Protein phosphorylation and redox sensing in chloroplast thylakoids
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Transduction of light dependent signals to redox sensitive kinases in photosynthetic membranes modulates energy transfer to the photochemical reaction centres and regulates biogenesis, stability and turnover of thylakoid protein complexes. The occupancy of the quinol-oxidation site of the cytochrome bf complex by plastoquinol and the redox state of protein thiol groups act as elements of the signal transducing chains.

Introduction
Photosynthetic electron flow is a process whereby photooxidation of water by the photochemical reaction centre of photosystem II (PSII) serves as a source of electrons for the reduction of the plastoquinone pool. Oxidation of plastoquinol and reduction of plastocyanin are mediated by the cytochrome bf complex. Plastocyanin oxidation via the light driven photosystem I (PSI) activity (Figure 1) generates NADPH needed for the carbon fixation process. Light, while being the source of energy for photosynthetic organisms, is also potentially dangerous under aerobic conditions. If light harvesting is not balanced by light energy utilisation and dissipation toxic radicals are formed, leading to oxidative damage to proteins, lipids and pigments of the thylakoid membrane [1].

Light-induced phosphorylation of LHCII, the major light-harvesting chlorophyll a/b protein, was shown to be related to the energy distribution between the two photosystems [2,3]. Signalling between light reception and phosphorylation of LHCII was found to involve the redox state of the plastoquinone pool; reduced plastoquinone (plastoquinol) leads to kinase activation [3,4]. Later observations revealed that cytochrome bf also plays a role in the kinase activation, and that several subunits of the PSI complex can undergo phosphorylation [5*]. Recent progress in our understanding of thylakoid protein phosphorylation will be reviewed here, with emphasis on its redox-dependent control and implication in stress-related responses, light acclimation and regulation of gene expression.

Redox activation of thylakoid protein phosphorylation
The pool of reduced plastoquinone molecules serving as the only activator of the thylakoid protein phosphorylation has been a central ‘dogma’ in photosynthesis research [3,5*,6]. Recent data, however, suggest a more elaborate mechanism for kinase activation stressing the importance of the cytochrome bf complex and of a plastoquinol molecule bound to the bf complex’s quinol oxidation site (Qo site) [7,8**]. Kinase activation in thylakoids has traditionally been induced either by illumination or by addition of reductants in darkness [5*]. These treatments reduce all the electron transport components and, therefore, do not permit identification of the specific redox sensors involved. This problem was overcome when it was found that protein phosphorylation could be induced by a short transient acidification (pH 4.3) of thylakoid membranes in darkness [7]. This treatment shifts the plastoquinone/plastoquinol equilibrium to a more reduced state. As a consequence, two of the cytochrome bf electron carriers, the Rieske Fe-S centre and cytochrome f, both components of the high potential electron path of this complex, as well as plastocyanin are also reduced ([7], Figure 1). The plastoquinol pool is subsequently rapidly re-oxidised by ambient oxygen while the Rieske Fe-S centre, cytochrome f, plastocyanin as well as the plastoquinol bound at the Qo site remain reduced in the dark. The active state of the kinase persists as long as a plastoquinol molecule interacts with the Qo site of the reduced cytochrome bf complex ([7], Figure 1). Two experiments gave credence to the existence of such a plastoquinol redox sensor [8**]. Displacement of plastoquinol from the Qo site by the plastoquinol antagonist 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) deactivated the kinase (Figure 2a) even when the plastoquinol pool was still reduced. Furthermore, the kinase could be deactivated by a single light flash, causing only one oxidation/reduction turnover of the PSI (Figure 2b). This initiates a downstream electron transfer via plastocyanin and the high potential path components of the cytochrome bf complex and leading to the oxidation of the Qo-bound plastoquinol by withdrawal of a single electron.
The concept of transformation of a redox sensing event at the Qo site into a signal for kinase activation has recently gained support from protein structure determinations. These include atomic resolution of the mitochondrial cytochrome bc1 complex [9] and of the luminal domain of the cytochrome bc1 complex [10]. The two complexes are analogous in their general function and structure. These three-dimensional data combined with mutagenesis and mechanistic studies have revealed dramatic structural changes in the Rieske Fe-S protein of cytochrome bc1 upon binding of ligands to its Qo site [11]. These changes are also likely to occur in cytochrome bc1 and potentially act as a structural signal mediating the redox sensing event to the kinase activation. This novel concept views the kinase–cytochrome bc1 interaction as being analogous to a signal transducing system. The cytochrome complex acts as the receptor, the plastoquinol pool as the mediator or agonist, and plastoquinol at the Qo site as the ligand. This model re-emphasises previous biochemical data suggesting a transient contact between the kinase and cytochrome bc1 [5]. Because the kinase active site is exposed at the stromal side of the thylakoid membrane, where the amino-terminal threonine residues of the substrate proteins (PSII and LHCII) are located, the
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Figure 2

(a) Autoradiogram of 32P-labelled polypeptides illustrating the major thylakoid phosphoproteins, the D1 and D2 reaction centre proteins of PSII (including dimer forms), CP43 (which binds chlorophyll a), minor chlorophyll a/b binding protein CP29, major chlorophyll a/b binding proteins LHCII, an uncharacterised 12 kDa protein and the 9 kDa phosphoprotein of PSII (PsbH). Deactivation of protein kinase activity due to displacement of plastoquinol from the Qo site of the cytochrome bc complex with the antagonist DBMIB is shown. (b) Deactivation of LHCII phosphorylation through oxidation of the Qo-bound plastoquinol by a single-turnover flash to PSI in the presence of the PSII inhibitor diuron 

Relationship between the occupancy of the Qo site of cytochrome bc complex by plastoquinol (PQH₂) and the kinase activation/deactivation. (a) Autoradiogram of 32P-labelled polypeptides illustrating the major thylakoid phosphoproteins, the D1 and D2 reaction centre proteins of PSII (including dimer forms), CP43 (which binds chlorophyll a), minor chlorophyll a/b binding protein CP29, major chlorophyll a/b binding proteins LHCII, an uncharacterised 12 kDa protein and the 9 kDa phosphoprotein of PSII (PsbH). Deactivation of protein kinase activity due to displacement of plastoquinol from the Qo site of the cytochrome bc complex with the antagonist DBMIB is shown. (b) Deactivation of LHCII phosphorylation through oxidation of the Qo-bound plastoquinol by a single-turnover flash to PSI in the presence of the PSII inhibitor diuron 

Protein phosphorylation in light-related processes

The involvement of a mobile phosphorylated LHCII in the reversible association of LHCII with PSII, thus balancing the excitation energy between the two photosystems (state transition), has been extensively reviewed [5•,6,14•]. A major theme of our review is that the short-term regulation of light harvesting may not be the most significant physiological function of thylakoid protein phosphorylation, but that this post-translational modification has broader effects on the photosynthetic process.

Protein phosphorylation was recently shown to regulate repair of photoinhibitory damage to PSII [15•]. Light stress leads to oxidative damage of the D1 protein subunit of the PSII reaction centre [16,17]. Repair of photosynthetic function requires replacement of the damaged D1 protein via degradation, synthesis of a new protein copy, and its integration into PSII. Under light stress the proportion of phospho-D1 increases [15•] although this form of the protein has been shown to be a poor substrate for proteolysis. Studies both in vitro [18] and in vivo [19] have demonstrated that a photodamaged D1 protein can be proteolysed only after its dephosphorylation. The co-ordination between protein phosphorylation and proteolysis provides a temporal delay between D1 degradation and the subsequent integration and assembly of the newly synthesized protein [15•]. Lack of such co-ordination would lead to decomposition of the entire PSII complex. Indeed, stabilisation of nascent D1 protein is related to the rate of degradation of the photodamaged protein [1].

The small 9kDa phosphoprotein (PsbH) also seems to be important for maintaining stability of PSII because a Chlamydomonas psbH-deletion mutant is impaired in the assembly of this complex [20]. Furthermore, phosphorylation of PSII polypeptides was shown to increase their stability in plants suffering from combined magnesium and sulphur deficiency stress [21].

Protein phosphorylation may influence the turnover of the D1 protein via a general structural effect. The PSII complex assumes a dimeric structure in situ [22], but monomerisation occurs under light stress as part
of the repair process [23,24]. Recent studies, however, indicate that in their nonphosphorylated form dimers can be converted to monomers, but not when they are phosphorylated [25].

The degradation of LHCII, which occurs when plants acclimatise from low to high light intensities, also appears to be regulated by protein phosphorylation [26]. By analogy with the D1 protein, there is a transient phosphorylation of LHCII and only after dephosphorylation does photooxidation occur (J Webster, D-H Yang, personal communication). This demonstrates that phosphorylation of LHCII is not only involved in short-term acclimation of light-harvesting but may also play an equally important role in the long-term regulation of the PSII antenna.

Another novel feature concerns phosphorylation of the PSII subunit CP29. Maize in which this chlorophyll-binding protein was not phosphorylated exhibited increased sensitivity towards cold stress [12,27]. Phosphorylation of isolated CP29 revealed alterations of its spectral characteristics, which were ascribed to conformational changes [28,29].

Phosphorylation-induced conformational changes have also been suggested for LHCII on the basis of spectroscopic analyses of its amino-terminal domain [14*,30*]. It was suggested that these conformational changes are mechanistically more important than repulsion from surface charges for the reorganisation of the mobile LHCII antenna and the associated state transitions.

**Reversible protein phosphorylation in vitro and in vivo**

The majority of studies on thylakoid protein phosphorylation have been performed in isolated membranes, where all electron carriers become rapidly reduced in the light. Accordingly, the kinase system becomes fully activated and the phosphorylation rate of LHCII and PSII increases with elevated levels of irradiance (Figure 3). In *vivo*, however, the situation is different because carbon dioxide serves as an electron sink and, consequently, the electron carriers are in a steady state of reduction. Recently, the levels of thylakoid phosphorylation in *vivo* have been determined in whole leaves by immunological quantification of phosphothreonine residues [31**]. Phosphorylation of the PSII proteins in *vivo* reached saturation with increasing irradiance in accordance with the situation in *vitro* (Figure 3). In contrast, maximal phosphorylation of LHCII was achieved at low light intensities, and was followed by a decrease at moderate light levels. At high light intensity the level of phospho-LHCII was found to be quite low [31**]. Related to these observations, it was found previously that LHCII phosphorylation was deactivated in photoinhibited *Chlamydomonas* cells [32]. As LHCII in *vivo* exhibits a transient maximal phosphorylation at relatively low light intensities [31**], state transitions could only be of relevance within a relatively narrow light intensity interval. Thus, the importance previously ascribed to the state transition process may need to be questioned, although its significance under low light and various stress conditions may still be a factor.

**Figure 3**

Schematic representation of the dependence of LHCII and PSII phosphorylation on light intensity *in vitro* and *in vivo*. The phosphorylation of LHCII reaches a maximum at low light intensity in both isolated thylakoids and intact leaves. A further increase in irradiation causes decrease in the LHCII phosphorylation level *in vivo* but not *in vitro*. In contradistinction, phosphorylation of PSII proteins requires higher light intensities and remains constant upon further increase in the irradiation in both *in vivo* and *in vitro* systems. Adapted from [31**].
of complexity, dephosphorylation of D1 protein targeted for degradation in photodamaged PSII was found to be light-stimulated, in contrast to phospho-D1 in functional reaction centres [19].

**The search for kinases and phosphatases**

The identity of the substoichiometric thylakoid protein kinases and phosphatases is still unknown, although available data suggest they are membrane bound [5*]. Presently, three putative thylakoid protein kinases with apparent molecular masses of 53 kDa, 66 kDa [5*] and 58 kDa [36,37*] have been found and highly enriched, active kinase fractions have been isolated [38]. Surprisingly, the putative 58 kDa kinase was inferred not to be associated with cytochrome bf but with the PS II complex [36,37*]. The significance of this observation, which would suggest distinct differences in the enzymology between LHCII and PSII phosphorylation, needs further attention.

A 29 kDa protein phosphatase from the chloroplast stroma capable of dephosphorylating LHCII was recently purified [39]. A thylakoid membrane phosphatase, was shown to interact with a newly discovered 40 kDa cyclophilin-like protein (TLP40) located in the thylakoid lumen and possessing peptidyl-prolyl cis-trans isomerase protein folding activity [40**]. TLP40, which contains two putative phosphatase-binding sites, was shown to influence thylakoid protein dephosphorylation (Figure 1). These results [40**] have revealed an unexpected regulation of dephosphorylation in thylakoids that possesses analogies to the cyclophilin-modulated calcineurin-dependent protein dephosphorylation in mammals [41].

**Redox regulation of gene expression**

As the turnover of the D1 protein is redox dependent via protein phosphorylation it is not surprising that the expression of *psbA*, the chloroplast gene encoding this protein, is also subject to redox control. The initiation of *psbA* transcription is regulated by phosphorylation of two sigma-like transcription factors [42*]. The kinase is associated with a chloroplast RNA polymerase and is itself regulated by phosphorylation. This led the authors [42*] to propose a phosphorylation cascade that connects the gene expression to a redox-regulated thylakoid protein kinase. Of particular interest is a specific activation of *psbA* pre-RNA splicing induced by light in *Chlamydomonas* [43**]. This splicing was abolished by inhibitors of photosynthetic electron transport, and was absent in nonphotosynthetic mutants. While it is not clear how electron transport stimulates the splicing, involvement of the thioredoxin system in the reduction of thiol groups was proposed [45*]. A new insight into the redox regulation of *psbA* mRNA translation [44] emerged recently with the identification of a protein disulphide isomerase that is able to modulate the association of the *Chlamydomonas* chloroplast polyadenylate-binding protein with the 5′-untranslated region of the mRNA. Possibly *psbA* mRNA translation can be regulated by photosynthetic activity via this disulphide isomerase and its reduction by thioredoxin [45*].

Whether the expression of other plastid genes is redox regulated remains to be established. Transcription of the nuclear *cab* gene, encoding LHCII, was proposed to be coupled to light-intensity via the redox state of plastoquinone and possibly to activation of a thylakoid protein kinase [46].

Redox sensing and protein phosphorylation in chloroplasts, despite their prokaryotic origin, display major differences from these processes in prokaryotic organisms. First, in the chloroplasts, regulation is controlled by reductive signals, while in prokaryotes it operates mostly via oxidative events [47,48*,49]. Second, eukaryotic Thr/Ser protein kinases are present in thylakoids, unlike the two-component signalling pathways which involve His/Asp phosphorylation in prokaryotes [14*,47]. It is attractive to speculate that the nuclear-encoded redox-regulated chloroplast Thr/Ser kinases, which originally evolved in the ancestor of current plastid containing cells, have replaced the prokaryotic kinases of the symbiont from which the chloroplast has evolved.

**Conclusions**

Light-driven redox reactions provide natural signals for self-regulation of photosynthetic electron flow. The first instance of such regulation concerned energy transfer via reversible phosphorylation of a mobile LHCII antenna. More recent data, however, suggest that redox-controlled protein phosphorylation in chloroplasts has a much wider physiological significance as exemplified by its involvement in the regulation of gene expression and stress-related responses. The processing of precursor proteins and their transmembrane translocation may represent events where protein phosphorylation will prove to be essential. Transport of proteins through the thylakoid membrane is a process in which protein phosphorylation could be important.

The redox signalling system in chloroplasts is unique, considering its reductive nature and that an electron transfer complex, cytochrome *bf*, acts as a sensor. The regulation of protein phosphorylation by thiol reduction, possibly via the ferredoxin-thioredoxin system, represents a second feedback loop of the redox control generated by the electron flow.

An ultimate understanding of thylakoid protein phosphorylation and its regulation still awaits the identification and characterisation of the kinases and phosphatases involved. This challenge will require the combination of advanced biochemical and genetic approaches, including the use of accumulated sequence information from databases of expressed sequence tags from *Arabidopsis* and other organisms.
References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
•• of outstanding interest


A novel mechanism for thylakoid protein phosphorylation redox control is presented. It involves a single plastoquinol molecule binding to the quinol oxidation site of the cytochrome b complex with a reduced high potential path. The protein kinase activation and deactivation analysis included determination of the redox states of the electron transport components by low temperature electron paramagnetic resonance, laser flash photolysis, optical spectroscopy and inhibitor studies.


The structure of the lumen-side domain of the thylakoid Rieske protein at 1.83Å resolution is presented. A structure-based comparative sequence analysis of the cytochrome b1 and bc1 groups of Rieske soluble domains is made. The biological implications of these data are discussed in line with a previously determined structure of the lumen-side domain of cytochrome f.


Variable conformations of the Rieske iron-sulfur protein of the cytochrome bc1 complex were identified in different crystal forms, which were induced upon binding of inhibitors to the Q0-site of the complex. Long-distance movement of the head domain of the Rieske subunit during electron transfer from quinol bound in cytochrome b to the heme of cytochrome c1 is proposed. Two distinct binding domains for inhibitors at the site are proposed to reflect an additional movement of the quinol and its reaction intermediates during oxidation within the binding site. An animation illustrating the movement of the Rieske protein is available on World Wide Web URL: http://arc-gen1.life.uiuc.edu/bc-complex/node


A minireview on state transitions and structural effects of LHChI phosphorylation that proposes the existence of a prokaryotic protein phosphorylation system in thylakoids.


A concise overview of the proteases of the chloroplasts with emphasis on protein turnover induced by high light levels. The regulation of D1 protein degradation and turnover by reversible protein phosphorylation is summarised.


The role of CP29 phosphorylation with respect to efficiency and stability of the PSII reaction centre and its antenna at low temperature was studied using phosphorylating and nonphosphorylating maize genotypes. It is suggested that phosphorylated CP29 performs its regulatory function by a shift of the distribution of the excitation energy from the reaction centres towards the antenna.


Spectroscopic techniques were used to study a synthetic peptide of the LHClI amino-terminal region as well as the native chlorophyll a/b binding protein. The results revealed that phosphorylation of LHClII may regulate its dynamic role during the state transitions through structural changes in the protein–protein interactions of surface-exposed domains rather than via surface charge repulsion between phosphoproteins.


An immunological approach using a polyclonal phosphothreonine antibody is introduced for the analysis of thylakoid protein phosphorylation in vivo. The LHClII polypeptides were found to have a different phosphorylation pattern in vivo with respect to increasing irradiance as compared to previous in vitro phosphorylation studies. The maximal phosphorylation in vivo only occurred at low light intensities, far below the light intensity required for optimal growth, and then drastically decreased at higher light levels. The results raise new questions with respect to the physiological significance of LHClII phosphorylation and its regulation.


Phosphorylation of LHClII in vivo is effectively inhibited by propyl gallate while having only a limited effect on PSII protein phosphorylation. Using this inhibitor, it is demonstrated that LHClII dephosphorylation is insensitive to light in vivo. Taken together with previous findings that PSII protein dephosphorylation is light stimulated, the authors suggest the presence of multiple thylakoid phosphatases.


A 58 kDa protein kinase is closely associated in substoichiometric abundance with a core complex of PS II. It could be resolved from the PS II complex and was shown to be active in the absence of the cytochrome b/f complex.


A 40 kDa cyclophilin-like protein/cyclophilin cis-trans isomerase was found to be present in the thylakoid lumen. The protein comprises a carboxy-terminal peptidyl-prolyl cis-trans isomerase catalytic domain and an amino-terminal region with a potential protein kinase domain. It functions as a co-immunoprecipitate with a thylakoid phosphatase activity and also to exert an effect on the dephosphorylation of several thylakoid phosphoproteins. This finding, revealing an analogy to the effects of calcineurin, shows that regulation of thylakoid protein dephosphorylation is more complex than previously anticipated.


A serine-specific protein kinase activity assigned to a 54 kDa polypeptide is described to be associated with the major chloroplast RNA polymerase and to phosphorylate sigma-like transcription factors in vitro. The polymerase-associated kinase is itself influenced by phosphorylation and dephosphorylation, which led the authors to propose that it is a part of a signalling cascade that controls chloroplast transcription in vivo via protein phosphorylation.


It is shown for the first time that Chlamydomonas psbA pre-RNAs, containing one or more introns, accumulate in wild-type cells in the dark. Exposure of cells to light increases the splicing efficiency of the psbA introns approximately 6-10-fold. The effect of light is specific for the psbA introns, because the levels of unspliced 23S pre-RNA did not decrease. The light stimulation of psbA pre-RNA processing was abolished by inhibitors of photosynthetic electron transport. Nonphosphorytrophic mutants, including a tscA-lacking PSI mutant, did not show evidence of light-stimulated RNA processing. The light response was restored in photosynthetic transformants that had been complemented with the tscA gene encoding a protein involved in the trans-splicing events that produce the mRNA for the subunit Ia of photosystem I.


A chloroplast protein disulphide isomerase copurifying with a polyadenylate-binding protein was shown to modulate the association of the latter protein with the 5′-untranslated region of the psbA mRNA of Chlamydomonas. A light/dark regulation of the psbA mRNA translation is proposed to be controlled by modulation of the protein disulphide isomerase redox state via thioredoxin.


A Rhodobacter capsulatus transcription factor, CrbT, responsible for aerobic repression of expression of genes encoding bacteriochlorophyll, carotenoid, and light harvesting complex II, was heterologously overexpressed, purified and shown to be biologically active. CrbT binds to conserved palindromic sequences that overlap the –10 and –35 promoter regions of the bchC operon. Binding of CrbT to the bchC promoter region was also found to be redox dependent, with CrbT exhibiting an approximately five-fold higher binding affinity under oxidising versus reducing conditions.