (reviewed in (Vener, 2001; He et al., 2004). The structure of TLP40 includes a cyclophilin-like C-terminal segment of 20 kDa, a predicted N-terminal leucine zipper, and potential phosphatase-binding sites flanking the leucine zipper (Fulgosi et al., 1998; Vener et al., 1999; Vener, 2001). The isolated protein possesses peptidyl-prolyl cis-trans isomerase protein folding activity (Fulgosi et al., 1998). TLP40 is localized in the thylakoid lumen, interacts with the inner surface of the thylakoid membrane, and regulates the activity of the PS II-specific protein phosphatase (Fig. 1). As judged from immunoblotting analyses, TLP40 appears to be confined predominantly to the unstacked thylakoid regions, the site of protein integration into the photosynthetic membrane (Fulgosi et al., 1998; Vener et al., 1999). It was proposed that TLP40 has a dual role in protein folding catalysis and in trans-membrane

regulation of the PS II-specific protein phosphatase (Fulgosi et al., 1998; Vener et al., 1998, 1999; Vener, 2001). The recent finding that the major PPIase protein-folding activity in the soluble lumen is associated with the cyclophilin TLP20 (Edvardsson et al., 2003; Romano et al., 2004) but not with TLP40 supports the suggestion of a specialized regulatory function for TLP40.

It has been found that binding of Cyclosporin A, an inhibitor of PPIases, to TLP40 activated thylakoid phosphatase, while PPIase substrates, prolyl-containing oligopeptides inhibited protein dephosphorylation (Fulgosi et al., 1998; Vener et al., 1999). These experiments required thylakoids be ruptured first to expose the lumenal membrane surface where TLP40 is located (Vener et al., 1999). Thus, TLP40 may act as a regulatory subunit of the PP2A-like membrane phosphatase, modulating activity of the latter at the outer thylakoid surface. This regulation likely operates via reversible binding of TLP40 to the inner membrane surface (Fig. 2). Indeed, significant heat shock-induced activation of the phosphatase coincided with a temperature-induced release of TLP40 from the membrane into the thylakoid lumen (Rokka et al., 2000). Moreover, induction of the phosphatase activity by TLP40 release from the membranes was confirmed

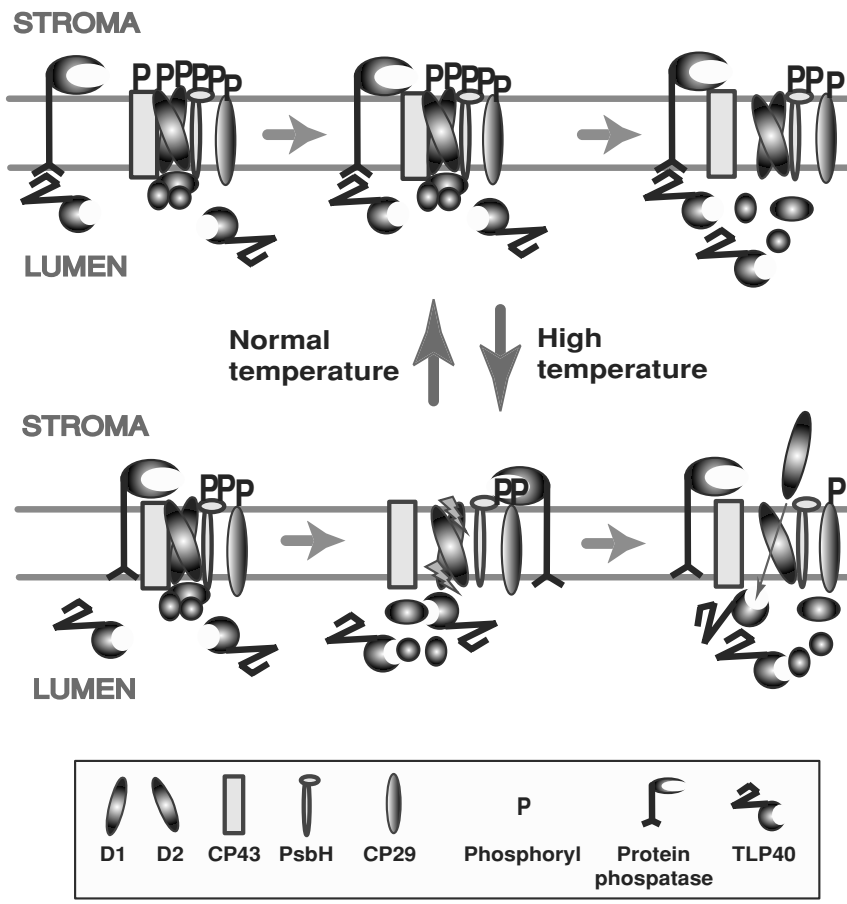

Fig. 2. Schematic illustration for trans-membrane regulation of PS II-specific membrane protein phosphatase by cyclophilin TLP40. TLP40 is present in the lumen in soluble and membrane-bound forms. The membrane-bound TLP40 interacts with the lumen-exposed region of the membrane phosphatase, which suppress the phosphatase activity at the opposite stroma-exposed surface of the membrane. At high temperatures (35°C and 42°C, for spinach), TLP40 is released from the membrane into the lumen, the phosphatase becomes highly active and dephosphorylates CP43, D2, and D1 polypeptides of PS II in a time scale of several minutes. The dephosphorylation leads to acceleration of PS II repair cycle. The release of TLP40 from the membrane also increases its PPIase protein folding activity in the lumen, which can support functional conformation of oxygen evolving complex polypeptides, as well as folding of newly inserted D1 protein at elevated temperature. Thus, coordination of the phosphatase and PPIase activities at the opposite sides of the thylakoid membrane may orchestrate PS II biogenesis.

by phosphatase assays using intact thylakoids, solubilized membranes, and the isolated protein phosphatase (Rokka et al., 2000).

F. Trans-Membrane Signaling by the Phosphatase and PS II Biogenesis

Signaling from TLP40 to the protein phosphatase has been proposed to be involved in the coordination of PS II dephosphorylation with protein folding (Rokka et al., 2000; Vener, 2001). Both processes are required for protein turnover of PS II reaction centers. A model outlined in Fig. 2 summarizes the experimental data on the possible trans-membrane signaling by the PS II-specific thylakoid protein phosphatase. The protein phosphatase is suggested to have a single transmembrane span and a short extension into the thylakoid lumen (Vener et al., 1999; Vener, 2001). The active sites of the phosphatase and TLP40 PPIase are situated on opposite sides of the thylakoid membrane (Fig. 2). Accordingly, the dephosphorylation of D1, D2, and CP43 proteins by the phosphatase proceeds at the stroma-exposed surface of the membrane. TLP40 is localized in the thylakoid lumen and distributed between the membrane and the soluble fractions (Fulgosi et al., 1998; Vener et al., 1999). Thus, the PPIase protein folding activity of TLP40 may be restricted to the proteins of the lumen and the lumen-exposed domains of integral thylakoid membrane proteins. The dissociation of TLP40 from the membrane surface upon abrupt elevation of temperature activates the PS II-specific protein phosphatase (Fig. 2). The phosphatase may rapidly dephosphorylate CP43, D2, and D1 proteins and allow for disassembly of the PS II monomers migrated to the nonappressed regions of thylakoids. The dephosphorylation may trigger degradation of D1, its substitution by a newly synthesized polypeptide, and the following steps of biogenesis and final assembly of functional PS II. In parallel with these events, the release of TLP40 from the membrane may increase protein-folding activity in the lumen that may ensure the sustained active conformation of the oxygen evolving complex polypeptides at high temperatures, as well as accelerate folding of the proteins newly inserted into the luminal space. The reversible interaction of TLP40 and the membrane phosphatase provides a potential molecular mechanism for trans-membrane signaling and synchronization of the degradation, synthesis, assembly, and folding of PS II polypeptides at both sides of the thylakoid membrane. A strong coordination of the numerous steps of PS II biogenesis and assembly is obviously required for maintenance of the functional photosynthetic machinery under stressful environmental conditions.

G. Reversible Phosphorylation of LHCII Polypeptides and State Transitions

The major polypeptides of the PS II light harvesting antenna, LHCII, undergo dynamic light- and redox-dependent reversible phosphorylation both in vitro and in vivo (Allen, 1992; Vener et al., 1995; Rintamäki et al., 1997; Fleischmann et al., 1999; Haldrup et al., 2001). For two decades, the major role for LHCII phosphorylation was ascribed to balancing of absorbed light energy distribution between the two photosystems, also called state transitions (Allen et al., 1981; Allen, 1992; Allen and Mullineaux, 2004; Allen and Forsberg, 2001). According to this concept, phosphorylation of the mobile pool of LHCII polypeptides leads to their detachment from PS II and migration to PS I (Allen, 1992). However, later studies have revealed that phosphorylation of LHCII in vivo occurs only at rather low light intensities and decreases dramatically at higher irradiances corresponding to normal plant growth (Rintamäki et al., 1997; Haldrup et al., 2001; Rintamäki and Aro, 2001). Thus, it has been proposed that LHCII phosphorylation could regulate the balance of excitation energy distribution just under moderate light intensities (Aro and Ohad, 2003). Moreover, the discovery of a deficiency in state transitions in *Arabidopsis* plants lacking the PSI-H or PSI-L subunits but exhibiting functional LHCII phosphorylation questioned a regulation of state transitions by LHCII phosphorylation (Lunde et al., 2000; Haldrup et al., 2001). When PSI-H was absent, LHCII was not able to attach to PS I and state transitions did not occur even though LHCII was highly phosphorylated (Lunde et al., 2000). In agreement with this, two recent studies have demonstrated that a significant fraction of LHCII interacting with PS I was not phosphorylated (Snyders and Kohorn, 2001; Zhang and Scheller, 2004). The dephosphorylation of LHCII associated with PS I may also result from the action of the LHCII-specific protein phosphatase (Elich et al., 1997). Despite the findings listed above, involvement of LHCII phosphorylation in state transitions cannot be completely ruled out (Allen and Forsberg, 2001). Nevertheless, the original paradigm of a mobile LHCII bound to PS II or PS I in dephosphorylated or phosphorylated form, respectively, certainly has to be modified.

Transient phosphorylation of the mobile LHCII polypeptides may be required to overcome a potential barrier for their detachment from PS II. The fate

of the released LHCII in the membrane may then be unrelated to its phosphorylation state. It is probable that significant light-induced structural changes in LHCII polypeptides (Zer et al., 1999, 2003; Garab et al., 2002) could be more important than phosphorylation for the determination of the interacting partners of free LHCII. In this respect, the light-dependent dynamics of the protein complexes in the photosynthetic membrane are not compatible with the oversimplified model featuring only two states, with LHCII attached to PS II and phospho-LHCII attached to PS I. Phosphorylation of LHCII polypeptides may regulate just some individual steps in the reversible and flexible interactions of these antenna proteins with both photosystems.

A regulatory role for reversible phosphorylation has also been proposed in the process of LHCII degradation when the antenna size of PS II is reduced upon acclimation of plants from low to high light intensities. Phosphorylated LHCII polypeptides were found to be poor substrates for proteolytic degradation during this process in comparison with the unphosphorylated LHCII (Yang et al., 1998). In this respect, phosphorylation may delay degradation of LHCII in a way similar to that demonstrated for D1 protein. However, an involvement of LHCII phosphorylation in the regulation of the size of light-harvesting antennae during acclimation still remains to be proven.

H. Differential Phosphorylation of CP29

The phosphorylated CP29 isolated from cold-treated maize were N-terminally blocked against N-terminal chemical sequencing (Bergantino et al., 1995), and was probably acetylated as was shown later for CP29 (LHCb4.2) from *A. thaliana* (Hansson and Vener, 2003) and for CP29 from the green alga *C. reinhardtii* (Turkina et al., 2004). Nevertheless, the phosphorylation site in maize CP29 has been localized to threonine residue number 83 (Table 1) by mapping of proteolytic fragments of the protein (Testi et al., 1996). The site of phosphorylation in CP29 (LHCb4.2) from *Arabidopsis thaliana* and in CP29 from *C. reinhardtii* (Turkina et al., 2004) has been localized to a threonine residue at position 6 of the mature proteins (Table 1). Interestingly, CP29 from *C. reinhardtii* revealed a unique characteristic. In contrast to all known nuclear-encoded thylakoid proteins, the transit peptide in the mature algal CP29 was not removed but processed by methionine excision, N-terminal acetylation, and phosphorylation on threonine 6 (Turkina et al., 2004). The N-termini of the mature CP29 from *Chlamydomonas* and *Arabidopsis* have a significant sequence similarity around their phosphorylation sites. The importance of this phosphorylation was proposed as the reason for the unique retention of the transit peptide in the mature algal CP29 (Turkina et al., 2004). The difference between phosphorylation of CP29 in *Arabidopsis* and in green algae versus phosphorylation of CP29 in maize lies in the physiological conditions inducing these modifications. Phosphorylation of *Arabidopsis* and algal proteins was found under standard growth conditions, while phosphorylation of maize CP29 was induced only by high light in the cold and on the other threonine residue. Notably, we have recently detected multiple and differential phosphorylation of CP29 depending on the environmental conditions (M.V. Turkina and A.V. Vener, unpublished data). Thus, phosphorylation of CP29 protein that is localized between PS II dimers and LHCII trimers in this photosystem (Yakushevska et al., 2003) may likely be involved in a number of responses to the changing environment.

IV. PsaD: the First Phosphoprotein in PS I

In a recent study on in vivo thylakoid protein phosphorylation in *A. thaliana* plants under normal growth light conditions, PsaD was identified as the first phosphoprotein in PS I (Hansson and Vener, 2003). The site of PsaD phosphorylation in vivo was mapped to the first threonine at the N-terminus of the mature protein (Table 1). The PsaD protein is essential for a functional PS I in plants and is required for the proper assembly and stability of this photosystem (Haldrup et al., 2003). PsaD is a hydrophilic protein that has no stable three-dimensional structure in solution (Antonkine et al., 2003), but forms a well-defined three-dimensional structure when bound to PS I (Fromme et al., 2001; Antonkine et al., 2003). Thus, the significant structural changes and flexibility of PsaD, together with its control position at the electron donor site of PS I, may rely on regulatory mechanisms operating via protein phosphorylation. The finding of PsaD phosphorylation opens a new direction in the investigation of possible PS I regulation by protein phosphorylation.

V. Phosphorylation of Other Thylakoid Proteins

A. TMP14: a Previously Unknown Thylakoid Phosphoprotein

During mass spectrometric characterization of phosphorylated peptides released from thylakoid

membranes of *A. thaliana* by trypsin (Hansson and Vener, 2003), a phosphopeptide from a previously uncharacterized protein, TMP14, was identified (Table 1). This protein is annotated as "expressed protein" in the *Arabidopsis* database and as a potential membrane protein with two trans-membrane regions in the database for *Arabidopsis* membrane proteins. It was named TMP14 for thylakoid membrane phosphoprotein of 14 kDa (Hansson and Vener, 2003). TMP14 is encoded by the nuclear gene At2g46820 in *Arabidopsis* and has homologous proteins encoded in the genomes of other plants and cyanobacteria. All of these proteins contain two potential trans-membrane helices and well-defined signaling peptides with a high predicted probability for chloroplast targeting. Experimental results confirmed localization of TMP14 in the thylakoid membranes of chloroplasts. Topology prediction for the plant proteins places the N-terminus of TMP14 on the stromal side of the thylakoid membrane, which is in agreement with phosphorylation of TMP14 at the membrane surface exposed to chloroplast stroma. The phosphorylation site in TMP14 was confined to one of two N-terminal threonine residues in the sequenced peptide. These residues correspond to the positions 65 and 66 in the sequence of the precursor protein. The discovery of phosphorylated TMP14 in the photosynthetic membrane raises questions about the function of this protein, its binding partners, association with the photosynthetic protein complexes, and the role of its in vivo phosphorylation.

B. TSP9 and Light-Induced Cell Signaling

The nature of a phosphoprotein with a relative electrophoretic mobility of 12 kDa (Bhalla and Bennett, 1987) has remained elusive during two decades of studies on redox-dependent protein phosphorylation in plant thylakoid membranes. This protein has recently been characterized as a novel plant specific protein and called TSP9 for thylakoid soluble phosphoprotein of 9 kDa (Carlberg et al., 2003). Genes encoding homologous "unknown" proteins were found on chromosome 3 of *Arabidopsis* and rice as well as in ESTs from more than 20 different plant species but not in any other organisms. TSP9 is a very basic protein. Mass spectrometric analyses revealed the existence of non-, mono-, di-, and tri-phosphorylated forms of TSP9 and phosphorylation of three distinct threonine residues in the central part of the protein (Table 1). The nature of this modification was transient with steady increase of the protein phosphorylation level upon illumination (Bhalla and Bennett, 1987; Carlberg et al., 2003). Light-induced phosphorylation of the protein was associated with partial release of phosphorylated TSP9 from the thylakoid membrane, which is in contrast to all other known thylakoid phosphoproteins tightly bound to the membrane (Carlberg et al., 2003).

The phosphorylation-dependent transient association of TSP9 with the photosynthetic membrane suggests a possible role of this protein in chloroplast signaling (Fig. 3). Plants acclimate to changes in environmental light quality and intensity by an adjustment of photosystem stoichiometry and size of the light-harvesting antennae. Regulation of expression of both chloroplast-encoded and nuclear-encoded photosynthetic genes in photosynthetic organisms by chloroplast redox signals, involving the redox state of plastoquinone, has been demonstrated (Escoubas et al., 1995; Pfannschmidt et al., 1999, 2001). Since plastoquinone also controls the activation of membrane protein kinases (Vener et al., 1998), repression of nuclear-encoded *cab* genes for light-harvesting polypeptides may thus be coupled to the redox status of plastoquinone via a thylakoid protein kinase that phosphorylates a protein dissociating from thylakoids (Escoubas et al., 1995). TSP9 is a potential candidate for such a plant cell signaling component (Fig. 3).

VI. Regulation and Role of Thylakoid Protein Phosphorylation in a Physiological Context

The recent application of analytical techniques for detection of thylakoid protein phosphorylation in vivo has uncovered a complex regulation of this process. The cooperative regulation of LHCII phosphorylation by plastoquinone reduction and the ferredoxin-thioredoxin system has been demonstrated (Rintamäki et al., 2000; Martinsuo et al., 2003). Feeding of pea leaves with glucose induced LHCII phosphorylation in darkness, which demonstrated that sugar metabolism or signaling exerted a control over phosphorylation of LHCII polypeptides (Hou et al., 2002). Phosphorylation of CP29 in winter rye has been shown to be induced by high light specifically at low temperatures (Pursiheimo et al., 2001). Phosphorylation of the D1 protein in the higher plant *Spirodela oligorrhiza* grown under natural diurnal cycles of solar irradiation has been shown to undergo circadian oscillation (Booij-James et al., 2002). These oscillations were out of phase with the period of maximum light intensity. However, light resettled the phase in the circadian rhythm of D1 phosphorylation

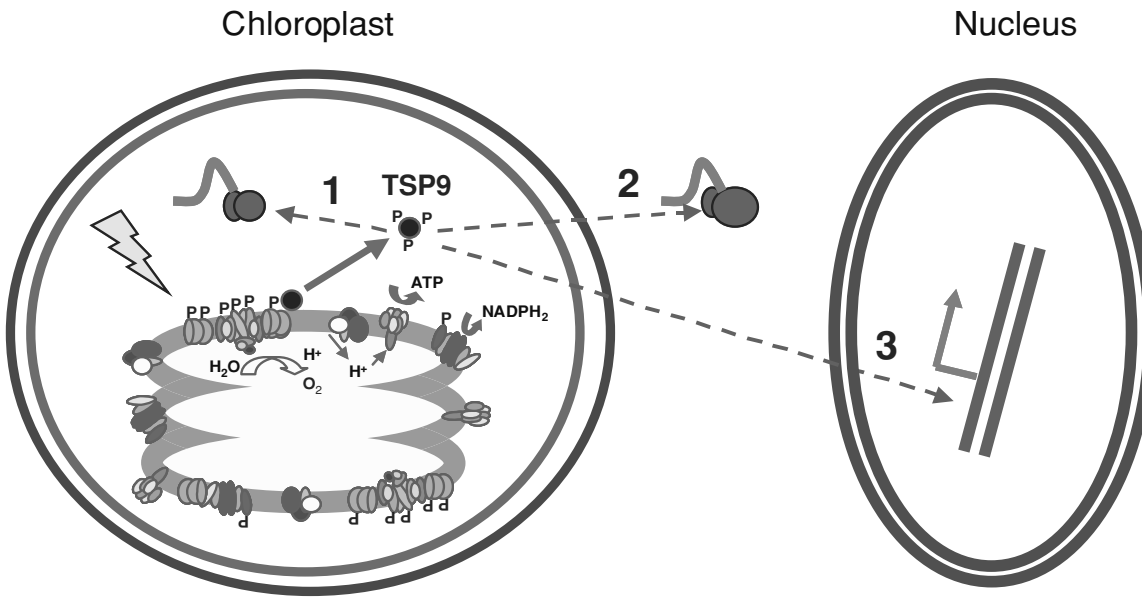

Fig. 3. Potential model for light-induced cell signaling by phosphorylation of TSP9 and its release from the thylakoid membrane. Upon illumination of thylakoids, TSP9 is phosphorylated at three distinct threonine residues and then released from the membrane. TSP9 is a basic protein that can potentially regulate transcription or translation of photosynthetic proteins. The model suggests three signaling options for the released TSP9: 1) regulation of protein expression in the chloroplast; 2) regulation of protein synthesis in the cytosol; and 3) regulation of gene expression in the nucleus.

(Booij-James et al., 2002). Specific fast dephosphorylation of D1, D2, and CP43 polypeptides of PS II in response to abrupt elevation of temperature has been demonstrated (Rokka et al., 2000; Vener et al., 2001). These findings clearly call for further studies of thylakoid protein phosphorylation in vivo to understand the physiological implications of this phenomenon. The differential changes in protein phosphorylation under variable environmental conditions are also indicative of a multifunctional involvement of this posttranslational modification in regulation and adaptive responses of the photosynthetic apparatus.

The complexity of thylakoid protein phosphorylation in a physiological context implies additional hurdles to revealing the enzymes and other molecular factors involved in reversible phosphorylation of thylakoid proteins. A multiple control of LHCII phosphorylation at the redox level alone has already revealed involvement of (i) plastoquinone reduction (Allen et al., 1981; Allen, 1992), (ii) plastoquinol binding at the Qo site of the cytochrome bf complex (Vener et al., 1995, 1997; Zito et al., 1999; Finazzi et al., 2001), (iii) plastoquinol oxidation at the Qo site and rapid reoccupation of the site with a new plastoquinol molecule (Hou et al., 2003), and (iv) the thiol redox state (Rintamäki et al., 1997, 2000; Carlberg et al., 1999; Martinsuo et al., 2003). At present, it is not clear how these events may influence any of the three TAK kinases (Snyders and Kohorn, 1999, 2001) or the two Stt7-like kinases (Depege et al., 2003) shown to be important for phosphorylation of LHCII polypeptides. The most feasible strategy to understand the role of each individual protein kinase is to use kinase-gene knockout plants for in vivo characterization of thylakoid protein phosphorylation to reveal the substrates for each kinase. The regulation of the individual kinase(s) involved in phosphorylation of distinct thylakoid proteins could then be studied in a more focused way. Examination of the in vivo protein phosphorylation patterns in mutant plants lacking individual regulatory components, like TLP40, TSP9, subunits of cytochrome *bf* complex, and thioredoxin-like proteins, should reveal the regulatory network for the reversible phosphorylation of photosynthetic proteins and signaling cascades controlling expression of these proteins.

The present level of knowledge on thylakoid protein phosphorylation favors different regulatory, adaptive, and signaling functions for reversible phosphorylation of individual proteins upon changing physiological conditions including stress. The recent progress in creating publicly available plant knockout lines and development of the analytical techniques for characterization of in vivo protein phosphorylation will likely lead to rapid progress in the understanding of

the multiple physiological regulatory functions of reversible and environmentally-modulated phosphorylation of thylakoid proteins.

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